PONTIFICIAE ACADEMIAE SCIENTIARVM SCRIPTA VARIA

45

SEMAINE D'ETUDE

SUR LE THÈME

CELLULES NERVEUSES, TRANSMETTEURS ET COMPORTEMENT

9-14 OCTOBRE 1978

ÉDITÉ PAR

RITA LEVI-MONTALCINI

PONTIFICIA
ACADEMIA
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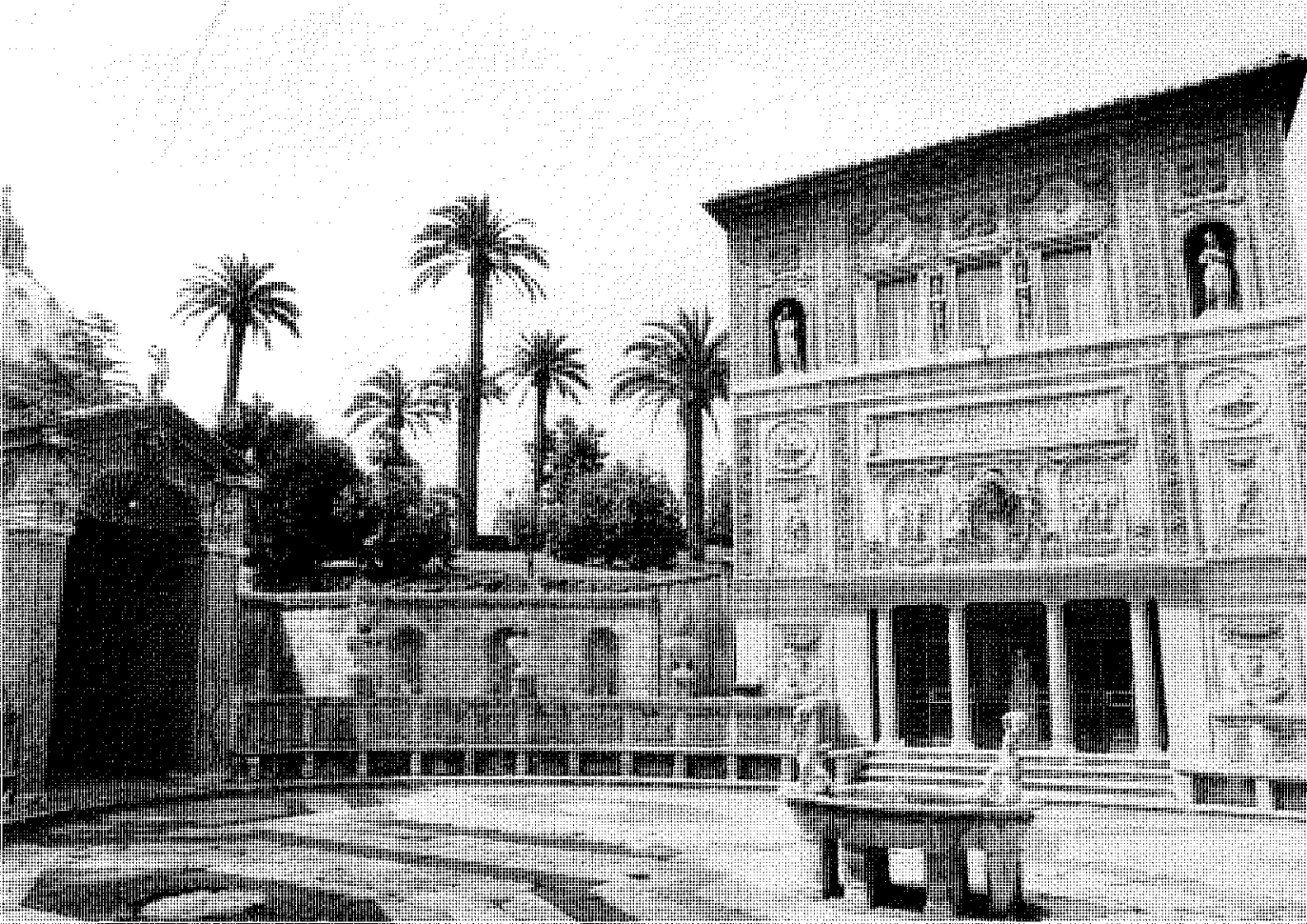
EX AEDIBUS ACADEMICIS IN CIVITATE VATICANA

MCMLXXX



PONTIFICIAE ACADEMIAE SCIENTIARVM SCRIPTA VARIA

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STUDY WEEK

ON

NERVE CELLS, TRANSMITTERS AND BEHAVIOUR

OCTOBER 9-14, 1978

EDITED BY

RITA LEVI-MONTALCINI

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FOREWORD

This Study Week on Neurobiology is the sixteenth organized by the Pontifical Academy of Sciences and was held at its seat, the Casina Pio IV, in the beautiful Vatican Gardens. It comes at an appropriate moment when surprising new facts are described and new interpretations are given to some of the most important aspects of our biological knowledge. It is a blooming field wherein the nerve cells, transmitters and behaviour results of yesterday can easily be substituted by those of today, and may be reversed by those of tomorrow. The effervescence of this field of research can be seen in the diversity of approaches, methods and technical skill, presented during this 5-day Meeting. From the study of cells, some of them serving as special models for particular studies, to that of the specialisation in split and intact brain, a large road has been covered by the participants in the Study Week. Certainly, not all the problems, suggestions and answers have been focussed during our Meeting. Its purpose, however, was perfectly achieved and all the presentations show the importance of the new approaches and how much our knowledge on brain mechanisms has been extended to the molecular level. It is safe to say that during the previous Study Week on "Brain and Conscious Experience", held in 1964, also a land-mark in this field, we could have hoped that a more detailed scrutiny of nerve function could be expected, but it would be difficult to imagine the progress achieved in the recognition of the intimate nature of some of the ways by which nerve cells interact. Certainly, much has still to be done and new frontiers of research will become necessary. One may say, however, that this Study Week has repeated the success of the one held in 1964.

This success is due to the tireless zeal and devotion with which our Academician Rita Levi-Montalcini, overcoming difficulties of all sorts, has dedicated herself to the task of organizing the Meeting and editing its Proceedings. Thanks to Rita Levi-Montalcini, whose discovery of the Nerve Growth Factor is a real cornerstone in the history of neurobiology and, I

am sure, in that of embryonic development, our meeting surpassed all our expectations.

Conveying to my colleague, the eminent Academician Rita Levi-Montalcini, the expression of my gratitude, I want to emphasize also in very warm terms how much we are indebted to Father Enrico di Rovasenda and Mrs. Michelle Porcelli Studer for all the help they have given to the success of the Study Week, before, during and after its realization.

Finally, I also want to thank Mrs. Gilda Massa for the transcription of all the discussions and Mr. Silvio Devoto for his technical assistance.

CARLOS CHAGAS

President of the Pontifical Academy
of Sciences

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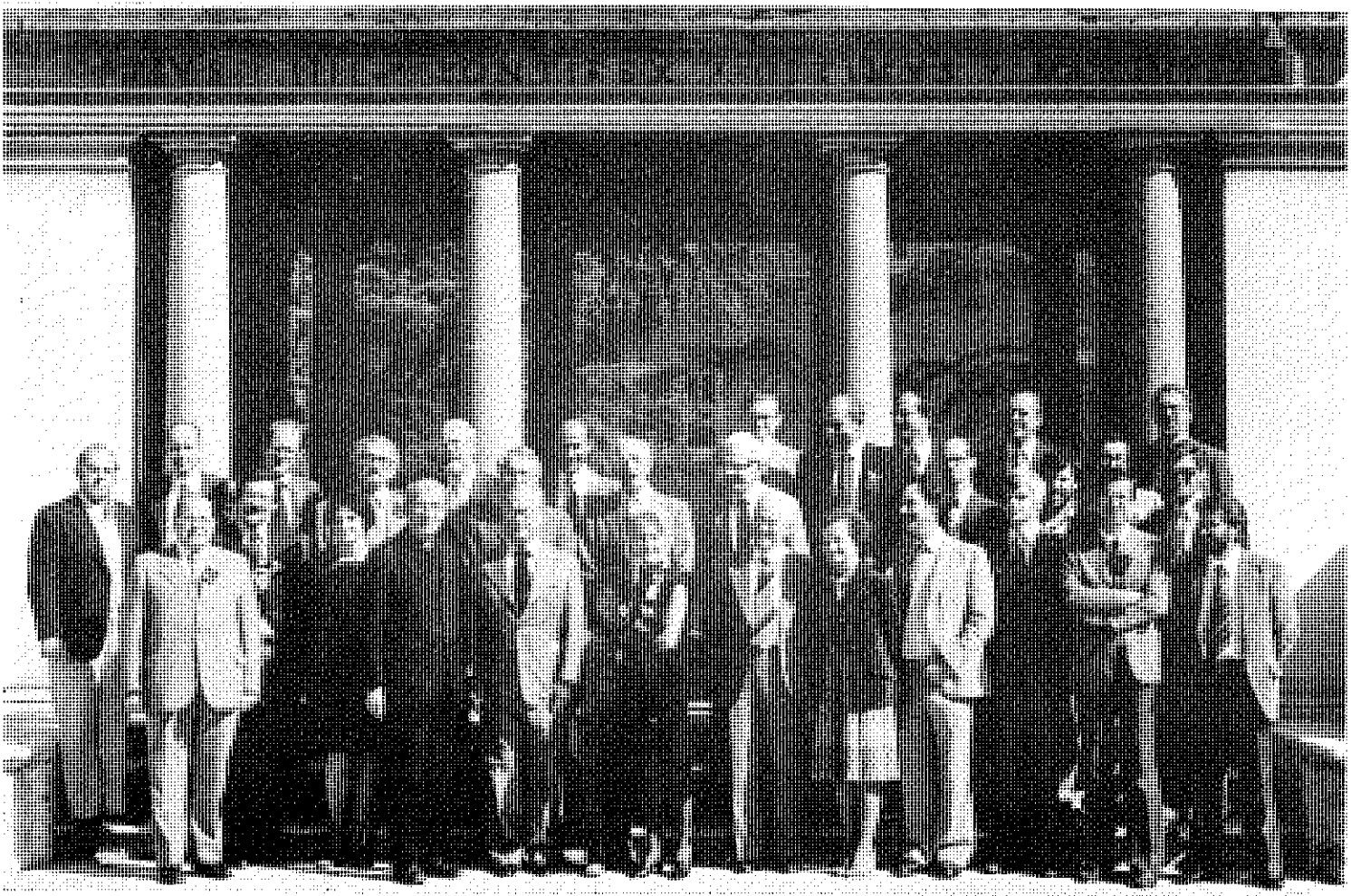

Participants in the Study Week

SCIENTIFIC PAPERS AND DISCUSSIONS

NERVE CELLS, NEUROTRANSMITTERS AND BEHAVIOUR

RITA LEVI-MONTALCINI

INTRODUCTION (Pre and after thoughts)

By way of introduction, I shall make some comments on the selection of the topics and on the content of each section of this Study Week Conference on Neurobiology.

At variance with a previous one of this same series, which took place fourteen years ago at the Pontifical Academy and centered entirely on the Problem No. 1 of Neurobiology, the Problem of Brain and Consciousness, the present one was aimed at a survey of recent progresses and perspectives in the areas that have been under intensive exploration in this last one and a half decade. It is easy to predict that they will remain at the center of attention also in the years to come. The recent developments in some of these sectors, set in fact the stage for a deeper analysis of problems which have been under investigation, ever since the nervous system became one of the favorite objects of experimental analysis, toward the beginning of the century, while at the same time developments in other areas of neurobiology, uncovered entirely new horizons, unforeseen and unforeseeable to our predecessors. According to the acute and admirable essay by T. S. Kuhn on "The Structure of Scientific Revolutions", the former sectors fit in the general field of research considered by the author under the heading of "Normal Science as Puzzle Solving" and are defined as "a highly cumulative enterprise eminently successful in its aim, characterized by the steady extension of the scope and precision of scientific knowledge" (1, p. 52). The other sectors which would better fit in the definition by the same author of "crises" which "begin with the blurring of a paradigm and the consequent loosening of the rules for normal research" (1, p. 84) represent the main object of the whole conference and bring to light the tremendous interest and effervescence which are

now pervading a field which used to be one of the most static and deserted ones, among the different areas of biological sciences until few decades ago, and well justified the pessimistic statement by one of the leading authorities in molecular biology, that "our present knowledge of the nervous system is in an exceedingly primitive state" (2). This field that only recently became known as the field of Neurosciences or of Neurobiology, assembles together the most diversified problems related to the fundamental unit of the nervous system, the nerve cell, the geometry and ontogenesis of neuronal circuits and the structural and functional organization of the most admirable organ devised by nature; the nervous system, be this the brain and ganglia of a beetle, or the vastly more intricate cerebro-spinal axis of a human being. Labelling of the field which used to be partitioned in different sectors, known as neuroanatomy, neurophysiology and behaviour, with a unique term, was more than a lip service to acknowledge the common denominator of these different areas of neurobiological research. It brought to the fore the tremendous advantage of keeping students with different inclinations and background, in close contact with each other, exchanging information and presenting at the same time the "outsiders" with a unique front far more alluring than a collection of loosely bound areas, encumbered with a forbidding and cumbersome nomenclature introduced by old anatomists to define at the macro and microscopic levels, brain areas and an enormous constellation of nerve cells interconnected by even more formidable neuronal circuits. A most gratifying result of this unplanned strategy, was to arouse the interest of investigators of other scientific branches to look over the fences and join forces with experts in different sectors of the unified neurobiological field. The newcomers: biochemists, molecular biologists, biophysicists and even theoretical physicists, not only moved in this area in progressively larger number, but even bent over the benches and labored at the microtome, or inspected at the inverted and at the electron microscope nerve cells cultured under different conditions, to acquire a first hand knowledge of the basic units of the nervous system and of their interconnections with their target neuronal or non-neuronal cells.

The unfailing enthusiasm of old timers (and tbe writer acknowledges with regret her belonging to this category) and tbe refreshingly different and more sophisticated approach of the neopbytes, transformed in a few years this static field in one of the more rapidly moving frontiers of biology. It does not seem either too optimistic or too presumptuous to consider the present status of neurobiology similar in many respects

to that which characterized the field of physics at the beginning of the century, and to that of molecular biology in the glorious early fifties, when "top secrets" of living matter were unveiled by a handful of most shrewd and sharp brains. Even if the field of neurobiology cannot still pride itself with any world-shacking discoveries, as those that characterized the two above mentioned areas of scientific endeavour, there are all too good motives, why even the present massive investment in this field, should have yet not produced any of the extraordinary tradition-shattering revolutions (to borrow again a terminology from T. S. Kuhn) which all of a sudden opened new horizons in the inanimate and animate world. The reasons, to mention only some of them, are that the nervous system with its billions of cells connected by far more numerous neuronal circuits, and the endless diversity of structure and function of its cellular units, presents the student with problems unmatched by any other biological objects, which were instead so successfully scrutinized by molecular biologists. In the case of the nervous system, the most formidable and possibly never to be solved problem, besides those already mentioned, is the brain-consciousness problem which will be discussed, with no claim to suggest a solution and not even a promising approach, in the last section of this conference.

The selection of the topics, as well as their somewhat arbitrary division in five sections, proved to be rather difficult, particularly for what concerns the material to be discussed in the first three days, devoted to problems related to nerve cells, neurotransmitters and to the newly discovered field of endogenous morphomimetic peptides and their receptors. Not only each one of these topics would have easily filled all the three sections of this study week and, even so, we would have barely touched upon some of the problems listed in each one of them, but the results of *in vivo* and *in vitro* studies of nerve cells, cell-to-cell communication through the release of neurotransmitters and receptors activation, are so deeply interwoven, as to make questionable any criteria aimed at drawing a partition line between these closely linked topics. Likewise the selection of the areas to be discussed in the every-day broadening field of nerve cell biology and brain functions, was a matter of personal preference, rather than of any priority of the chosen over the regretfully neglected areas.

In a way of justification for such an arbitrary selection, I shall briefly consider the topics discussed in each section.

The first section is devoted to two unrelated aspects of nerve cell biology, which share however in common a tremendous debt of gratitude

to one of the most versatile and at the same time simple techniques: the tissue culture technique devised at the beginning of the century by an experimental embryologist, R. G. Harrison (3) to provide evidence in favor of the hypothesis that nerve fibers or axons, are the product of single cells, and to solve in this way the long and bitterly fought battle between supporters of the so-called neuronal theory and those who claimed that all nerve cells are interconnected (the reticularists) in an inextricable syncitium where each cell loses its individuality and becomes part of a massive global connection of nerve cells and their axons. The question was definitely settled by Harrison's *in vitro* experiments, but neither the time nor the techniques were ripe for tackling more sophisticated problems and asking more subtle questions. Only four decades later, the technique which had in the meantime been perfected, even if used mainly to explore the growth pattern and differentiation of animal cells at the morphological level, all of a sudden revealed its tremendous potentialities, thanks to the discovery that it was possible to isolate and culture different cell lines originating from single cells and exploring their growth and differentiation under different experimental conditions. While this technique opened an entirely new field to students of normal and neoplastic cells, it was of no advantage to the neurobiologist who is dealing with non-proliferating "non-clonable" cells. Nevertheless it was the classic old-fashioned hanging-drop technique which was to reveal the existence and chemical nature of the Nerve Growth Factor and made in this way possible the isolation and identification of this protein molecule (4).

The second common feature of these two different lines of investigations discussed in the first section, is that both depart from the beaten routes of "Normal Science" as defined by T. S. Kuhn and signed the beginning of promising approaches to old and new neurobiological problems. In the impossibility of considering in detail the most significant results reported in this section I shall only briefly comment on some studies directed to investigate the mechanism of action of NGF on its target nerve cells and on a pheochromocitoma cell line, and on two other investigations dealing with neurological properties and synapses connections between hybrid neoplastic cells and myogenic cell lines.

Studies on the mechanism of action of NGF extensively pursued in recent years by P. Calissano and co-workers, took their start from the discovery that NGF induces the *in vitro* polymerization of a precursor dimeric protein known as tubulin in contractile filaments designated as microtubules (5). Subsequent studies by the same group gave evidence

for a similar effect elicited *in vitro* by NGF on the microfilaments precursor, actin. Since the earliest and most striking *in vivo* NGF effects on its target cells, is a tremendous increase in both contractile proteins, which in turn results in enhanced axonal production, these investigators considered the possibility that the NGF tubulin and the NGF-actin interaction may be a primary event in the chain of reactions called forth by NGF in its target nerve cells.

Biochemical, physico-chemical and ultrastructural studies reported in this article give support to this provocative hypothesis which has the additional merit of having revived the interest of neurobiologists in contractile proteins not only for their recognized role in antero- and retrograde axonal transport, but for the multiple and diversified functions displayed by the dimeric protein tubulin and actin and by their polymerized products in the nerve cell life.

The article by L. A. Greene *et al.* further extends studies initiated by this author and his co-workers (6) on the NGF nerve fiber promoting outgrowth from a pheochromocitoma cell line known as PC 12, which in the absence of NGF exhibits typical chromaffin features whereas in its presence in the culture medium, transforms into nerve cells undistinguishable from sympathetic cells. Since the NGF nerve growth promoting effect is of a temporary nature and its withdrawal from the culture medium results in the reverse phenomenon, namely in the transformation of nerve cells in chromaffin cells, this cell line has provided a most valuable model where to explore the mechanism of action of NGF at the molecular level. In their article Greene and co-workers discuss the results of ingenious experiments aimed at answering the question whether the transformation of chromaffin in nerve cells called forth by NGF in the PC 12 line is due to a modulatory or instructive role of this molecule. They come to the conclusion that the two roles are not incompatible with each other: one or the other would materialize according to the prior and present status of the responsive cells.

The articles by M. Nirenberg *et al.* and by C. N. Christian and P. G. Nelson illustrate the advantage of applying a most rigorous biochemical-molecular approach to classic problems of neurobiology. Only molecular biologists could in fact have conceived the idea of utilizing hybrid cells derived from neoplastic neuronal and glial parental cell lines to explore problems of synapse plasticity and synapse formation between these cells and muscle cells. Not only these cells proved to offer a most favorable model system to explore these processes but they lent themselves to additional studies of other basic neurobiological questions such

as: activation of receptors by different ligands, membrane potential and acetylcholine release from the activated cells.

The similarity between the sequence of some of the effects elicited by receptor mediated effects in these hybrid neoplastic cells, including neurotransmitter storage and release, turning on and off of synapses, lends support to the belief that these cells behave in a substantially similar way to conventional nerve cells and can therefore offer a lead to the study of more specific neurological activities such as simple form of learning, memory retention, habituation, tolerance, dependence and sensitization, as it is suggested in the article by M. Nirenberg and co-workers.

The second section is devoted to one of the most rapidly growing and at the same time changing fields of neurobiology: the field of definition and identification of neurotransmitters, which after decades of neglect, have now monopolized the interest of biochemists, neurophysiologists, neuropharmacologists and, of late also that of experts in immunochemistry who see in this technique a powerful tool to visualize the intracellular localization of ascertained and putative neurotransmitters. It is quite appropriate that this section which now includes beside the classic and new neurotransmitters, also related agents defined as co-transmitters and neuromodulators, should open with the report of the historical and present-day status of one of the most interesting "classic" neurotransmitters, gaba-aminobutyric acid (GABA) which only very slowly came to the forefront of neurobiology for its outstanding role in diversified brain functions. The dramatic history of its discovery and of the long and difficult route followed by GABA to obtain its overdue recognition is told by its discoverer: Eugene Roberts. In the same section R. Paoletti discusses some of the newly discovered roles of prostaglandins in the central nervous system. Although no claim (as far as I know) has yet been made that these amazing derivatives of arachidonic acid, perform the function of neurotransmitters, these evanescent agents which as recently stated by W. E. M. Lands (7, p. 633) "modulate and modify a growing list of physiological events" are known also for the activity that they display in numerous brain functions. Paoletti and his co-workers provided strong evidence for the interference of exogenous PGs with convulsive and tremorogenic drugs, thus adding a most important piece of evidence for the notion that prostaglandins "although ubiquitous" are "pervasive, having influenced all biological disciplines" (8, p. 64).

Different and significant developments in the field of "classic" neurotransmitters: Noradrenaline and GABA, are discussed by two other speakers: G. Levi and M. Raiteri who have analyzed on a comparative

basis the processes of synthesis, storage, release and re-uptake of these two agents, giving evidence for the diversity and versatility of biological mechanisms set in motion in the synthesis, activation or inactivation of agents such as Noradrenaline and GABA endowed with vastly different properties and functional activity. J. Burnstock and T. Hökfelt, using different models and strategies, challenge Dale's principle that each neurone has the ability to synthetize, store and release only one transmitter substance. This revolutionary, though not yet generally accepted rejection of one of the most solidly established dogmas of neurobiology (9) serves the purpose of shaking the too comfortable feeling that nerve cells behave according to rigid man-established rules and that their assignments have, once for all, heen determined by unmodifiable genetic programs. In his long and most informative essay, Burnstock also discusses his previous and present evidence for a neurotransmitter role of purinergic agents and raises the challenging question whether our concept of neurotransmitters should not be extended to other neuronal activities, such as those of activating or repressing other nerve cell activities by modulating the transmission of the nerve impulse from these cells, or releasing trophic factors essential for the very subsistence of other neuronal or non-neuronal cells. T. Hökfelt *et al.* survey another area which owes to the senior author some of the most significant contributions in the identification of peptide neurotransmitters, achieved through the exploitation of immunohistochemical techniques. While the release of hypophysiotropic peptides from hypotbalamic nerve centers and their transmission from the nerve end terminals to the target cells through the intermediate agency of vascular channels, has been known ever since the discovery of the neurosecretory activity displayed by these cells, the mechanism of activation of postsynaptic cells, has heen considered as altogether different from that which occurs through the conventional nerve fiber-mediated transmission of humoral agents from typical neurons to other neuronal or non-neuronal recipient cells. In the present article Hökfelt presents compelling evidence in favor of the concept that synthesis, storage and release of peptides does not necessarily utilize the hlood mediated transmission, but also the direct nerve fiber humoral transmission. The identification of peptides releasing nerve cells in other brain areas besides the hypothalamic centers, further adds to the perplexity of where (and if) one should draw a demarcation line between conventional and neurosecretory neurones.

The third short section is devoted to the discussion of some aspects of a phenomenon which, only a few years after its discovery has already

captured the interest and stirred emotion not only among experts in the field of neurobiology but also in outsiders for its far reaching implications in the areas of basic and clinically applied neuroscience. The scientist who deserves great merit for the discovery that a peptide which became known as enkephalin and other like peptides represent endogenous ligands to naturally occurring opiate receptors, H. W. Kösterlitz and two other most competent investigators in this field, L. Terenius and B. Hamprecht, will present some facets of this newly opened field which deserves to be listed among "those extraordinary episodes" defined by T. S. Kuhn as "scientific revolutions" that represent "the tradition shattering complements to the tradition-bound activity of normal science" (1, p. 6).

In retrospection it seems that it was wise to devote the fourth long and intense section to a field that can instead be listed among those belonging to "Normal Science", in the best sense given hy Kuhn to this terminology which in the same way as the definitions of a line of research as "orthodox" or "classic" (this second term as Viktor Hamburger once remarked has a slightly derogatory connotation, specially when uttered by young investigators), is meant to indicate research pursued according to solidly founded concepts along the lines traced by the great masters of the past and present generation.

The six essays dealing with events which characterize the ontogenesis of nerve cells interaction with their target organs under different normal and experimental conditions, the biochemical hasis of synapses formation, the interaction hetween nerve fibers and their receptors and the mechanism of denervation changes which follow surgical axotomy, are blended in such harmonious way as to leave the reader with the regret that this unfolding story of one of the most intensely studied and central issues in neurobiology, should not have heen carried even farther. Among the six beautifully presented essays by G. Filogamo, V. Hamburger, E. Giacobini, D. Fambrough, D. Purves and A. Cangiano, I wish to call attention to the excellent presentation by Viktor Hamburger, who devoted his article to clarify concepts and carry in depth the analysis of the significance of the terms "prespecification" and "plasticity", from the vantage viewpoint of his recent most revealing work in this area. In reading his article, I could not but agree again with T. S. Kuhn who states that "few people who are not actually practitioners of a mature science, realize how much mop-up work of this sort a paradigm leaves to be done or quite how fascinating such work can prove in the execution" (1, p. 24). I would like to add that this kind of activity is not only fascinating, but of tremendous value at a time like ours, when

technical jargon which prevails among "science practitioners" particularly among those of the young generation, has not only suppressed the pleasure of reading scientific reports presented according to identical stereotyped rules, but has resulted in a total disregard for the exact significance of words and concepts with the result of increasing the confusion in an expanding field such as that of neurobiology.

The fifth and last section brings back to the stage the Problem No. 1 of neurobiology, namely the problem of brain higher functions, as exemplified by those of the human brain.

It would be presumptous for an outsider, even if a life-long admirer of the extraordinary progresses achieved in this field, as the writer, to comment on each of these five remarkable presentations which discuss some of the most interesting features of hemispheric specialization in the human brain. I shall only mention that this last section opens with a most enlightening report by Sir John Eccles on endogenous potentials and their significance in relationship to the mind-brain problem. This discovery restores our faith in the control that human beings can exert on their own brain, a faith that the present scientific trend and some sad considerations on human interactions, has recently been severely shaken!

One can now by way of conclusion, ask whether and to what extent we have achieved the not easy task of presenting in a 5-day conference, the major topics of the enormously expanding field of neurobiology. I shall leave the answer to the readers. I may only mention that—at least according to the intentions—the unifying thread that runs through these apparently loosely related or unrelated topics, was the belief that advances in each one of these diversified areas, cannot but benefit the overall understanding of problems related to brain structure and function. These in turn are the prerequisite for answering the oldest and most urgent quest of the human brain to know why it knows and to gain control over its own function.

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NEW FEATURES OF THE NERVE GROWTH FACTOR-TARGET CELLS INTERACTION

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INTRODUCTION

Studies performed in recent years on the Nerve Growth Factor-target cells interaction, shed light on some new features of the growth response elicited by the Nerve Growth Factor (NGF) which markedly broaden the spectrum of action of this molecules and at the same time provide evidence for the all powerful and unique role of this molecule in: *a*) counteracting otherwise lethal effects produced by different toxic compounds; *b*) in guiding nerve fibers along a NGF concentration gradient in the developing organism and *in vitro*; c) in channelling differentiative processes of cells stemming out from the same precursors toward the neuronal rather than glandular cell line. The unifying thread of these three processes is somewhat tenuous, and the reader may wonder whether such widely different effects elicited by NGF have anything in common and, even more, if one expect to elucidate the mechanism of action of NGF by extending the analysis to hitherto unknown features of the growth response elicited by this protein molecule, rather than focussing the attention on only one, possibly the most significant of these effects. This has in fact been the strategy followed by students of hormonal action who, once they had identified the source of origin of a given hormone and the anabolic or morphogenetic response elicited in the target cell or cells, narrowed their analysis to the study of the interaction of the hormone and of the receptive system, following the principle that all scientific exploration requires reductionism in order to make the problem manageable. But

the NGF, for all the efforts spent in the study of its origin, effects and mechanism of action, still defies the reductionism approach and forces us to continue the jig-puzzle game in the hope to eventually obtain enough information to reconstruct the whole range of action of this remarkable protein molecule. It is gratifying that the problem which we face with NGF is the same as that faced by other students of specific growth factors which came to the attention after the discovery of NGF. One is therefore justified in submitting the hypothesis that NGF as well as other specific growth factors belong to a category different from that of traditional hormones and it is wise for the time being to refrain from the temptation to apply to it, the same procedures used in the studies of other hormones, ignoring the features of the growth response which depart from those in line with this concept.

In the following we shall first list in a summary fashion the main effects elicited by NGF in *in vivo* and *in vitro* systems. We shall then consider the new features of the response mentioned above.

Source of origin and physico-chemical properties of NGF

As reported in original and in a large number of review articles, the evidence for the existence of a factor endowed with nerve growth promoting activity on embryonic sensory and sympathetic nerve cells came from the casual observation that dorsal root ganglia (Bueker, 1948) and sympathetic ganglia (Levi-Montalcini and Hamburger, 1951) undergo marked volume increase upon transplantation of two mouse sarcomas known as sarcomas 180 and 37 in 3-day chick embryos. This factor identified in a protein molecule and labelled from its growth promoting activity as "Nerve Growth stimulation Factor" (Cohen *et al.*, 1954) later shortened in "Nerve Growth Factor" (Levi-Montalcini, 1964) was then discovered in much larger amount in two other biological sources: the snake venom and the mouse salivary glands (Cohen and Levi-Montalcini, 1956; Levi-Montalcini, 1958). These findings afforded the possibility of isolating and characterizing the growth promoting molecule released from these excretory glands. Studies initiated at Washington University by S. Cohen and then pursued in the Laboratory of Cell Biology in Rome gave evidence for the physico-chemical properties of this molecule isolated from the mouse salivary glands and identified at first in a protein with a molecular weight of 44,000 (Cohen, 1960). With the advent of the techniques of ion-exchange chromatography and gel filtration, it became possible to obtain NGF samples of sufficient purity to permit structural

analysis of the salivary NGF. By a modification of the procedure devised by Cohen, Bocchini and Angeletti (Bocchini and Angeletti, 1969) obtained in two steps a biologically active moiety with a sedimentation coefficient of 2.5S and an apparent molecular weight of 30,000. Experiments by Zanini and Angeletti indicated that the NGF could be separated in two fractions of 20,000 and 14,000 molecular weight (Zanini *et al.*, 1968). Following a different procedure, another group reported that NGF activity can be isolated from mouse salivary gland homogenate in association with a high molecular weight species having a sedimentation coefficient of S (Varon *et al.*, 1967). Upon incuhation at mildly acidic or alkaline conditions this 140,000 molecular weight complex, dissociates in three subunits designated as α, β and γ subunits. Only the β suhunit is endowed with NGF activity. The original hypothesis submitted by these authors that the α and γ subunits potentiate the biological NGF activity of the β subunit (Varon and Shooter, 1970) has recently been abandoned and replaced with the hypothesis that the role of the 7S complex is related to the biosynthesis, storage and protection of NGF (Server and Shooter, 1977). The subunit structure of 2. 5 7 NGF first reported by Zanini and Angeletti (Zanini *et al.*, 1968) was re-examined by R. H. Angeletti (Angeletti and Bradshaw, 1971) and R. H. Bradshaw who found that it represents a dimer of two identical subunits bound together by noncovalent bonds in a tight complex which is difficult to dissociate. The native protein which is composed of two subunits, has a molecular weight of 26,518; each of the polypeptide chains contains 118 amino acids and has a molecular weight of 13,259, possessing amino terminal serine and carboxyl terminal arginine. The three disulfide bridges apparently impart particular rigid structure to the NGF molecule, as indicated by its striking resistance to enzymatic, chemical and heat denaturation (Angeletti *et al.*, 1973).

An important step in the elucidation of the structural properties of NGF has been achieved by its crystallization (Wlodawer *et al.*, 1975). These crystals are exagonal bipyramids, up to 0.7 mm long and 0.25 mm across. Since the biological identity and unicity of a protein resides in its entire three dimensional structure, which comprises not only the amino acid sequence (primary structure), but also its folding (secondary) and superfolding (tertiary structure) to form a globular entity, X-rays analysis of NGF crystals, a method which has provided invaluable information on three dimensional structure of proteins, should allow to extend our knowledge of NGF structure.

Morphological and metabolic effects of NGF

Four features characterize the *in vitro* response to NGF of sensory and sympathetic embryonic ganglia and of their dissociated nerve cells: *a*) the formation of a dense fibrillar halo around intact ganglia; *b*) the rapidity of the growth response which becomes apparent in 6 hours and reaches its peak in 16-24 hours; *c*) the geometrical stereotyped pattern of nerve fiber outgrowth from stimulated ganglia (Fig. 1); and *d*) the sur-

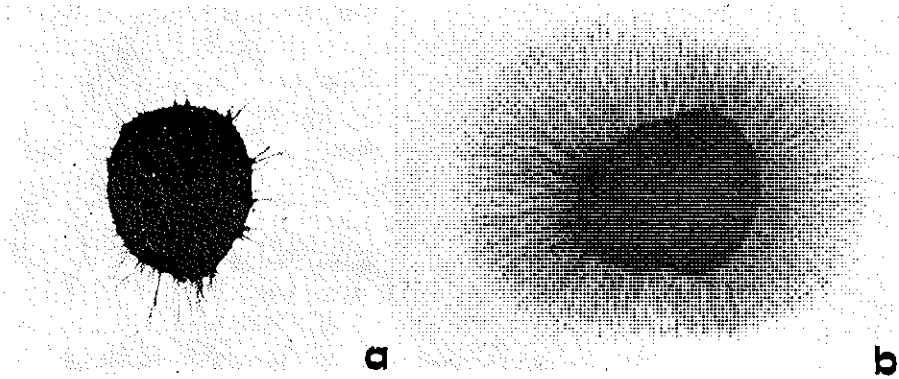

Fig. 1. Compares two sensory ganglia dissected out from a 8-day chick embryo and cultured for 10 hours in medium deprived of NGF (a) or with 10 ng of NGF (b) (1 biological unit). Notice in b the dense fibrillar halo of nerve fibers around the ganglion.

vival for indefinite periods of time of dissociated sensory and sympathetic embryonic cells cultured in otherwise inadequate culture media if a few nanograms of NGF are added to these media (Levi-Montalcini and P. U. Angeletti, 1963).

At the ultrastructural level, the most outstanding as well as the earliest *in vivo* and *in vitro* effects are a massive increase in neurotubules and neurofilaments in the cell perikarya and in their axons (Levi-Montalcini *et al.*, 1968). These findings focussed attention on microtubule and microfilament proteins for their possible role in mediating the NGF action. A direct effect on *in vitro* polymerization of tubulin, the precursor of microtubules was discovered by P. Calissano and C. Cozzari (Calissano and Cozzari, 1974). More recent studies by P. Calissano revealed a similar NGF effect on polymerization of actin, the microfilament precursor (Calissano *et al.*, 1975; Calissano *et al.*, 1978). The results of these studies which bring to light some highly specific aspects of these interactions, raise

the question of their significance and possibly primary role in the chain of events triggered by NGF in its target cells.

Injections of the salivary NGF in newborn rodents at the doses of 10 μg/gr of body weight, result in a striking volume increase of sympathetic ganglia up to 6-10 fold that of controls (Fig. 2) (Levi-Montalcini

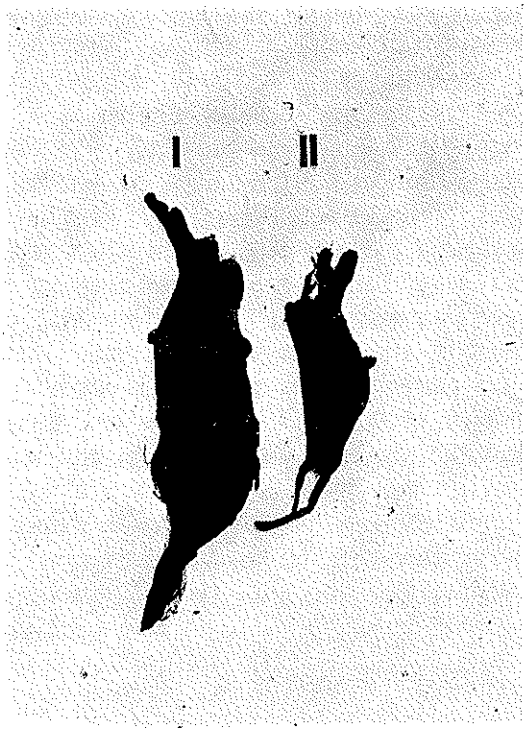

FIG. 2. Whole mounts of 7-day old littermate mice injected since birth with NGF (I) and with physiological solution (II). × 14.

and Booker, 1960; Levi-Montalcini, 1966). This effect is due to a marked increase in size and number of individual neurons. The question whether the hyperplasia is due to a mitogenic effect on sympathetic nerve cell precursors or to a decrease of the naturally occurring death of a percentage of nerve cells during post-natal stages, cannot be considered definitely settled at present but the latter alternative supported by J. Hendry (Hendry and Campbell, 1976) is gaining strong support.

At the metabolic levels NGF was shown to enhance all anabolic

processes: protein and lipid synthesis are increased as indicated by enhanced incorporation of labelled amino acids and acetate (Angeletti *et al.*, 1964a; Liuzzi *et al.*, 1967). Fractionation of labelled proteins extracted from ganglia incubated in presence of NGF, showed that NGF selectively enhances the synthesis of acidic proteins (Gandini-Attardi *et al.*, 1967), a finding of interest and in line with the observation of a massive and early increase in neurofibrillar material mentioned above. Glucose oxidation is stimulated primarily through a direct oxidative pathway (Cohen, 1960; Angeletti *et al.*, 1964; Angeletti *et al.*, 1968). No *de novo* DNA synthesis is detectable whereas RNA synthesis is markedly enhanced after 2-4 hours as indicated by uridine incorporation studies and by comparison of the total RNA content in ganglia cultured with and without NGF (Angeletti *et al.*, 1965). Actinomycin D added to the incubation medium at a concentration which almost completely inhibits RNA synthesis prevents most of the events elicited by NGF but the nerve fiber outgrowth stimulated by NGF was only moderately impaired (Parlow, 1969; Larrabee, 1972; Levi-Montalcini and Angeletti, 1971). Of particular interest, since it shows that the growth factor does not only enhance differentiative and metabolic processes but also stimulates the specific function of sympathetic nerve cells was the finding of a NGF effect on enzymes involved in the synthesis of the noradrenergic transmitter, noradrenaline. A marked increase in the synthesis of thyrosine hydroxylase, the key limiting enzyme in the synthesis of NA was demonstrated by Thoenen *et al.* (Thoenen *et al.*, 1971).

Growth inhibition of sympathetic nerve cells by immunochemical and pharmacological procedures

a) *Immunosympathectomy*

The selective destruction of sympathetic nerve cells produced in neonatal rodents and other mammals by a specific antiserum to NGF first reported in 1960 (Cohen, 1960; Levi-Montalcini and Booker, 1960) emphasizes the central role played by NGF in the life of these cells. This effect which deprives the animal of the sympathetic system without interfering with its somatic development and vitality became known ever since 1961 as "immunosympathectomy" (Levi-Montalcini and Angeletti, 1966). Although the precise mechanism by which the antiserum displays its action is not known, the cytotoxic effects have been studied in details at the structural and ultrastructural levels. Inspection at the light micro-

scope of sympathetic ganglia showed already three days after the first injection, a large number of dead cells and cell debris. Immature neurons are much smaller than controls and the cells are almost deprived of ribonucleic-acid as shown by the failure to stain with basic dyes and by the fact that nucleoli are barely visible. While dead cells are found in increasing larger number, satellite cells are intact and appear at first even more numerous than in control ganglia. Subsequently also satellite cells undergo regressive changes and the ganglia are reduced to diminutive sclerotic nodules detectable only with the aid of the dissecting microscope (Fig. 3). Electron microscopic studies gave evidence of the extra-

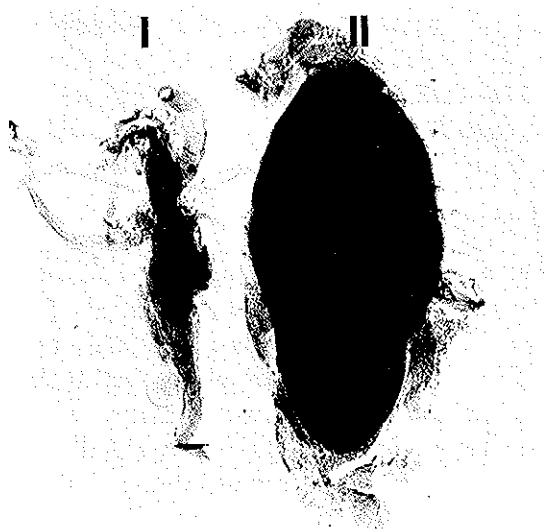

FIG. 3. Whole mounts of superior cervical ganglia of 10-day old littermate mice injected since birth with a specific antiserum to NGF (I) and with physiological solution (II). × 45.

ordinary rapid effect elicited by the antibodies to the NGF. The most precocious alterations, already apparent 2 hours after the injection of NGF-AS, consist of marked changes in the fine structure of the nucleoli: in proximity to the altered nucleoli, the chromatin clumps in large areas and appears considerably denser than in controls. The only cytoplasmic alteration which is apparent in these early stages consists of some disorganization of the ribosomes. In immediately subsequent stages, 4 and 12 hours after the injection of the antiserum, the nuclear and cytoplasmic

materials become intermixed. Vacuolization, pyknosis, rupture of the plasma membranes and other signs of cytolysis are seen in the large majority of neurons which become surrounded by macrophages and histiocytes (Levi-Montalcini *et al.*, 1969). In adult rodents the antiserum to NGF induces marked atrophic changes of the ganglionic cell bodies characterized by loss of neurofibrillar materials, marked decrease of catecholamine uptake and its decreased content in peripheral organs (Angeletti *et al.*, 1971). These effects are however of a temporary nature at variance with those produced in neonatal rodents where the destruction of sympathetic ganglia is irreversible. Recent electrophysiological studies on adult guinea pigs injected with antiserum to NGF for a 4-5 day period, showed depression of intracellular recorded synaptic response within 4-5 days from the end of antiserum administration. Synapses counted in E.M. sections from same ganglia showed only half as many contacts as in control ganglia (Niä and Purves, 1977).

b) *Chemical sympathectomy*

The possibility of selectively destroying by means of pharmacological agents sympathetic nerve cells in neonatal rodents, was first reported from our laboratory in 1970. It was shown that injections of 6-hydroxydopamine (6-OHDA) a dopamine derivative which in adult animals produces a degeneration of the synaptic vesicles in adrenergic nerve endings and a long-lasting blocking effect of sympathetic transmission of tbe nerve impulse (Thoenen and Tranzer, 1968; Tranzer and Thoenen, 1968), in neonatal animals on the contrary produces the destruction of immature sympathetic nerve cells. This process became know as chemical sympathectomy (Angeletti and Levi-Montalcini, 1970a; Angeletti and Levi-Montalcini, 1970b). The destruction of immature sympathetic nerve cells by 6-OHDA seemed at first to lend support to the hypothesis that the shorter distance between the peripheral ending of the growing axon and the cell body was responsible for a diffusion of tbe dopamine derivative from the end terminal to the cell perikaryon and for its irreversible damaging effect on the cell cytoplasm or nucleus (Angeletti and Levi-Montalcini, 1970). Subsequent E.M. studies did not give support to this hypothesis. It was in fact found that daily injections of 6-OHDA do not cause any alteration in the cell compartments of sympathetic immature cells. 6-OHDA accumulates in the nerve end terminals in neonatal as well as in adult rodents and

blocks the two-way transport system which accounts for the release of the neurotransmitter and the re-uptake of noradrenaline as well as for the uptake and retrograde axonal transport of trophic factors released by peripheral tissues (Levi-Montalcini *et al.,* 1975; Aloe *et al.,* 1975). It is this latter effect, namely the inability of the developing nerve fibers to avail themselves of the trophic factors released by peripheral tissues that accounts for the irreversible lesions produced by 6-OHDA to the immature sympathetic nerve cells. A similar situation occurs upon surgical transection of postganglionic axons in fully differentiated or immature developing nerve cells. The former suffer of only temporary reversible damages upon disconnection from their end organs; the latter undergo irreversible degenerative changes which result in death upon surgical postganglionic axotomy (Hendry, 1975).

The marked increase in vulnerability of developing sympathetic nerve cells as compared to the fully differentiated neurons, already well apparent in the differential damaging effects inflicted by antibodies to NGF in sympathetic nerve cells of neonatal or adult rodents (Levi-Montalcini *et al.,* 1972), came in sharp relief also in studies with pharmacological agents which selectively accumulate in other cell compartments, such as guanethidine and vinblastine. The former gains access to the postganglionic noradrenergic neuron, through uptake via the membrane pump. Guanethidine accumulates mainly outside the NA storage granules exerting a direct toxic effect on mitochondria, blocking Ca^{++} binding to phospholipids and inhibiting phosphorylation and calcium uptake in these organelles; the latter effect leads to an increased level of free Ca^{++} that causes toxic effects culminating in the cell death. Injections in newborn rodents of guanethidine at doses of 20-30 μg/gr of body weight call forth massive destruction of immature sympathetic nerve cells; the end effects are of the same range and severity as those induced by antibodies to NGF or by injections of 6-OHDA, but the intracellular localization of lesions produced by guanethidine are markedly different from those elicited by AS-NGF and by 6-OHDA. They are apparent at the E.M. toward the third day and increase progressively in severity and range in subsequent days. Dilation and rupture of mitochondria followed by widespread lesions of the endoplasmic reticulum are evident in all immature neurons between the third and fourth day of treatment. At 8 days practically no intact nerve cells are seen in any section and the residual population amounts to less than one tenth that of controls (Eränkö O. and Eränkö L., 1971; Angeletti *et al.,* 1972; Angeletti and Levi-Montalcini, 1972).

A third drug, vinblastine was recently the object of studies directed to examining its effects in fully differentiated and immature sympathetic nerve cells. The vinca alkaloid prevents microtubules and microfilaments polymerization and in this way interferes with processes which are essential in the most diversified cell functions such as cell replication; anterograde and retrograde axonal transport. While fully differentiated sympathetic neurons undergo reversible damages, the immature nerve cells are destroyed by daily injections of 1.0 nM vinblastine per gr. of body weight (Calissano *et al.*, 1976; Menesini-Chen *et al.*, 1977; Johnson, 1978).

Since other nerve cells are not damaged by injections of any of the three pharmacological agents considered above: 6-OHDA, guanethidine and vinblastine, it has been possible to selectively destroy the sympathetic system of rodents by injecting neonatal mice and rats with one or the other of these compounds and to obtain animal colonies deprived of the sympathetic function but otherwise vital and healthy as untreated littermates.

Recent developments

a) *Protective effect of NGF against 6-OHDA, guanethidine and vinblastine*

Experiments reported in detail elsewhere (Levi-Montalcini *et al.*, 1975; Aloe *et al.*, 1975; Calissano *et al.*, 1976; Menesini-Chen *et al.*, 1977; Johnson, 1978; Levi-Montalcini, 1974) showed that the simultaneous treatment of neonatal rodents with each one of the above agents: 6-OHDA, guanethidine or vinblastine and NGF, does not only protect the immature nerve cells from the otherwise destructive effect of these agents but results in overgrowth of sympathetic ganglia which, in the case of the combined 6-OHDA and NGF treatment, far exceeds the volume increase produced hy treatment with NGF alone (Fig. 4). This paradoxical effect which has been the object of detailed structural ultrastructural and biochemical studies, has been shown to result from an extraordinary production of collaterals from chemically axotomized nerve fibers. Nerve cells prevented from establishing structural and functional connections with end organs by the accumulation of 6-OHDA in the synaptic endings, but geared to a higher than normal metabolic activity by the daily supply of NGF, produce an excess of axonal material which is channelled in collateral branches stemming out from the proximal segment of the axotomized

fiber (Fig. 5). Discontinuation of the NGF treatment results in the abrupt death of sympathetic nerve cells deprived of this factor essential for their survival, that is normally supplied by end organs.

These experiments called attention on the dual role played by peripheral end organs and tissues on nerve cells providing to their innervation: the first which is generally acknowledged, is to release trophic factors essential for growth and differentiation of the innervating nerve cells.

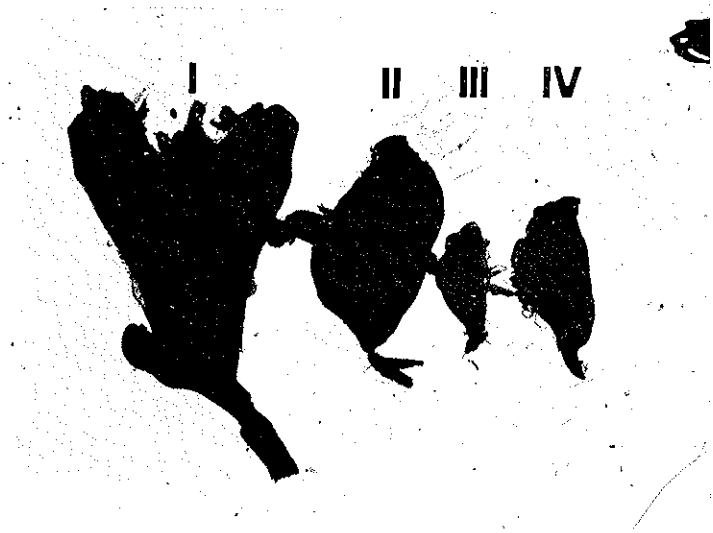

FIG. 4. Superior cervical ganglia of 12-day old littermate rats injected with NGF and 6-OHDA (I), with NGF alone (II), with 6-OHDA (III) and with physiological solution (IV). (From: Aloe *et al.*, reference 2). × 8.

The other that is seldom if ever mentioned, is to prevent further elongation of the fiber and production of axonal material once the nerve fibers have established permanent structural and functional connections with their end organs. Upon surgical or chemical disconnection obtained by mechanical or chemical procedures, this inhibitory effect is removed and an extraordinary production of collateral branches takes place, thus showing that nerve cells are endowed with far broader potentiality in producing nerve fibers than it is apparent in the intact system.

NGF likewise suppresses the cytotoxic effects of guanethidine in sympathetic nerve cells of neonatal rodents (Johnson and Aloe, 1974). The toxic effects of this compound upon its selective accumulation in immature and fully differentiated noradrenergic nerve cells (Heath *et al.*,

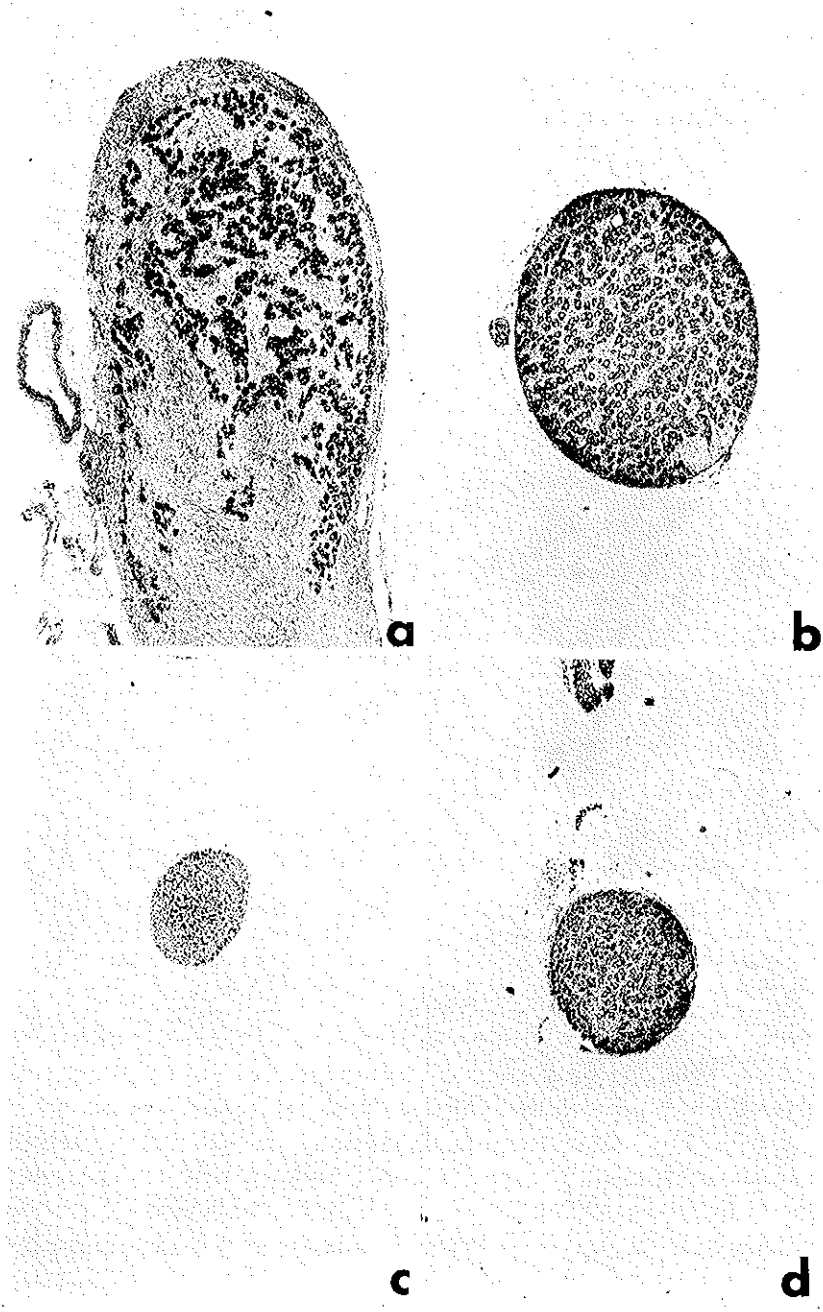

FIG. 5. Largest cross sections of superior cervical ganglia of 19-day old littermate rats treated since birth with NGF and 6-OHDA (a), NGF (b), 6-OHDA (c) and physiological solution (d). × 45.

1972; Heath *et al.*, 1973; Kostrazeva and Jacobowitz, 1974; Angeletti *et al.*, 1972) are attributed to inhibition of oxidative phosphorylation and calcium uptake into the mitochondria leading to an increase of free Ca^{++} which exherts its damaging effects in the developing nerve cell, and in long range experiments also in the fully differentiated neuron (unpublished experiments Aloe). The ability of NGF to overcome the

FIG. 6. Whole mounts of superior cervical ganglia of littermate mice injected from the day of birth to the seventh day with: NGF (I), physiological solution (II), vinblastine (III) and vinblastine + NGF (IV). Notice the volume increase of ganglion in mouse injected with vinblastine + NGF. Explanation in text. × 95.

cytotoxicity of guanethidine may be a reflection of a primary NGF effect: increased efflux of Ca^{++}. Very low levels of free Ca^{++} may affect several intracellular processes such as assembly and organization of filamentous proteins (mainly microtubules and actin-like filaments) which in turn may result in widespread degenerative effects. This admittedly tentative hypothesis of the NGF protective effects against guanethidine is now the object of experimental *in vitro* studies.

The third protective effect of NGF against the lethal vinblastine effects as demonstrated at first in our laboratory (Menesini-Chen *et al.*, 1977) (Fig. 6) and more recently by Johnson (Johnson, 1978) was the object of *in vitro* and *in vivo* experiments which offer a plausible explana-

tion of this effect. The noxious effects of vinblastine treatment are localized at first in the nuclear compartment, in particular in the nucleolus where the nucleolonema undergoes segregation into components of different electron density. This effect is followed by alteration of the chromatin which exhibits increased density and appearance of round dense bodies. At 36 hr most neuronal cells show widespread alterations in the nuclear and cytoplasmic compartment (Fig. 7). Cross sections of the postganglionic nerve showed marked axonal swelling, disappearance of MTs and formation of electron-dense inclusions. Nerve cells of newborn mice injected simultaneously with NGF and vinblastine exhibited none of the noxious effects produced at the structural and ultrastructural levels by the vinca alkaloid (Fig. 8). In fact the ganglia underwent marked volume increase as when treated with only NGF and the hypertrophic nerve cells and their axons did not show any of the toxic effects produced by vinblastine. Studies *in vitro* on the interaction of vinblastine and tubulin and vinblastine and microtubules suggest a plausible explanation for this NGF protective effect. It was in fact shown in previous studies (Wilson *et al.*, 1976) and confirmed in our laboratory (Menesini-Chen *et al.*, 1977) that tubulin has two binding sites for vinblastine, a low affinity site ($K = 8 \times 10^4 M^{-1}$) responsible for the paracrystalline arrangement of microtubules and a high-affinity site ($K = 6{,}2 \times 10^6 M^{-1}$) which when occupied, blocks the process of assembly and is presumably responsible for the vinblastine inhibitory action on cell division. The high affinity site is inaccessible to the alkaloid in the intact MT, but is freely accessible in their precursor rings and dimers. The *in vitro* resistance of MT-NGF complex to vinblastine and the *in vivo* analogous protective action could be visualized as an indirect consequence of the action of NGF on MT stability. This factor, by shifting the equilibrium between tubulin and MT toward the latter, would favor the state of tubulin refractoriness to the binding of vinblastine. It should be added that the integrity of MTs in a developing sympathetic nerve cell is a condition *sine qua non*, for its further development and differentiation. The key role played by these organized structures in antero and retrograde axonal transport processes has been unequivocably proved (Hendry *et al.*, 1974; Iversen *et al.*, 1975, Paravicini *et al.*, 1975).

Suppression of retrograde transport prevents the access to the cell perikarya of trophic factors released by end organs, which are essential to the cell development. Evidence has also been presented that in the case of sympathetic nerve cells the factor synthetized and released by end organs is NGF. It is tempting to subunit the hypothesis that the vinca

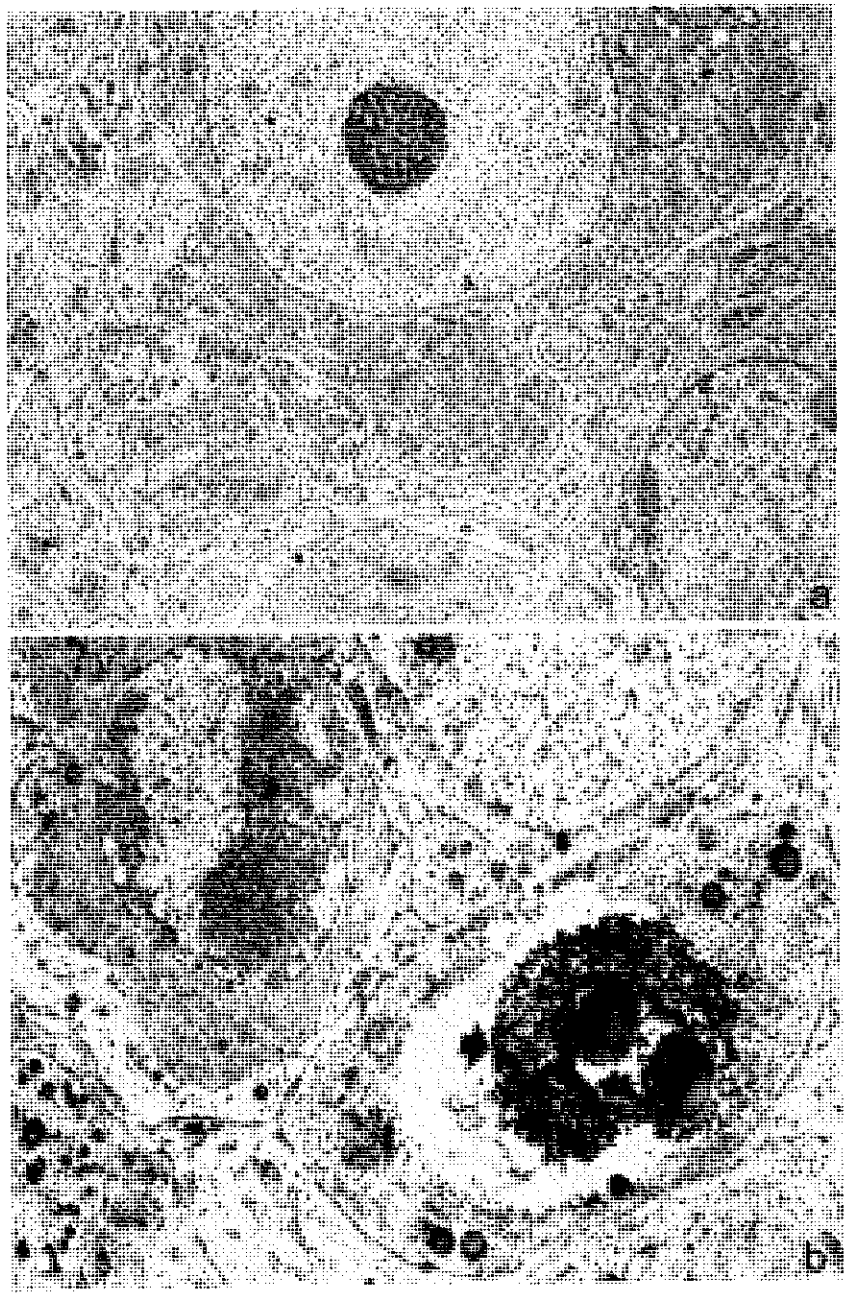

FIG. 7. Electron micrographs of sympathetic nerve cells in superior cervical ganglia of 36 hour old littermate mice injected since birth with physiological solution (a) and with vinblastine (b). Notice the extensive degeneration particularly advanced in the nuclear compartment of the vinblastine injected pup (arrow). × 3200.

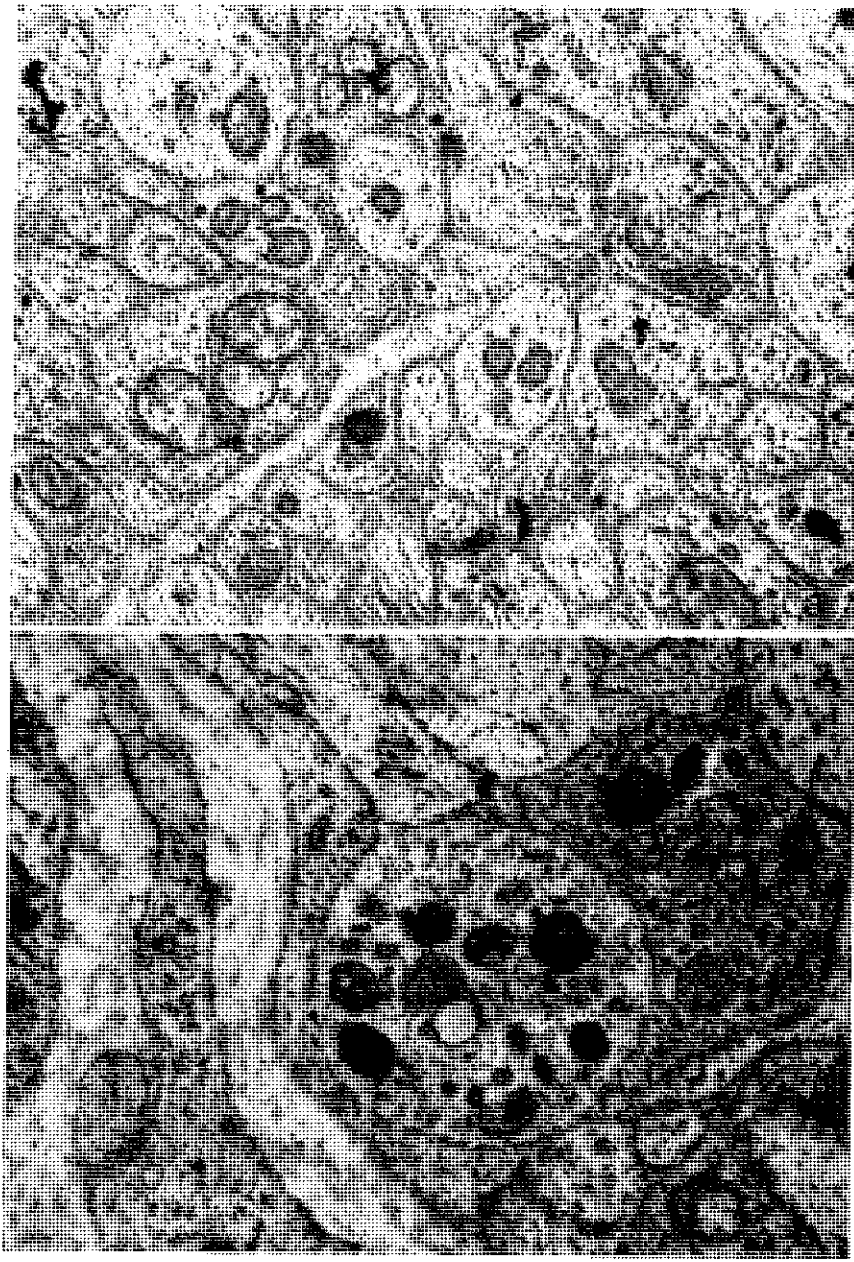

FIG. 8. Electron micrographs of 36 hour old littermate mice injected since birth with NGF + vinblastine (a) or with vinblastine alone (b). Both micrographs show transverse sections of axons in the superior cervical ganglion. Notice the excellent state of axons in NGF + vinblastine injected mouse (a) and the swelling and replacement of microtubules and mitochondria with electron dense bodies in the axons of the vinblastine injected mouse. × 18.000.

alkaloid destructive effects on immature sympathetic nerve cells and the neutralization of these effects by combined NGF injections, are due respectively to the disintegration of microtubules and to their integrity in vinblastine, or in vinblastine and NGF combined treatment. Preservation of microtubules in the dual treatment, makes possible the NGF transport from peripheral tissues even in presence of vinblastine.

b) *A NGF neurotropic effect*

If the above results established the dependence of the sympathetic immature nerve cells from NGF and the protective action exherted by this protein molecule against lethal effects produced by different pharmacological compounds, studies to be considered here have a direct bearing on another problem which is unique of the nervous system: the building of neuronal circuits in the neural tube and in the brain and the establishment of connections between nerve centers and the periphery. The problem whether the establishment of specific connections between axons and peripheral end organs are rigidily programmed in all their most minute details and unmodifiable, or whether growing nerve fibers are endowed with some plasticity which allows for deviations from pre-established routes, in response to chemical signals issued during ontogenesis and regeneration from neuronal and non neuronal cells, has remained unanswered ever since Cajal raised this question in his masterful analysis of neurogenetic and regenerative events in the vertebrate nervous system (Cajal, 1913). The isolation and characterization of chemical agents endowed with such properties represents a task far above the resolving power of the techniques available to the biochemist. The discovery of the Nerve Growth Factor and of its key role in growth and differentiation of sympathetic nerve cells, offered for the first time the possibility of approaching the problem and answering this question. Here we shall only mention the main results of this investigation which provided unequivocable evidence for a neurotropic role of NGF on growing sympathetic fibers. If, as we believe, it is justified to consider the NGF-target cells interaction as a valid model of interaction between developing nerve cells and their end organs, then the results to be briefly summarized offer the first proof for the directing role played by humoral factors released by end organs on growing nerve fibers.

The experiments reported in detail elsewhere (Levi-Montalcini, 1976; Menesini-Chen *et al.*, 1978; Levi-Montalcini *et al.*, 1978) consisted in the injection in the developing brain of neonatal rodents of NGF with the

aid of a mouth micropipette. The injections repeated daily for a ten-day period were well tolerated. The brain and spinal cord with attached cephalic and spinal roots, sensory and sympathetic ganglia were dissected out in both control and intracerebrally NGF-injected neonatal mice and rats, processed according to the histofluorescence technique, sectioned serially and examined at the ultraviolet microscope. All experimental cases showed markedly larger and more intensely fluorescent sympathetic ganglia than controls, and large and strongly fluorescent fiber bundles in the dorsal funiculi of the spinal cord and in the lateral and ventral aspects of the medulla oblongata. This latter finding seemed at first to lend support to the hypothesis of a growth effect elicited by NGF on central monoaminergic neurons. Comparative studies of the locus coeruleus and of other smaller noradrenergic cell aggregates in the brain stem of experimental and control littermates showed however no apparent size differences in these centers. Also there was no topographical relationship between the fluorescent fiber bundles and the monoaminergic neurons of the locus coeruleus and other nuclei. The origin of these fibers from the sympathetic paravertebral ganglia became apparent from the inspection of the entire neural tube and adjacent structures. Fibers emerging from these ganglia and easily recognizable on account of their intense fluorescence, invaded the spinal roots in large numbers. Those which entered the ventral roots, innervated blood vessels in the choroid and subarachnoid space in much larger number than in controls. An even more striking deviation from normality was apparent in dorsal roots. These roots are barely discernible in controls at the ultraviolet illumination due to the presence of only two exceedingly thin fluorescent fiber bundles lining the non-fluorescent sensory fibers which form the main bulk of these roots. In NGF-injected animals, the entire dorsal roots appear intensely fluorescent as a result of the large number of noradrenergic fibers which enter these roots soon after their emergence from the adjacent sympathetic ganglia (Fig. 9). The fluorescent fibers run across the sensory ganglia where they loose their compactness and spread all over the neuronal cell population; they reassemble again as they leave the ganglia and enter into the spinal cord where they take up a position in the lateral and dorsal funiculi (Fig. 10). Once inside the neural tube, the fluorescent fiber bundles become ascendant and reach the rostral level of the spinal cord. Fibers originating in the superior cervical ganglion gain access in the brain stem through the roots of the X and IX cephalic nerves (Menesini-Chen *et al.*, 1978) (Fig. 11). At the middle level of the medulla oblongata the adrenergic fiber tract segregates into two dorso-lateral and ventro-lateral

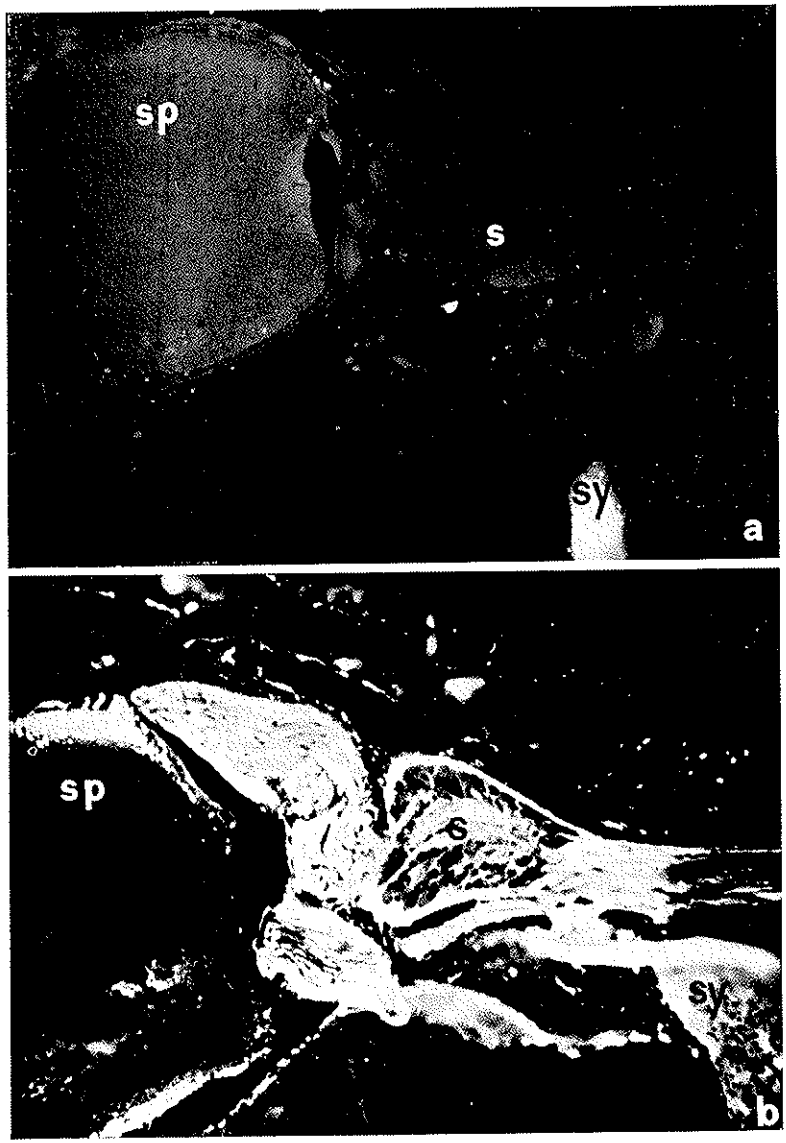

FIG. 9. Microphotos at the fluorescent microscope of transverse sections of the spinal cord (sp), sensory (s), sympathetic ganglion (sy), and adjacent tissues in two 12-day old littermate rats injected since birth intracerebrally with vehicle solution (a) or with NGF (b). Notice in the NGF injected pup the intense fluorescence of sensory sympathetic ganglia, postganglionic root and dorsal funiculus. × 45.

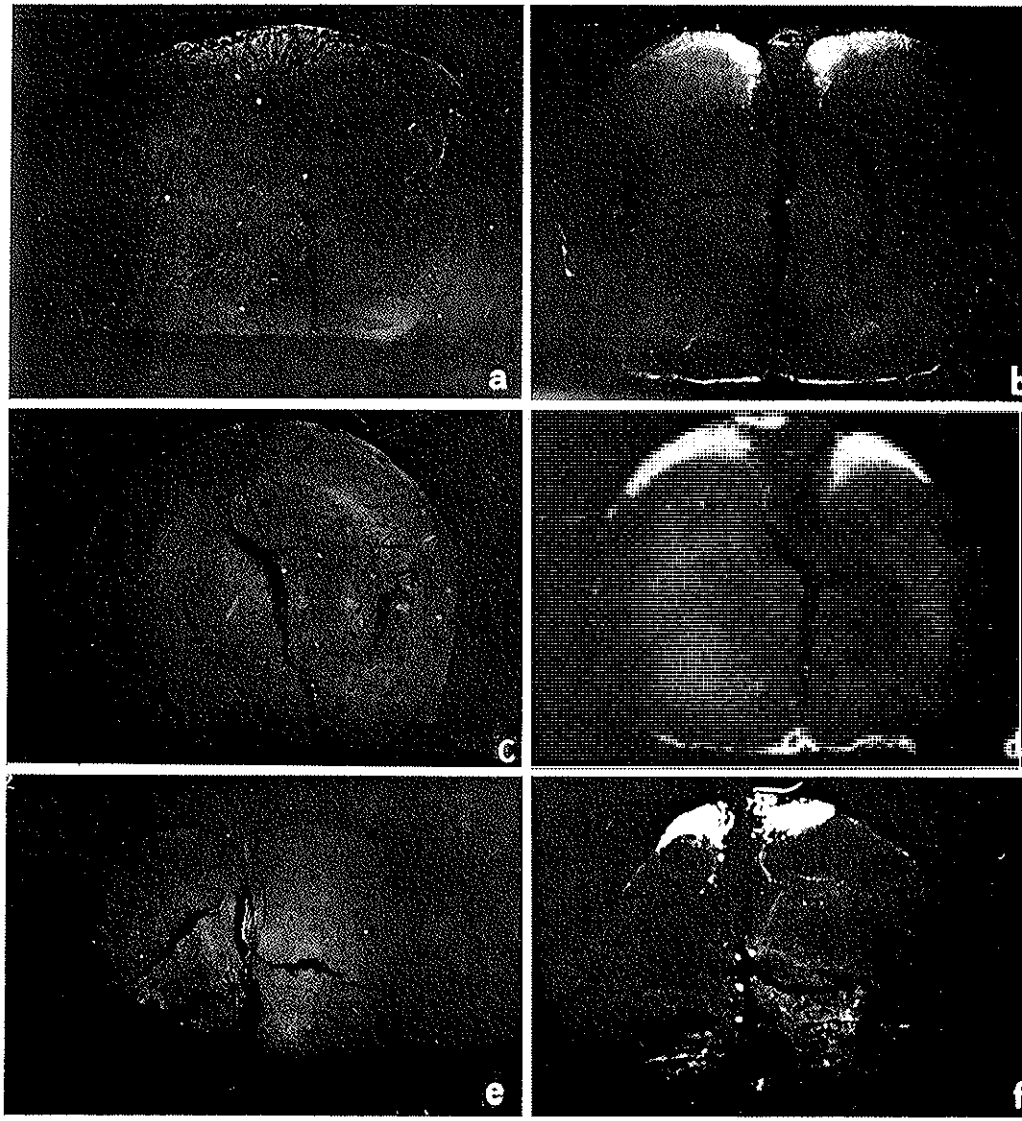

FIG. 10. Microphotos at the fluorescent microscope of transverse sections cut at different levels of the spinal cord of two 10 day old littermate rats injected intracerebrally since birth with vehicle solution (a, c, e) or with NGF (b, d, f). Notice the two triangular shaped highly fluorescent areas in correspondence of the dorsal funiculi in the NGF injected pup (b, d, f). × 32. Explanation in text.

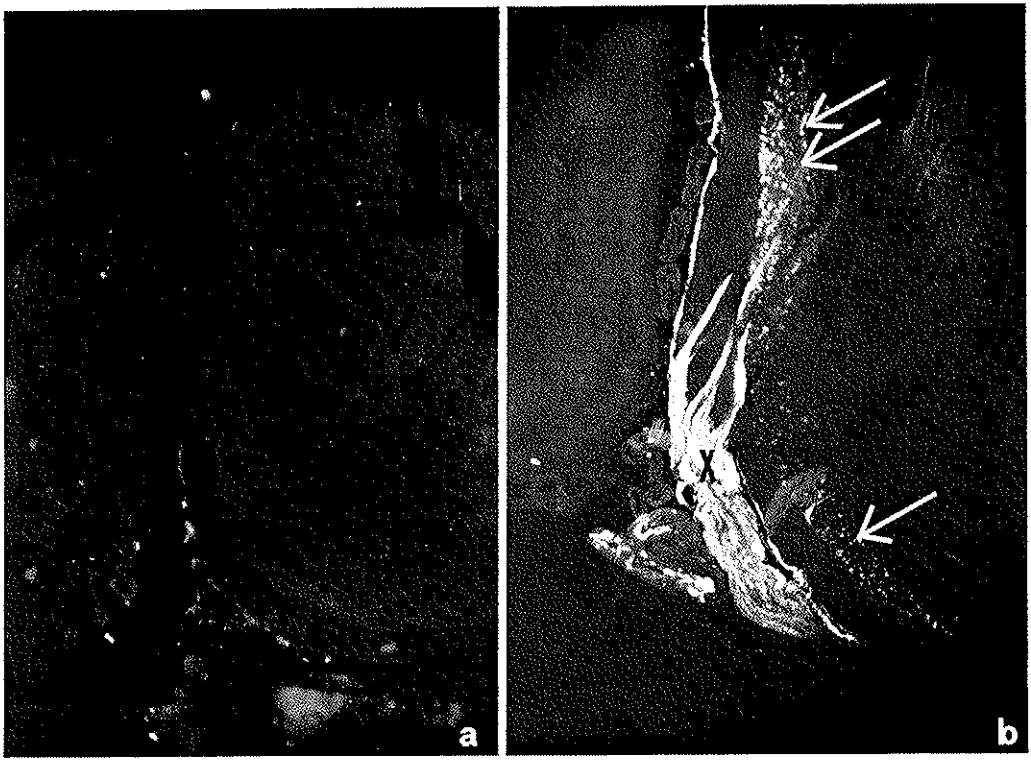

FIG. 11. Microphotos at the fluorescent microscope of the lower level of the medulla oblongata in two 14-day old littermate rats intracerebrally injected since birth with vehicle solution (a) or with NGF (b). Notice the intensely fluorescent postganglionic root of the tenth nerve (x) and the accumulation of fluorescent fibers in the lateral cordons of the medulla oblongata (arrows) in the NGF intracerebrally injected pup (b). × 25.

funiculi which decrease progressively in width and end at a distance from the locus coeruleus without establishing any connection with it nor with other fluorescent or non-fluorescent systems. Discontinuation of the NGF intracerebral NGF treatment results in the progressive fading away and complete disappearance of these fiber bundles; a result that suggests that these fibers do not belong nor find acceptance in the CNS where they are ignored as intruders by intrinsic neuronal systems. In two elegant *in vitro* series of experiments, new and unequivocable evidence was provided in other lahoratories for the role played by NGF in directing growing nerve fibers along a NGF concentration gradient. In order to test this effect R. Campenot devised a three-chamber culture system separated from each other by a virtually impermeable barrier obtained by

a layer of a sterile silicone grease which prevented fluid diffusion from one to the other chamber but permitted the neurite penetration beneath the divider, along scratches made on the bottom of the culture dish. Nerve cells were seeded in the central chamber in a medium enriched with NGF, while the two lateral chambers were filled with a culture medium enriched or devoid of NGF. Neurites gained access into the NGF-rich chamber but not in the other. Withdrawal of NGF from one side chamber, after the neurites had already entered into it, resulted in the degeneration of the axonal branches even if the cells of origin persisted in good condition in the central NGF containing chamber (Campenot, 1977). More recently the same problem was object of reinvestigation by P. C. Latourneau who used a somewhat different *in vitro* approach. Sensory neurons from dissociated dorsal root ganglia of chick embryos were cultured within semisolid matrices containing different concentration gradients of NGF which diffused from an adjacent source. A preferential orientation of approximately 60% of the tips of the fibers and an enhanced extension of fibers up NGF concentration gradients were observed. The oriented response appeared similar in degree to the chemotactic response of leukocytes or bacteria. The author came to the conclusion that the oriented response is not a concentration-dependent trophic response to NGF and that chemotaxis is a regulatory factor in neuronal morphogenesis (Letourneau, 1978).

Thus these *in vivo* and *in vitro* experiments provided strong evidence for the role played by chemical factors in directing the growing tip of nerve fibers. Since NGF is released in minute amounts from peripheral tissues which receive noradrenergic innervation, one can extrapolate from the above findings and see in this release, the tropic factor which directs the fibers toward their correct destination and provides for their subsistence once these connections are established.

c) *NGF transforming effect of normal immature and neoplastic chromaffin cells in sympathetic nerve cells*

A glandular cell which shares the origin as well as a number of properties with the sympathetic neuron, known as the chromaffin cell, is also receptive to NGF under two conditions: neoplastic transformation or early differentiation stages. In 1976 L. A. Greene and A. S. Tischler (Greene and Tischler, 1976) demonstrated that a cell line derived from a rat pheochromocytoma tumor, referred to as PC12, responds to NGF by acquiring properties characteristic of sympathetic nerve cells, such as

production of neurites, electrical excitability, storage and release of catecholamine. Withdrawal of NGF from the culture results in retraction of neurites, loss of other neuronal properties and resumption of active proliferation typical of neoplastic cells. Shortly thereafter K. Unsicker *et al.* found that *in vitro* explants of immature chromaffin cells dissociated from rat adrenal medulla, cultured in presence of NGF acquire biochemical and morphological properties of sympathetic nerve cells (Unsicker *et al.*, 1978). These *in vitro* NGF effects could be due, at least in part, to the fact that the chromaffin cells were subtracted to the well known inhibitory influence of the glucocorticoid hormones which in the adrenal gland are released from adjacent cortical cells. *In vivo* experiments performed in our laboratory provided definite evidence for a NGF transforming effect of immature chromaffin cells in sympathetic neurons, even in the intact gland where these cells are in close contact with cortical cells which synthetize and secrete glucocorticoid hormones. NGF was injected in 16-17 day old rat foetuses and the injections were resumed immediately after birth and repeated daily until the tenth post-natal day. This treatment resulted in dramatic morphogenetic changes of the adrenal glands: the whole chromaffin cell population of the medulla underwent transformation in sympathetic cells while chromaffin cells in the cortical section of the gland were in large part replaced by nerve fibers emerging from the sympathetic neurons of the medulla as shown in Fig. 12 (Aloe and Levi-Montalcini, 1979).

Concluding remarks

Three decades after the discovery that the immature sympathetic nerve cell depends for its further growth and differentiation from a protein molecule released in quantal amount from a large number of neoplastic and normal cells, and is synthetized and secreted in much larger quantities by some excretory glands, the analysis of this stimulus-response system is still in progress and keeps us wondering whether this molecule is not endowed with a much wider role than it was first suspected. The new features of the NGF-target cells interaction which came recently to light, hardly fit in the concept that NGF enhances growth of cells which have already engaged along a pre-programmed differentiation line. The property of this molecule to confer to immature sympathetic nerve cells the faculty to withstand the otherwise lethal action of different compounds and even to cause their increase in size and axonal production, its neurotropic effect and the transformation of immature glandular cells

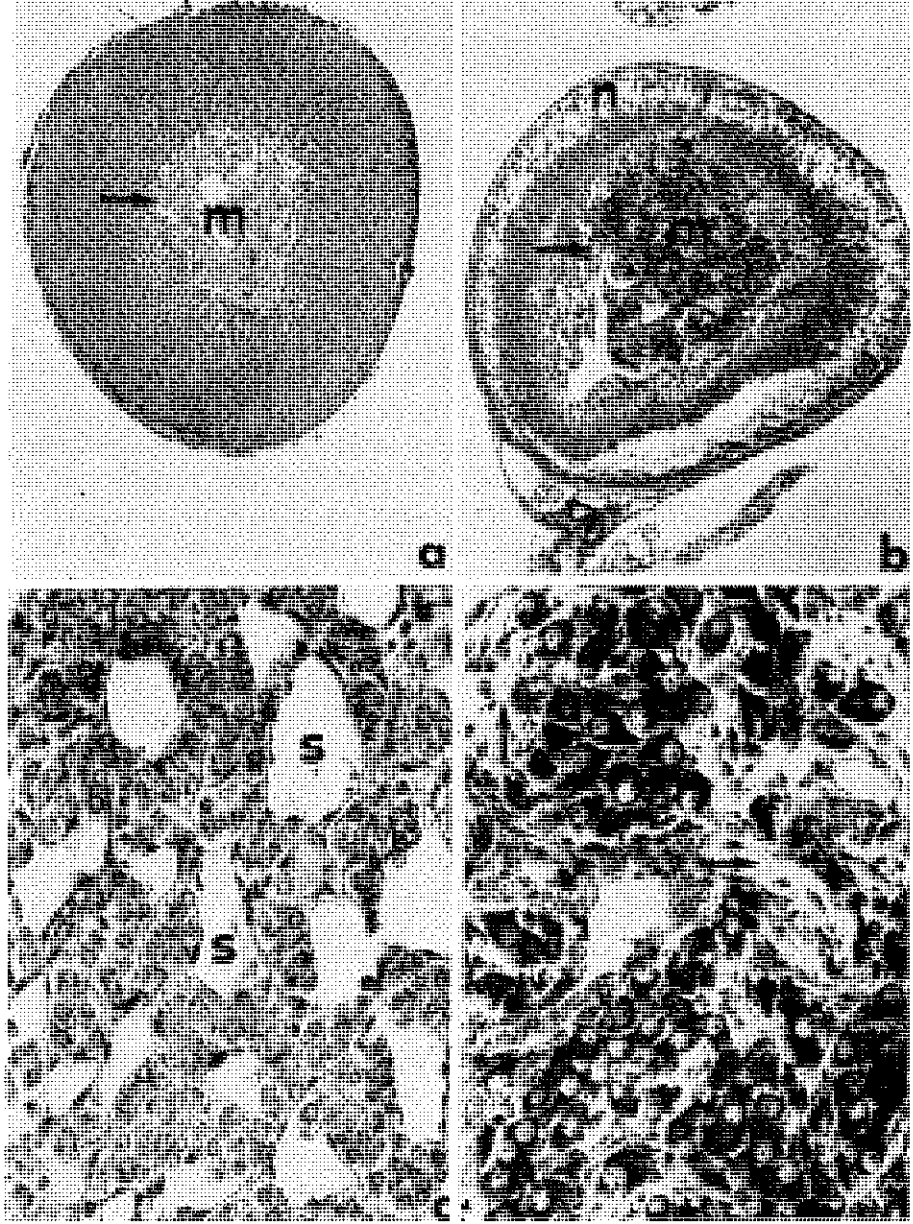

FIG. 12. Effect of NGF foetal and post-natal injections on the adrenal glands of 10-day old rat pups. Microphotos a, b compare the two largest sections of the adrenal gland of a saline injected pup (a) and of a NGF injected littermate (b). Arrows in both sections point to adrenal medulla (m) and to replacement of chromaffin with sympathetic nerve cells in the NGF-treated pup. Notice marked reduction of the cortical layers in the NGF treated infant rat. In the external cortical zone, large nerve fiber bundles produced by the sympathetic neurons (n) in the medulla replace the cells of the cortical glomerular layer. Toluidin blue stain. × 53. Microphotos c, d compare at higher magnification the chromaffin cells in the medulla of the control gland (c) and in that of the NGF treated littermate (d). Notice large nerve cell aggregates and nerve fiber bundles (arrows) which have obliterated the venous sinuses (s) in the medulla of the NGF treated gland. Toluidin stain. × 345.

in sympathetic nerve cells, reveal a wide range of heteromorphic and apparently unrelated responses which give evidence for a hitherto unsuspected plasticity of sympathetic and related cell precursors. If it is justified to visualize the sympathetic nerve cell as a valid model of other nerve cells which have so far defied analysis in view of their far less approachable position, then the above results would acquire new significance in the ever broadening panorama of neurobiology.

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DISCUSSION

TERENIUS

You said that this is not a survival factor. What is it then?

LEVI-MONTALCINI

The term "survival factor" which unfortunately has been introduced in the literature to define the action of NGF on its target cells, is not only very vague but misleading, since the activity of this protein molecule which displays an essential role in the life of embryonic sensory and embryonic and fully differentiated sympathetic nerve cells is much more extensive and diversified than this term would imply. NGF does not only permit the survival *in vitro* of embryonic sensory and sympathetic nerve cells in media inadequate for their growth, but stimulates their overgrowth and axonal production during early developmental stages *in vivo,* and calls forth increase in size and enhanced function in the fully differentiated sympathetic nerve cells. Two other outstanding properties of NGF which do not fit in the concept of a "survival factor" are: its neurotropic and its transforming effects. The first was clearly documented by the penetration of sympathetic nerve fibers into the cerebrospinal axis of neonatal rodents injected intracerebrally with NGF and by some *in vitro* experiments which I also mentioned; the second was proved by the NGF transforming effect discovered by L. Green of pheochromocytoma cells in sympathetic nerve cells *in vitro.* Recently we proved that this effect materializes also in rat foetuses injected with NGF.

NELSON

In the studies of central neurons and their sensitivity to NGF, to what degree has a particular period of sensitivity to or a requirement for NGF been looked for?

LEVI-MONTALCINI

Studies on different cell populations in the brain of chick embryos injected intracerebrally with NGF at different developmental stages and on noradrenergic centers in the brain of fetuses and infant rats, did not give evidence of any growth or differentiation enhancing effect elicited by NGF on these nerve cells.

ROBERTS

Has any definite agreement been reached about the origin of an excessive number of nerve cells in sympathetic ganglia of NGF injected infant rats? Is NGF a mitogenic factor or is it not?

LEVI-MONTALCINI

I believe that the problem whether NGF is endowed with a mitogenic activity should for the time being be considered still unsettled, although I am inclined to agree with J. Hendry that the evidence gathered so far does not favor this hypothesis. The numerical increase of sympathetic nerve cells in neonatal rodents injected with NGF can in fact be explained as the result of decrease in the normally occurring death of a large number of immature nerve cells under the action of NGF, rather than to a NGF mitogenic effect.

REVOLTELLA

To be precise, we found that mouse 2.5S NGF exhibits a mitogenic effect on cloned murine C1300 neuroblastoma cells *in vitro*. We have no evidence of such an effect on normal cells or *in vivo*.

HÖKFELT

Did you analyze your adrenal sympathetic neurons also under an electromicroscope? What kind of vesicle populations did they have?

LEVI-MONTALCINI

Yes, we examined at the electron microscope the transformed chromaffin cells in sympathetic neurons in neonatal and infant rats injected during fetal life with NGF. The cytoplasmic compartment exhibits features characteristic of both cell types: one sees in fact a rather large number of electron dense vesicles identical to those of chromaffin cells and a large number of densely packed microtubules, microfilaments and neurofilaments which one only finds in nerve cells treated with NGF. The nuclei instead show the typical appearance of nuclei of sympathetic nerve cells with wheel-shaped nucleoli different and much larger than those of chromaffin cells.

HAMPRECHT

Did you say that 6-hydroxydopamine intervened with the retrograde transport of NGF?

LEVI-MONTALCINI

The effect that you mention is not called forth by the dopamine derivative, 6-hydroxydopamine (6-OHDA), but by vinblastine. This vinca alkaloid produces its toxic effects by interfering with the polymerization of microtubules and other filamentous proteins. In developing sympathetic nerve cells, vinblastine produces the disruption of microtubules and microfilaments which play a most important role in antero as well as in retrograde axonal transport. We believe that the death of sympathetic nerve cells in neonatal rodents injected with NGF is due to the block of retrograde axonal transport of NGF from peripheral end organs. The same cells survive and undergo excessive growth only if provided with exogenous NGF which finds access to the cell perikarya through the cell membrane receptors.

HAMPRECHT

But in the case where you used vinblastine and NGF, you got this tremendous increase in size.

LEVI-MONTALCINI

The extraordinary volume increase of sympathetic ganglia that I mentioned and you quote, is the result of the dual NGF and 6-OHDA combined treatment of neonatal rodents and not of NGF and vinblastine. Ganglia of neonatal rats injected with NGF and 6-OHDA for a three-week period undergo a 30 fold volume increase as compared to ganglia of control littermates. This effect is mainly due to the excessive collateral fibers production by the axotomized axons and is typical of the dual NGF and 6-OHDA treatment but not of guanethidine or of vinblastine injected together with NGF.

CAN NERVE GROWTH FACTOR (NGF) PLAY A TRANSCRIPTION-DEPENDENT "INSTRUCTIVE" ROLE DURING DEVELOPMENT? EVIDENCE FROM STUDIES WITH CLONAL PHEOCHROMOCYTOMA CELLS

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ABSTRACT

The PC12 clonal line of pheochromocytoma cells is a particularly useful model system for studying the mechanism(s) of action of NGF. When grown without NGF in the presence of serum, such cells display the differentiated noradrenergic and cytochemical properties associated with adrenal medullary chromaffin cells. After treatment with NGF, PC12 cells cease mitosis and acquire many of the morphological (e.g. presence of neurites) and electrophysiological (e.g. action potentials) properties of normal sympathetic neurons. Thus, among the advantages of the PC12 line for NGF-related studies are that the cells respond to but do not (in contrast to normal neurons) require the factor for their survival and that it is possible to observe the initial effects of the factor on previously unexposed cells. In this paper we discuss recent experiments with PC12 cells that suggest: 1) that NGF can affect target cells not only by promoting their survival and modulating the degree of expression of pre-existing phenotypes, but can also "instruct" cells to display new phenotypes; 2) that initiation of such events requires two separable effects of NGF—one of which is RNA-synthesis-requiring and the other of which is transcription-independent; 3) that NGF may play a role in regulating phenotypic expression not only in post-mitotic neurons, but also in sympathicoblasts.

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INTRODUCTION

The biological effects of nerve growth factor (NGF) on responsive sympathetic and dorsal root ganglionic neurons have been well described, in large part via the efforts of Levi-Montalcini and her colleagues (Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968; Levi-Montalcini *et al.*, 1972). These responses include stimulation of neurite outgrowth (Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968; Levi-Montalcini *et al.*, 1972), increase in somatic volume (Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968; Levi-Montalcini *et al.*, 1972), increase in the levels of enzymatic activities involved in the synthesis of neurotransmitters (Thoenen *et al.*, 1971) and maintenance of survival (Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968; Levi-Montalcini *et al.*, 1972; Levi-Montalcini and Angeletti, 1963). Such actions have been demonstrated both *in vivo* and *in vitro* (Levi-Montalcini, 1966; Levi-Montalcini and Angeletti, 1968; Levi-Montalcini *et al.*, 1972; Levi-Montalcini and Angeletti, 1963).

The means whereby NGF promotes its effects on its target cells may be conceptualized under several possible categories. For example, one possibility is that some or all of the effects of NGF are "*permissive*" in that by merely maintaining viability, NGF allows or permits a neuron to carry out differentiated programs which are either endogenously programmed or directed by other factors. For example, Partlow and Larrabee (1971) suggested that neurite outgrowth by cultured sympathetic neurons which occurs in the presence of NGF may not be due to a direct action of the factor, but is rather indirectly due to its prevention of cell death. From this point of view, by merely preserving cell viability, NGF would permit neurite outgrowth to occur. A second possible action for NGF is as a *modulator* of phenotypic behavior. That is, NGF may not only permit a target cell to express differentiated properties, but could actively modulate (i.e. increase or decrease) the degree to which a given property is expressed (Varon S., 1975). However, the types of phenotypic properties expressed by the neuron would be specified by intrinsic or extrinsic signals other than NGF. An example of this concept is the work of Chun and Patterson (1977), who found that the *choice* of neurotransmitter properties shown by neonatal rat sympathetic neurons in culture is dependent on factors other than NGF but that NGF could modulate the *extent* to which such neurotransmitter properties were expressed. A modulatory role of NGF would also be consistent with the observed *in vivo* effects of exogenously administered NGF in increasing neuronal somatic diameter,

neurite outgrowth and tyrosine hydroxylase activity. The third possible action of NGF is an "*instructive*" one—that is, that NGF can itself cause the target cell to express new or different phenotypic properties. Until recently, there has been little evidence for such a possible action of NGF.

For any of the actions of NGF—permissive, modulatory or instructive—there is tbe issue as to the mechanism(s) whereby such effects are promulgated. In a broad sense, two possible categories for the mechanism of action can be distinguished. First, NGF could affect target cells by transcription-independent means. That is, it is possible that the effects of NGF on phenotypic expression are not mediated via synthesis of specific gene products. Evidence for such a mechanism has first come from the experiments of Partlow and Larrabee (1971). These workers showed that although neurite outgrowth from explanted ganglia requires the presence of NGF, such NGF-dependent outgrowth can occur even in the presence of levels of actinomycin-D sufficient to inhibit greater than 90% of cellular RNA synthesis. Further evidence for local effects of NGF have come from the elegant experiments of Campenot (1977), in which neurite elongation in culture was shown to require the physical presence of NGF at the nerve ending. In addition, Calissano and colleagues (1976) have provided evidence that NGF may directly interact with and regulate the state of polymerization of molecules such as tubulin and actin *in vitro*. This has led to the suggestion that such local interaction may be in part responsible for NGF's effects on neurite elongation (Calissano *et al.*, 1976). The second possible mechanism of NGF's action is at the level of the genome, i.e. that the action of the factor requires RNA synthesis. Although this possibility is intuitively appealing, conclusive evidence for such a mechanism has been difficult to obtain.

The intent of this paper is to summarize recent work from our laboratory that 1) demonstrates instructive as well as modulatory actions of NGF and 2) that provides evidence that initiation and maintenance of such actions require two separable actions of NGF, one of which requires RNA synthesis and the other of which does not. In addition, we shall discuss the possible relevance of such findings to the role of NGF in development of the peripheral nervous system.

Before beginning our discussion, however, it is necessary to describe the experimental system with which studies were carried out—an NGF responsive clonal line of rat pheochromocytoma cells.

PC12 pheochromocytoma cells. Pheochromocytomas are considered to be the neoplastic counterparts of adrenal medullary chromaffin cells. A transplantable tumor of this type was induced in a male rat several years

ago by Warren and his colleagues (1972). Subsequent studies have revealed that cells from this tumor (as well as from human pheochromocytomas) exhibit many of the ultrastructural and noradrenergic features associated with normal chromaffin cells (Warren and Chute, 1972; De Lellis *et al.*, 1973; Chalfie and Perlman, 1976). In 1973, cells from this rat tumor were adapted to monolayer tissue culture (Tischler and Greene, 1975) and a continuous single cell clonal line was obtained (Greene and Tischler, 1976). This line was designated PC12. Characterization of this line revealed that PC12 cells growing in nutrient medium supplemented with horse and fetal bovine sera also closely resemble normal chromaffin cells (Greene and Tischler, 1976; Greene and Rein, 1977 a; Greene and Rein, 1977 b; Greene and Rein, 1978). These properties are summarized in Table 1.

TABLE 1. *Adrenal Medullary Chromaffin Cell Properties of PC12 Clone*

Presence of chromaffin granules
Argentaffinic
Intense formaldehyde-induced fluorescence
Enzymes for synthesis and degradation of norepinephrine
Storage of catecholamines
Stimulated release of catecholamines (by elevated K^+ or by nicotinic cholinergic stimulation)
Short-term regulation of catecholamine synthesis
Depolarizing responses to acetylcholine

NGF response of PC12 cells. While PC12 cells resemble adrenal chromaffin cells both anatomically and functionally, evidence has accumulated over the past several years that the line can also respond to nerve growth factor. Significantly, several of these responses involve changes in the phenotypic properties of the cells and are as follows:

1. *Cell division.* PC12 cells replicate in medium containing serum (15%) with a doubling time of 2-4 days. However, after several days of treatment with NGF, the cells cease replication and become non-dividing (Greene and Tischler, 1976).

2. *Morphology.* In the absence of NGF, PC12 cells, like normal medullary chromaffin cells, are round to ovoid in shape and entirely lack processes (see Figure 1). Within several days of exposure to NGF, the

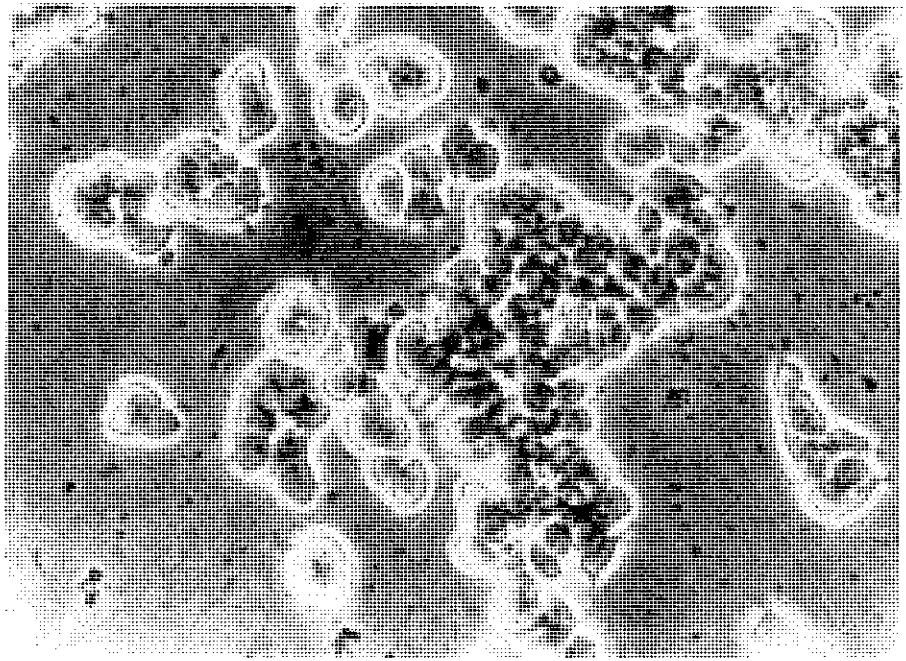

FIG. 1. Phase contrast photomicrograph of PC12 cells before treatment with NGF.

cells begin to put out processes which progressively elongate to form a neuronal-like network (Greene and Tischler, 1976) similar to that elaborated by normal sympathetic neurons in culture (see Figure 2). Such processes appear to be true neurites. Ultrastructural studies reveal them to contain bundles of microtubules and neurofilaments (Greene and Tischler, 1976; Tischler and Greene, 1978). As with normal neurons, outgrowth of neurites from PC12 cells appears to require assembly of microtubules, since it is reversibly blocked by agents such as colchicine (0.1 μg/ml) and nocodazole (0.5 μg/ml) which prevent polymerization of tubulin. Furthermore, dose-response curves reveal that stimulation of maximal levels of neurite outgrowth from PC12 cells requires a minimum level of about 10 ng/ml of 2.5S NGF. This concentration is about one B.U. and is similar to the "physiologic" concentration of NGF present in serum of rats. It is also of interest to note that the effect of NGF on neurite outgrowth in this system appears to be quite specific. Treatments such as cyclic AMP analogues, DMSO, low serum, and mitotic inhibitors, which produce process outgrowth in murine neuroblastoma cultures do not induce neurite outgrowth from PC12 cells (Greene and Tischler, 1976).

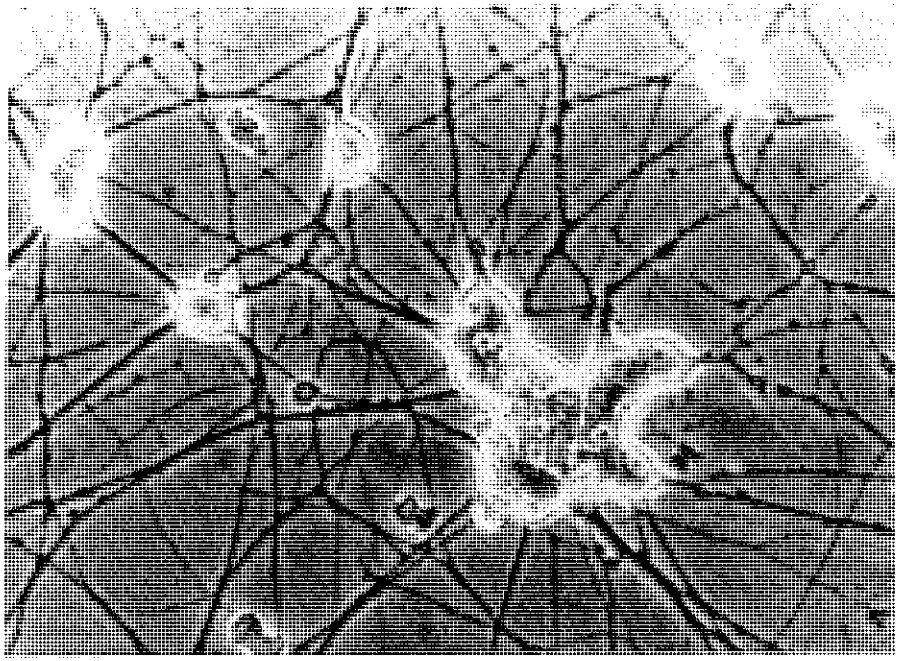

FIG. 2. PC12 cells treated with NGF for 11 days.

3. *Ultrastructure.* Aside from causing rearrangement of the types of cytoplasmic structures which are present in PC12 cells prior to treatment, NGF also induces the presence of qualitatively new structures (Greene and Tischler, 1976; Tischler and Greene, 1978). These are small (20-26 nm) vesicles which are generally present as aggregates within varicosities and endings of processes as well as in cell bodies of NGF-treated cells. These structures could be synaptic vesicles of the type present in normal autonomic neurons and/or could be involved in membrane synthesis or turnover.

4. *Electrophysiologic properties.* Dichter *et al.* (1977), using intracellular recording techniques, studied the electrophysiologic properties of PC12 cells grown in the presence or absence of NGF. Significantly, non-NGF-treated PC12 cells were not electrically excitable. In contrast, with cultures that had been treated with the factor for several weeks, it was possible to elicit active responses (action potentials) from nearly every cell tested. Furthermore, it was found that the proportion of electrically excitable cells increased progressively with time over tbe first several weeks of NGF treatment. Acquisition of excitability did not appear to

be an indirect consequence of the effect of NGF on cell division or on neurite outgrowth since (a) it was possible to find excitable cells that did not possess neurites and (b) blockade of cell division with a mitotic inhibitor did not in itself induce excitability (such mitotically blocked cells did, however, acquire excitability after treatment with NGF).

Given the *de novo* appearance of the properties mentioned above after treatment with NGF, it seems clear that NGF is acting in an "instructive" sense; that is, NGF induces the expression of qualitatively new phenotypic behavior in PC12 cultures. Since PC12 cells respond to but do not require NGF for their survival in serum-containing medium, such effects could not be "permissive" in nature. Furthermore, since such properties were not detectable prior to NGF treatment, the factor could not be acting in a "modulatory" fashion.

While NGF may thus function in an instructive manner on PC12 cells, there is also evidence for specific modulatory control in such cultures. Several examples are as follows: 1) *Cell size.* NGF treatment results (over several weeks) in a 3-5-fold increase in the volume and protein content of PC12 cells. 2) *Acetylcholine sensitivity.* Studies with PC12 cells on release of neurotransmitter evoked by nicotinic cholinergic agonists (Greene and Rein, 1977 b) and of responsiveness to iontophoretically-applied acetylcholine (Dichter *et al.*, 1977) indicate that NGF treatment results in a large increase in cholinergic sensitivity. 3) *Specific protein synthesis.* Electrophoretic analysis of polypeptides synthesized by PC12 cells before and after treatment with NGF reveal very few changes in the relative proportions of major cellular proteins (McGuire *et al.*, 1978). Several quantitative alterations have, however, been detected. Among these are specific increases in the relative levels of a protein of apparent molecular weight (on SDS gels) of 80,000 (J. C. McGuire and L. A. Greene, 1979) and of a surface-exposed glycoprotein of apparent molecular weight 230,000 (McGuire *et al.*, 1978). 4) *Uptake of amino acids.* Treatment of PC12 cultures with NGF results in a rapid (commencing within 15 minutes) increase in the rate of uptake of amino acids (McGuire and Greene, 1978). This effect appears to be selective in the sense that NGF treatment did not alter uptake of other small molecules (uridine, thymidine or norepinephrine) for which the cells also possess transport mechanisms.

Use of PC12 system to study the mechanism(s) of action of NGF. If, as argued above, NGF can act both in a modulatory and instructive sense, the question arises as to whether such control involves merely transcription-independent mechanisms or whether there is also a requirement for specific gene regulation. PC12 cultures provide a particularly advantageous

system in which to study such issues, particularly in comparison with normal neurons. First, although PC12 cells can respond to NGF, they, unlike normal neurons (Levi Montalcini and Angeletti, 1963), do not require NGF for their survival in serum-containing medium. Thus, PC12 cells that are not treated with NGF provide a stable, non-dying population that can be used as adequate controls in metabolic studies. Second, prior to experimental intervention, normal responsive neurons have been exposed to NGF *in vivo* and have already (probably because of such exposure) grown neurites. Thus, for *in vitro* experiments such cells are already "experienced" with regard to NGF treatment and the neurite outgrowth which they undergo in culture in response to NGF can be regarded as regeneration. In contrast, PC12 cells can be maintained for many generations in the absence of detectable levels of NGF. Thus, upon introduction of NGF into PC12 cultures, one can study the steps leading to *de novo* initiation or generation of neurites. Moreover, PC12 cells can be pre-treated with NGF for one-two weeks and then divested of their neurites by mechanical means. Such cells can be used (analogously to normal neurons) to study regeneration of neurites (Greene, 1977).

Evidence for a transcriptional requirement for neurite generation. To test whether initiation of neurite outgrowth requires transcription, PC12 cultures were exposed to NGF in the presence of three different inhibitors of RNA synthesis. Each of these inhibitors—actinomycin-D, cordycepin and camptothecin—has a different mechanism of action. In each case, neurite generation was blocked at levels of drug which appeared to have little effect on cell viability during the time course of the experiment (Burstein and Greene, 1978). Confirmation of cell viability and vitality was obtained with the camptothecin-treated cultures. The effects of this inhibitor on RNA synthesis have been shown to be completely and rapidly reversible (Horwitz, 1975). Accordingly, removal of camptothecin from the cultures (even after a week of treatment) was followed by initiation of neurite outgrowth (Burstein and Greene, 1978). Such experiments thus suggest that *de novo* synthesis of RNA is required for NGF-stimulated initiation or generation of neurite outgrowth.

Experiments have also been carried out on the effect of camptothecin on NGF-induced increase in synthesis of the 80,000 mol wt protein and 230,000 mol wt glycoprotein. In each case, induction of synthesis was selectively blocked by the drug (McGuire *et al.*, 1978). Thus, synthesis of new RNA appears to be required for some of the modulatory as well as instructive effects of NGF.

Evidence for non-transcriptional effects of NGF. As previously mentioned, it is possible to study NGF-dependent regeneration of neurites by PC12 cells that have been pre-treated with the factor for 1-2 weeks. In such a case (unlike initiation of neurite outgrowth which has a lag of 1-2 days), neurite regeneration occurs within 24 hrs (Greene, 1977). Such neurite outgrowth also requires NGF; in the presence of the factor, 80-90% of the cells regenerate neurites within 24 hrs, while without the factor only 5-15% do so. This situation is thus analogous to the NGF-dependent neurite regeneration which normal sympathetic neurons undergo in culture. In contrast to NGF-induced *generation* of neurites, NGF-stimulated *regeneration* of neurites by PC12 cells is not blocked by RNA synthesis inhibitors even at 1000-fold higher levels of drug (Burstein and Greene, 1978). This result is similar to those obtained hy Partlow and Larrabee (1971) with normal sympathetic neurons and provides corroborative evidence for a non-transcriptional effect of NGF which is required for neurite outgrowth from PC12 cells. Interestingly, NGF-stimulated amino acid uptake by PC12 cells was also found to be insensitive to camptothecin (McGuire and Greene, 1978).

If initiation of neurite outgrowth requires RNA synthesis while regeneration of neurites does not, then the question arises as to the transition between these two capacities. That is, how does NGF "prime" responsive cells for rapid, transcription-independent neurite outgrowth? One model (Burstein and Greene, 1978) for this is that exposure to NGF treatment causes responsive cells to accumulate RNA-synthesis-dependent material(s) that is required for neurite outgrowth. Addition of inhibitors of RNA synthesis to cultures which have not been previously exposed to the factor would thus block such accumulation and therefore inhibit neurite outgrowth. However, once the cells have been exposed to NGF long enough to cause a sufficient store of such material to accumulate, then neurite regeneration (by normal sympathetic neurons as well as PC12 cells) could occur even when RNA synthesis (and continued accumulation) is blocked. This model is supported by experiments in which NGF-pretreated (i.e. primed) cells were subcultured and then incubated for various lengths of time in the absence of NGF before being re-exposed to the factor in the presence of inhibitors of RNA synthesis. As the cells were deprived of NGF for increasingly longer times, their capacity for rapid, transcription-independent neurite regeneration became progressively less (Burstein and Greene, 1978). Thus, withdrawal of NGF led to time-dependent loss of priming.

Priming of PC12 cells in the presence of NGF does not require neurite outgrowth. That is, when the cells are exposed to NGF in even spinner-suspension culture (in which they do not attach to a substrate and consequently do not possess neurites), they also acquire the capacity for rapid, transcription-independent neurite outgrowth when they are plated on a collagen-coated substrate in the presence of NGF.

Possible nature of RNA-synthesis-dependent effects of NGF. If initiation of neurite outgrowth by NGF requires RNA-synthesis-dependent accumulation of certain material, then the question arises as to the nature of such material. Two possibilities would seem to be most likely. One is that one of several species of RNA itself plays a required role in neurite outgrowth. It has been suggested, for example, that RNA could play a structural role in organization of microtubules (Heidemann *et al.*, 1977). A second possibility is that neurite outgrowth requires protein which is the translational product of RNA whose synthesis is regulated by NGF. Intuitively, given the dramatic effects of NGF on the phenotype of PC12 cells, one might expect a rather striking effect of NGF on their protein composition. However, analysis of PC12 cells by two-dimensional isoelectric focusing x SDS gel electrophoresis reveals that NGF treatment causes no qualitative and only very few quantitative alterations in the relative proportions of the 1000-or-so peptides resolved by this method (McGuire *et al.*, 1978). Such findings suggest that promotion of neurite outgrowth by NGF takes place in large part via rearrangement of the types of structural molecules which the cell already makes prior to treatment with the factor. This again underscores the presence of a non-transcriptional role for NGF. Despite the absence of major alteration in protein composition in response to NGF, several quantitative changes have been detected. As previously mentioned, these include increases in levels of an 80,000 mol wt protein and in a 230,000 mol wt surface-exposed glycoprotein (McGuire et al., 1978). Significantly, such increases are specifically blocked by the RNA-synthesis-inhibitor, camptothecin. It is also highly possible that NGF causes changes in the levels of other proteins that are present in only a small number of copies per cell and which were therefore not detected by electrophoretic analysis. Such minor proteins could play an important regulatory (rather than structural) role in responses to NGF, and in particular, in directing neurite outgrowth and elongation. The search for such molecules is currently under way.

Relevance of PC12 system to normal development. Most of the well-documented effects of NGF on the nervous system have been on post-mitotic cells. In each case—increase in cell size, increased neurite out-

growth, elevation of enzyme activity—the effects of the factor appear to be either modulatory or permissive. Although it has been proposed (Levi-Montalcini, 1966; Bjerre and Björklund, 1973) that NGF can also affect the development of sympathicoblasts (i.e. the replicating precursors of post-mitotic sympathetic neurons), the evidence for this has not heen conclusive.

We have presented evidence here that NGF can both cause cessation of replication and alteration of phenotype of a pheochromocytoma cell line. The overall effect of the factor is to cause a dividing chromaffin-like cell to acquire the differentiated phenotypic properties of non-dividing sympathetic neuron. Such findings have implications for the role of NGF in early development of the sympathetic nervous system. Both chromaffin cells and sympathetic neurons are derived from the neural crest and are thought to share a common precursor "stem" cell. During development this stem cell appears in turn to give rise to the immediate precursors of chromaffin cells and sympathetic neurons known as pheochromoblasts and sympatheticoblasts, respectively. The pheochromocytoma cell could be viewed as being the neoplastic counterpart of the pheochromoblast or could, given the plasticity of neoplastic cells, have some of the properties of a sympathicoblast or even the pluripotency of a common precursor stem cell. If such an analogy holds, then the present findings suggest that NGF could affect the phenotype of such cells during normal development. That is, NGF, as it does to pheochromocytoma cells, could instruct mitotic precursor cells (i.e. sympathicoblasts) to cease replication and to take on the phenotypic behavior of mature sympathetic neurons. Once such cells become post-mitotic, NGF would still affect their properties by means of permissive and modulatory interaction.

One last point of interest is the question as to the effect of NGF on the phenotype of normal adrenal chromaffin cells. NGF has been shown to elicit neurite outgrowth from PC12 as well as from other pheochromocytoma cells in culture (Tischler and Greene, 1975). Moreover, there is evidence that cultured adrenal medullary cells can also produce neurites in presence of the factor (Unsicker *et al.*, 1978). Yet, adrenal medullary chromaffin cells do not possess neurites *in situ*. Moreover, PC12 cells growing in rats as solid tumors also do not exhibit neurite outgrowth (Greene and Tischler, 1976) even though they are presumably exposed to circulating NGF. One possible explanation for this is that although normal medullary chromaffin cells (or their precursors) may have the capacity to respond to NGF, they are inhibited from doing so *in situ*. It has been suggested that the *in situ* inhibitor could be corticosteroids (Lempinen,

1964), which are known to be at high concentrations in the adrenal medulla. Experimental evidence has recently appeared which supports this possibility (Unsicker *et al.*, 1978). Significantly, however, corticosteroids do not block NGF-induced neurite outgrowth in PC12 cultures (Tischler and Greene, 1975; Greene and Tischler, 1976). Such a possible difference in behavior of PC12 cells from normal adrenal medullary cells could derive from the tumor properties of pheochromocytoma cells. A second possible explanation for the lack of *in vivo* response of adrenal medullary or PC12 cells to NGF could be cell-cell interaction. Recent experiments indicate that the ability of PC12 cells to initiate neurite outgrowth and to become primed in the presence of NGF is inhibited in high density cultures. A similar effect could occur *in vivo* where adrenal medullary and PC12 cells form a tight, three-dimensional matrix.

Conclusions and Summary

On the basis of our experiments with NGF-responsive clonal PC12 pheochromocytoma cells, we have provided evidence that NGF can not only modulate the degree of expression of existing phenotypes, but can also instruct appropriate target cells to express new phenotypic behaviors. The initiation of such effects by NGF requires two separable mechanisms, one of which requires RNA synthesis and the other of which does not. The effect of NGF on cell division and phenotypic expression in PC12 cells supports the possibility of a role for NGF in the differentiation of neuroblasts in the peripheral nervous system.

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DISCUSSION

HAMBURGER

Among the many interesting experiments, there was one which interested me most particularly, and that was the case in which after withdrawal of NFG the cells resumed proliferation. I would like to ask whether this is a resumption by cells which have previously grown neurites or whether there were reserve cells in the culture which were responsible for this. I must say that the pictures did not show any room for cells which did not send out fibers. So, am I right in assuming that actually cells which were differentiated would become proliferative again?

GREENE

That is the assumption. The evidence for this is that at least 90% of the cells undergo neurite outgrowth in response to NGF and that the rate at which the cells re-enter the cell cycle after removal of NGF is consistent with the entire population dividing. That is, if there is only a small subpopulation that resumes cell division, one would expect the growth rate of the entire population to have a much lesser slope than we found experimentally. There is also another piece of evidence. If one takes NGF-treated cultures, lets them resume cell division by removing NGF and then re-treats with NGF, the cells again respond with neurite outgrowth. But you are quite right. We have not rigorously proved that the cells that resume mitosis after removal of NGF were neurite-bearing.

ROBERTS

Is there any specificity for the presence of descending noradrenergic innervation in the spinal cord?

LEVI-MONTALCINI

There is no evidence that the sympathetic nerve fibers that gain access into the neural tube upon intracerebral NGF injections in neonatal rodents, follow a specific route. They find accommodation in the dorsal funiculi, most likely because they enter into the spinal cord and brain stem through the

dorsal or cephalic routes and the dorsal funiculi in the spinal cord as well as the latero-ventral funiculi in the brain stem offer the most convenient and close "built-in" path for the ectopic fibers inside the neural tube.

BURNSTOCK

You showed that these cells have many properties of adrenergic neurons when you use NGF. Have you looked to see if there is any choline acetyltransferase? Because many developing sympathetic neurons contain synthesizing enzymes for both adrenergic and cholinergic transmitters.

GREENE

Yes, we have looked for cholinergic enzymes. Let me preface this. What I believe Dr. Burnstock is referring to is the finding that under appropriate conditions cultured sympathetic neurons can also synthesize substantial amounts of acetylcholine. If one looks at pheochromocytomas, they also have high levels of choline acetyltransferase and of acetylcholine. We do indeed believe that PC12 cells synthesize both catecholamines and acetylcholine (Greene L. A. and Rein G., "Nature", *268*, 349-351, 1977).

BURNSTOCK

You showed agranular vesicles and that is one of the reasons I asked the question.

GREENE

Exactly, of course. I suspect that many of the agranular vesicles are cholinergic. One finds that if one compares NGF-treated cells with untreated cells, the specific activities of choline acetyltransferase are comparable. However, the NGF-treated cultures had several-fold higher levels of acetylcholine. This would be consistent with the induction by NGF of appropriate cholinergic storage vesicles.

We originally isolated about 30 different clones from the tumor. All respond to NGF and, so far as we can tell, do so in a similar fashion. Furthermore, Arthur Tischler has reported that human pheochromocytoma cells also respond to NGF in a similar way.

PURVES

I wonder if you could elaborate on the role that you think your results have in the normal development. What I have in mind is this: that normally

those that become ganglion cells send out neurites and the cells that are going to become chromaffin cells don't. Do you think this implies some sort of differential access to NGF release in the two cell types?

GREENE

There are several possibilities. One is differential access to NGF. A second is differential access to inhibitory factors. It is quite possible that there is something which inhibits the cells from responding to NGF. One which has been mentioned, I think by Lempinen originally, was that perhaps corticosteroids, which are found at high concentrations in the adrenals, inhibit chromaffin cells from responding to NGF. In a recent paper in PNAS Unsicker et al. ("Proc. Nat. Acad. Sci. USA", *75*, 3498-3502, 1978) presented evidence that if one puts chromaffin cells in culture, they will grow neurites in the presence of NGF, but will be inhibited from doing so by dexamethasone. I must say that this possibility is not consistent with our experiments on pheochromocytoma cells. If one grows PC12 cells in the presence of a wide range of concentrations of corticosteroids, they still respond to NGF. Of course, as tumor cells this aspect of their behavior may differ from that of normal chromaffin cells. There is a third possibility that I can bring up and this has to do with cell-cell interaction. We have noticed in our experiments that when PC12 cells are at very high density, they do not respond well to NGF. We wonder if perhaps within the adrenal gland, where the chromaffin cells are very tightly packed, one might have the same effect. This possibility reminds me of an experiment that Dr. Calissano did when he was visiting with us in Boston. I believe, Pietro, that you found that NGF was taken up by PC12 cells at a much higher rate when the cultures were at a high density. Perhaps at very high density the cells are taking up and possibly destroying NGF at a very high rate and this is why they do not respond. Also, when one puts PC12 cells into a rat, they form tumors. Presumably such tumors are exposed to circulating NGF, yet the tumor cells do not put out neurites. Perhaps again it is a matter of cell density.

NIRENBERG

From the two-dimensional gels that you showed, it looked as though there were numerous differences in proteins. And with clear vesicles appearing one might expect a number of protein new components to come up or many at least quantitative changes to come up.

GREENE

We have examined a large number of these two-dimensional gels and we really do not see very many consistent qualitative changes. We do see some quantitative changes, but these are relatively small in number. Some of the latter changes we believe are due to the fact that NGF causes the cells to stop division. There must be a number of proteins whose syntheses are affected by cell division. Also, your point is well taken that it would seem from our structural data that there must be some new proteins. All I can say is that such changes in synthesis would probably be among the ten-thousand non-abundant proteins that are not detected by the two-dimensional gel system. But I agree that one would expect to find changes in the synthesis of other proteins in addition to the ones we have shown. Our major point, nevertheless, is that the relative synthesis of abundant structural proteins is probably unaltered in response to NGF treatment, even in the quantitative sense.

KOSTERLITZ

Have you any information as to whether or not neuroactive peptides are present in your cells?

GREENE

I only have information on substance P, which is not present. I do not know about any other active peptides.

PURVES

Do these cells synapse with one another?

GREENE

We presently have no electrophysiologic or morphologic evidence that PC12 cells synapse with one another. Schubert *et al.* ("Proc. Nat. Acad. Sci. USA", *74*, 2579-2583, 1977), however, have published evidence that PC12 cells can form cholinergic synapsis with striated muscle in culture.

CHAGAS

I would like to know what happens when NGF sources are exhausted.

GREENE

Yes, we have such evidence. The point is, if there is material whose synthesis is stimulated by NGF, then, as suggested by Dr. Chagas, this material ought to be diminished by turnover when synthesis is blocked or when NGF is withdrawn from the cultures. To test this, we have done two types of experiments. One experiment was to treat NGF-treated cultures with the transcription inhibitor camptothecin for 24 hrs before the cells were passaged for regeneration. We found that such pretreatment blocked the capacity of the cells for transcription-independent neurite regeneration. The second type of experiment was as follows. PC12 cells were allowed to grow neurites in the presence of NGF. The cells were then passaged and maintained for various lengths of time in the absence of NGF. After this time, NGF was added back and 24 hrs later the cultures were scored for the proportion of cells that regenerated neurites. The idea of this experiment was that when cells are deprived of NGF, the synthesis of the postulated material should fall to control levels and the level of the material should fall due to turnover. This would in turn result in loss of the ability for rapid regeneration of neurites. This was indeed what we found (Burstein D. E. and Green L. A., "Proc. Nat. Acad. Sci. USA", *75*, 6059-6063, 1978). The longer the cells were deprived of NGF, the less the cells had the ability to regenerate neurites. In fact, we were able to estimate a half-life for this loss of about 16 hrs. This is the evidence we have that cells not only become primed by NGF, but that maintenance of priming requires both transcription and the presence of NGF.

NIRENBERG

When you take NGF-treated cells and withdraw NGF, did the cells seem to be synchronized with respect to their ability to divide?

GREENE

We have done one experiment on this which suggests that they are reasonably well, but not perfectly, synchronized. This is, however, only the result of a single experiment.

NIRENBERG

How long does it take before they double?

GREENE

This depends on the passage number. For very early passage numbers, the doubling time is about four days. For late passages, the doubling time is closer to two days, presumably because of selection for the fastest growing cells.

NIRENBERG

So it takes between two and four days.

GREENE

It takes about 3 or 4 days before the cells re-enter the cell cycle.

BURNSTOCK

Did you say that if you put these cells with muscle they formed processes?

GREENE

This has been reported by Schubert *et al.* ("Proc. Nat. Acad. Sci. USA", *74*, 2579-2583, 1977).

BURNSTOCK

Have you compared their interaction with a potentially densely innervated muscle (for example atrium) with a potentially sparsely innervated muscle (for example uterus)?

GREENE

We have not done this experiment. So far, our own experiments have concentrated on the PC12 cells themselves. Perhaps in a few years, we or someone else will look into the interaction of PC12 cells with other cell types in mixed cultures.

STUDIES ON THE MECHANISM OF NGF-INDUCED NEURITE GROWTH

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INTRODUCTION

Nerve cell differentiation is accomplished through two major essential processes: arrest of cell division and neurite growth. Although it is still unclear whether these two events are induced by a unique signal, it is reasonable to think that this operator(s) acts through a common intracellular mechanism which, following a precise temporal pattern, switch of cell division and starts one of the most impressive changes in cell morphology, i.e. growth and elongation of the axon. Among several intracellular structures, filamentous proteins known as microtubules (MTs, 240 Å diameter) and microfilaments (MFs, 50 Å diameter) are the most likely organelles to partake and play a key role in these two cellular events. It is now widely recognized that MTs and MFs participate as force generating structures in all cell phenomena involving motility (for a review see Goldman *et al.*). In nerve cells, MTs and MFs are actively engaged in mitosis, intracellular transport of substances, change in cell morphology with special reference to neurite growth, exo- and endocytosis. Thus, it is clear that every signal acting or favouring the arrest of cell division and process formation must somehow operate through a control of the function of these two contractile elements.

The discovery by Levi-Montalcini *et al.* (see this volume) of the protein nerve growth factor (NGF) has provided the most valuable tool to investigate the molecular events which accompany the early steps of nerve cell differentiation. Thus NGF not only induces neurite growth but,

in target cells like pheochromocytoma, it also arrests cell division (Greene *et al.*, this volume). Both NGF effects could be realized through different mechanisms.

a) NGF may trigger or stimulate the synthesis of substances instrumental to the process of polymerization of tubulin or actin the precursor elements of MTs and MFs. Some proteins have been isolated and found to favour MTs assembly (Cleveland *et al.*, 1977) or affect actin polymerization and organization (for a review see Korn, 1978).

b) NGF may alter the permeability of cytoplasmic membrane to cations which are known to affect *in vitro* the state of MTs or MFs. A typical example is Ca^{++} which inhibits MTs assembly and activates actomyosin systems. Changing its intracellular concentration or distribution would in turn modulate the functional state of the contractile elements.

c) A third possibility, which we have investigated in our laboratory extensively, is that NGF interacts directly with tubulin and/or actin and changes some of their physico-chemical properties. In this article we will briefly summarize the major features of NGF-tubulin complexes, describe in more detail the action of this growth factor on actin purified from brain and discuss how these interactions could be related with the mecbanism of arrest of cell division and neurite growth.

Materials and methods

NGF, in the 2.5S form was purified as described by Bocchini and Angeletti (1969). Actin from brain was purified with the method described by Bray and Thomas (1976). Analysis of [^{3}H] NGF binding to actin, measures of [^{32}P]-ATP idrolysis and preparation of HMM were performed as described by Calissano *et al.* (1978).

Abbreviations used

NGF	nerve growth factor
HMM	heavy meromyosin
F-actin	filamentous actin
G-actin	globular, unpolymerized actin
MT	microtubule made of tubulin
MF	microfilament made of actin

Results

The finding which demonstrated a direct effect of NGF on tubulin and actin is shown in Fig. 1. When this growth factor is added to a 105.000 × g supernatant of an homogenate of chick embryo brain and incubated for 20-30 min at 37°C, there is an increase in the turbidity of the protein solution. Centrifugation and SDS acrylamide gel electrophoresis of the proteins responsible for this increased turbidity demonstrates that the

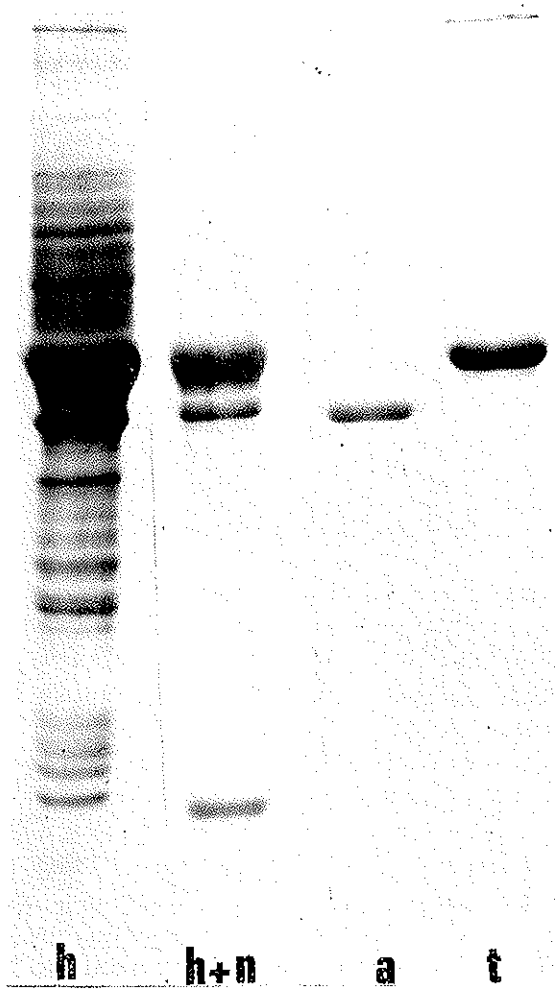

Fig. 1. SDS acrylamide gel electrophoresis of the precipitate of chick embryo brain induced by NGF.
3 g of 12 days chick embryo brain were homogenized in a glass homogenizer with two volumes of buffer A (150 mM NaCl, 0.2 mM of $MgCl_2$, ATP and dithiothreitol and 10 mM Tris-Cl buffer pH 7.4). The homogenate was centrifuged for 90 min at 105.000 × g, the pellet was discarted and the supernatant (5.4 mg proteins/ml) divided in two 0.15 ml aliquots. To one of these samples 50 μg of NGF in 2 mM acetate buffer pH 5 was added while another aliquot was mixed with buffer alone. The two solutions were incubated 20 min at 37°C, layered on 0.5 ml of the buffers used for homogenization + 20% sucrose and centrifuged at 105.000 × g for 30 min. The pellet was dissolved in 1% SDS 8 M urea - 1% mercaptoethanol and analyzed in electrophoresis.
h homogenate of the 105.000 supernatant of chick brain extract
h + n pellet of the homogenate incubated with NGF and centrifuged as described
a actin purified from brain according to Bray and Thomas (1976)
t tubulin purified from calf brain with 3 cycles of polymerization-depolymerization.

pellet is formed by three major constituents which comigrate with purified preparations of tubulin, actin and NGF. This finding clearly showed that NGF can quite selectively precipitate the two major constituents of microtubules (MT) and microfilaments (MF). The apparent analogy of this effect with that previously observed with the antimitotic and anti-microtubular agent vinblastine (Marantz *et al.*, 1969) will be discussed later. A direct interaction between NGF and the precursor elements of MTs and MFs deserved a careful analysis on the specificity and possible involvement in the mechanism of action of the growth factor.

Table 1 reports the summary of the experiments performed on NGF-tubulin interaction. These studies indicate that NGF binds to a site dif-

TABLE 1. *Properties of NGF-Tubulin complexes*

Diss. K. = 1.5×10^{-8}M	(Calissano *et al.*, 1976)
Stoichiometry =	(Calissano and Cozzari, 1974)
NGF-D 2:2	(Monaco *et al.*, 1977)
NGF-MT 1: <20	(Calissano and Cozzari, 1974)
No detectable inhibition by: Colchicine (0.1 mM), Ca^{++}, Mg^{++}, GTP (1.0 mM) Na^{+} (1.0 M)	(Levi *et al.*, Monaco *et al.*, 1977) (Levi *et al.*, 1975)
Rate of assembly = Increased	(Calissano *et al.*, unpublished)
Rate of disassembly = Decreased	(Monaco *et al.*, 1977; Menesini Chen *et al.*,
Resistance to vinblastine in vitro and in vivo	1977)

NGF

D ⇄ MT

ferent from that of colchicine, vinblastine or GTP. Ionic interactions do not seem to play an important role in NGF binding to tubulin since this event also occurs in 1,0 M NaCl or (data not reported) in 40% $(NH_4)_2SO_4$. NGF markedly influences the equilibrium between the pool of tubulin dimers and the MTs. In its presence the rate of assembly is increased and MTs with bound NGF are much less susceptible to cold depolymerization. Thus, when a solution of MTs is rapidly brought to 0-2°C, within 1-2 min no more intact tubules are seen at an electron microscope observation, while in the presence of NGF some intermediate structures, mainly rings, and several MTs are still detectable even after 30 min. Altogether these data would indicate that NGF shifts the equilibrium between dimers and tubules in favour of the latter. Whether it acts

on the nucleation or on the elongation of microtubules remains still to be fully elucidated, although there are indirect indications that NGF acts on the first step i.e. in the nucleation process.

We consider now in more detail the interaction of NGF with actin. Previous work (Bray and Thomas, 1976) had shown that brain actin is not structurally very different from muscle actin in terms of molecular weight and peptide map. At variance with the latter, however, actin from brain does not readily polymerize in partially purified preparations, either because an inhibitor of this process is present in brain extracts or because brain actin has a higher threshold of polymerization. Whatever the explanation, NGF added to this partially purified actin confers to it the ability of assembling at a much faster rate and in larger amounts (Fig. 2). In the presence of this growth factor, actin precipitates and is recovered in the pellet already after 5′ incubation, while in its absence it takes 70 min to have a comparable amount of F-actin in the bottom of the centrifugation tube. As it will be shown in Fig. 4, the pellet formed by NGF actin complexes is not an amorphous aggregate hut a highly organized supramolecular structure. Fig. 3 reports a binding analysis of the growth factor to 4 preparations of fully purified brain actin performed in conditions which do not allow polymerization. At saturation there are 0,7-0,95-1,0-1,25 moles of NGF bound/G-actin with an average of 0,95 moles/mole. When the extent of NGF bound is evaluated with G-actin in conditions for polymerization the ratio is much lower being 1 NGF/7-10 G-actin molecules. These findings suggest that during the process of F-actin formation a pool of NGF molecules is displaced from the filaments and made free, potentially ready for new interactions, or alternatively, that NGF favours initiation of polymerization which then proceeds spontaneously. In both cases the stoichiometry of NGF to F-actin is lower than that to G-actin. The growth factor not only favours F-actin formation, but it also induces its organization to form paracrystalline structures (Fig. 4). They are formed by several single filaments lining in parallel arrays. The actin filaments are in register as shown by the periodicity detectable approximately every 380 Å. A more detailed analysis of NGF actin complexes reported in Fig. 5 showing the optically filtered images of the preparation shown in Fig. 4, reveals an interesting feature of these paracrystals. They exhibit an additive periodicity superimposed on the actin elical structure suggesting that NGF coordinates the actin filaments to form hexagonal arrangements similar to those seen in muscle or non muscle cells where actin forms contractile units. In NGF induced paracrystals, in fact, there are additional meri-

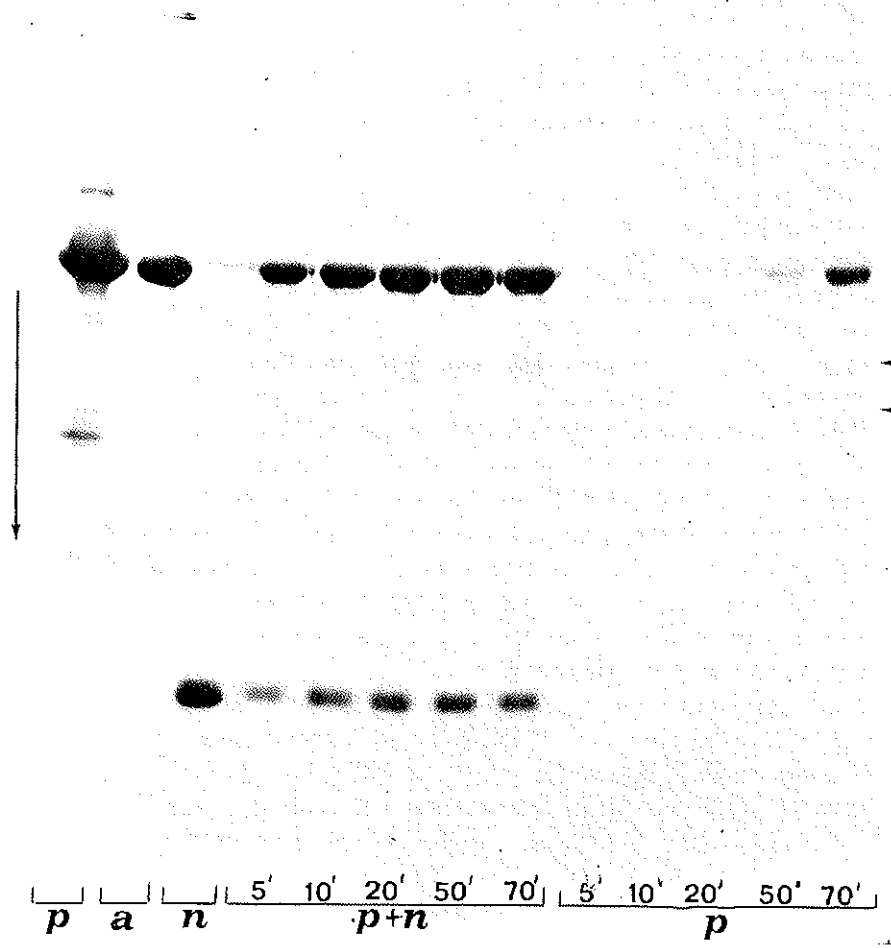

FIG. 2. Sodium dodecyl sulfate/acrylamide gel electrophoresis of actin-NGF complexes. A 12 day chick embryo extract of 40 brains was chromatographed on a G-100 Sephadex column as described (Bray and Thomas, 1976). The pool containing actin with a protein concentration of 0.35 mg/ml was divided into two 10-ml aliquots to which 300 µl of 10 mM acetate buffer, pH 5.0, plus or minus 1.0 mg of NGF and a trace amount of [^{3}H] NGF were added. The two samples were incubated at 37°C, and at time intervals of 5′, 10′, 20′, 50′, and 70′ min 2 ml were withdrawn, layered on 1.0 ml of buffer also containing 10% sucrose, and centrifuged at 165.000×g for 60 min at 20°C. The supernatant was carefully removed and the pellets were dissolved in 0.1 ml of 0.1 M NaOH for protein determination and, subsequently, treated with urea/sodium dodecyl sulphate/mercaptoethanol for electrophoretic analysis. P, G-100 Sephadex actin pool (50 µg of total protein) before incubation. A, 15 µg of actin purified according to Bray and Thomas (1976). N = 15 µg of purified NGF. P+N and P are pellets obtained after incubation for the time indicated in the presence and absence of NGF, respectively. An identical aliquot (50 µl) of each sample was put in each acrylamide well. The arrows on the right point to some minor components having molecular weights of 30,000 and 22,000-25,000 (from P. Calissano *et al.*, 1978).

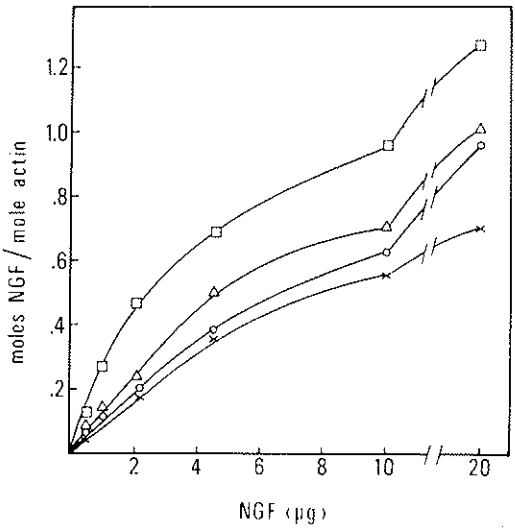

FIG. 3. Binding analysis of [^{3}H] NGF to brain actin. Binding of [^{3}H] labelled NGF to 4 different preparations of G-actin, purified with the method described by Bray and Thomas (1976), was performed incubating different concentrations of NGF with a constant amount of actin (15 μg) in buffer A (see Fig. 1) without NaCl. After incubation, an equal volume of 70% $(NH_4)_2SO_4$ was added, mixed and after standing at 8°C for 30 min, centrifuged at 20.000×g for 30 min. The supernatant was removed and the [^{3}H] NGF precipitated in the pellet counted and taken as a measure of NGF bound to actin, after subtracting the contributions of the growth factor sedimented in the absence of actin. This portion never exceeds 7-12% of the total NGF.

dional reflections, attributable to NGF, at an axial spacing of about 9 nm. Similar arrangements have been observed in actin containing filaments from sea urchin egg where helical actin is coordinated by a 55.000 dalton protein.

An obvious and most important question is: how do these organized structures interact with thick filaments of myosin, the other element of a contractile unit? This is an important question since some other cases of induced paracrystalline organization were found to activate myosin ATPase to a lower extent than actin filaments alone (Korn, 1978). As can be seen in Fig. 6, on the contrary, NGF-F-actin complexes activate myosin ATPase much better than F-actin alone. It is unclear at present whether this potentiation is the consequence of an increased amount of F-actin, the only form of the protein capable of activating myosin, or is the expression of the cooperative interaction between G-actin molecules settled in the potentiating conformation by the bound NGF.

It is well known that when HMM is added to F-actin filaments, in the absence of ATP, the thin filaments appear "decorated" by HMM which gives them the aspect of arrowhead structures (Huxley, 1973). This property has been widely employed not only to reveal the presence of F-actin in non muscle cells, but also to provide information on its polarity. It was of interest to assess the polarity of the single filaments

forming the NGF actin paracrystals. To our surprise, addition of HMM in conditions for decorating F-actin i.e. in the absence of ATP, induces a complete disappearance of the highly organized structure seen before; paracrystalline structure are no longer detectable and the single microfilaments forming a bundle are far apart from each other. Evidently, HMM dissociates tbe NGF actin complex. It has to be ascertained

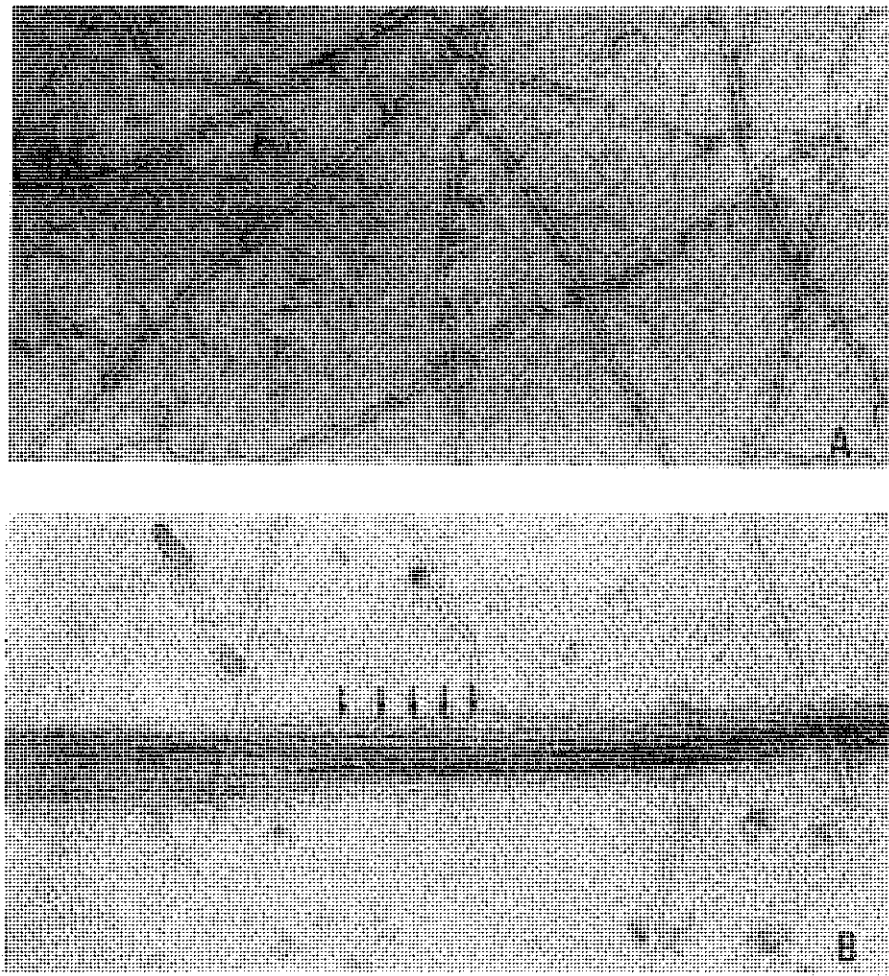

FIG. 4. NGF-F-actin complexes. 100 µl of actin purified from brain at a concentration of 0.3 mg/ml in buffer A (see Fig. 1) was mixed with 10 µl 2 mM acetate buffer plus (B) or minus (A) 10 µg of NGF. The mixture was incubated for 20 min at 37°C and a sample of 20 µl negatively stained with 1% uranil acetate. Magnification = × 140.000. The arrows in B point to cross striations detectable at approximate distance of 380 Å.

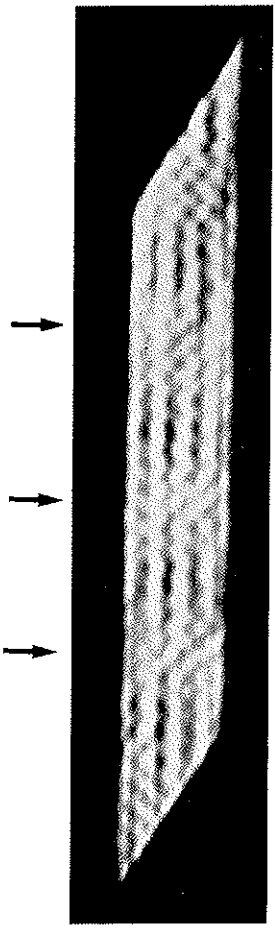

FIG. 5. Optically filtered image of the NGF induced paracrystal shown in Fig. 4.
It can be seen that the paracrystal is formed by several F-actin filaments (each formed by two long pitch helical chains wound round each other). The long pitch strands can be seen forming elongated loops. The arrows point to the stripe positions of the crossover repeat.

whether this effect is achieved through a displacement of NGF bound or through a detachment from each other of F-actin-NGF complexes. Preliminary experiments would indicate that, in the presence of HMM, a portion of NGF detaches from F-actin filament and is made free in the incubation mixture, possihly ready for new interactions.

DISCUSSION

The finding that NGF interacts *in vitro* with tubulin and actin and changes some of their physicochemical properties poses the question if this event may also occur in target cells and how it could be related with the mechanism of NGF induced neurite growth. We may wonder, for

example, how do we fit the specificity of NGF action with the well known wide distribution of tubulin and actin? And how can this growth factor, which acts in catalytic concentrations, affect the properties of these contractile elements which represent between 5 and 20% of total soluble proteins? Finally, what is the mechanism which brings about the physical contact between the externally added NGF and these proteins?

To answer these questions we may attempt to depict a series of subsequent steps in the NGF target cell interaction. Most of these steps have already been shown to occur while some remain open to demonstration. The specificity of NGF action may simply be conferred on the cells by specific receptors. Indeed, in all NGF target cells specific binding sites for this growth factor have been found and thoroughly investigated (Herrup and Shooter, 1973; Banerie *et al.*, 1973). NGF bound to its receptor may come in contact with a pool of tubulin and/or actin either present in the membrane (Blitz and Fine, 1974) or in the cytoplasmic side of the

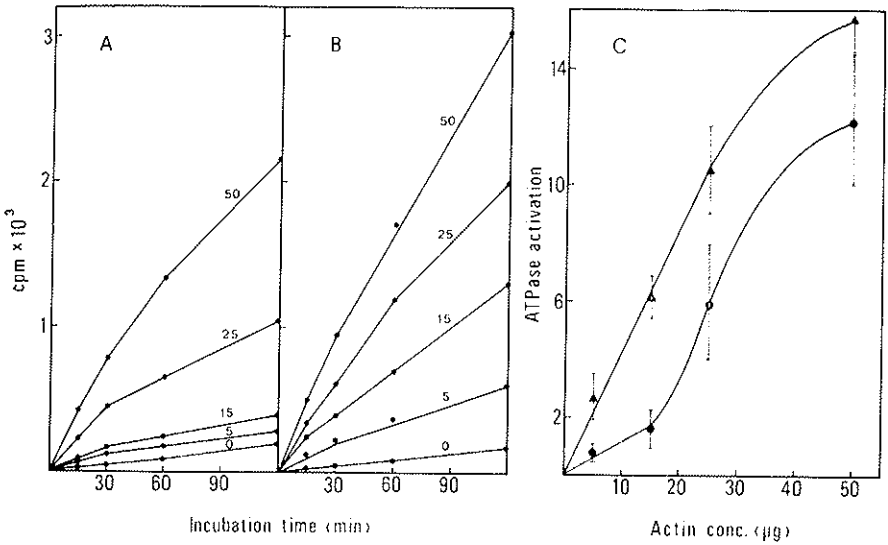

FIG. 6. Activation of HMM ATPase by different actin or NGF-actin concentrations. Brain G-actin (1.0 mg/ml) was allowed to polymerize in the presence or absence of NGF as described in Fig. 4. Different aliquots (ranging between 5 and 50 µg) were then transferred to tubes containing HMM (0.1 mg/ml) in a final volume of 0.25 ml of phosphate buffer containing 2 mM $MgCl_2$ and 0.4 mM $CaCl_2$. The reaction was started by addition of 1.0 mM ATP final concentration and trace amounts of [^{32}P] ATP [^{32}P].
(A) Actin alone; (B) actin-NGF complexes, at the concentration (µg) indicated in the figure. (C) Fold increase of ATPase by different concentrations of actin alone (●) or plus NGF (▲) calculated by taking an average of the three determinations made at the time intervals of each sample tested. Error bars indicate ± SD from Calissano *et al.*, 1978.

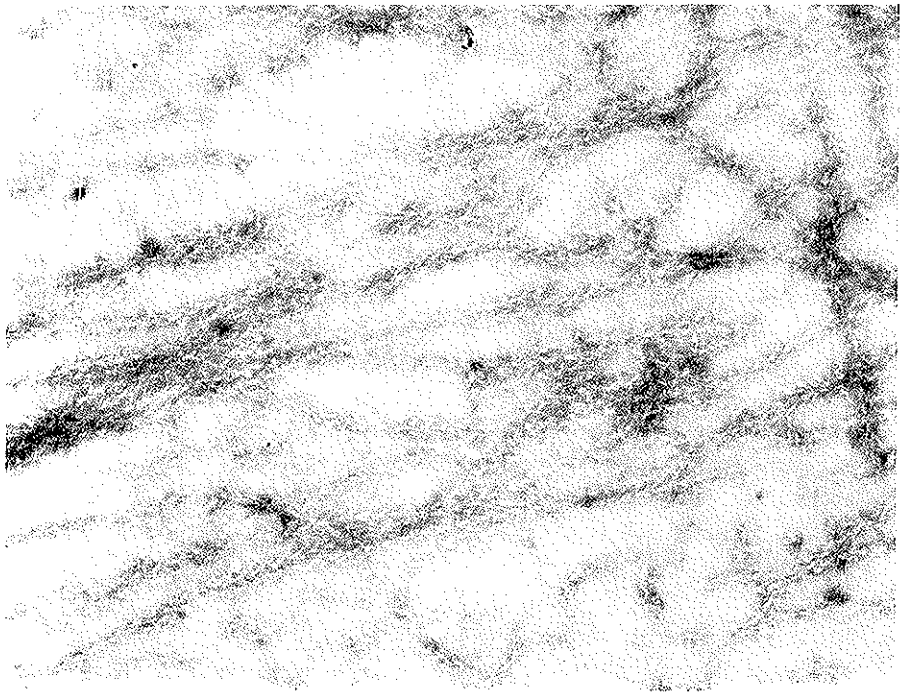

FIG. 7. HMM decoration of NGF-MF complexes. Sixty microliters of G-actin (0.7 mg/ml) was mixed with 0.15 M NaCl and 15 μg of NGF and allowed to polymerize for 60 min at room temperature. Aliquots, 10 μl each, were then mixed with 100 μl of HMM (0.6 mg/ml) in 7 mM phosphate buffer, pH 7.0/100 mM KCl, allowed to incubate for 60 min at room temperature, and negatively stained. Notice the absence of the usual paracrystalline structures seen in Fig. 4 and the presence of a more dissociated array of several MFs (×24.000) from Calissano *et al.*, 1978.

cell. This hypothesis postulates that NGF undergoes some sort of internalization into the target cell. Several experiments performed with a clonal line of a rat pheochromocytoma which responds to NGF (Greene *et al.*, this symposium) have shown that a large fraction of the total NGF bound to its receptors becomes tightly bound, resistant to trypsin digestion and cross reacts with monospecific ^{125}I-labelled antibodies only when the cells, after fixation in paraformaldehyde, are treated with triton to solubilize the cytoplasmic membrane. These experiments suggest that indeed a pool of NGF is internalized and has, on a theoretical ground, the possibility of interacting with membrane bound or cytoplasmic proteins. It is now widely demonstrated that both tubulin and actin are present in the context of the membrane of nerve cells (Blitz and Fine,

1974; Bhattacharyya and Wolff, 1975) although the biological significance of this presence is still obscure. One possibility is that they constitute a pool of molecules in functional contact with the larger cytoplasmic fraction. If we assume that a formation of NGF-tubulin or NGF-actin complexes occurs after binding of this growth factor to its receptors, we may now wonder how they could participate and play a key role in the process of neurite growth, also in view of the small amount of this growth factor (1-10 ng/ml) sufficient to trigger this event. Knowing the approximate concentration of this growth factor bound and probably internalized in PC12 cells at saturation of receptors (1-3 ng/10^6 cells) and the concentration of tubulin and actin (1-5 μg/10^6 cells) we may calculate an approximate ratio of 0.2-1.0 NGF to 10^3 contractile molecules. This ratio would be at least an order of magnitude higher ($1:10^2$) if we postulate an interaction confined to certain areas of the cell or to the cytoplasmic membrane. Since an MT 1.0 μm long is made of 1.6×10^3 dimers or an MF of the same length is formed by 3.6×10^2 G-actins, we may calculate that in an intact cell approximately 1.0 NGF might be bound to each contractile unit or, in the case of a confinement to certain areas of the cell, it may exceed that ratio and it could also be present along their entire length or playing some other role. How can NGF affect some properties of MTs and or MFs in such low ratio? An excellent analogy may be drawn with vinblastine, a vinca alkaloid widely used in clinical therapy as an atimitotic drug for its capacity of altering the structure and function of MTs. This substance exerts an *in vitro* inhibitory action on tubulin polymerization at relatively high concentrations (10^{-4}-10^{-6}M) while *in vivo* 50% of its antimitotic action has been calculated to occur at vinblastine/tubulin ratio of 1/200-1000 i.e. with largely substoichiometric amounts (Wilson *et al.*, 1976). The most convincing hypothesis on the *in vivo* mechanism of action of this alkaloid, as well as of other antimicrotubular agents, is that they bind only to the terminal end of a growing tubule and inhibit further addition of new tubulin dimers by capping the MT (Margolis and Wilson, 1977). As a consequence, the equilibrium between MTs and dimers is rapidly shifted toward the latter and the end result is an induced depolymerization. An analogous situation may be postulated to occur for NGF, with the crucial difference that this growth factor has an opposite effect on MTs since it shifts the equilibrium, at least *in vitro,* toward the organized form. Thus, the effects of NGF on tubulin *in vitro* have been up to now obtained with stoichiometric or slightly substoichiometric amounts, but *in vivo* NGF may act in a vinblastine-like fashion, at much lower concentrations, even because it

may be sufficient to interact with a small pool (e.g. the portion membrane bound) to control and modulate the totality of tubulin or actin. The opposite effects of NGF and vinblastine on tubulin may explain the antagonism that the growth factor exerts, *in vitro* and *in vivo* on the destructive action of this vinca alkaloid (Monaco *et al.*, 1977; Menesini Chen *et al.*, 1977). It is worth mentioning, also, that in view of the higher affinity for tubulin, NGF, if present at the same concentration as vinblastine, has better chances of forming complexes with tubulin than the vinca alkaloid.

We may now attempt to visualize how NGF-tubulin, NGF-actin complexes or both may participate and possibly guide the process of neurite growth. As mentioned in the introduction, this event is intimately connected with the structural organization of MTs and MFs. A large body of evidence shows that hoth structures are present in the differentiating neuron. While MTs are mainly confined to the cytoplasmic area running radially from the perinuclear zone toward the cell periphery (Marchisio *et al.*, 1978) and converging into the neurites without establishing contacts with the edge of the growth cone, MFs are more peripherally located and are always associated with motile areas (Small *et al.*, 1978) in the growth cone and its microspikes. If we confine our hypothesis to the events occurring in this area of the growing neurites we may postulate that NGF comes in contact with a pool of actin either in the globular (G) or filamentous form (F). Assuming that this growth factor acts like *in vitro,* the end result of this interaction is an increased amount of F-actin and its organization in bundles or paracrystals. These structures, similar to stress fibers, have been postulated to provide the basic elements necessary to allow cyclic movements generated by a sliding between MFs anchored to the cytoplasm and mobile filaments (e.g. myosin) in the interior of the cell (Huxley, 1973). As the front part of the growth cone is pushed forward by the internal pressure generated by the shearing between bundles of MFs and internal structures, new attachment and nucleation sites for actin filaments might be created by a pool of NGF released after actin-myosin interaction and consumption of ATP. The whole cyclic process of NGF binding to actin, induced polymerization and assemblage to form bundles, followed hy detachment of NGF after myosin interaction, may provide a transient, reversible system of contraction utilized by the growth cone as a saltatory mechanism of movement and exploration of the environment.

As for NGF-tubulin interplay, it can be better visualized for the pool of the growth factor actually penetrated into the cell cytoplasm. Here

NGF bound to tubulin could play, for instance, an important role in the control of cell division, through stabilization of the MTs of the mitotic spindle. Arrest of cell division, an essential step in nerve cell differentiation, could be achieved by NGF, at variance with antimicrotubular agents like vinblastine or colchicine, with a mechanism of stabilization (Table 1) instead of disassembly of MTs. In this way, stabilized MTs could still participate in the many functions required for the process of nerve cell differentiation and axonal growth. Although we realize that an actual interplay *in vivo* between NGF, tubulin and actin is still to be proved, this hypothesis provides a molecular model for the mechanism of action of NGF which does not involve a second messenger (no convincing evidence has been provided for changes in cAMP or Ca^{++} in NGF target cells) and can be proved or disproved with a precise experimental approach.

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DISCUSSION

BURNSTOCK

It has already been mentioned this morning by Rita Levi-Montalcini that guanethidine can cause destruction of adrenergic neurons. If you look at how it achieves this in culture (or *in vivo* for that matter) you see that it causes retraction of the fibres before it finally kills the whole cell. Now Rita and her colleagues have shown that NGF opposed this extraordinary retractive effect of guanethidine. Would you care to speculate about the mechanisms involved here?

CALISSANO

I wonder whether the precise mechanism of action of guanethidine is known. If we assume that its primary action is exerted on mitochondria where it inhibits their capacity of accumulating intracellular Ca^{++}, then the effect exerted by guanethidine in neurite retraction and degeneration and the antagonism by NGF in this event may be visualized as follows. Guanethidine induces a release of Ca^{++} from mitochondria; Ca^{++} in turn will cause, as it does *in vitro,* a disassembly of MTs which play an essential role in neurite growth and elongation. As a final consequence, neurite will retract and degenerate. NGF could antagonize guanethidine by stabilizing MTs in their assembled state as it does *in vitro.* In this way it could prevent disassembly of MT and consequent neurite retraction.

PURVES

NGF has a trophic effect independent of the presence or absence of neurites. Do you imagine that this is, in some sense, related to what you talked about?

CALISSANO

At the present state of our knowledge we do not know whether NGF trophic action and NGF-mediated neurite growth are achieved by a unique mechanism or, alternatively, by two distinct actions. I wish to stress, however, that an interaction of NGF with contractile proteins (tubulin and actin)

in a living cell could set in motion and control many different intracellular events so that, depending upon the particular metabolic and differentiative state of the cell, NGF could exert a simple trophic action or it could also play an essential role in neurite growth.

HAMPRECHT

Tubulin assembly consists of two processes: nucleation and growth. Which one is affected by NGF?

CALISSANO

I do not have a precise answer. My guess is that NGF affects only the nucleation step.

HAMPRECHT

May I ask a second question? You mentioned that NGF affected calcium flux through the membranes - influx or efflux?

CALISSANO

No, this is a hypothesis. I would say there are no proven theories against the idea that calcium fluxes are affected by NGF.

NIRENBERG

Does NGF affect the electrophysiological activity of nerve cells?

CALISSANO

I think that perhaps Dr. Green can better answer this question.

GREENE

No, I cannot really answer the question. In preliminary experiments there were no changes in the resting potential as measured with micro-electrodes. On the other hand, recording from cells, as you know, does not really give the true resting potential of the cell. So, that could be an artifact. So I really cannot answer the question.

HAMPRECHT

Phalloidin causes the formation of F-actin. Does it also cause the release of NGF from actin to which previously NGF was bound?

CALISSANO

We bave not done that experiment, yet. We tried to follow the effect of phalloidin on NGF-binding to specific receptors and we found no detectable action by this peptide on NGF binding.

GREENE

There is a question that we have discussed before many times. That is the question of charge interaction, which you alluded to. NGF is a very basically charged molecule and there is some evidence that basically charged molecules can also interact with tubulin. I wonder if you could elaborate on this issue.

CALISSANO

This is a crucial problem which we have faced since the beginning of our investigation on NGF-tubulin or NGF-actin interaction. How specific is this binding also in view of the net different charges which the two molecules have and which could play a role in favouring their reciprocal association? Definition of specificity in this interplay is purely operational i.e. it is based on a series of physico-chemical measurements which we have obtained in a test tube. From this point of view the interaction is quite specific. Thus, it is not inhibited by higb ionic strength (1.0 M NaCl), it exhibits a precise stoichiometry and it must occur at a specific site in tubulin which is not common to that responsible of binding colchicine, vinblastine, GTP or Ca^{++}. Moreover, as shown in my presentation NGF changes some physico-chemical properties both of tubulin and actin favouring their supramolecular organization. In conclusion, at present state of our investigation, it is in my opinion futile trying to define NGF interplay with tubulin and actin as specific or non specific. The clear cut answer to this problem will only come when and if we will be able to demonstrate that indeed NGF comes in contact with these proteins in its target cells. If that will be the case, any aspect of the *in vitro* interaction will be important and deserving the term "specific". In the opposite case all these studies will simply represent a peculiar *in vitro* phenomenon with no essential relevance to the mechanism of action of NGF.

FAMBROUGH

Just a comment about electrical responses to the peptide hormones. I have done a few experiments with a colleague of mine to see if there are any large, fast responses to nerve growth factor or epidermal growth factor, and there do not seem to be any easily measured fast responses. But, of course one has to do a very careful and detailed analysis to learn about some of the other possible responses.

I have a question. Do you know what the problem is in familial dysautonomia, which I think is related to nerve growth factor or responses to nerve growth factor? Perhaps Dr. Levi-Montalcini could answer this question.

LEVI-MONTALCINI

Familial dysautonomia is a complex syndrome characterized by a large number of impaired functions such as corneal anaesthesia, poor taste discrimination, deficient vestibular reflexes, insensitivity to pain and temperature indicative of sensory abnormalities and prominent and widespread deficiency of sympathetic and parasympathetic systems. It has been claimed by some authors that patients suffering from this disease have a decreased NGF content in their blood. However the results of NGF determinations in the blood of human individuals or animals by radioimmunoassay, vary from one to another laboratory by one and even more orders of magnitude and are therefore devoid of significance. Of more interest in this connection, are some recent findings by Luigi Aloe in our laboratory. He found that rat foetuses injected with a specific NGF antiserum, develop after birth severe deficiencies of sensory, motor and sympathetic functions, are retarded, smaller than controls and if they reach maturity show sexual disfunctions. Most of these last symptoms are not part of the genetic disease known as familial dysautonomia and for this reason I do not believe that they lend support to the hypothesis that familial dysautonomia results from impaired synthesis of NGF in human patients, although this hypothesis cannot be dis-missed at present.

NELSON

I wonder if you would just comment on whether you feel that stabilization of cytoskeletal proteins is sufficient to account for this extraordinary NGF effect on nerve fiber outgrowth or do you see another class of phenomena necessary to explain those findings?

CALISSANO

First of all I wish to stress that stabilization of microtubules by NGF is not the only event occurring in NGF-tubulin interplay; rather, it seems to me as a sort of end product of the general effect exerted by NGF, which consists in shifting the equilibrium between dimers and microtubules toward the latter. In connection with your question, my answer is yes: I do believe that if we assume that NGF does in a living cell what it does *in vitro* on tubulin and actin, then the multiple effects called forth by this growth factor could be simply mediated via an interaction with these two proteins. If one considers the many essential intracellular events controlled by the tubulin or actin in their organized form (cell division, cell shape, axonal growth, receptor motility, exo and endocytosis, etc.) it is reasonable to assume that a protein changing some of their physico-chemical properties may in turn control several major intracellular events. I wish to add that when I think of the mechanism of action of NGF as compared to some hormones, I visualize its action not as mediated through the typical amplification system of a second messenger which in its turn activates in a cascade reaction, several other proteins, typically enzymes of a given metabolic pathway. I think that NGF itself could play a role as second messenger controlling several functions not through a cascade reaction which involves many different proteins but controlling the function only of one or two proteins (e.g., tubulin and/or actin) which, however, play a fundamental role in several intracellular events as those that I have mentioned above.

PURVES

There is always the problem of the mechanism of action of anti-NGF; I wonder if what you have described sheds some light on this mechanism?

CALISSANO

I think that the action of antibodies to NGF in destroying sympathetic nerve cells *in vivo* or in inducing neurite retraction and degeneration *in vitro* could simply be achieved by binding and neutralizing circulating NGF or NGF present in tissue culture. In this fashion antibodies would remove the factors which play *in vitro* an important role in stabilizing the structure and favouring the function of those elements (microtubules and microfilaments) which are known to partake of several intracellular functions including neurite growth.

PURVES

If that is the case, shouldn't one see some morphological correlates of the effects of anti-NGF that you described? In fact, one sees nuclear effects first, as I think you showed earlier this morning.

CALISSANO

The fact that one of the first effects noticeable after NGF antibodies addition is in the nuclear area does not exclude that they are achieved through a removal of a pool of NGF interacting with tubulin. Thus, perinuclear sites of tubulin assembly are present and well documented in, e.g., neuroblastoma cells, and NGF itself has been shown in recent studies to accumulate in the nucleus of target cells. An alternative possibility is that nuclear damages elicited by NGF antibodies are achieved through some still unknown mechanisms perhaps involving complement activation.

CHAGAS

One of your first slides showed that there were some molecules. Is that real evidence or just a guess?

CALISSANO

Up to now it is just a guess since we do not know, as yet, whether patching is a process induced and following NGF binding to its receptors or whether it is induced by the NGF antibodies employed to localize NGF on the surface of the cells.

TUMOR SPECIFIC SURFACE PROPERTIES OF MALIGNANT C1300 NEUROBLASTOMA CELLS

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THE MURINE C1300 NEUROBLASTOMA

The murine C1300 neuroblastoma is a transplantable tumor which arose spontaneously in the body cavity of an albino, male, newborn A/Jax mouse, and was tentatively identified as a neuroblastoma (Dunham and Stewart, 1953). It was originally maintained by serial transmission in susceptible syngeneic mice and then, about ten years ago, adapted to grow in continuous culture (Augusti-Tocco and Sato, 1969; Schubert *et al.*, 1969). When inoculated subcutaneously into an A/Jax mouse the cells give rise to a solid tumor at the site of the inoculum and the mouse eventually succumbs to the large progressively growing tumor mass. The biochemical and morphological characteristics of these cells show striking resemblances to the human neuroblastoma, a major neoplasm in children (Prasad and Kumar, 1974).

Cells of the C1300 NB line were the first examples of neural [1] cells displaying reversible differentiation in cell culture (Seeds *et al.*, 1970), and for many years have been considered as an *in vitro* model for the growth, differentiation and function of sympathetic nerve cells. The cells grow *in vivo* and *in vitro* in a relatively homogeneous form and can be easily cloned. *In vitro* most clones grow in suspension in a round almost anaplastic form; some clones, however, when grown in culture vessels to which they can attach, at a low serum concentration, or when cultured

[1] The term "neural" is used here simply as meaning "of the nervous system".

in the presence of one of the many varied chemical inducing agents, undergo neuronal differentiation. They rapidly emit cytoplasmic processes and acquire metabolic and morphological properties characteristic of mature neurons (Fig. 1) (Nelson *et al.*, 1969; Schubert *et al.*, 1969; Blume *et al.*, 1970; Harris and Dennis, 1970; Schubert and Jacob, 1970; Seeds *et al.*, 1970; Furmanski *et al.*, 1971; Harris *et al.*, 1971; Kates *et al.*, 1971; Nelson *et al.*, 1971; Schubert *et al.*, 1971 a; Schubert *et al.*, 1971 b; Amano *et al.*, 1972; Minna *et al.*, 1972; Prasad, 1972; Richelson, 1973; McMorris and Ruddle, 1974; Gilbert, 1976; Kimhi *et al.*, 1976; Bertolini *et al.*, 1977; Ishii *et al.*, 1978). Morphologically differentiated cells can express new membrane structures (Glick *et al.*, 1973; Prasad and Kumar, 1974; Truding *et al.*, 1974; Prasad, 1975; Glick, 1976) and antigenic determinants (Akeson and Herschman, 1974 a; Akeson and Herschman, 1974 b) not detectable on uninduced cells grown in suspension. The availability of these homogeneous populations of cloned cells in large quantities has facilitated the study of dissected type-specific cell functions as well as the study of cell surface components which mediate the complex cellular interactions occurring in the development of the nervous system. Different clones may express various differentiated phenotypic traits to differing extents either in suspension or in response to the same inducing agent. Thus even if coordination of differentiated phenotypes and neural properties may occur in these cells after the induction of cell differentiation (Minna *et al.*, 1972), each differentiated phenotype should probahly be viewed as the result of positive or negative genetic controls rather than the result of a co-ordinated sequential phenotypic expression in the course of a programmed neural differentiation (McMorris and Ruddle, 1974).

Cell Surface Antigenic Markers

Of particular relevance is the identification of the surface constituents of tumor nerve cells as differentiation markers, serving to identify cell populations of the same type, and to study correlations between neural specific and tumor-associated surface antigens. Several immunological markers have been identified on C1300 NB cells (Table 1). These are termed antigens because they have been defined by serological techniques, but this does not imply that they are necessarily antigenic in the A/Jax mouse. Xenogeneic antisera have usually been raised in rabbits or rats against whole NB cells, either in the undifferentiated or in the differentiated form. Anti-species specificities are then absorbed from the sera on

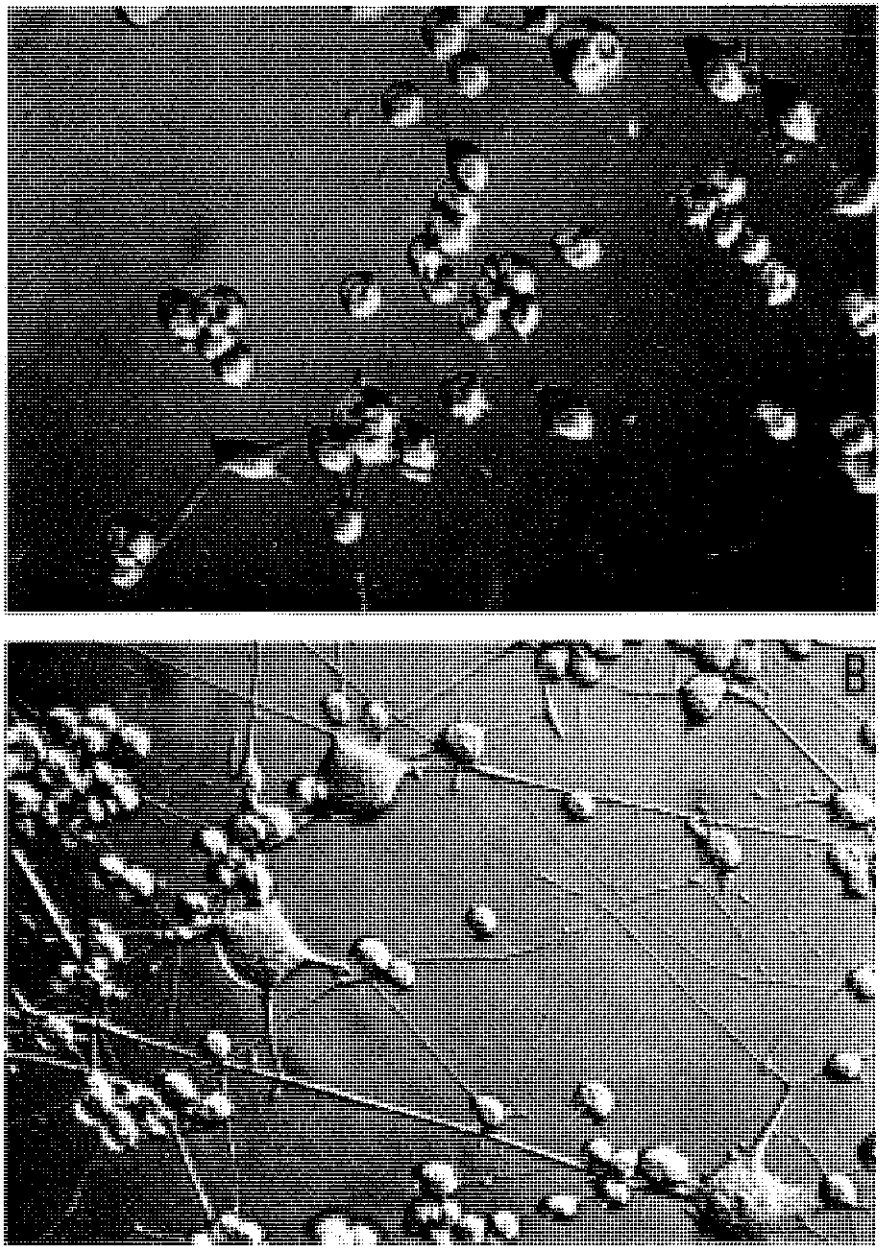

FIG. 1. Photomicrographs of C1300 neuroblastoma cells (clone NB1R) grown in suspension (A) or in monolayer (B). (A) the cells were grown in bacteriological petri dishes in Dulbecco's modified Eagle's medium (EDM) with 20% FBS in an atmosphere of 10% CO_2 and air. (B) the cells were grown in Falcon petri dishes in EDM supplemented with only 1% FBS: under these conditions the cells attach to the culture vessels, cease to divide rapidly, emit extensive cytoplasmic processes and neuronal-type cell differentiation occurs. FBS = Fetal Bovine Serum.

TABLE I. *Distribution of surface antigens on mouse C1300 neuroblastoma and other tissues, as detected serologically.*
Legend: H-2[a] = histocompability, haplotype a; Thy = Thymus and brain antigen; Pc = Plasmacell antigen(s); Sk = Skin cell-associated antigen(s); Ly = lymphocyte-associated antigens; MuLV = murine leukemia C-type RNA

	H-2	Thy-1	Pc	Ly-1	Ly-2,3
C1300					
— solid tumor	+	±	+	—	—
— tissue culture line:					
non-attached "undifferentiated"	±	—	±	—	—
attached "differentiated"	+	—	+	—	—
Foetal brain	±	±	+	—	—
Adult brain	+	+	+	—	—
Kidney	+	—	—	—	—
Sperm	±	—	—	—	—
Embryo	+	—	±	—	—
Neuronal tumors	±	±	±	—	—
Glial tumors	+	?	?	—	—
Plasma cells	+	—	+	—	—
Lymphocytes (spleen)	+	+	—	+	+
Erythrocytes	±	—	—	—	—
Epidermal cells	+	—	—	—	—
Antisera raised in:	M	M Rb Rat	M Rb	M Rb	M Rb

M = Mouse; Rb = Rabbit; Rat = Rat; G = Goat; Mns = Mouse natural serum antibodies.

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4) Chaffee and Schachner, 1978.
5) Esber et al., 1979.
6) Faraggiana et al., 1978.
7) Fields et al., 1975.
8) Fiorani et al., 1978.
9) Martin, 1974.

virus (Gross); MBA = mouse brain antigen; HFB = human foetal brain antigen (s); CerS.A. = cerebellar specific antigen(s); Ran 1 (common) = rat nervous antigens; NS = nervous system antigens; MuLV TAA = antigen(s) detected on NB cells but also on C-type RNA viruses; TA = NB-tumor associated transplantation antigen (? = no clear evidence).

		Neuronal restricted					Glial restricted			Non-restricted			NB-Tumor restricted	
MuLV gp71	MuLV p30	MBA-1	MBA-2	HFB	CerSA	Ran	NS-1	NS-2	NS-4	NS-3	NS-7	NS-A	MuLV TAA	TA
+	+	+	?	±	—	—	?	?	—	+	+	±	+	+
+	+	±	—	+	—	—	?	?	—	±	±	+	+	+
+	+	+	—	+	—	—	?	?	—	+	+	+	+	+
+	±	+	+	+	?	+	—	—	+	—	+	+	+	+
—	—	+	+	+	+	+	+	+	+	+	+	+	—	—
—	—	+	+	—	+	+	—	—	—	+	±	—	—	—
±	±	—	+	?	+	—	—	—	+	—	+	—	±	—
±	±	?	+	?	?	+	—	—	+	—	+	+	±	—
±	±	+	+	+	+	+	—	—	—	+	+	—	±	—
±	±	—	—	—	—	+	+	+	+	+	+	—	±	±
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
±	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
—	—	?	—	—	—	—	—	—	—	—	—	—	—	—
M G Mns	M G	Rat	Mns	Rb	Rh	M	M	M	Rb	Rh	Rb	Rh	M Rb	M Rb
2, 6, 10	6	9	10	1	17	3, 7	12	13	14	15	4	16	2, 5, 6, 8, 18	

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17) Seeds, 1975.
18) Terman et al., 1975.

normal A/Jax cells (Akeson and Herschman, 1974 a; Akeson and Herschman, 1974 b; Schachner, 1974; Schachner and Wortham, 1975; Faraggiana *et al.*, 1978; Fiorani *et al.*, 1978; Esber *et al.*, 1979; Berwald-Netter and Kulakoff, 1979). Mouse antisera reacting with C1300 NB cells have been produced in several laboratories, either by inoculating viable cells into strains of mice not susceptible to the tumor growth, or by inoculating aldehyde fixed, or Rx inactivated cells into A/Jax mice (Schachner, 1974 Schachner and Wortham, 1975; Faraggiana *et al.*, 1978; Esber *et al.*, 1979). Alternatively, antisera can be raised in A/Jax mice by inoculating cell membranes purified from C1300 NB cells (Berwald-Netter and Kulakoff, 1979). These immunizations protect the mice against subsequent challenge with viable C1300 NB cells. However, tumor growth is enhanced if the inactivated cells or cell membranes are inoculated at the same time or after the viable cell challenge (Bertolini *et al.*, 1976). A/Jax antibodies reacting with C1300 NB cells have also been obtained from mice with growing C1300 NB tumors (Oldstone, 1975; Terman *et al.*, 1975; Faraggiana *et al.*, 1978).

These antisera with anti-tumor specificity have proved to be directed mainly against four classes of antigens: 1) antigens associated with C-type RNA virus (core and coat proteins) (Schachner, 1973; Martin and Martin, 1975 a; Martin and Martin, 1975 b; Faraggiana *et al.*, 1978; Berwald-Netter and Kulakoff, 1979); 2) antigens similar to, or the same as, antigens present on other neural or non-neural tissues (normal, embryonal or neoplastic) (Martin and Martin, 1975 a; Martin and Martin, 1975 c; Schachner, 1974; Schachner and Wortham, 1975; Esber *et al.*, 1979); 3) histocompatibility antigens (Schachner, 1973; Berwald-Netter and Kulakoff, 1979; Procicchiani *et al.*, 1979) and 4) antigens present in all cells of C1300 NB lines tested, but not detected on normal tissues. These latter antigens will be referred to as tumor-associated transplantation antigens (TA) (Martin and Martin, 1975 b; Terman *et al.*, 1975; Faraggiana *et al.*, 1978; Berwald-Netter and Kulakoff, 1979; Butler and Revoltella, 1979; Esber *et al.*, 1979).

All known cell lines of C1300 NB release little or no C-type RNA virus particles, while in most clones A-type virus RNA particles can be clearly seen (Stringner and Wivel, 1973; Bosman *et al.*, 1975; Martin and Martin, 1975 b). Several clones of C1300 NB, however, express large quantities of antigens cross-reacting with C-type RNA (Gross) virus associated antigens, particularly the coat glycoprotein gp 69-71 and the core protein p30 (Schachner, 1973; Faraggiana *et al.*, 1978; Berwald-Netter and Kulakoff, 1979; Butler and Revoltella, 1979). This raises a

series of still unanswered questions about the nature and origin of these molecules. In addition to possible roles in tumor growth (see later) they may have a role in influencing normal cell differentiation (Huebner and Todaro, 1969; Huebner *et al.*, 1970; Temin, 1971; Temin and Baltimore, 1972). Evidence in support of this hypothesis has been provided by the detection in normal embryonic tissues of C-type RNA virus particles and virus specific proteins, which are usually not expressed in adult tissues (Feldman *et al.*, 1967; Huebner *et al.*, 1970; Fowler *et al.*, 1972; Strand *et al.*, 1974; Del Villano and Lerner, 1976; Lieberman and Sachs, 1978). In view of the known polymorphism of several C-type virus associated antigens (e.g. the gp 69-71 glycoprotein (Del Villano and Lerner, 1976; Elder *et al.*, 1977), a relationship has been proposed between viral gene expression and certain pathways of cell differentiation and neoplasia.

Two neuronal antigens have been recognized on neuroblastoma cells. These have been named mouse brain antigens (MBA) 1 and 2. MBA 1 is present on undifferentiated and on differentiated C1300 NB clones, and on normal mouse brain cells, but not, for instance, on rat nerve cells (Martin, 1974). Antibodies to MBA 1 have been raised in rats but not, as yet, in mice (Esber *et al.*, 1979). MBA 2 was discovered in a mouse neuroblastoma designated NB1 derived from an adrenal metastasis of a primary ovarian teratoma which arose spontaneously in a C_3H/He Icrf mouse (Martin and Martin, 1975 c). MBA 2 has been found on brain and kidney cells of various mouse strains, in normal gray matter from 7-10 day old mouse embryos, and on brain tissue from a variety of species. It has also been detected on human kidney tissue and on human neuroblastoma cells, but not on mouse or human glial cell lines and not on other cell lines of mouse neuroblastoma (Martin and Martin, 1975 a). It has been suggested that MBA 2 is a neuroembryonic antigen which demonstrates a highly restricted interspecies variability (Esber *et al.*, 1979). Table 1 resumes the characteristics of neuronal associated antigens and focuses attention on the neuronal antigens detectable on neuroblastoma cells.

Mouse allo-antisera raised against private $H\text{-}2^a$ antigenic determinants proved to react with C1300 NB cells. Usually these anti-H-2 antibodies revealed significant differences between the various C1300 NB lines and clones (Berwald-Netter and Kulakoff, 1979; Revoltella and Butler, 1979 b). In general histocompatibility antigens are poorly expressed in NB cells, just as they are poorly expressed in normal mouse neural cells

(Vitetta and Capra, 1978) where they first become detectable in 16 day old embryos.

TA antigens have been detected in all lines and clones of C1300 NB so far tested, using both xenogeneic and isogeneic antisera (Esber *et al.*, 1979; Berwald-Netter and Kulakoff, 1979; Butler and Revoltella, 1979; Revoltella and Butler, 1979 b). The specificity of anti-TA antisera was proved by radioimmunological and immunofluorescence assays after extensive absorption on normal A/Jax cells and on primary cultures of normal and embryonic sympatbetic cells. The antisera retained anti-C1300 NB activity but did not react with H-2, virus associated proteins, or neural antigens.

The reactivity of the various antisera with the different clones of C1300 NB did not parallel each other, suggesting that the antigens are on distinct molecules (Butler and Revoltella, 1979; Revoltella and Butler, 1979 b). An example of non-coordination between H-2 and TA in different NB clones and somatic cell hybrids is reported in Fig. 2.

Preliminary evidence has indicated that anti-neuroblastoma monoclonal antibodies obtained *in vitro* from hybrid cells cloned after somatic cell fusion between mouse plasmacytoma cells and spleen lymphoid cells from rats or mice immunized against C1300 NB cells may provide a very useful tool for the characterization of unique and shared cell surface neuroblastoma antigenic determinants (Kennett, 1979; Revoltella and Butler, 1979 b).

Variations in membrane antigen expression during cell differentiation

There is clear evidence that neuroblastoma cell differentiation results in antigenic alterations on the cell membrane (Glick *et al.*, 1973; Akeson and Herschman, 1974 a; Truding *et al.*, 1974; Revoltella and Butler, 1979 b). The fact that these changes are concomitant with other physiological and morphological modifications of the cells strongly suggests that the new membrane antigens must play a role in the function of the differentiated cell. Many changes that occur at the cell periphery following differentiation have been widely documented since the description of the phenomenon of process formation and elongation described by Seeds *et al.* (Seeds *et al.*, 1970). Most of these changes involve the exocytoskeleton or the endocytoskeleton of the cell. Much less is known about the membrane integral proteins which are associated with the

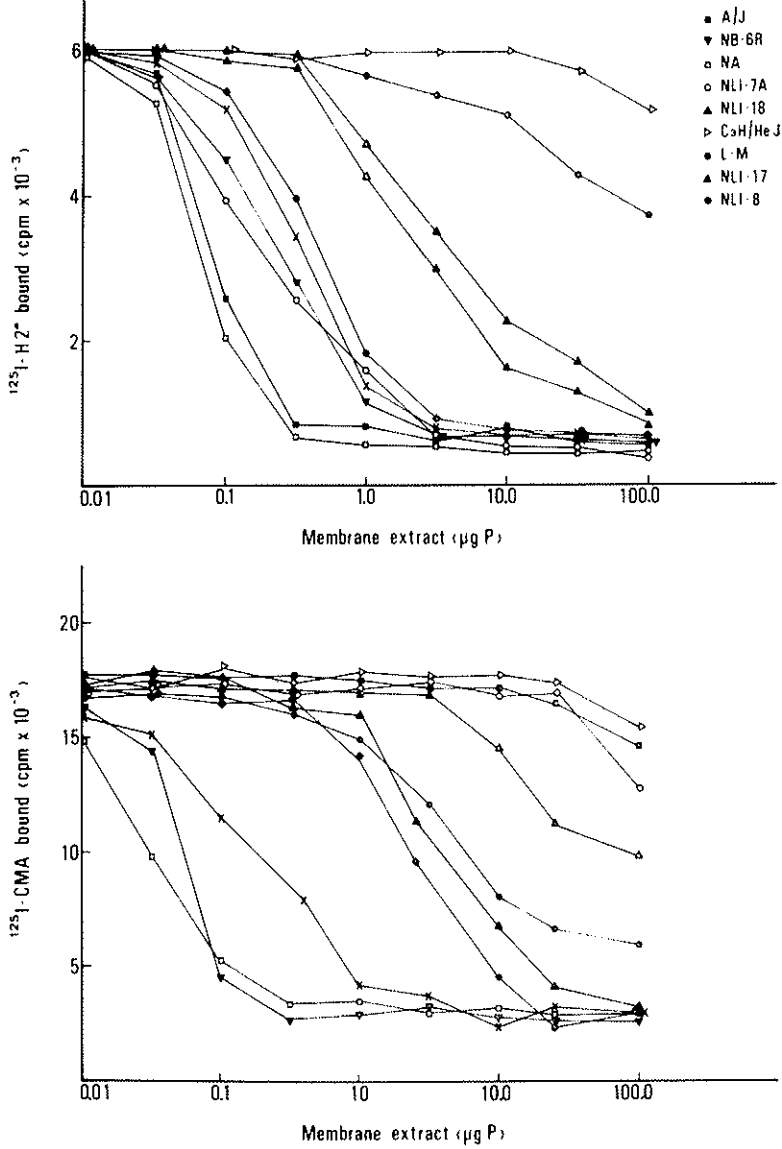

FIG. 2. Analysis of histocompatibility and tumor type transplantation antigen in membrane extracts of neuroblastoma cell clones (NA, NB1R, NB6R) and hybrids. The hybrid clones (NLI-7a, 8, 17, 18) were formed from the fusion of neuroblastoma clone NA (H-2^a haplotype, an HGPRT$^-$ clone) and the mouse fibroblastic cell line L-M (H-2^k haplotype, a TK$^-$ clone) (McMorris and Ruddle, 1974). Measurement was carried out by competition radioimmunoassay, as described by Ihle *et al.* (1974). For measuring H-2 antigen, 20 ng of [^{125}I] - B10.A (= H-2D4) (prepared essentially as described by Strober *et al.*, 1970) and (B10.A. AKM × 129/J) anti (B10.A) antiserum (anti-H-2D4) at 1:200 dilution were used. For measuring tissue-type transplantation antigen, 30 ng of ^{125}I-labelled solubilized crude membrane antigen (CMA, prepared as described previously by Faraggiana *et al.*, 1978) and a rabbit anti NB6R antiserum at dilution 1:500 (Fiorani *et al.*, 1978) were used. As controls, membrane extracts were also prepared from spleen, kidney, and brain of adult male A/Jax and C3H/HeJ mice, respectively named A/J and C3H/HeJ. Membrane extracts from different clones and hybrids (prepared as for CMA) were added as competitors in the amount indicated. (Note the different competing capacity of A/J and NLI-7a membranes).

phospholipidic bilayer and regulate the social behaviour of the cell undergoing morphogenesis and differentiation.

The normal regulator of growth and differentiation of mouse sympathetic cells has been identified and characterized as the protein, Nerve Growth Factor (NGF) (Levi-Montalcini *et al.*, 1954; Cohen, 1960; Angeletti and Bradshaw, 1971; Levi-Montalcini, 1976). Most clones of C1300 NB bind NGF (Levi-Montalcini *et al.*, 1974; Revoltella *et al.*, 1974 a; Revoltella *et al.*, 1974 b; Revoltella *et al.*, 1975; Diamond *et al.*, 1976), but in only a few cases can a definite effect of the binding be demonstrated (Revoltella and Butler, 1979 a). We have used two clones, called NB1R and NB6R (Bertolini *et al.*, 1977), which respond in different ways to the inducer, NGF, in order to test the relationship between virus-associated, neural, histocompatibility and tumor-associated antigens during induction of tumor cell differentiation.

NB1R and NB6R clones grown in the undifferentiated forms in 20% fetal bovine serum (FBS) express C-type RNA virus associated envelope gp 69-71 and core p 30 antigens, TA antigen(s) and neural associated antigens on their surface. They do not express the histocompatibility private determinant H-2D4 as determined by a variety of methods. In order to study the effects of NGF on the two clones with minimal interference from unknown serum factors the FBS in the culture medium was lowered to 0.1%. In the absence of NGF under these conditions the cells of both clones almost completely cease to divide, and differentiate morphologically. When NGF at an optimum concentration of 5-20 ng/ml was added at the same time, the responses of the two clones were completely different. After a lag phase of a few hours, cells of clone NB1R started to incorporate ^{3}H-Thymidine into DNA and to divide. Most cells remained in suspension, and, if NGF was continuously supplied to the medium, the growth rate remained exponential for several cell generations (Fig. 3) (Revoltella and Butler, 1979 a). Our observations suggested that NGF enhanced expression of the two viral antigens, but had no effect on the neural antigens, TA or H-2D4 with respect to NB1R cells in suspension culture without NGF. In contrast, cells of clone NB6R ceased to divide apparently immediately or after one cell generation. Thereafter, the cells were found in stationary phase similar to cells grown in 0.1% FBS without NGF. The effect of the NGF was seen in the lengthened time of survival of the cells under these conditions: 4-6 days in the absence of NGF, over 40 days if NGF was continuously supplied. On differentiation in the presence of NGF, expression of TA antigens on NB6R cells remained unaltered, neural antigen expression increased, and

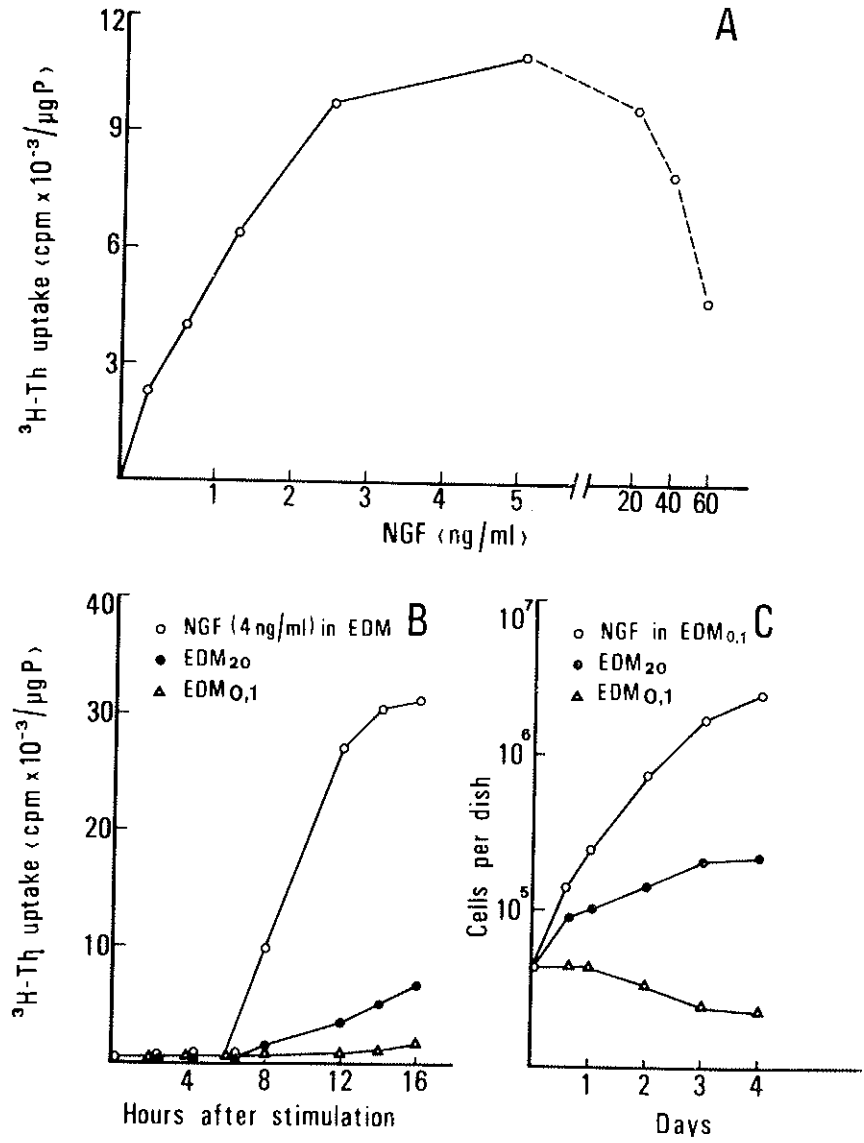

FIG. 3. Effects of NGF on clone NB1R cell growth. A. Effects of NGF added on tritiated thymidine (^{3}H-Th) uptake, after 72 hours of culture in EDM supplemented with only 0.1% FBS. B. Time course of ^{3}M-Th stimulation on clone NBIR cells under different culture conditions; ○ NGF 4 ng/ml in EDM supplemented with 0.1% FBS ($EDM_{0.1}$), ● in EDM supplemented with 20% FBS (EDM_{20}) and, △ in $EDM_{1.0}$ without added NGF. C. Similar experiment to B, but using viable cell count as measure of NBIR cell growth. FBS = Fetal Bovine Serum.

H-2D4 became detectable on the cells. Viral antigen expression was also enhanced during the first 5-8 days but then decreased again in the mature cells (Revoltella and Butler, 1979 b). These results are summarized in Table 2. In order to verify whether the increase of H-2D4 and neural antigen(s) was specifically due to the NGF, the cells were also incubated

TABLE 2. *Allo-antigens expressed on clones NB1R and NB6R grown in suspension or in the differentiated forms as detected by indirect immunofluorescence staining and antibody dependent cytotoxicity tests with mono-specific antisera* (*From:* Revoltella *and* Butler, *1979 b*)

Clones	Culture conditions *	H-2D4	Neural antigens (anti A/Jax brain)	MuLV gp 71	MuLV p30	TA
NB1R	in suspension EDM_{20}	—	±	+	+	+
	in suspension $EDM_{0.1}$ + NGF	—	±	+ +	+ +	+
	adherent ($EDM_{0.1}$) (4 d.)	+	+ +	+ +	+	+
NB6R	in suspension EDM_{20}	—	±	±	±	+
	adherent $EDM_{0.1}$ (4 d.)	—	+	+ +	+ +	+
	adherent $EDM_{0.1}$ + NGF: 4d	+	+	+ +	+	+
	adherent $EDM_{0.1}$ + NGF: 12d	+ +	+ +	+	+	+
	adherent $EDM_{0.1}$ + NGF: 30d	+ +	+ +	+	+	+

* Cells were grown in Dulbecco's modified Eagle's medium (EDM) supplemented with 20% FBS (EDM_{20}) in 10% CO_2 and air atmosphere. Alternatively EDM was supplemented with 0.1% FBS ($EDM_{0.1}$), with or without NGF (10 ng/ml) (See the text and Fig. 3). (Results are reported on an arbitrary scale: —, negative; ±, dubious; +, positive; + +, very positive).

alone in 0.1% FBS or in the presence of other inducers. Dexamethasone was an effective inducing agent even in 10%-20% FBS. H-2D4 and neural antigen expression were both induced, and a correlation was always seen between the proportion of morphologically differentiated cells and the proportion of cells positive for H-2D4 (Fig. 4) (Revoltella and Butler, 1979 b). However, H-2D4 was not detected in cells induced by low serum concentration or by various other inducing agents. Neural antigens were always induced, as were the viral antigens. These results

	Viable cells ($\times 10^4$) dish	% of cells differentiated	% of cells with H-2D4
Solvent control (1% ethanol)	160 ± 10	25 ± 2	2 ± 1
Desamethazone, 10 µg/ml	32 ± 6	42 ± 5	36 ± 4
, 50 µg/ml	26 ± 4	66 ± 4	50 ± 4
Testosterone, 10 µg/ml	148 ± 5	18 ± 3	2 ± 1
Estradiol, 10 µg/ml	156 ± 3	15 ± 2	2 ± 1
Androstenedione, 10 µg/ml	108 ± 2	22 ± 2	2 ± 1

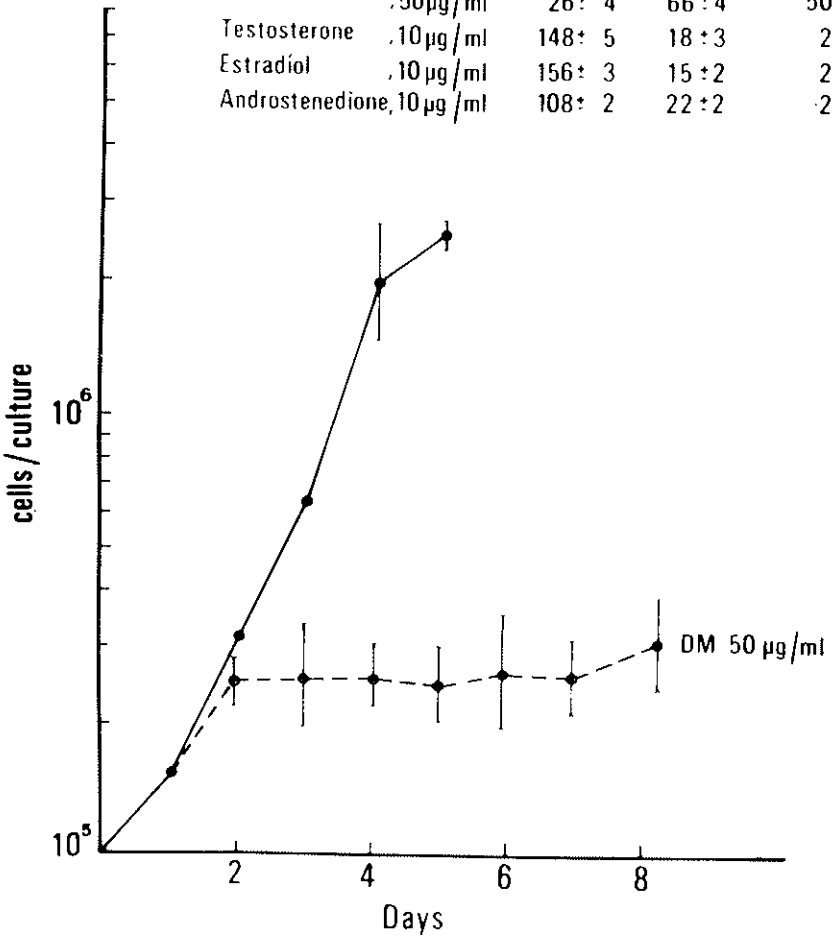

FIG. 4. Effects of different inducing agents on clone NB6R cell growth and differentiation. NB6R cells were grown in EDM supplemented with 10% FBS (without γ-globulins) without (●—●) and with desamethazone (●——●), or other compounds, for various days. Viable cells and percentage of adherent, differentiated cells were counted. Cells which expressed H-2D4 were detected by immunofluorescence staining. As control the cells were also maintained in culture with ethanol, the solvent used for dissolving the inducers. (From: Revoltella and Butler, 1979 b).

(Table 2) demonstrated that the lengthening of the stationary phase and morphological differentiation are not necessarily associated with H-2D4 expression in this clone.

Shedding of the viral antigens and TA antigens into the culture medium could be demonstrated by radio-immunoassay or lymphoproliferative assays (Revoltella and Butler, 1979 b). The antigens were first detectable in the supernatants after 4 hours. With frequent changes of medium supplemented with NGF it was found that shedding from these cells followed a cycle of about 8 hours (Fig. 5).

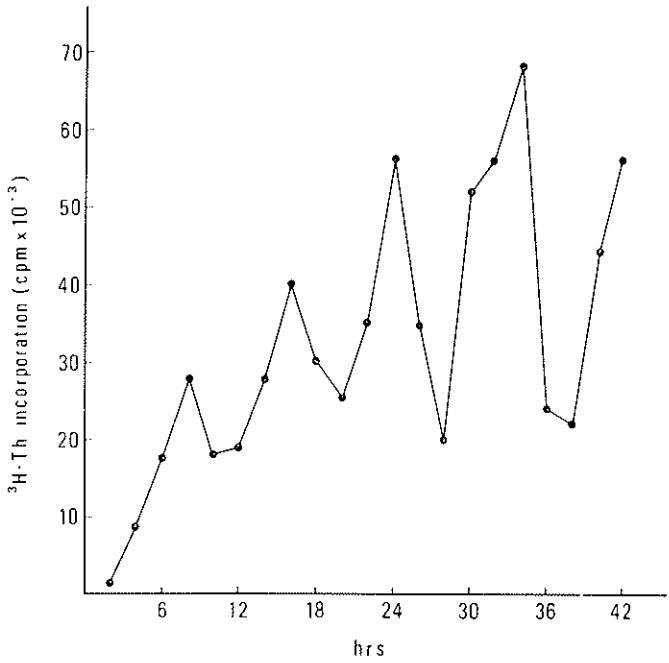

FIG. 5. Tumor antigen release dynamics. Stimulation of ^{3}H-Thymidine (2 hrs pulse) incorporation into 2×10^6 syngeneic spleen cells from primed A/Jax mice (inoculated s.c. 7 days previously with 2×10^6 fixed NB6R cells) by supernatants from cultures of 1×10^6 NB6R cells harvested at the time intervals indicated (From: Revoltella *et al.*, 1979 b).

MODULATION OF CELL GROWTH BY ANTIBODIES

In order to test whether specific antibodies had any modulatory effect on C1300 NB cell growth, in accordance with Prehn's hypothesis of tumor immunostimulation (Prehn, 1971; Prehn, 1972; Shearer *et al.*, 1973; Shearer *et al.*, 1975), we studied the effect of polyvalent anti-C1300 NB antibodies and monospecific antibodies against specific antigenic determinants on the growth of cloned C1300 NB cells in suspension or in monolayer.

Several NB clones isolated from a common C1300 NB line, and hybrids formed from the fusion of a mouse ($HGPRT^-$) NB clone and a mouse fibroblastic (TK^-) cell clone were tested. In most of the clones tested, which were selected for their demonstrated differences in dissected expression of H-2D4, neural-associated, viral-associated and TA antigens (i.e. quantitiation, time of emergence during differentiation and/or shedding capacity), there was a correlation between antigen expression and the effect of the antisera. When the appropriate antigen was strongly expressed the serum, as expected, was highly cytotoxic, but when the antigen was poorly expressed or not detectable the cells were stimulated to divide (Revoltella and Butler, 1979 b). Fig. 6 shows an example of this effect with NB6R cells in suspension (day 0) or induced to differentiate by NGF (day 1 to 5).

Immunogenicity of C1300 NB cells in vitro and in vivo

Evidence has been provided from several laboratories (Martin and Martin, 1975 b; Martin and Martin, 1975 c; Martin and Martin, 1975 d; Oldstone, 1975; Terman *et al.*, 1975; Revoltella *et al.*, 1976; Phillips *et al.*, 1977; Faraggiana *et al.*, 1978; Procicchiani *et al.*, 1979) that autogenous immunity to C1300 NB cells exists in syngeneic A/Jax mice. Spleen lymphoid cells from normal mice or from mice with a growing C1300 NB tumor recognize C1300 NB cells in unidirectional mixed cell cultures, as shown by tests of lymphoproliferation or cytotoxicity. In the lymphoproliferative assay spleen lymphoid cells from the mice incorporate ^{3}H-Thymidine in response to stimulation by an optimal responder to stimulator ratio of fixed C1300 NB cells or to solubilized antigens from these cells (Revoltella *et al.*, 1976; Procicchiani *et al.*, 1979; Revoltella *et al.*, 1979 a). Spleen cells from tumor-bearing mice respond earlier and incorporate more ^{3}H-Thymidine than spleen cells from normal mice. Work is in progress in our laboratory with the aim of solubilizing and characterizing antigenic components from NB tumor cell membranes, and evaluating their biological and *in vitro* and *in vivo* immunological properties. So far, a few fractions have been solubilized by non-ionic detergents or papain digestion. These fractions retain tumor specificity as determined by a variety of sensitive and specific immunological assays (Revoltella *et al.*, 1976; Faraggiana *et al.*, 1978; Butler and Revoltella, 1979). The nature of these antigens has yet to be determined but some of them seem to be associated with virus antigens while others seem to correspond to the TA antigens (Faraggiana *et al.*, 1978; Butler and Revoltella, 1979).

Morphologically the first event in cell-cell interactions consisted of cell adherence of lymphoid cells and activated macrophages to the neuroblastoma cells with formation of rosettes (Fig. 7) (Fiorani *et al.*, 1978; Procicchiani *et al.*, 1979). Light and transmission electron microscopy were applied in combination with ^{51}Cr release cytotoxicity assays to fol-

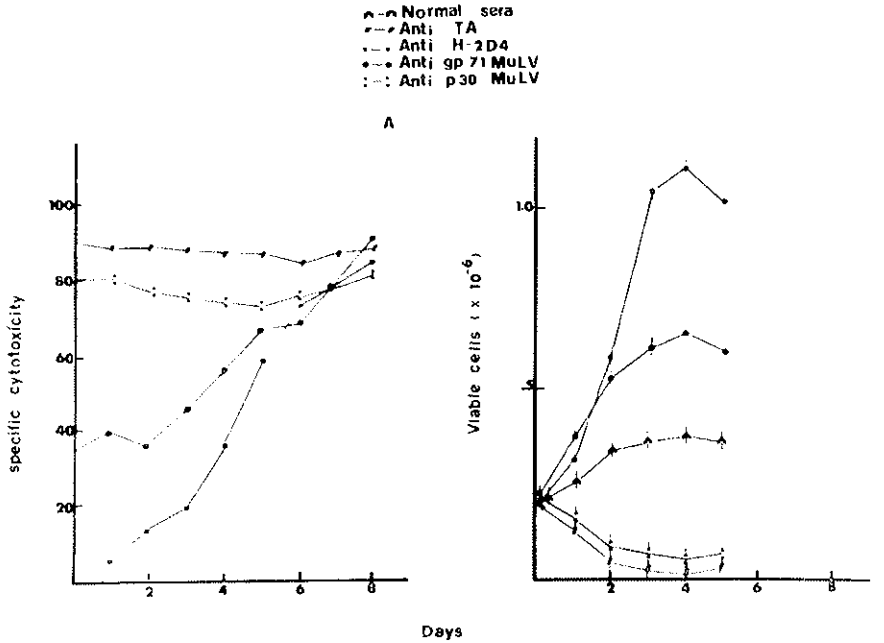

FIG. 6. A. Effect of different antisera on ^{51}Cr release from prelabeled NB6R cells grown in culture for various times in EDM supplemented with 0.1% FBS and stimulated with 10 ng/ml of NGF. The heat decomplemented antisera (56° C for 30 min): rabbit anti-NB6R.TA (1:15); mouse anti-H-2D4 (B10.A-AKM × 129/J) anti (B10.A) (1:20); goat anti C-type RNA virus associated antigens [goat anti MuLV gp 71 (1:50) and goat anti MuLV p 30 (1:10)] were incubated with 2×10^6 pre-labeled NB6R cells in the presence of 1:10 normal rabbit serum (pre-absorbed on NB6R cells) as complement. After 1 hr incubation at 37° C, cytotoxicity was measured in the cell culture fluid supernatants. Per cent specific cytotoxiticy was expressed as:

$$\frac{(^{51}Cr \text{ release in experimental}) - [^{51}Cr \text{ release in controls (NB cells alone)}]}{[\text{Maximum } ^{51}Cr \text{ release } (+ 5\% \text{ SDS})] - [^{51}Cr \text{ release in controls (NB cells alone)}]}$$

values are the mean of triplicates (SD was less than 5% and is not reported).
B. Viable cell counts of NB6R cells treated with the same antisera (the same as in A) or with normal serum. Values are the means of triplicate cell counts performed with a haemocytometer ± standard error of the means (brackets). To facilitate cell counting, the cells were washed once in PBS without Ca and Mg, treated with 0.05% trypsin for 5 min and counted in EDM containing 0.04% erythrosine-B dye. Experimental cells were grown in EDM supplemented with 2.0% FBS and containing 10 ng/ml NGF; the medium was not changed throughout the whole experiment. (From: Revoltella and Butler, 1979 b).

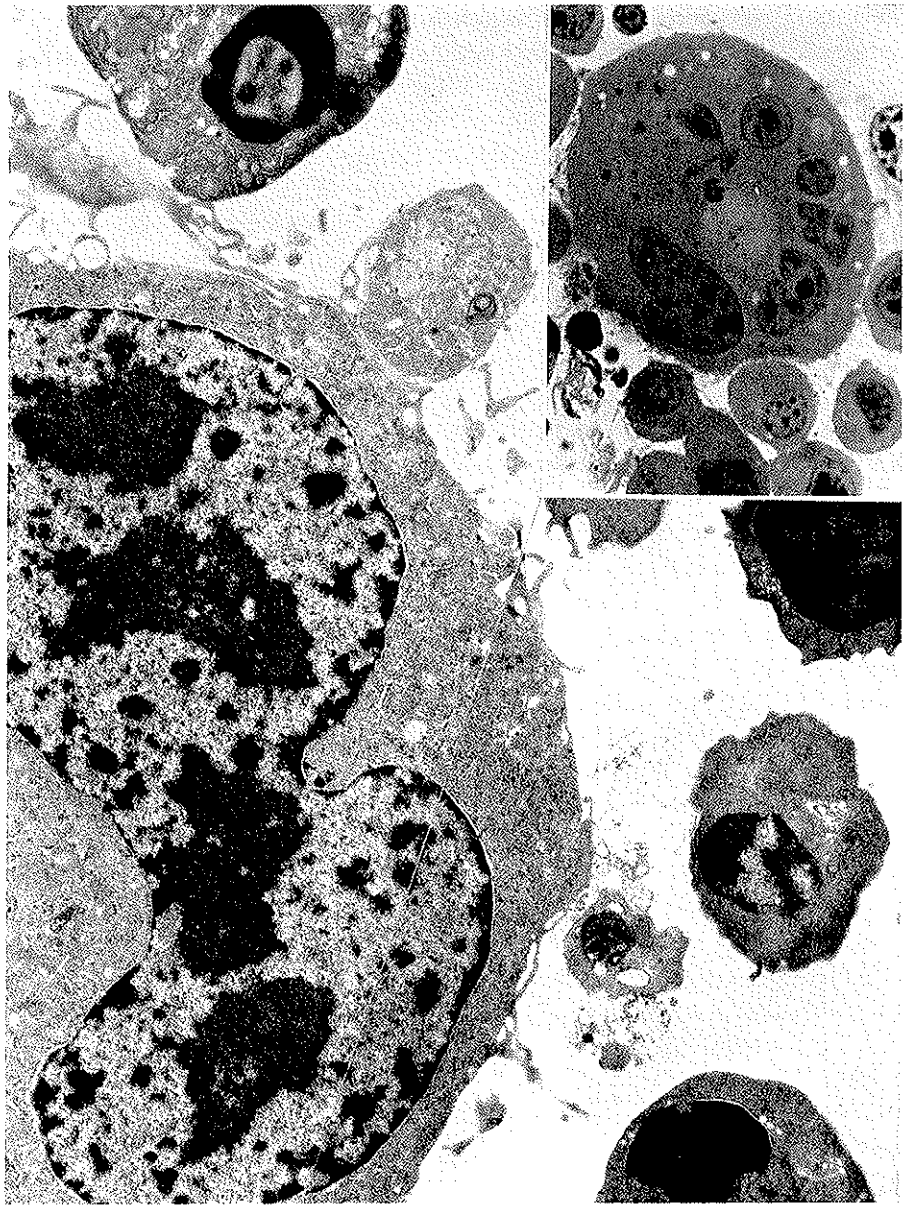

FIG. 7. Ultrathin section of a rosette-forming NB6R cell after 30 min of cell contact with sensitized syngeneic spleen lymphoid cells. Cytoplasmic processes are departing from the NB6R cell toward the lymphocytes and lymphoblasts; a few vesicles containing mitochondria, ribosomes and amorphous material are in close contact to the tumor cell. In the insert, a semifine section showing a rosette-forming polynuclear NB6R cell, stained by toluidine blue (from: Procicchiani *et al.*, 1979).

low the time course of the reaction after rosette formation. With unsensitized lymphoid cells rosettes were formed but target cell morphology remained in general unchanged. With sensitized lymphoid cells target cells were seen to undergo severe degeneration, initially in the mitochondria and, later, when ^{51}Cr release became significant, in the formation of large surface blebs and protrusions (Fig. 8): this cellular-type reaction is believed to reflect the *in vivo* process of lymphoid cell mediated immune reaction, probahly the most important event controlling tumor growth.

In addition to cell-mediated immune response, A/Jax mice have been shown to be capable of mounting a humoral immune response to C1300 NB tumors (Terman *et al.*, 1975; Faraggiana *et al.*, 1978; Revoltella *et al.*, 1979 a). The mice produce circulating antibodies against a variety of the antigens present on the tumor cells. It is a remarkable fact that in all mouse strains but also in humans, primates, felines, cattle, etc., there are natural antibodies directed against C-type virus associated antigens, particularly gp 69-71, and other potentially inactivating serum factors (Ihle *et al.*, 1974; Aaronson and Stephenson, 1974; Bendinelli *et al.*, 1974; Batzing *et al.*, 1974; Ihle *et al.*, 1974; Nowinski and Kaehler, 1974; Hanna *et al.*, 1975; Lee and Ihle, 1975; Levy *et al.*, 1975; Aoki *et al.*, 1976; Louie *et al.*, 1976; Stepbenson *et al.*, 1976; Bartholomew *et al.*, 1978). This chronic autogenous anti-C-type RNA virus reactivity changes with age, more significantly in some strains of mice than in others (Nowinski *et al.*, 1975). Strain A/Jax mice which have naturally high serum reactivity against C-type RNA virus are, nevertheless, susceptible to C1300 NB growth. It has been suggested that these antibodies may have enhancing or blocking activity *in vivo* (Hellström *et al.*, 1968; Jose and Seshadri, 1974; Oldstone, 1975; Faraggiana *et al.*, 1978). We have performed immunopathological studies in 2-3 month old A/Jax mice inoculated subcutaneously with 2×10^6 viable cloned C1300 NB cells. All of the mice developed a palpable tumor within 6-12 days and died within 28-30 days.

A summary of the immunological response of the mice inoculated with NB6RC cells is reported in Table 3. Levels of antibodies capable of reacting with ^{125}I-laheled MuLV gp 69-71 were originally quite high in the untreated mice but decreased during the course of the tumor growth. Shortly after the tumor cell inoculum, however, the mice began to produce detectable quantities of antibodies reacting with MuLV p30 core protein and with TA antigen solubilized from C1300 NB cells. Circulating immune complexes were detectable from about day 8-14. In addition, as

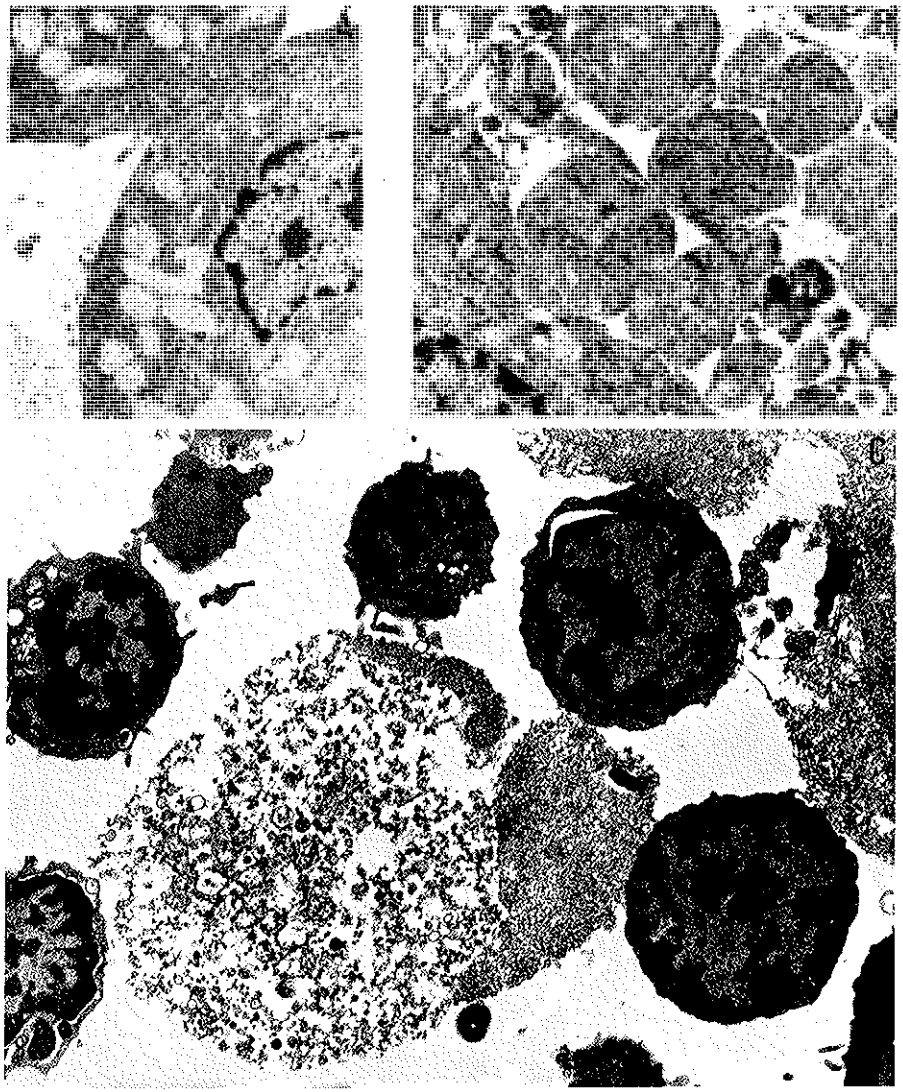

FIG. 8. (A) Ultrathin section of two NB6R cells showing clear disorganization of cristae in the mitochondria. (B) bleb formation from a rosette-forming NB6R cell after 30 min of mixed culture with primed syngeneic spleen lymphoid cells. (C) Ultrathin section showing complete cytolysis of rosette-forming NB6R cells (From: Procicchiani *et al.*, 1979).

TABLE 3. *Comparative summary of immunological response of mice inoculated sc. 2×10^6 viable NB6R tumor cells* (*From:* Revoltella *et* al., 1979 a).

Results are reported on an arbitrary scale; — negative, ± dubious, + positive, ++ very positive.

	Days After Tumor Injection												
	0	2	4	6	8	10	12	14	16	18	20	28-30 (Death)	
Tumor growth *	—	—	—	1/30	6/30	21/30	30/30	30/30	30/30	30/30	30/30	18/18	3/3
Spleen cells response to NB6R:													
1. Lymphoproliferation	+	+	+	+	+	++	++	++	++	++	++	+	+
2. Cytotoxicity													
4 hrs	±	++	++	±	—	—	—	—	—	—	—	—	—
24 hrs	±	++	++	++	++	++	++	+	+	+	+	±	±
Effects of sera from tumor bearing mice on spleen lymphocytes from normal mice:													
1. Lymphoproliferation	—	—	±	±	+	+	+	+	+	+	+	+	+
2. ADCC (on NB6R target cells)	±	++	+	±	—	—	—	—	—	—	—	—	—
Immunofluorescence in kidneys (Anti Mouse IgG and $C_3/\beta 1c$):													
1. Normal mice	—	—	—	—	—	—	—	—	—	—	—	—	—
2. Immunized mice	—	—	—	—	—	—	—	—	—	—	—	—	—
3. Tumor bearing mice	—	—	—	—	±	++	+	±	—	—	—	—	—

* Number of mice with tumors over number of living mice.

previously reported by Oldstone (Oldstone, 1975), we found that a transient glomerulopathy may develop in these mice after about 8-12 days. Despite an apparently normal histologic appearance the nephropathy was clearly demonstrated by electron microscopy and classified as a focal mesangiopathic glomerulopathy (Germuth and Rodriguez, 1975; Faraggiana *et al.*, 1978). Deposits of 7S G-immunoglobulins and C3 complement were detected in the kidneys, and the antibodies recovered from the kidneys proved to react with MuLV p30 virus core protein and C1300 NB TA antigens. Other clones of C1300 NB behaved in a similar fashion but with a different time scale (Faraggiana *et al.*, 1978).

In another series of experiments (Revoltella *et al.*, 1979 a) we studied the effect of sera from tumor-bearing mice on the *in vitro* response of unsensitized lymphoid cells against C1300 NB cells. Two assays were used: the lymphoproliferative assay and an assay of antibody dependent cell-mediated cytotoxicity (ADCC). Early sera (2-6 day) from mice inoculated with NB6RC cells had no effect on the lymphoproliferative assay, but were stimulatory in ADCC. On the other hand, later sera stimulated the lymphoproliferative response but were ineffective in ADCC. The shift from one phase to the other occurred at about the same time as deposits of immune complexes were first detected in the kidney glomeruli.

Clonal variability in vitro

The finding that different clones which were all isolated from the same original C1300 NB cell line exhibited different tumorogenic and immunogenic properties *in vivo* suggested the attempt to reproduce *in vitro* spontaneous genetic variation in NB cells. Spleen lymphoid cells from specifically sensitized syngeneic or allogeneic mice were induced to bind to and form rosettes with NB6R cells. Rosette formation usually led to tumor cells lysis but, occasionally, electron microscopy revealed lymphoid cells spontaneously incorporated by the target cell, lysed and their material re-utilized (Fig. 9). Using specific genetic markers it was shown that sub-clones of hybrid cells were produced (Tables 4, 5). These sub-clones had modified expression of surface markers, altered capacity to stimulate lymphoid cells *in vitro,* and modified malignancy in A/Jax mice (Revoltella *et al.*, 1979 b). Provided that this phenomenon is not simply a biological curiosity which occurs *in vitro,* it may play an important role in determining (together with differentiation and mutation

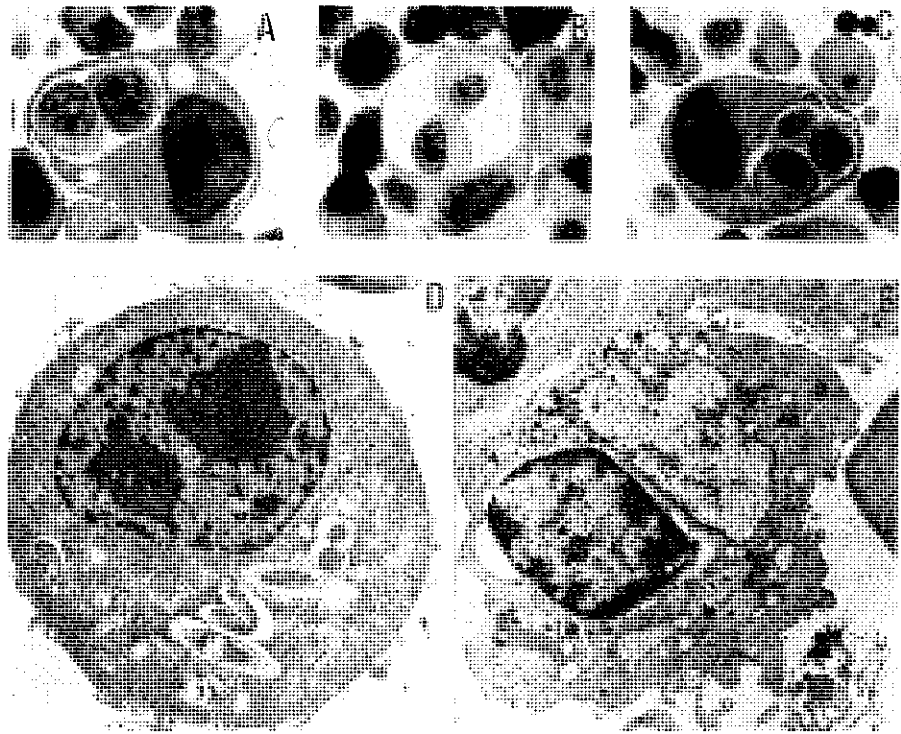

FIG. 9. Spleen lymphoid cells from sensitized A/Jax mice added to syngeneic NB6R cells in mixed culture, *in vitro*. (A-C) semi-fine sections showing two or three lymphoid cells within a vacuole comprised in the cytoplasm of a rosette-forming NB6R cell. (D) Electron micrograph showing intracytoplasmic remnants of cellular digests. (E) Ultrathin section of an unusual NB6R cell showing two nuclei. One nucleus is apparently comprised in a vacuole which is surrounded by an intact membrane. The cytoplasm of the intact NB6R cell also contains several patches of amorphous electron dense material (From: Procicchiani *et al.*, 1979; Revoltella *et al.*, 1979 b).

events produced by various forms of pressure) the *in vivo* character of the tumor. Continuous variation in the cell population composing the tumor mass would undoubtedly cause unpredictable changes in the tumor-host relationship.

CONCLUDING REMARKS

Antigenic analysis of C1300 NB cells has been mainly directed to the identification and characterization of antigens associated with the malignant character of the tumor; that is, antigens which could clearly distinguish the tumor cells from their normal counterparts and precursors.

	Gradient	Morphology	Glucose phosphate isomerase
Lymphocytes			fast
NB6R			slow
band A	0-10%	Lymphocytes, cell debris	fast
band B	10-15%	Lymphocytes, blasts, monocytes	not tested
band C	18-20%	NB6R	slow
band D	20-22%	NB6R, rosettes, polynucleocytes	slow, fast and intermediate
band E	22-24%	Rosettes	slow, fast and intermediate

TABLE 4. *Spontaneous fusion and formation of hybrids between C1300 neuroblastoma cells in mixed cultures. Spleen lymphocytes from C_3H/HeJ mice, were added to NB6R cells in suspension cultures. After 20 min incubation the mixed cells were centrifuged and examined for rosette formation. About 35% of NB6R cells formed rosettes (i.e. were surrounded by several lymphocytes). The cell suspension was then applied to a discontinuous albumin density gradient and centrifuged at 400 g for 60 min at R.T. Cells stratified in four major (A-D) and one minor (E) band of the gradient. Cells recovered from bands C, D, E, were transferred into culture for 24 hrs in EDM_1. The non adherent cells were discarded and the adherent neuroblastoma cells were resuspended after trypsin-EDTA treatment. The Table reports the GPI phenotypes in C_3H/HeJ lymphocytes and NB6R cells and in NB cells recovered from the bands of the discontinuous BSA gradient and then selected by adherency in culture. The cell populations from bands D and E contain hybrids, as demonstrated from the presence of both parental forms of GPI and a presumptive hybrid (heteropolymer) enzyme* (*From:* Revoltella et al., *1979 b*)

However, many important questions remain unanswered, particularly concerning their origin and inter-relationships at the genetic coding level, and their possible role in relation to cell growth, DNA duplication, and cell physiology or morphogenesis. Modulatory changes in antigen expression in cells undergoing differentiation, the appearance of new antigens, or of antigens which are present during some stage of normal embryonal development, the discontinuous synthesis and shedding of viral-associated and other antigens, all appear to be relevant, but the mechanisms underlying their relationship mostly remain to be elucidated.

The resemblance between tumor cells and embryonic cells is certainly not only confined to a few common antigens. They show similar high responsiveness to certain chemical inducing agents. They have similar

Cells	Absorption tests H-2					Direct citotoxicity tests MuLV (Gross)	
	H-2.32 ($H\text{-}2^k$)	H-2.4 ($H\text{-}2^a$)	H-2.8	A/J anti C_3H/HeJ	C_3H/HeJ anti A/J	anti gp69-71	anti p30
NB6R	—	+	+	—	+	+	+
NA (HGPRT-)	—	+	+	—	+	+	+
NB cells from BSA gradient:							
band C	—	+	+	—	+	+	+
band D	±	+	+	+	+	+	+
band E	+	+	+	+	+	+	+
C_3H/HeJ lymphoid cells	+	—	+	+	—	±	—

TABLE 5. *Cell surface alloantigens (H-2 and MuLV) of neuroblastoma and spleen lymphoid cells in culture as determined by direct and absorption analyses. Summary of results* (*See legend of Table 4*) (*From:* Revoltella et al., *1979 b*)

properties in morphogenetic movements and adhesiveness, and, above all, are similar in their high rate of cell division. Expression of embryonic gene products is not necessarily a pathological symptom. For example, antigens common to fetal liver and hepatomas are also reported to occur in the process of normal liver regeneration which follows a surgical lesion (Stomilawski-Birenocajg *et al.*, 1967). The appearance of fetal antigens in normal tissues may be a normal manifestation of proliferation. Thus the essential difference from normality in tumor cells would be their lack of responsiveness to the normal switch-off mechanisms of cell proliferation.

It has been recently proposed, from careful analysis of a number of widely differing eukaryotic cells, that normal differentiation operates in a stochastic manner. The first or commitment program should require the presence of an inducer acting on the target cell and irreversibly committing it to the second program, the expression program (Gusella and Hausman, 1976). Deficiencies in either program might be the real distinction between a tumor cell and a normal one. In support of this hypothesis, it is generally found that tumor cells in general lack either the capacity to bind or to respond to the normal inducing/regulating agent of their normal counterparts. The establishment of clones of a tumor cell line which differ in this competence represents a useful system to test the hypothesis directly. The selection of two clones of C1300 NB

which show opposing responses to NGF is a clear example of the disarrangement of normal regulation in these tumor nerve cells.

How and why the program of normal nerve cell differentiation operates, and why it is altered in NB cells are still unanswered questions. The gene products of C-type RNA viruses may influence normal cell growth, development and differentiation. There is evidence that C-type RNA viral genomes are integrated and vertically transmitted within the genomes in many animal species (Tooze, 1973; Aaronson and Stephenson, 1974; Stephenson *et al.*, 1976). To quote Huebner's words (Huebner and Todaro, 1969 ".. Should it (the C-type viral genome) carry a message to replicate, it could conceivably be useful during the well regulated period of embryogenesis, while derepression in differentiated cells later in life leading also to cell replication could prove disastrous". Thus type-C RNA virus may play a role in modifying normal cell competence for the induction of cell differentiation or perhaps, in the differentiation process itself (Lieberman and Sachs, 1978).

The findings: 1) that in single clones of C1300 NB cells the control of expression of histocompatibility antigens, virus associated antigens, and differentiation and tumor-type associated antigens is dissected or at least only partly co-ordinated; 2) that different clones express virus-associated and other antigens to different extents; 3) that autogenous immunity exists in A/Jax mice to C-type RNA virus associated antigens and the animals can be specifically stimulated against virus and non-virus antigens on the surface of the syngeneic NB cells; 4) that this type of auto-immune response to the tumor may be either cytotoxic or stimulatory to different clones of the same tumor line, depending on the expression of particular antigens on the individual cells; suggest the possibility of epigenetic controls on cell growth and development mediated through specific structural components on the cell surface, both *in vivo* and *in vitro*. This immunological approach to the problem of tumor growth will hopefully also provide new insights into the possible relationships between embryonic cell differentiation and malignancy.

Summary

Murine C1300 neuroblastoma (NB) cells have been examined as a model for the study of tissue and tumor specific surface antigens and immune responses to such antigens associated with differentiation and oncogenesis. Specific anti-NB cellular and humoral *in vivo* immune responses have been demonstrated in immune and NB-tumor bearing mice. Analysis

of the anti-NB antibodies in their sera showed that they were mainly directed against: 1. mouse C-type RNA virus-associated antigens; 2. tissue-specific antigens; 3. antigenic determinants of the histocompatibility complex, and 4. tumor-associated transplantation antigen(s).

In vitro, NB cells change surface antigen exposure and acquire new structural membrane components during differentiation. Two NB clones respond in different ways to stimulation with mouse nerve growth factor (NGF). Clone NB1R cells continue to grow and divide in suspension even in medium with low fetal bovine serum, whereas clone NB6R cells, under the same conditions, are induced to differentiate. We have demonstrated differences in specific antigen expression (time of emergence and exposure and/or shedding into the culture fluid) in these clones growing in suspension or differentiating in monolayers. With fixed doses of anti-NB antibodies directed against specific membrane antigens, cells of various NB clones and hybrids which exhibited either low or high surface expression of the corresponding antigens, were either stimulated to cell division, or lysed. This may give some clues to the means by which NB cells *in vivo* escape the host immunological control despite the autogenous immunity which exists in strain-A mice. Further evidence is also provided by this model of the derangement of normal control mechanisms of differentiation in NB cells.

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DISCUSSION

FAMBROUGH

You showed a table in which you listed the percent of H-2 positive cells after treatments with different steroids and the percent of branching cells. You said that those two parameters were correlated, but the correlation seemed poor to me, except perhaps in the case of dexamethorfan.

REVOLTELLA

These data were reported in Fig. 4. We showed that out of several other steroids tested, dexamethazone induced growth inhibition, morphological cell differentiation and concomitant histocompatibility (H-2) antigen expression on cloned murine neuroblastoma cells. With dexamethazone the effect was dose dependent and the correlation between process formation and H-2 expression was always very strict. Cells from the same clone tested on growth phase, did not express H-2. Similarly, other steroids which did not inhibit cell growth, did not induce H-2. These findings thus confirm and extend a recent report by Sundquist et al. ("Exp. Cell Res.", *113*, 375, 1978), who showed that dexamethazone on different mouse neuroblastoma cells similarly induced cell growth inhibition and morphological differentiation, *in vitro*. In both cases the cells were grown in culture medium supplemented with 20% agammaglobulinemic fetal calf serum. H-2 was revealed immunologically, by indirect immunofluorescence assay and radio-immunoprecipitation assays on solubilized cell membranes, using anti H-2.4 monospecific alloantisera.

GREENE

Other than H-2A, do you have any evidence as to the distribution of any of these antigens in the normal nervous system?

REVOLTELLA

Again, I stress the point that most of our assays to detect surface markers are immunological assays and that we are using as probes antibodies raised in rabbits, goats or mice against cloned NB cells as well as mono-clonal antibodies from hybridomas ohtained from somatic cell fusion between a mouse plasmacytoma and primed anti-neuroblastoma spleen leukocytes. In regard to the

H-2 antigens, we looked for private and public H-2^a specificities, to rule out possible non-specific reactivities of our H-2 alloantisera. Now, to answer your question, H-2 on neuroblastoma cell membrane is distinct from other structural markers which are detectable also on nerve cells either from embryonic or adult nerve cells. For example: lectin receptors (e.g. for PHA); the NGF receptors; a group of high molecular weight glycoproteins also found on brain and kidney from adult A/J mice, but not in muscle and liver cells; a group of surface antigens reacting with antibodies specific to C-type murine leukemia, virus-associated antigens (envelope glycoproteins and core proteins) which are highly expressed on mouse neuroblastoma cells and in embryonic normal nerve cells but at a low level in adult nerve cells.

GREENE

What about some of the other tissue antigens, other than H-2?

REVOLTELLA

A list of known antigens neuronal-restricted or not was presented in Table 1 of my presentation. In our laboratory our present attention is directed mainly to isolate and characterize immunologically and chemically three different antigens on NB6R cell membranes, which are on distinct molecules other than H-2. The first one is a non-inducible species non-specific glycoprotein, approximately 80,000 m.w., which shows cross-reaction with anti C-type mouse leukemia virus envelope glycoproteins. The second antigen is an inducible high molecular weight tissue-type associated glycoprotein which can be precipitated with anti-brain alloantibodies. The third antigen is a low molecular weight papain-cleaved product from neuroblastoma membranes which retains a strong alloantigenic property for sensitized syngeneic spleen leukocytes, e.g. from mice primed against C1300 neuroblastoma cells.

NELSON

That's another question. On the mitogenic effect of NGF on this particular cell line, I noticed your control cells were not growing; so under what conditions was this experiment done?

REVOLTELLA

The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and antihiotics, at 37°C and 10% CO_2 in air. The cells were washed twice, by centrifugation, in medium without

serum, and plated again. NGF was eventually supplemented, while the controls were in medium with only low or no serum. The mitogenic effect of NGF is easily measured in medium containing 0.1-0.2% serum, and also in serum-free medium. However, in this latter case one cannot exclude that after cell washing a certain level of serum factors is still present in the culture. Therefore, we don't know yet for certain, whether the mitogenic effect induced by NGF on NB1R cells was a direct effect of the protein factor or was obtained through the activation of other serum factors following NGF interaction. We are now attempting to adapt our cells to grow in a serum-free defined culture medium to circumvent this problem.

SYNAPSE PLASTICITY

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Two model systems were used to study the process of synaptogenesis: clonal cell lines of neural origin and neurons dissociated from embryos and cultured *in vitro*.

Twenty-three neuroblastoma or hybrid cell lines were tested for their ability to synthesize and release acetylcholine and form synapses with rat striated muscle cells and clonal muscle cells. Six cell lines form synapses with muscle cells with high frequency; whereas 17 cell lines were found which have defects in synapse formation. Rates of acetylcholine synthesis were 34-463 pmol acetylcholine/min/mg/homogenate protein; intact cells incubated with [^{3}H]-choline contained 55-1,600 pmol [^{3}H]-acetylcholine/mg/protein and released 80-6,400 fmol [^{3}H]-acetylcholine/min/mg protein into the medium. Five kinds of decets were detected with cell lines that form few or no synapses: 1) little or no acetylcholine synthesis, 2) large dense core vesicles present but not clear vesicles, 3) clear vesicles present but not large dense core vesicles, 4) little or no action potential Ca^{++} ionophore activity, and 5) a defect in another step required for stimulus-dependent acetylcholine secretion.

The ability of hybrid cells to form synapses was found to be regulated. Growth of hybrid cells in the presence of dibutyryl-cAMP increased the concentration of intracellular acetylcholine, the abundance of vesicles, the amount of acetylcholine released from cells in response to excitory stimuli, the efficiency of synaptic communication, and the number of synapses formed. These effects also were obtained with cAMP, but not with cGMP.

The effects of putative neurotransmitters and hormones on intracellular cAMP or cGMP levels, cell membrane potential, and acetylcholine secretion from cells were determined. At least 10 species of receptors were detected on synapse competent cell lines. Activation of receptors for serotonin, $PGF_{2\alpha}$ acetylcholine, bradykinin, neurotensin, or angiotensin resulted in secretion of acetylcholine from cells. Receptor mediated increases in cAMP or cGMP levels had no immediate effect on acetylcholine secretion from cells. However, growth of hybrid cells for 24 or more hours in the presence of ligands of receptors that are coupled to the activation of adenylate cyclase and/or exposure of cells to other inhibitors of cyclic nucleotide phosphodiesterase resulted in increase in cAMP levels of cells and mimicked all regulatory effects of dibutyryl-cAMP on acetylcholine storage and release from cells. These results show that cell lines with and without synapse defects can be generated and that receptor mediated reactions that activate adenylate cyclase and elevate cAMP levels of cells regulate the storage and stimulus-dependent secretion of acetylcholine, thereby regulating synapse formation and the flow of information across synapses. Synapses were turned on or off slowly over a period of days, which suggests that cAMP, directly or indirectly, regulates the acquisition of components that are required for synaptic activity.

We previously showed that activation of NG108-15 opiate receptors or muscarinic acetylcholine receptors results in inhihition of adenylate cyclase and that exposure of cells to morphine or carbamylcholine reduces cAMP levels of cells initially, but gradually, over a period of 24-48 hours, cAMP levels increase and return to the control value due to a compensatory, long-lived increase in the specific activity of adenylate cyclase. Subsequent studies have shown that NG108-15 cells possess presynaptic α_2-receptors and that exposure of cells to 1 μM norepinephrine similarly reduces cAMP levels initially, and that cAMP levels slowly return to the control value over a 10 hour period as the specific activity of adenylate cyclase increases. The cells then are dependent on norepinephrine to inhibit the elevated adenylate cyclase activity. Withdrawal of norepinephrine or blockade of the α-receptors results in a 4-9 fold increase in intracellular cAMP. Approximately 8 hours are required for the elevated enzyme activity to return to the control value. Cyclic AMP levels are elevated during the withdrawal period, and cells are supersensitive to ligands for other species of receptors, such as PGE_1, that activate adenylate cyclase. The demonstration that 3 species of receptors which mediate inhibition of adenylate cyclase also evoke persistent increase in adenylate cyclase activity suggests that the phenomenon is a general one, and that

other species of receptors that inhibit adenylate cyclase also may act as dual regulators of the enzyme.

The hypothesis that activation of adenylate cyclase may lead, conversely, to a reduction in adenylate cyclase activity also was tested. Incubation of NG108-15 cells with PGE_1 for 12 hours resulted in 60-80% decreases in basal adenylate cyclase specific activity and NaF-, Gpp,NH)p-, 2-Cl-adenosine, and PGE_1-stimulated activities. Basal and PGE_1-stimulated adenylate cyclase activities of cells exposed to PGE_1 decayed exponentially with half-lives of 6 hours. On withdrawal of PGE_1, adenylate cyclase activity slowly increased and returned to the control value over a period of 24 hours; cyclohexamide inhibited the increase in adenylate cyclase activity $> 90\%$. These results show that activation of adenylate cyclase leads to a loss of enzyme activity and that the recovery of enzyme activity to the control value requires protein synthesis and approximately 24 hours of incubation.

These long-lived, receptor-mediated effects on adenylate cyclase activity, acetylcholine storage, stimulus-secretion coupling, and the demonstration that synapses can be turned on or off by regulating acetylcholine release, have properties that resemble those expected for simple forms of learning and memory, such as habituation, tolerance, dependence and sensitization, but whether synapse plasticity in a cultured cell system is related to behavioral phenomena is not known.

Cultured neurons dissociated from chick embryo retina and spinal cord also were used to study the process of synapse formation. Both intact chick retina and cultured retina cells were shown to have high choline acetyltransferase activity and abundant nicotinic and muscarinic acetylcholine receptors. ^{125}I-Labeled α-bungarotoxin and 3-[^{3}H]-quinuclidinyl benzilate, which bind with high affinity and specificity to nicotinic or muscarinic acetylcholine receptors, respectively, were used as probes to determine the properties of the receptors, the number of binding sites, and their distribution within the retina during embryonic development. Most of the nicotinic acetylcholine receptors and all of the muscarinic receptors of chick retina were localized in layers within the inner synaptic layer of the retina; 11 layers were distinguished within the inner synaptic layer of chick retina on the hasis of muscarinic and nicotinic acetylcholine receptor concentrations and acetylcholinesterase activity. The layers appear in an ordered sequence during development with respect to temporal and positional relationships. These results and those of others show that neurites of the same type sort out from neurites of other types on the basis of species of receptor, transmitter, or enzyme of transmitter me-

tabolism. A possible mechanism for generating sets of stratified or columnar neurons with similar properties and relating one set to another by cross-linking neurons of the same type to one another via synaptic connections was proposed.

The specificity of synapse formation by dissociated chick embryo retina neurons was examined by culturing retina cells with inappropriate synaptic partner cells, such as striated muscle cells which possess nicotinic acetylcholine receptors. The results show that neurons are generated in chick embryo retina that are able to form synapses with striated muscle cells and then lose the ability to form synapses with a half-life of 21 hours. These neurons first appear in chick retina on the sixth day of embryo development, and are most abundant on the eighth day, comprising perhaps 8% of the retina cell population. Almost all myotubes are innervated after coculturing retina and muscle cells for only 2 hours. However, the mismatched synapses between retina neurons and muscle cells are transient and slowly disappear over a period of 8 days. Neurons lose the ability to form new synapses by the 16th day, but not the ability to synthesize and secrete acetylcholine. Cultured retina neurons also form synapses in abundance with other retina neurons (approximately 1×10^9 synapses/mg of protein), and synapses between retina neurons were found after all synapses between retina neurons and muscle cells had been terminated.

Preparations of neurons from spinal cord, which presumably contain motorneurons that normally innervate striated muscle cells, also formed synapses with muscle cells *in vitro* but the number of synapses remained constant during subsequent culture. Thus, spinal cord neurons either form stable, long-lived synapses with muscle cells or attain a steady state wherein rates of synapse formation and termination are equal. These results show that populations of cholinergic neurons from retina and spinal cord differ in the rate of synthesis of synapses with muscle cells and probably also in the rate of synapse termination, and that populations of synapses can be selected on the basis of differences in synapse turnover rates. The results suggest that part of the specificity of synaptic circuits may be acquired by a process of selection.

A factor extracted from chick embryo retina and spinal cord was found to agglutinate rabbit erythrocytes *in vitro*. The amount of agglutinin activity varies markedly during embryonic development in the spinal cord, rising to a peak on the 10th day of embryonic development and then decreasing 7-fold by the time of hatching. The factor was first detected in 10 day embryo retina and increases in concentration until the 16th day

in ovo. Despite differences in hemagglutination activity and the patterns of development, both the retina and spinal cord lectins exhibit the same specificity for saccharides. Lactose was the most potent inhibitor of hemagglutination found (half-maximal inhibition with 2×10^{-5}M lactose).

To identify retina molecules required for synaptogenesis or communication across the synapse, we have used the technique recently introduced by Milstein and coworkers of monospecific antibody synthesis by clonal spleen cell x myeloma hybrid cell lines formed by fusion of mouse myeloma cells with mouse spleen cells immunized against retina cells. Large quantities of monospecific antibodies can be obtained. Hybridoma cell lines were obtained which synthesize relatively homogeneous monospecific antibodies of high titer directed against cell membrane antigens of cells in retina and brain which were not detected with numerous other tissues. These antibodies bind to molecules that are specific markers for certain cells in the nervous system. Hybridoma A2B5 synthesizes an antibody directed against a molecule in plasma membranes of cell bodies of retina neurons which was not detected on axons or dendrites: whereas, other cell lines synthesize antibodies directed against membrane molecules both cell bodies and processes of retina cells. Further characterization of antibody A2B5 and other monospecific antibodies directed against retina or neuroblastoma cell surfaces are in progress, including evaluation of their effects on neural functions.

DISCUSSION

GIACOBINI

In the first part of your lecture you emphasized the importance of the phosphodiesterase-adenylcyclase system and cyclic AMP, and you drew a relationship between this activity and the formation of the synapse. Then, somewhat later you introduced a new concept, that is, cyclic AMP as being connected to the release of acetylcholine. You also mentioned, in one sentence, the depolarizing effect of acetylcholine. My question is: Is the effect of acetylcholine that of a depolarizing agent during synaptic formation in converting this cell population from synaptic non-forming to synaptic-forming?

NIRENBERG

No, I did not mean to imply that depolarization is necessary for forming the synapse. If the cells synthesize acetylcholine, they release acetylcholine and in fact, we see depolarizing potential in the muscle cells but there is no evidence that depolarization is needed for it.

GIACOBINI

What is then the signal? How does the release carry on the message? What kind of signal? How do you envision the importance of neurotransmitter release on synaptic formation?

NIRENBERG

You mean release of acetylcholine from the neuron?

GIACOBINI

Right.

NIRENBERG

Well, if the cells do not release acetylcholine, they do not talk to the muscle cells.

GIACOBINI

O.K., so it is depolarization. That is an effect of neurotransmitter on the receptors. But you do not have evidence of this effect.

NIRENBERG

Oh yes, we measured that in our assay system.

GIACOBINI

But you do not have neurophysiological evidence that depolarization is actually important in order to establish synaptic connection?

NIRENBERG

Our assay is to stick a microelectrode in a muscle cell and measure depolarizing potential. We also can put another microelectrode in the hybrid cell and stimulate the cell electrically and measure the depolarizing potentials in the muscle cells and—I think Dr. Nelson will discuss this more—maybe I am not sure exactly what your question is.

GIACOBINI

You have answered my question. You say that depolarization, that is the effect of the neurotransmitter on the receptor is important, so I wonder what happens if you block the receptor. Have you done any experiment blocking the receptor and looking at synaptic formation?

NIRENBERG

Yes, we have. We have done many checks. D-tubocurarine, α-bungarotoxin or even β-bungarotoxin presynaptically inhibit the transmission across the synapse. There are papers in the literature that say that synapses will form under these conditions. I do not know if this is true because at the time those papers were published it was not known if synapses could form, so fast, within 30 minutes, for example.

GIACOBINI

I will bring some evidence that *in vivo* you can indeed block the receptor from the beginning and you will obtain almost a normal—well, normal as far as number of synapses; however, as far as biochemistry is concerned, highly abnormal—synaptic formation.

BURNSTOCK

Formation of synapses depends, of course, not only on nerves but also on muscle. You say that cyclic AMP increases synapse formation. It is interesting that in the smooth muscle system, cyclic AMP delays the dedifferentiation of smooth muscle cells; in other words it acts as a differentiating factor. One wonders, therefore, whether the muscle is more able to receive synapses because of the cyclic AMP.

NIRENBERG

We have looked at this and done experiments, for example, retreating the hybrid cells alone, with the same agents that I have shown, and then adding to the muscle cells, in the presence and absence of the agents, and although many more experiments ought to be done, all that the results suggest is, I think, that they show a pre-synaptic effect and an increase in the ability of the neuronal type cell, the hybrid cells, to store acetylcholine and to release acetylcholine when stimulated. There may well be also other effects. But we see a very large pre-synaptic regulation in synapse formation that is connected with the ability of the cells to release transmitter.

BURNSTOCK

Just one other comment. In the smooth muscle system, synapses continue to form even if neurotransmitter antagonists are present. This appears to differ from the situation you describe for ganglionic synapses.

PURVES

I wonder if a possible explanation for the window phenomenon that you described might be as follows. Some neurons are independent, in early development, of a trophic factor which is going to maintain them in later development and maturity; your results might represent this period of independence, rather than an influence on synapse formation *per se*.

NIRENBERG

Well, it is entirely possible, but when we measure cholinacetyltransferase activity in the cultures, we see an increase in this activity and that remains high. There is no death of cholinergic neurons. This is indirectly proved by the finding that transmitter synthesis and release into the medium remain high, even though those neurons no longer form synapse with muscle cells.

A working hypothesis is that those cholinergic neurons form in fact synapses with other retinal neurons and we see a segregation of retinal neurons into even monolayer cultures, and in cultures where these neurons aggregate and form balls, which consist almost entirely of retinal neurons.

PURVES

The failure to see cell death does not rule out the general idea in the sense that—as we heard this morning—there is considerable evidence for local effects on neurites, without gross damage to parent neurons.

NIRENBERG

But in many of these experiments we have tried to eliminate this possibility by using many retinal neurons—just covering the surface of the cell with retinal neurons—so that extensive neurite outgrowth is not needed to find an appropriate place to innervate. They cover the surface of the plate. I think that we would see synapses if they could in fact form; but we see segregation of cells in the case of the retinal cells. Retina cells adhere well to other retina cells and form aggregates, which in monolayer cultures would sort out from muscle cells.

NELSON

Might this be due to competitive situations? There is abundant evidence that throughout development neurons can establish new and even atypical synapses. Have you plated at very low density of retinal cells to see if the effect is an absolute inability to make connection with muscle or a competitive, preferential interaction of the retinal cells with one another?

NIRENBERG

We have plated a large number, we have looked at cell concentration in a systematic way, and at every cell concentration that we have looked at, we find the same thing. I do not think it is a question of cell concentration although I think cell concentration obviously will influence the aggregation of retina—the ability of retina neurons maybe to sort out from other neurons. We have not done a critical experiment—that we ought to do really—and that is to plate single retina neurons down in the absence of other retina neurons. We talked about it a lot, and it is a good experiment to do, but we have not done that yet. Maybe that is the way to test it.

Probably I did not understand the question of bungarotoxin synapses. Generally *in vivo* using bungarotoxin after twelve days the synapses are completely absent or, if they are present, they are in a very small number.

GIACOBINI

Did you investigate the relationship between receptor formation and developmental stage?

NIRENBERG

Yes. Matt Daniels has looked, in collaboration with Steve Vogel, at nicotinic acetylcholine receptor localization in the retina as a function of development. Now the earliest synapses that you can recognize by electromicroscopy appear in chicken retina on the 13th day of embryonic development. We see synapses starting, first appearing on the 6th to 7th day of embryonic development, and it is all over at 16 days. Daniels has done electromicroscopy on these cocultured cells and does not find well differentiated mature-looking synapses formed between retinal neurons and muscle cells. What he does find is that some retina neurons can form diminutive junctions between retina cells and muscle cells—there are occasional vesicles but not the collection of vesicles that he finds in presynaptic elements. But the electrophysiologic experiments clearly show that functional synapses can form, at a time when synapses are still not recognized by electromicroscopy. So I think that electromicroscopy is detecting very late events in synapse formation. It is clear that functional synapses form before you can recognize them by current electromicroscopic criteria.

GIACOBINI

I do agree with the latter. However, with regard to ganglionic synapses (ciliary ganglion synapses *in vivo*), bungarotoxin does *not* prevent the formation of synapses, it only modifies it. You have, as showed in my presentation, some changes in the acetylcholine-cholineacetylase-receptor system, but synapses are formed in the ganglia.

NIRENBERG

Yes, if we add α-bungarotoxin to the retina synapses, the transmission is blocked. β-bungarotoxin similarly will first increase the responses of the net frequency and then slowly over a period of an hour or so, eventually

totally inhibit spontaneous synaptic potentials in muscle. With the ganglion there are binding sites for nicotinic acid for α-bungarotoxin present, that seem to be highly specific binding sites which may be a part of the receptor but they are not functional with respect to depolarization, as probably Dr. Fambrough will discuss.

KOSTERLITZ

Do you know whether the α-adrenoceptor which stimulates adenylate cyclase formation belongs to the presynaptic or postsynaptic type of receptor?

NIRENBERG

It has the properties of a presynaptic alpha-receptor, although the criteria at this stage are not definitely established.

KOSTERLITZ

That is very interesting. In my talk I shall give some evidence of interaction of presynaptic opiate receptors and α-adrenoceptors in the morphine-dependent guinea-pig ileum. This interaction leads to a complete loss of sensitivity to clonidine in the withdrawn state, but the sensitivity is normal as long as morphine is present in the organ bath. It fits in very well with your observations.

RAITERI

Have you done any measure of high affinity uptake of choline in concomitance with the synapse formation and the increase in the release of acetylcholine?

NIRENBERG

Yes, we have, and there seem to be both high affinity and low affinity sites in the cell lines that we have looked at—we have looked at some of the high frequency synapse formation cell lines—we see an increase in the concentration of intracellular choline as the cells differentiate, but the data apparently are not good enough really to say whether we are getting a reduction or an increase in the number of high affinity uptake sites. However there are some technical difficulties with these experiments, and so the precision of our assays is not terribly good thus far. So I hesitate to make a strong statement just now.

SYNAPSE FORMATION OF CONTINUOUS CELL LINES

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Cells of the neuroblastomas X glioma hybrid clone NG108-15 were discovered to form functional cholinergic synapses when cocultured with embryonic myotubes (Nelson, Christian and Nirenberg, 1976; Puro and Nirenberg, 1976). Subsequently, functional synapses were found when myotubes were cocultured with cells of the S20Y neuroblastoma cell line, the NG108-15 hybrid cell line (Christian *et al.*, unpublished observations), and the PC12 pheochromocytoma cell line (Schubert, Heinemann and Kidokoro, 1977). The growing number of continuous cell lines shown to be synaptically competent (Puro and Nirenberg, 1976) suggests that many continuous cell lines are synaptogenic when sufficiently differentiated and cocultured with an appropriate target cell.

Functional synapses also form in systems in which the postsynaptic cells are from continuous cell lines. Spinal cord cells form synapses with the clonal myogenic cell line L-6, derived from rat hind limb cells (Kidokoro and Heinemann, 1974), and the myogenic cell line G-8, derived from mouse muscle (Christian *et al.*, 1977). Biclonal synapses, in which both the presynaptic and postsynaptic elements are clonal cell lines, have been produced by coculturing cells of cholinergic and myogenic cell lines (Christian *et al.*, 1977; Schubert, Heinemann and Kidokoro, 1977).

CLONAL CELL LINES IN THE STUDY OF SYNAPTOGENESIS

The advantages and disadvantages of cell culture in neurobiology have often been discussed (Nelson, 1975; Fischbach and Nelson, 1977). The synapses between nerve and muscle *in vitro* do not develop into fully

mature connections, and identifiable circuits which mimic the specificity of connections found *in vivo* are not yet available in culture systems. The present state of cell culture technology, therefore, most advantageously lends itself to the study of the early events in synaptogenesis at the cellular and molecular levels.

An important advantage of cell culture in the study of synaptogenesis is the opportunity to study presynaptic and postsynaptic cells in isolation, and to observe the modification of each cell's properties as a result of their interaction with one another. In addition, cell cultures have proven useful for the extraction and identification of molecules which may be involved in intercellular interactions.

The use of clonal cell lines permits a genetic approach to synaptogenesis (Nelson, 1978). The isolation of clones with synaptogenic defects may help in the identification of important processes and factors in synapse formation. If synaptogenic factors have a high degree of synaptic specificity and different classes of neurons produce different synaptogenic factors, clonal cell lines may be useful in obtaining substantial quantities of a specific factor, as clonal myeloma cells were used in analyzing the molecular structure and specificity of immunoglobulins (Kabat, 1976).

A PRESYNAPTIC CLONE: PROPERTIES OF THE NG108-15 CELL

The synaptically competent clonal cell line NG108-15 was derived by somatic cell hybridization (see Nirenberg *et al.*, Hamprecht, this volume). The parent cell lines were the mouse neuroblastoma clone N18TG-2 and the rat glioma clone C6BU-1. Both parent cell lines had negligible levels of Choline Acetyltransferase (CAT), the rate limiting enzyme in the synthesis of acetylcholine. Cells of the hybrid clone NG108-15 were found to have high levels of CAT activity. Synapses were sought by co-culturing NG108-15 cells with striated embryonic mouse myotubes. Because dibutyryl cAMP (dBcAMP) induced the formation of neurites (Daniels and Hamprecht, 1974) and increased the endogenous levels of CAT activity in NG108-15 hybrid cells, the cells were pretreated with dibutyryl cAMP for at least 1 week before being added to the muscle cultures. When plated on muscle cells in the presence of dibutyryl cAMP, NG108-15 cells formed processes with varicosities and terminal swellings. NG108-15 cells making contact with myotubes were chosen for electrophysiological study.

Electrophysiology of synapse formation

Intracellular recording techniques had shown that in NG108-15 cells differentiated by dBcAMP, intracellular depolarization elicits action potentials in most cells. When co-cultured with myotubes, approximately 30% of hybrid cell myotube pairs were synaptically connected. An action potential in the hybrid cell elicited a depolarizing response in the myotube. These excitatory postsynaptic potentials could be elicited by hybrid action potentials within 3 days of adding the hybrid cells to myotube culture (Nelson *et al.*, 1978). Spontaneous postsynaptic potentials were observed within hours after the addition of NG108-15 cells to myotube cultures (Higashida, personal communication). Individual synaptic connections were found to persist for a period of at least 24 hours, the longest time point sampled.

As shown in Fig. 1, hybrid cell action potentials produced in a myotube depolarizations of varying amplitude and latency. The polarity of the elicited myotube depolarizations was reversed by an adjustment of the myotube membrane potential to more than – 10 mVolts. The myotube depolarizations elicited by hybrid cell action potentials were reversibly blocked by d-tubocurarine and irreversibly blocked by alpha-bungarotoxin (α-btx). The replacement of calcium ions in the recording medium with magnesium ions produced a reversible inhibition of synaptic transmission. Taken together, the evidence is overwhelming that the functional connections are due to a chemical synapse utilizing acetylcholine as a neurotransmitter.

Not all hybrid cell action potentials were followed by myotube depolarizations in synaptically coupled hybrid cell myotube pairs. Indeed, when the rate of failures was used to compute the quantal content, it was generally less than 1. Thus, myotube depolarizations elicited by hybrid cell action potentials are thought to represent the release of one quantum. Low quantal contents are a feature of all presynaptic continuous cell lines which form synapses, a situation similar to that found with developing embryonic neuromuscular junctions *in vivo* (Diamond and Miledi, 1962; Redfern, 1970; Bennett and Pettigrew, 1974). Whereas the quantal content increases with the development of the neuromuscular junction, the quantal content of NG108-15 cell myotube connections does not increase over a period of 3 weeks. It is of great interest to determine what additional culture conditions will modulate the connectivity in these co-cultures.

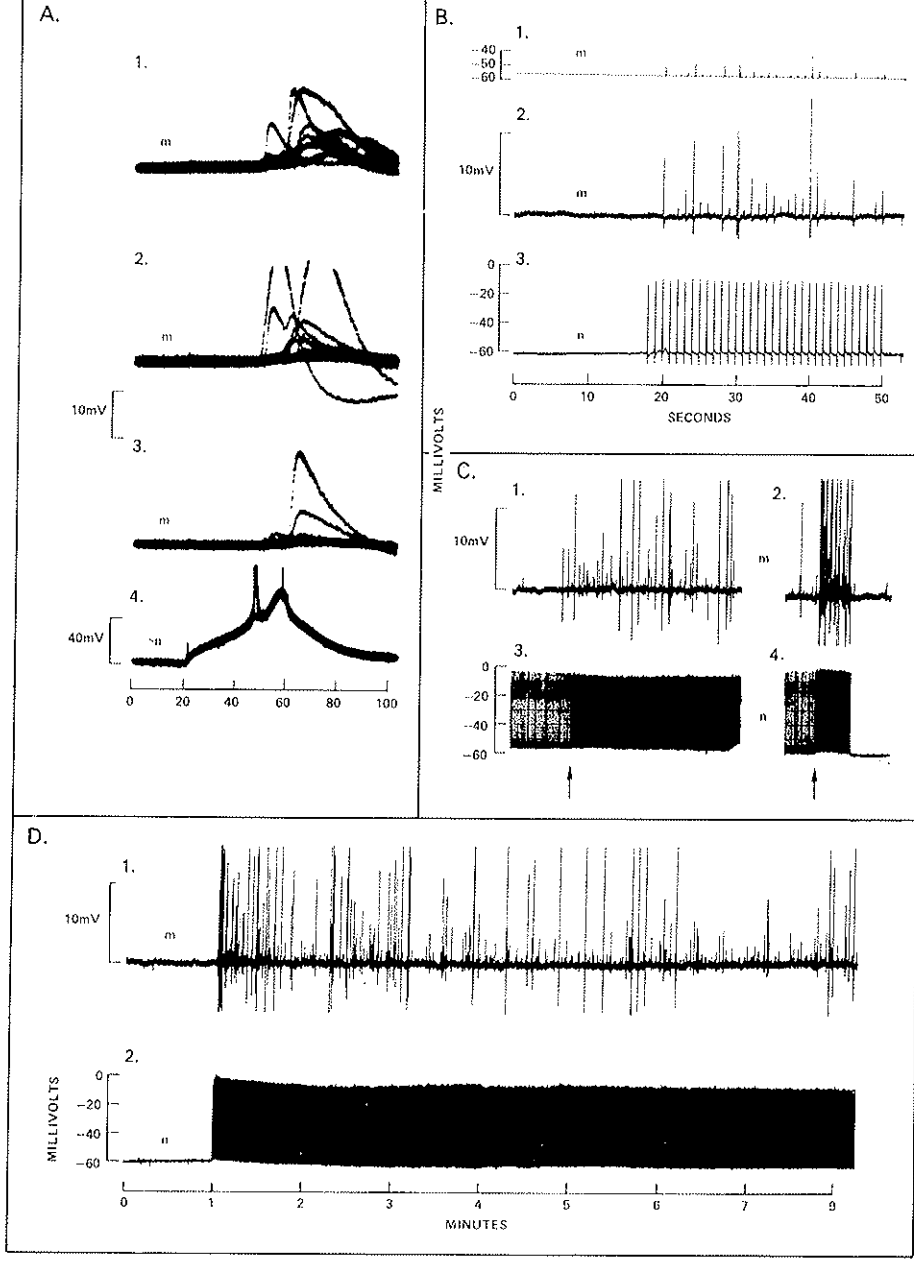

FIG. 1. Functional connections between NG108-15 cells and mouse myotubes. All traces marked with an n are intracellular records of an NG108-15 cell in which action potentials were elicited by intracellular depolarization. Traces marked with an m are intracellular records in an adjacent myotube. In A, multiple oscilloscope traces demonstrate depolarizing responses of varying amplitude and latency in an innervated myotube. The time base is in milliseconds. In B, a chart record demonstrates nearly one to one following between NG108-15 action potentials and myotube responses. Panel C shows the frequency potentiation of synaptic activity; at the arrows, the rate of NG108-15 cell action potentials was increased from 1/sec to 3/sec. Panel D demonstrates the fatiguability of a synaptic connection during continual stimulation at 3/sec (Nelson *et al.*, 1978).

Presynaptic pharmacology of the hybrid cell myotube synapse

When grown alone NG108-15 cells possess a variety of receptors for neuroactive compounds. Acetylcholine, dopamine, noradrenaline or 5-hydroxytraptamine (5-HT) applied to the membranes of NG108-15 cells produce depolarizations (Hamprecht, 1974; Myers and Livengood, 1975; Christian *et al.*, 1978 a; Nirenberg *et al.*, this volume). Either 5-HT or acetylcholine, when iontophoresed onto the surface of hybrid cells produced a graded depolarization. The effect was caused by an increase in the hybrid cell membrane conductance. The reversal potential for the response was approximately – 10 mVolts. The majority of hybrid cells tested were responsive to both 5-HT and acetylcholine, but some cells were responsive to only one of these compounds. In addition, many cells responded to iontophoretically applied dopamine with depolarizing responses. When expressed as mVolts of depolarization per nAmp of current passed through the iontophoretic pipette, the relative responsivity of the majority of cells was in the order: 5-HT $>$ acetylcholine $\gg$ dopamine.

By the iontophoretic application of large quantities of either of the three neurotransmitters, hybrid cell membrane potentials approached the reversal potential for the drug response. Following such applications, the cells were refractory to subsequent applications of the same neurotransmitter. This period of desensitization lasted for a period of minutes. Following desensitization to 5-HT, the NG108-15 cells were also refractory to dopamine, but not to acetylcholine. Cells desensitized to acetylcholine were still responsive to 5-HT. Consequently, although 5-HT and dopamine may activate the same receptors, 5-HT and acetylcholine appear to activate different receptors on NG108-15 cells.

There is no clear correlation between electrophysiologic evidence of agonistic activity and the effectiveness of agents which change cyclic nucleotide levels in hybrid cells. 5-HT, for instance, which produces dramatic electrophysiologic responses has no effect on cyclic AMP or cyclic GMP levels in hybrid cells (Matasuzawa and Nirenberg, unpublished observations). Intracellular levels of cyclic nucleotides in NG108-15 cells are modulated by the activation of receptors for opiates (Sharma, Klee and Nirenberg, 1975), PGE1 (Hamprecht and Schultz, 1973), or PGF2α (Matsuzawa and Nirenberg, unpublished observations). When tested in hybrid cell myotube co-cultures, morphine or the prostaglandin PGE1 had little effect on the membrane potential of NG108-15 cells. The prostaglandin PGF2α produced a depolarization of hybrid cells.

When 5-HT was applied to a NG108-15 cell, the depolarization of the hybrid cell soma was often accompained by synaptic responses in an adjacent myotube. The passive depolarization of the hybrid cell membrane to the level produced by application of 5-HT did not evoke synaptic responses in the myotube. Hence, 5-HT may elicit synaptic release from hybrid cells by some means other than hybrid cell depolarization. In a similar fashion, PGF2α, applied alone or with a phosphodiesterase inhibitor, evoked synaptic release when applied to hybrid cells.

In a number of cases, 5-HT elicited synaptic release only when applied to discrete areas on the processes of hybrid cells which made contact with myotubes. The application of the same amount of 5-HT to the hybrid cell soma, although producing a larger depolarization of the hybrid cell, did not elicit synaptic release. This method of focal application of 5-HT to NG108-15 cell processes was used to locate hybrid cell release sites for subsequent ultrastructural analysis.

In addition to eliciting synaptic release, 5-HT or PGF2α facilitated the release elicited by NG108-15 cell action potentials. During a continuous train of hybrid cell action potentials produced by intracellular pulses passed through a recording pipette, the application of either 5-HT or PGF2α produced an increase in the frequency of evoked responses. Passive depolarization of the NG108-15 cell membrane to levels attained during the application of either agent did not produce facilitation. The period of facilitation produced by 5-HT or PGF2α often outlasted the effect on the membrane potential. Hence, it is thought that facilitation of action potential elicited release from NG108-15 cells is not caused by hybrid membrane depolarization. At a cholinergic synapse in Aplysia 5-HT also causes facilitation, an effect thought to be mediated by cAMP (Kandel *et al.*, 1976). However, the mechanism of facilitation in NG108-15 cells may not involve cAMP, because 5-HT, as mentioned above, has no detectable effect on intracellular levels of cAMP, and PGE1, which increases the level of cAMP in hybrid cells, produces little synaptic release or facilitation.

Fine Structure of the hybrid myotube contact

When grown alone and treated with dBcAMP NG108-15 cells extend processes which contain parallel arrays of microtubules and 10 nm filaments (Daniels and Hamprecht, 1974). The processes also contain 60 nm clear vesicles which resemble those found at the presynaptic endings of cholinergic synapses, and larger, electron dense vesicles, which resemble the dense-core vesicles found in adrenergic neurons.

The ultrastructure of functionally connected hyhrid cell myotube pairs was studied (Nelson *et al.*, 1978). In some cases, release sites were located by the application of 5-HT to hybrid cell processes (see Fig. 2). After electrophysiological study, co-cultures were embedded and stained, the functional pairs relocated and multiple thin sections inspected by electron microscopy. NG108-15 cell processes were separated from myotube membranes in some cases by a gap of approximately 15 nm, and in other cases across a 50 nm gap which contained basement membrane-like material. The intracellular fine structure of the hybrid cell processes apposed to myotubes did not differ substantially from that of hybrid cells grown alone. The NG108-15 cells known to make cholinergic synapses contained dense core vesicles as well as small clusters of clear vesicles, some near areas of apposition to myotubes. The processes did not have the specialized presynaptic structures found to be associated with the release sites of nerve endings at the neuromuscular junction.

There is evidence that acetylcholine release from PC12 cells involves in part large dense core vesicles. It remains to be determined whether the clear vesicles or dense core vesicles mediate release from NG108-15 cells. The ultrastructure of NG108-15 cells is at least consistent with the 60 nm clear vesicles releasing acetylcholine. The relative paucity of either type of vesicle in the hybrid cell processes is consistent with the low quantal content and fatiguability of the hybrid myotube synapse.

Developmental interaction of pre- and postsynaptic elements

A variety of observations on developing synaptic systems *in vivo* indicate that powerful mutual inductive or trophic interactions occur between the presynaptic neuron and the postsynaptic muscle (Changeux and Danchin, 1977). The survival of the spinal cord motorneuron and the integrity of the muscle fiber appear to depend on the successful establishment of synaptic connections. Can one identify and characterize tbe molecules that mediate the nerve-muscle developmental interactions in synapse formation?

Intercellular regulation of Choline Acetyltransferase Activity

Choline acetyltransferase activity, found in presynaptic cholinergic neurons, increases substantially during the period these neurons establish functional synapses with target tissues. To study the possible cellular interaction between cbolinergic neurons and target tissues, dissociated

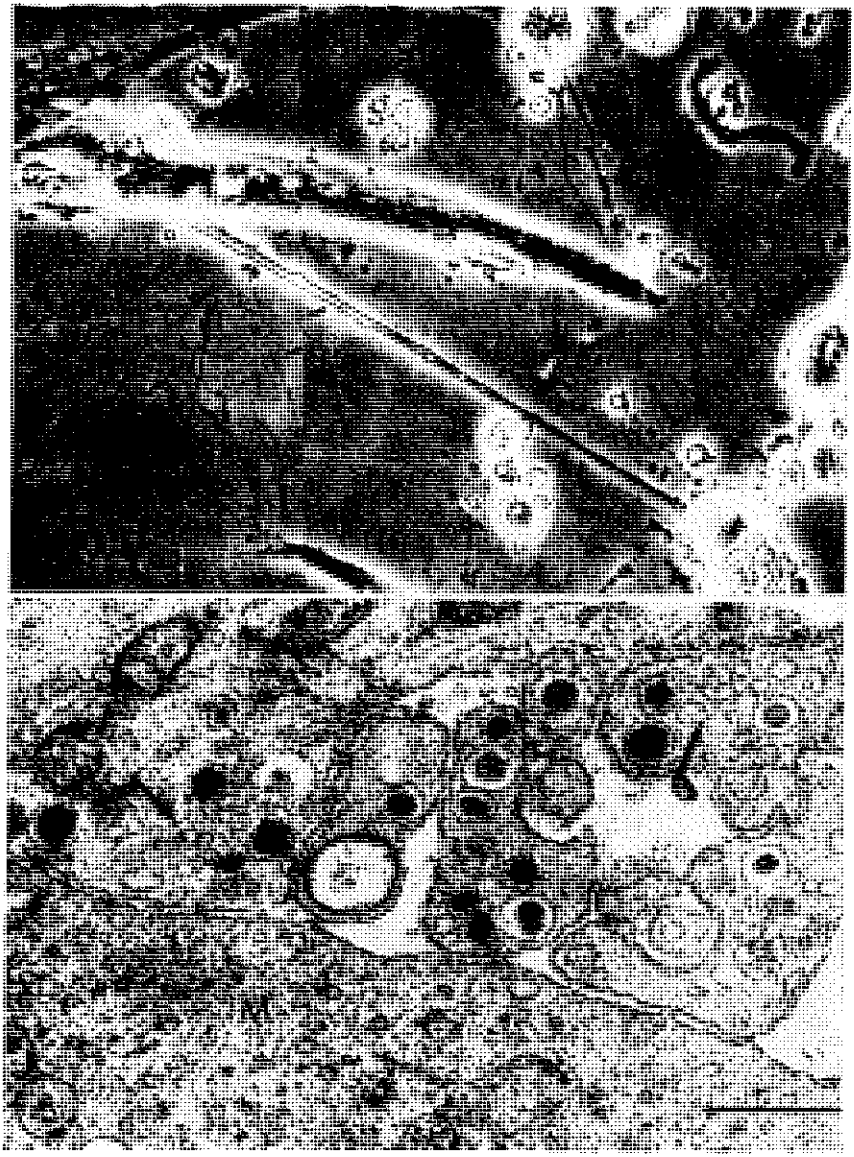

FIG. 2. Panel A is a phase contrast photomicrograph of a myotube functionally innervated by an NG108-15 cell process. Panel B is an electron micrograph of one of the contact regions between the myotube and nerve process. The arrows indicate clusters of clear vesicles (Nelson *et al.*, 1978).

mouse spinal cord cells were cocultured with embryonic mouse striated myotubes in a system show to form functional cholinergic synapses. The CAT activity of spinal cord cells cocultured with muscle cells increased by more than 10 fold when compared to spinal cord cells grown alone (Giller *et al.,* 1973).

Choline acetyltransferase in spinal cord cultures was also increased by the addition of medium conditioned by contact with myotube cultures (Giller *et al.,* 1977). CAT stimulating factor in muscle conditioned medium is a macromolecule with a molecular weight probably greater than 50,000 Daltons. The factor exhibits both tissue and species specificity. With mouse spinal cord cultures used as the assay system, CAT activity was increased by media conditioned by cultures of mouse striated myotubes, heart cells, kidney cells, or primary liver cells, but CAT activity was not enhanced by media conditioned by mouse skin cells or a secondary culture of liver cells (Godfrey, Schrier and Nelson, unpublished). Rat muscle conditioned medium enhanced CAT activity, but not chick muscle conditioned medium, even though chick muscle conditioned medium increases CAT activity in chicken spinal cord cultures (Popiela *et al.,* 1978).

A factor which increases CAT activity in cultured rat sympathetic ganglion cells has also been found in medium conditioned by rat muscle cells and other rat cell types (Patterson, Chun and Reichardt, 1977; Patterson, 1978). The species and tissue specificity of the factor stimulating CAT in rat sympathetic ganglion cells differs somewhat from the factor which stimulates CAT activity in mouse spinal cord cells. It remains to be determined if these factors are the same molecule.

CAT activity in the NG108-15 cells was also increased by a factor found in medium conditioned by mouse muscle cells (Godfrey, Schrier and Nelson, unpublished). The CAT activity expressed in 4 other continuous neuronal cell lines was not modulated by muscle conditioned medium. Although muscle conditioned medium and dBcAMP botb increase CAT activity in NG108-15 cells, they probably operate by different mechanisms. Conditioned medium increased NG108-15 cell CAT activity in the presence or absence of dBcAMP. Unlike treatment with dBcAMP, muscle conditioned medium did not promote the outgrowth of NG108-15 cell processes.

Regulation of Acetylcholine synthesis and release

The capacity of NG108-15 cells to synthesize and release acetylcholine was studied by direct biochemical measurement (McGee *et al.,* 1978; Nirenberg *et al.,* this volume). For this purpose, hybrid cells were grown

on the inside surface of capillary tubes, which permitted rapid perfusion and fast exchange of media with a minimum of mixing. Capillary tube cultures were labelled with tritiated choline and then washed by perfusion. Levels of ACh and choline in each perfusate fraction were compared to the levels which remained in the hybrid cells.

The metabolism of ACh was regulated by dBcAMP. As the NG108-15 cells differentiated during treatment with dBcAMP, there was an increase in the uptake of tritiated choline and the synthesis of acetylcholine.

During the perfusion of NG108-15 cells which had been labelled with tritiated choline, acetylcholine was released into the perfusion medium (Fig. 3). The rate of release of acetylcholine was increased by depolarizing the cells with perfusion solutions containing 80 mM potassium ions. Stimulated release of ACh was also obtained with veratridine; this effect was blocked by tetrodotoxin. Thus, part of the measured efflux of acetylcholine was due to depolarization of the cells and activation of voltage dependent sodium ionophores. The potassium stimulated release of ACh was blocked by replacing calcium with magnesium in the perfusion medium. The potassium stimulated release of acetylcholine was only detected after hybrid cells were treated for 3 days with dBcAMP.

Additional evidence that this biochemical measure of ACh release is related to the electrophysiologically measured synaptic activity in NG108-15 cell myotube co-cultures was obtained by studying ACh release evoked by neuroactive compounds. Perfusion media containing 5-HT or PGF2α evoked ACh release from NG108-15 cells, but PGE1 evoked little ACh release.

Although high potassium also stimulated the efflux of choline from NG108-15 cells, stimulated choline release differed from stimulated acetylcholine release. Potassium stimulated choline release was found in hybrid cells not treated with dBcAMP, and was not blocked by removing calcium ions from the perfusion medium. Although veratridine stimulated choline release, the effect was not tetrodotoxin sensitive. Moreover, choline was not released by 5-HT or PGF2α.

Therefore, NG108-15 cells grown in the absence of muscle cells can organize the presynaptic machinery required for the action potential elicited release of acetylcholine. In comparison with other synaptic systems, the relative efficiency of the synaptic process in the NG108-15 cell system is low. The NG108-15 cell converts a lower percentage of intracellular choline into acetylcholine than found in synaptosomes or

another cell line, the PC12 pheochromocytoma clone (Schubert and Klier, 1977). Moreover, the proportion of intracellular acetylcholine released by stimulation is lower than found in other systems. An important question is whether, in addition to dBcAMP, other factors will increase

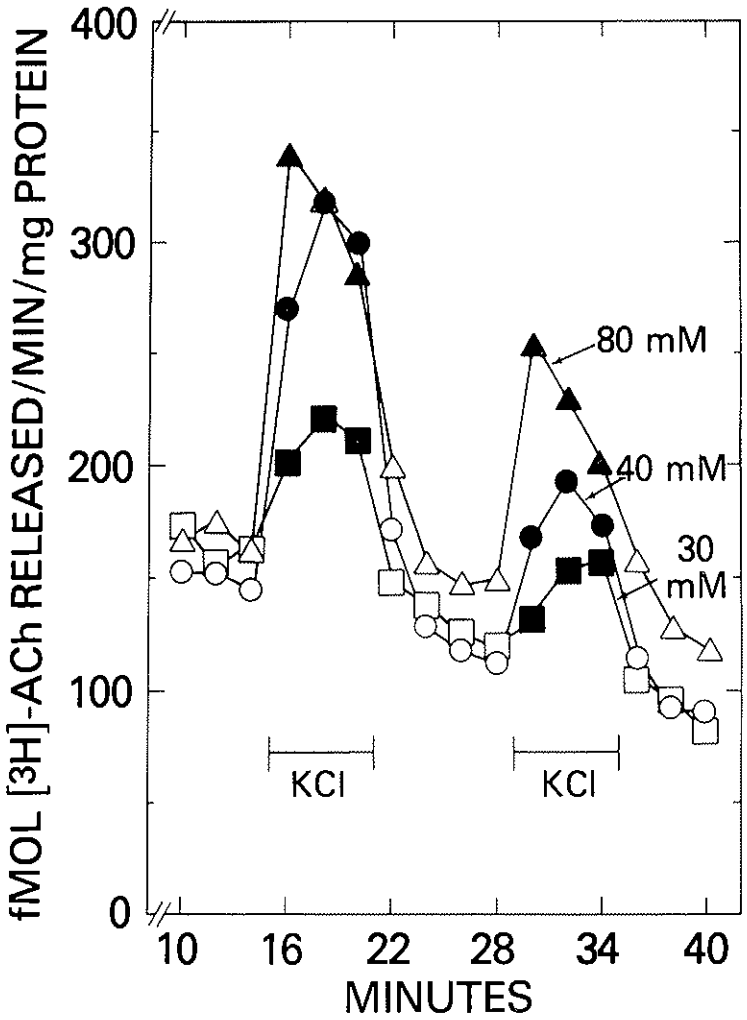

FIG. 3. Depolarization of NG108-15 cells by KCl increases the rate of efflux of acetylcholine. NG108-15 cells were treated for 11 days with dBcAMP, labelled for 45 minutes with tritiated choline and then perfused with choline free medium containing various concentrations of KCl (solid symbols). Each point represents the amount of acetylcholine in a fraction of the perfusate taken every 2 minutes (McGee *et al.*, 1978).

the relative efficiency of synaptic release in these cells. Will the factor from muscle cells which increases CAT activity in NG108-15 cells also increase the ability of these cells to release acetylcholine?

Long term modulation of synaptic connections

Because dBcAMP regulates many properties of the NG108-15 cell, its effect on synaptogenesis was determined. NG108-15 cells which had been predifferentiated in dBcAMP were added to mouse myotube cultures and given one of two anti-mitotic treatments in the presence of dBcAMP (Christian *et al.*, 1978). When treatment with cytosine arabinoside was used to suppress cell division, the maintenance of synaptic connections was dependent on the continual presence of dBcAMP. In co-cultures maintained in the absence of dBcAMP, only 16% of hybrid cell myotube pairs were synaptically connected, despite the maintenance of a normal differentiated morphology. By contrast, in co-cultures maintained in dBcAMP after treatment with cytosine arabinoside, 88% of pairs tested were synaptically connected. When co-cultures were X-irradiated to prevent cell division, over 60% of tested pairs were functionally connected in the absence of dBcAMP. Thus, the two anti-mitotic treatments, cytosine arabinoside and X-irradiation, produce populations of cells which differ in their capacity for synaptic modulation, and may be useful in studying the molecular mechanisms involved in synaptogenesis and the maintenance of synaptic connections.

Postsynaptic mechanisms in synapse formation

In vitro *Models of the Neuromuscular Junction*

There is a high degree of ultrastructural specialization of the presynaptic and postsynaptic cells at a synapse (see e.g. Fambrough, 1976). One aspect of this specialization at the neuromuscular junction is the higher concentration of junctional acetylcholine receptors (AChR) than extrajunctional AChR. The registration of presynaptic release sites with postsynaptic concentrations of acetylcholine receptor is clearly the result of interaction between the nerve and muscle, but the nature of the mechanisms *in vivo* is not known. Is a discrete area on the muscle cell marked as a site of innervation before actual contact by a nerve process? Does the nerve process induce a local accumulation of AChR on the muscle? It is clear that the electrical activity of the muscle cell modulates the rate of synthesis of AChR and the concentration of extrajunctional

AChR (Lomo and Westgaard, 1976), but activity-related modulation of muscle AChR does not explain the formation or persistence of the high concentration of junctional receptors. Consequently, researchers are turning their attention to mechanisms which affect the spatial distribution of AChR in the muscle cell membrane. We will consider three aspects of the distribution of acetylcholine receptors on muscle cells: localization, stabilization and registration.

Localization of acetylcholine receptors

The distribution of acetylcholine receptors on innervated muscle *in vivo* is non-random. At the junctional areas of the muscle cell membrane the average concentration of acetylcholine receptors is much higher than the concentration of extra-junctional receptors. On cultured myotubes, the distribution of AChR is nonrandom and developmentally regulated. After cultured myoblasts spontaneously fuse to form myotubes, the AChR which appear on the myotube membrane initially have a homogeneous distribution. After a few days in culture, approximately 10% of the myotube AChR are found in small areas of a high receptor concentration (Vogel, Sytkowski and Nirenberg, 1972; Sytkowski, Vogel and Nirenberg, 1973; Fischbach and Cohen, 1973; Bekoff and Betz, 1976; Axelrod *et al.*, 1976). In mouse myotubes, these acetylcholine receptor aggregates or "hotspots" are patches with an average diameter of 10 microns. Although the role of serum factors cannot be ruled out entirely, the formation of AChR aggregates on myotubes in the absence of innervation suggests that the myotubes have the machinery to modulate the distribution of acetylcholine receptors on their membranes.

NG108-15 cells modulate the localization of the acetylcholine receptor of myotubes (Christian *et al.*, 1978 b). Established cultures of embryonic mouse striated myotubes were plated with NG108-15 cells and treated in the same way as cultures shown by electrophysiologic means to form synapses. After 7 days of co-culture the distribution of nicotinic acetylcholine receptors was visualized by labelling the cultures with α-btx and then staining the bound toxin by an immunoperoxidase method. Muscle cultures grown with NG108-15 cells had a 3 fold increase in the number of acetylcholine receptor aggregates per myotube when compared to muscle cultures grown without NG108-15 cells.

NG108-15 cells produce a soluble factor which increases the number of acetylcholine receptor aggregates per myotube. Medium without serum was added to flasks of NG108-15 cells, removed after 24 hours and

added to myotube cultures. After 6 days of this daily treatment, the number of acetylcholine receptor aggregates per myotube increased nearly five fold when compared to myotube cultures which received daily feedings with fresh medium. A 3 fold increase in the number of AChR aggregates per myotube occurred within 24 hours after the treatment of muscle cultures with NG108-15 cell conditioned medium. The effect was not produced by the non-specific effect of proteins released by hybrid cells into the conditioned medium, because fresh medium plus 5% horse serum had little effect on the number of AChR aggregates per myotube. The acetylcholine receptor aggregating activity in NG108-15 cell conditioned medium is heat labile and is retained by a dialysis membrane.

The expression of AChR aggregating activity was tested in the two parent cell lines of the NG108-15 somatic cell hybrid. Medium conditioned by cells of the N18TG2 neuroblastoma parent cell line produced a 3 fold increase in the number of myotube receptor aggregates per myotube. Medium conditioned by cells of the glioma parent C6BU-1 had no detectable effect on the number of acetylcboline receptor aggregates per myotube. Thus, the parent cell line with neuronal properties also produced acetylcholine receptor aggregating activity.

The co-culture of myotubes with spinal cord cells or sympathetic ganglion cells, which form functional cholinergic synapses with myotubes, also produced an increase in the number of acetylcholine receptor aggregates per myotube. Medium conditioned by spinal cord cultures or sympathetic ganglion cell cultures also contained AChR aggregating activity (Shaffner, Christian and Daniels, unpublished observations). Media conditioned by cells of 2 fibroblast cell lines had no detectable AChR aggregating activity. There is no postsynaptic species specificity for AChR aggregation by NG108-15 cell conditioned medium. Medium conditioned by NG108-15 cells increased the number of acetylcholine receptor aggregates on muscle cells derived from mouse, rat or chicken muscle.

Mechanism of Acetylcholine receptor localization

Medium conditioned by NG108-15 cells increases the number of acetylcholine receptor aggregates per myotube by redistributing mobile acetylcholine receptors. The evidence is threefold. First, tbe increased number of AChR aggregates on myotubes treated with NG108-15 cell conditioned medium is not due to a comparable increase in the number of acetylcholine receptors. Myotubes treated with NG108-15 cell condi-

tioned medium showed a 3 to 4 fold increase in AChR aggregates per myotube over a period of twenty four hours, with no more than a 20% increase in the total number of myotube acetylcholine receptors. Second, the increase in aggregates per myotube caused by conditioned medium did not require the synthesis and insertion of new acetylcholine receptors into the myotube membrane, since it occurred in the presence of cycloheximide. Third, myotube cultures prelabelled with α-btx showed the same increase in the number of acetylcholine receptor aggregates per myotube as cultures treated with conditioned medium and then labelled with α-btx. Hence, the observed aggregates contained ACh receptors which were present in the myotuhe membrane before the addition of conditioned medium. Because the number of AChR aggregates per myotube in prelabelled cultures was increased 3 fold by NG108-15 cell conditioned medium, the number of labelled diffusely distributed receptors which became redistributed into AChR aggregates was increased by NG108-15 cell conditioned medium.

As determined by fluorescence photobleaching experiments (Axelrod *et al.*, 1976), the diffusely distributed acetylcholine receptors of cultured myotubes are mobile in the plane of the membrane, whereas the ACh receptors found in aggregates are nearly immobile. An ACh receptor aggregation factor may reduce the mobility of the myotube ACh receptors, either by directly cross-linking ACh receptors or by promoting the linkage of ACh receptors to the myotube cytoskeleton. We have no direct evidence as to the mechanism involved in the action of the AChR aggregation factor, but results from other systems suggest a number of testable hypotheses. It has been demonstrated that the spatial arrangement of surface membrane receptors can be affected by forces acting either on the external surface or on intracellular cytoplasmic systems. Cross-linking of receptors by extracellular divalent ligands induces the formation of receptor aggregates in lymphocytes and fibroblasts (Nicolson, 1976; Schreiner and Unanue, 1976). Antihodies to AChR increase the number of AChR aggregates on cultured myotubes and increase the rate of AChR degradation (Drachman *et al.*, 1978; Prives *et al.*, in press). High concentrations of surface membrane proteins are located near accumulations of membrane associated actin (Bourguignon, Tokuyasu and Singer, 1978). Covalent modifications of receptor molecules (such as phosphorylation or dephosphorylation) has been proposed as a mechanism for altering the affinity of receptor molecules for one another and hence changing the prohability of receptors forming aggregates (Gordon, Davis and Diamond, 1977; Teichberg, Sobel and Changeux, 1977).

Stabilization of the acetylcholine receptor

The half-life of acetylcholine receptors in cultured myotubes was not effected by the presence of NG108-15 cells (Sugiyama, unpublished observations). Cultures of mouse myotubes were labelled with radioactive α-btx and the rate of appearance of the radioactive label in the medium was used to determine the kinetics of the degradation of acetylcholine receptors. The loss of acetylcholine receptor from mouse myotubes grown alone demonstrated first order kinetics and suggested that there is predominately one population of receptors. The rate of degradation of AChR in myotube cultures grown with NG108-15 cells was nearly identical to that derived from myotubes grown alone. The number of acetylcholine receptors found in aggregates is probably less than 10% of the diffusely distrihuted receptors. Although the presence of NG108-15 cells does not increase the half-life of the majority of acetylcholine receptors, this smaller population of receptors may be stabilized by the presence of NG108-15 cells.

On muscle *in vivo,* the junctional ACh receptors are more stahle than extrajunctional ACh receptors (Chang and Huang, 1975; Brockes, Berg and Hall, 1976). At the diaphram neuromuscular junction, the half-life of degradation of junctional ACh receptors is at least six times the half-life of extrajunctional receptors. However, in cultured myotubes, the ACh receptors located in aggregates have no longer half-life than the diffusely distributed ACh receptors (Schuetze, Frank and Fishbach, 1978). The lack of an increased stability of the aggregated ACh receptors on cultured myotubes may indicate that the AChR aggregates are not precursors of junctional receptors. On the other hand, it may indicate that the initial interaction between nerve and muscle which redistributes the muscle cell acetylcholine receptors does not increase the stability of the receptor. The mechanism which stabilizes acetylcholine receptors at the neuromuscular junction may operate after the initial redistribution of acetylcholine receptors (see Burden, 1977a; Burden, 1977b), and may not be found in present myotube cultures.

Registration of Acetylcholine receptors

The most salient feature of the neuromuscular junction is that the ingrowing axon establishes contact with areas on the myotube membrane which contain high concentrations of AChR. A similar alignment of neurites and muscle cell AChR aggregates have been seen *in vitro* (Stein-

bach *et al.*, 1973; Cohen and Fishbach, 1977; Frank and Fishbach, 1977; Anderson, Cohen and Zorychta, 1977). Recent data suggest that this alignment of presynaptic and postsynaptic elements can result from the local induction of an AChR aggregate by an ingrowing neurite. When a neurite from a spinal cord explant was allowed to innervate a myotube which had been labelled with fluorescent α-btx, high concentrations of ACh receptors were seen to form under the ingrowing neurite (Anderson and Cohen, 1977). Thus a neurite can produce a local concentration of ACh receptors on the myotube it innervates. Because the visualized receptors were lahelled with α-btx before the addition of the spinal cord explants, the induced aggregates resulted from the lateral motion of existing myotube receptors.

What is the relation between a soluble ACh receptor aggregating factor and the accumulation of myotube ACh receptors under an innervating neurite? In co-cultures of NG108-15 cells and myotubes, some of the myotube AChR aggregates occur in registry with NG108-15 cell neurites. Moreover, the results of the electrophysiological mapping of myotube ACh sensitivities suggested that the NG108-15 cell presynaptic release sites are aligned with the small discrete areas of high acetylcholine sensitivity on mouse myotubes (Christian *et al.*, 1977). It is likely, therefore, that the release sites of NG108-15 cells are found in registry with local concentrations of myotube AChR. If the soluble AChR aggregation factor produces this registry, it may be released at hybrid cell release sites, or may be attached to the hybrid cell membrane at release sites. In either case, we postulate that a local concentration of the AChR aggregation factor produces a local concentration of the myotube AChR.

The general problem of synaptic registration

The alignment of presynaptic release sites and postsynaptic receptor aggregates is clearly not a phenomenon restricted to the nerve-muscle synapse. It seems reasonable to suppose that the mechanisms involved in such alignment at the neuromuscular junction would have counterparts in other synaptic systems. We make the working hypothesis that there is a family of aggregation factors with specificity for a variety of receptors. A neuron synthesizing a given neurotransmitter would be expected to make the aggregation factor specific for the receptor with which this neurotransmitter interacts. By means of a receptor specific aggregation factor, a neuron would organize the surface membranes of only those cells which have appropriate receptors for the presynaptic neuron.

We have not touched on the important questions of the maturation and stabilization of synapses following the initial registration or pre- and postsynaptic elements. Although they are insufficient to account for many of the processes of consolidation in synaptogenesis, we feel that the phenomena discussed in this paper are involved in the initial stages of synapse formation and play a critical role in the development of the nervous system.

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DISCUSSION

HÖKFELT

I was impressed by the first set of slides, where you had injected peroxidase and the possibility to follow the same axon at various places where it contacts different types of neurons. I was just wondering if the axons or the terminals always looked the same at the contact sites, i.e., if the synapses had the same structural features.

NELSON

Yes. As you know, it is difficult to make an absolutely complete reconstruction, but Dr. Neale is very persistent, she did get 60 to 80% reconstruction of several of these sets of contacts and they were always homogeneous for a given cell.

LEVI-MONTALCINI

Is anything known about the chemical nature of this factor?

NELSON

The aggregation factor is a macromolecule but has not been purified extensively as yet; we are in the process of attempting to do this.

ROBERTS

I wanted to mention that when you talk about receptor you really talk about a recognition protein and I think you have the channel, the pump and the transport system or degradative system if it is acetylcholine involved. Do you think that these various macromolecules would be organized at that site as well as just the recognitions site?

NELSON

A number of membrane components certainly are typically brought together at most synapses. In the system I described we are looking only at the receptor, but it would certainly be of great interest to see to what degree other membrane proteins are or are not co-aggregated with the receptor.

PURVES

If receptors are aggregating, then shouldn't the number of hot spots per myotube decrease during the course of innervation? The experiments of Monroe Cohen and others suggest that as labeled receptors aggregate under the nerves, extra-synaptic receptors disappear. Could you comment on this?

NELSON

The experimental data show that there is an increase in aggregates or "hot spots" in the absence of any overall increase in total acetylcholine receptors, so there must have been a decrease in diffuse, background receptors. We have not directly looked at this quantitatively but it should be done.

FAMBROUGH

I would like you to clarify your answer to Dr. Roberts' question, which I think was: do acetylcholinesterase, ion pumps and receptors coaggregate? My understanding is that ion pumps are not clustered at synapses the way receptors are, and that the esterase does not cluster simultaneously with the clustering of receptors during development and may not even be located in the plasma membrane.

NELSON

In general the postsynaptic membrane specialization undoubtedly involves more than just an accumulation of receptors. Our data do not deal with the question of co-migration or co-aggregation of any molecules other than the acetylcholine receptor. This is clearly an important question that needs to be addressed.

FAMBROUGH

That implies that these different components are separate; we also do not know whether the ion channel is a different thing from the finding in a fluorescent column.

HAMPRECHT

Is the effect of your factor reversible?

NELSON

We have not looked at the question of reversibility.

GREENE

I believe you have shown that a neurite can actually crawl along a muscle for quite a distance before forming a synapse. Would this imply then that there is no release of your factor along these other areas from which there is no formation of synapses or hot spots? If so, this would also imply that there must be a specific time when the neuron decides it is going to make a synapse.

NELSON

Steven Cohen at Bethesda looked physiologically at interactions between nerve and muscle at very early times following physical contact. By stimulating the nerve and averaging many responses, he was able to show that there were depolarizing responses in the subquantal (less than 50 μV) range. The best interpretation seemed to be that the nerve was releasing normal amounts of acetylcholine at regions of the muscle containing very low amount of acetylcholine receptor (AChR). This low AChR concentration could be shown by iontophoretic application of acetylcholine. Thus, the nerve can release acetylcholine in the absence of a well developed synapse.

GREENE

What about release of the factor itself? That is, there are many locations in addition to the point of synapse formation at which the neurite is in contact with the muscle. Presumably this would imply that there must be a specific release of the factor at a specific point along the neurite, or else you would have hot spots all along the places where the neurite comes in contact with the muscle.

NELSON

We do see such linear arrays of AChR; Anderson and Cohen showed the same thing.

PURVES

If the agent is in the medium, then wouldn't you expect to see hot spots developing extra-synaptically?

NELSON

Aggregates forming and becoming stable at sites of nerve contacts in co-cultures would presumably be due to a high concentration of the aggregation factor at these contact sites.

PURVES

But you would need something else, I think. Isn't it the case that new hot spots do not form in these experiments except where the nerve contacts muscle? Either they disappear, or they appear underneath the growing axon.

NELSON

I'm not sure to what degree the point has been made that there are no hot spots other than at contacts between nerve and muscle.

BURNSTOCK

I just wondered whether you get anything comparable to "rejection". In the system we use, "recognition" takes place, even when two nerves get to a cell at once. If another nerve fiber reaches the cell later, it is "rejected", perhaps by "switch-off" of the "recognition" system. Do you get multiple innervation and do you have any evidence of "rejection"?

NELSON

We do see multiple innervation in our cultures, but I don't know if hyper-innervation is less likely in a previously innervated fiber or not.

FAMBROUGH

There are two publications on the occurrence of larger numbers of hot spots on un-innervated muscle near nerve explants—one by Cohen and Fischbach and one by Betz and Beckoff. So it is probably true that there is some influence from a distance in culture. In relation to Dr. Burnstock's point, as far as I know all skeletal muscles are pluri-innervated at an early developmental stage, so even though it is difficult in culture to start asking how many connections there are on these fibers, you might expect it will be several per fiber, if the system is behaving normally.

HAMPRECHT

You find aggregates even in the absence of conditioned medium, so do you believe that the factor in the conditioned medium has some similar factor or some correspondent factor in the muscle cell? Or do you think that the conditioned medium factor activates a mechanism which is already somewhat active in the muscle cell?

NELSON

At present we have no way to distinguish between a single aggregation mechanism operating in the muscle cells under all conditions, and the possibility that different mechanisms are responsible for aggregate formation in the presence or absence of the aggregation factor.

γ-AMINOBUTYRIC ACID (GABA): A MAJOR INHIBITORY TRANSMITTER IN THE VERTEBRATE NERVOUS SYSTEM

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INTRODUCTION

For a number of years, beginning with my joining the staff at Washington University in St. Louis in 1946, I was interested in the study of amino acid metabolism in both normal and neoplastic tissues in experimental animals. The elegant chromatographic technologies available to us today had not yet been developed and the most advanced analytical procedures were those employing laborious, and not always absolutely specific, microbiologic assays. With the advent of two-dimensional paper chromatographic techniques, it became possible to examine the content of free or easily extractable ninhydrin-reactive constituents in animal tissues. Our earliest observations (Roberts and Frankel, 1950 a, b) showed that, in a given species at a particular stage of development, each normal tissue has a distribution of easily extractable ninhydrin-reactive constituents that is characteristic for that tissue, whereas quite similar patterns of free amino-acids are found in many different types of transplanted and spontaneous tumors.

Working during the summer of 1949 at the Roscoe B. Jackson Memorial Laboratory in Bar Harbor, Maine, where an unusually good selection

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of transplantable mouse tumors carried in a number of inbred mouse strains was available for study, I analyzed the free amino acid content of the C1300 transplantable neuroblastoma, then available only in solid form. I chromatographed several mouse brain extracts for comparison with the neuroblastoma and, much to my surprise, an unidentified and previously unobserved ninhydrin-reactive material was seen on the chromatograms. At most, only traces of this material had appeared in a large number of extracts of many other normal and neoplastic tissues previously examined, or in samples of urine and blood. This immediately excited my curiosity, and I reached the tentative conclusion that the unknown material was histidine. As is my inclination, I began to build a fanciful hypothesis about the role of histidine in brain, based on the presumption that the alleged histidine was prohably in a precursor pool for the formation of histamine.

Upon returning to my laboratories in St. Louis, my assistant, Sam Frankel, and I isolated the unknown material from suitably prepared paper chromatograms. A study of the properties of the substance revealed it to be γ-aminobutyric acid (GABA). The initial identification was based on the co-migration of the unknown with GABA on paper chromatography in three different solvent systems. Sidney Udenfriend, at that time also on the staff of Washington University, then made an absolute identification of the GABA in our extracts by the isotope derivative method. We submitted abstracts to the Federation meetings that year (1950) reporting the presence of GABA in brain (Roberts and Frankel, 1950; Udenfriend, 1950). An abstract reporting the presence of an "unidentified amino acid in brain only" appeared from the Houston laboratory of Jorge Awapara in the proceedings of the same meeting (Awapara, 1950). At the Federation meetings, where these findings were reported, I shared a room with Awapara who knew by that time that the unknown material he had found in brain was GABA.

In addition to GABA, work in both of our laboratories had revealed the presence of large amounts of taurine in extracts of many rodent tissues. Awapara and I agreed that my laboratory would continue to work on GABA, while he would put emphasis on the study of metabolism of taurine. It is interesting that only relatively recently taurine has been suggested to be a possible neurotransmitter (Davison and Kaczmarck, 1971) and has been shown to have some anticonvulsant action (Van Gelder, 1972). The first three complete papers dealing with the occurrence of GABA in brain all appeared in the same issue of the Journal of Biological

Chemistry (Roberts and Frankel, 1950; Udenfriend, 1950; Awapara *et al.*, 1950).

We were helped initially in the identification of GABA in brain extracts by the finding that GABA was found to be prominent among the soluble nitrogenous components of the potato tuber detectable by two-dimensional paper chromatography (Steward *et al.*, 1949). In fact, this caused me to write semi-facetiously in my notebook, "This proves that the brain is like a potato!". Unfortunately, little has happened to the state of the world since that time to change my mind. Of course, GABA had been found in nature before. Ackermann found it to be produced in putrefying mixtures by the action of bacteria in 1910 (Ackermann, 1910; Ackermann and Kutscher, 1910) and, subsequently, many reports have been made about the occurrence of GABA and/or its formation in bacteria, fungi, and plants. I was thrilled to receive a letter from Ackermann with congratulations on our first report of the presence of GABA in brain.

The only authentic sample of GABA that could be located at that time was in the chemical stockroom of the Department of Chemistry at the University of Illinois. Subsequently, we were able to make our own GABA by simple hydrolysis of a free and generous supply of 2-pyrrolidinone obtained from Cliff's Dow Chemical Company in Marquette, Michigan. In order to demonstrate conclusively that the precursor for GABA was glutamic acid in crude brain preparations (Roberts and Frankel, 1950), it was necessary to employ ^{14}C-labeled glutamic acid. No commercial sources were available. A sample of uniformly labeled L-glutamic acid isolated from a hydrolysate of algae grown in $^{14}CO_2$ was kindly furnished by Konrad Bloch, then on the staff of the University of Chicago.

Since L-glutamic decarboxylase (GAD) from other sources was known to require pyridoxal phosphate as a coenzyme (Gale, 1946), it was necessary to test this substance for its effect on the decarboxylation of glutamic acid in brain preparations. The only source of pyridoxal phosphate available to us, after much searching, was in the possession of Wayne W. Umbright, who gave us a generous supply of this cofactor.

Even in those times, I had faith that GABA must serve an important function in brain and had many fantasies about it. I remember vividly a conversation with a friend in which I jokingly said that GABA might possibly even play a role in epilepsy and schizophrenia. These possibilities now will be discussed more seriously in a subsequent section of this paper. There now are good reasons to believe that abnormalities in the function of GABA neurons might be involved in various neurologic and psychiatric disorders.

At the time of the discovery of GABA, I was immediately faced with a serious conflict. I was working in the Wernse Laboratories of Cancer Research in Washington University School of Medicine under E. V. Cowdry (deceased), a great scientist and a fine human being and, incidentally, a participant in the first study week of the Pontifical Academy of Science in 1948. I desperately wanted to work on the metabolism and function of GABA, but my obligations lay in the field of cancer research. Nonetheless, for almost three years thereafter, most of my research efforts were devoted to the study of GABA in brain. During that period I received much encouragement from Cowdry and never once was I criticized or reprimanded for diverting my efforts from the main thrust of his program. I am most grateful to this gentleman for his support and forbearance during the period I remained in his laboratory, and for his friendship in the subsequent years before his death.

For several years, the presence of relatively large amounts of GABA uniquely in the tissue of the central nervous system (CNS) of various species remained a puzzle. The great neurochemist Heinrich Waelsch (deceased) once discouragingly remarked that GABA was probably a metabolic wastebasket. My continuing efforts to convince some of the eminent neurophysiologists working at Washington University at that time to test GABA on various nerve preparations at the end of their planned experiments met with complete failure, even though I brought GABA solutions personally to their laboratories in the hopes of persuading them to test it.

In the first review on the subject in 1956 (Roberts, 1956), written after I had moved to my present position, I concluded in desperation, "Perhaps the most difficult question to answer would be whether the presence in the gray matter of the central nervous system of uniquely high concentrations of γ-aminobutyric acid and the enzyme which forms it from glutamic acid has a direct or indirect connection to conduction of the nerve impulse in this tissue". However, later that year, the first suggestion that GABA might have an inhibitory function in the vertebrate nervous system came from studies in which it was found that topically applied solutions of GABA exerted inhibitory effects on electrical activity in the brain (Hayashi and Nagai, 1956; Hayashi and Suhara, 1956). In 1957, from studies with convulsant hydrazides (Killam and Bain, 1957; Killam, 1957), the suggestion was made that GABA might have an inhibitory function in the CNS. Also in 1957, definitive evidence for an inhibitory function for GABA at synapses was derived from studies that established GABA as the major factor in brain extracts responsible for the

inhibitory action of these extracts on the crayfish stretch receptor system (Bazemore *et al.,* 1957). Within a brief period, the activity in this field increased greatly so that the research being carried out ranged from the study of the effects of GABA on ionic movements in single neurons to clinical evaluation of the role of the GABA system in, for example, epilepsy, schizophrenia, and various types of mental retardation. This warranted the convocation of a memorable interdisciplinary conference in 1959 at which were present most of the individuals who had a role in opening up this exciting field (Roberts *et al.,* 1960).

This first conference was the greatest learning experience of my life. From having spent most of my scientific career in the rather narrow confines of classical hiochemistry and the bare beginnings of molecular biology, I was thrust into the world of EEG, membranes, electrodes, voltage clamps, neuroanatomy, clinical seizures, neuroembryology and animal behavior. I had the privilege of meeting a numher of the world's leading neuroscientists among the participants, many of whom are still active scientifically and have remained close personal friends. What a mind-boggling intellectual feast! The meeting, itself, was overwhelming to a number of us. The sense of excitement was pervasive hecause we all sensed that a new era was beginning. The suhject of neural inhibition finally had returned to front stage and center after many years of languishing in the wings (for hackground and history see Roberts *et al.,* 1960; Diamond *et al.,* 1963; Eccles, 1969; von Euler *et al.,* 1968; von Békésy, 1967). It was obvious that much of the future progress in the field would depend on interdisciplinary efforts and that we all would have to begin to learn each other's languages and ways of thinking. It is true that at times the proceedings resembled what one imagines may have taken place at the Tower of Babel. However, we all shared the optimistic feeling that we could help each other learn enough so that effective communication soon would take place. For most of us this turned out to be true, and many students in the lahoratories of the participants reaped the benefit of the "new enlightenment". It was a particularly heartening social occasion because individuals from Australia, Canada, England, France, Hungary, Japan, United States, and the Soviet Union met in enthusiastic amity and forged long-lasting scientific and personal links.

In the years that followed there were good times and fallow periods. For some years many doubted that GABA was a true neurotransmitter. These doubts now have heen laid to rest. The status of this substance now is that of a major inhibitory transmitter in the vertebrate CNS and in some invertebrate and peripheral nervous systems. Great advances in

our understanding of neural circuitry based on neuroanatomic and physiologic observations gave us the opportunities and incentives to attempt to devise more specific ways of localizing GABA neurons *in situ,* eventually leading to the development of immunocytochemical tools for visualizing them both at the light and electron microscopic levels. Advances in techniques of cell fractionation, autoradiography, nerve tracing with isotopes and peroxidase, lesioning procedures, and clinical neurology led to the convocation of the second GABA meeting, seventeen years after the first one (Roberts *et al.,* 1976). Rapid developments in studies of general membrane and specific receptor properties, the effects of drugs on these properties and on ionic movements, and the role of the GABA system in disease states led to the organization of a meeting this year devoted solely to GABA (Krogsgaard-Larsen *et al.,* 1979) at which were present two individuals, David Curtis and I, who attended the first meeting and a number of individuals who either attended the second one or have worked with the attendees of the first two conferences. The intervening period of 28 years since the discovery of GABA has seen the flowering of much of modern neuroscience. There has been a great development of instrumental technologies and intellectual approaches during that period, and it has become more fashionable to participate in interdisciplinary experimentation and conferences, such as this one, than was the case in 1959. I have the feeling that we now have more than scratched the surface of knowledge of the function of GABA in nervous system function, but that many further advances remaining to be made in this field will have to take place hand in hand in a contingent fashion with other types of progress. A case in point is the necessity for definitive characterization of the major excitatory transmitters and visualization of the neurons using them. Of course, identification and characterization at the molecular level of the components of the receptor-ionophore-ion pump complexes related to actions of all transmitters is a *sine qua non* for our understanding of the underlying mechanisms involved.

It is my hope that the fundamental advances being made in this field eventually will lead not only to a better understanding of nervous system function but also to the development of substances and procedures that are useful in the alleviation of some neurologic and psychologic disorders and, at least in some instances, will help make some brains less "potato-like".

There now is a vast and bewildering literature dealing with GABA function and metabolism. I will not make an attempt to summarize it here, since this has been done in a number of recent books and reviews.

Instead, I will deal with some of our current work and some of our thinking in which we are attempting to help delineate further the role of GABA neurons in information processing in the vertebrate nervous system.

GABA NEURONS IN THE VERTEBRATE CNS

One of our major areas of concern is that dealing with the localization of GABA-releasing neurons. The demonstration that a synapse is a GABA-releasing one is not sufficient evidence to identify it unequivocally as an inhibitory synapse. Supporting physiological evidence always is necessary for such a functional assignment to be made. However, in almost all instances adequately studied to date the overall effects of GABA in the vertebrate nervous system, where GABA synapses have been proven to exist, have been found to be inhibitory either at pre- or postsynaptic sites. GABA typically produces an increase in membrane permeability to Cl^- ions that can be measured as an increase in membrane conductance. It is in this way that this naturally occurring transmitter can counteract the depolarizing action of excitatory processes to maintain the polarization of a cell at an equilibrium level near that of its resting value, acting essentially as a chemical voltage clamp. In most instances studied, GABA has been shown to exert a hyperpolarizing or inhibitory effect via the above mechanism. However, when there are relatively high intracellular Cl^- concentrations, GABA can produce a decrease in membrane potential or depolarization, a mechanism probably involved in presynaptic inhibition.

Until quite recently the localization of GABA neurons has been inferred by correlating microchemical, electrophysiologic, pharmacologic, and iontophoretic studies with what was known of the cytoarchitecture of specific regions of brain or spinal cord. Analyses of GABA contents and GAD activities have been performed in almost all identifiable brain structures and in the spinal cord. Many studies have combined biochemical analyses with lesioning procedures in correlating specific neural degenerations with losses of GAD and GABA. The distributions of the components of the GABA system also have been studied extensively by subcellular fractionation techniques in preparations from whole hrain or selected regions. Interpretation of results from the above types of analyses at the cellular level often suffer from the lack of definition attributable to the presence of myriad cells of different types in any dissected region; and it is difficult to make definitive conclusions about specific synaptic connectivities from them. Individual cell bodies of large neurons, such as Purkinje and Deiters' cells, and even portions thereof, have been dis-

sected out and subjected to microanalyses. In the latter instances it is not always possible to ensure absence of contaminating presynaptic endings from other neurons or of glial cell constituents, and therefore, their contribution to a particular measured chemical variable. The technical difficulties involved in the latter type of work also usually preclude the study of large numbers of neurons in this manner.

The above approaches, while yielding fundamental and valuable data, have not made it possible to obtain a definitive understanding of how GABA neurons might participate in information processing in different parts of the vertebrate CNS. Direct demonstration of GABA neurons and nerve endings now has been achieved by the visualization of glutamate decarboxylase (GAD), the enzyme that catalyzes the formation of GABA from glutamic acid, at the light and electron microscopic levels through the use of immunocytochemical peroxidase labeling procedures. The basic strategy employed by us, the chronology of its achievement, and the names of the participating scientists are shown in Table 1. Many appropriate citations to pertinent literature also can be found in the references cited as well as the relevant technical details. GABA neurons have been found to form axodendritic, axosomatic, axoaxonic, and dendrodendritic synapses

TABLE 1. *Immunocytochemistry of GABA-Related Enzymes*

Steps along the way	Date	Authors
1. Purification and Properties		
GAD		
Purification and characterization of glutamate decarboxylase from mouse brain	1973	Wu, Matsuda and Roberts
Electrophoresis of glutamic acid decarboxylase from mouse brain in sodium dodecyl sulfate polyacrylamide gels	1973	Matsuda, Wu and Roberts
Properties of brain L-glutamate decarboxylase: inhibition studies	1974	Wu and Roberts
GABA-T		
Purification and characterization of the 4-aminobutyrate-2-ketoglutarate transaminase from mouse brain	1973	Schousboe, Wu and Roberts
Subunit structure and kinetic properties of 4-aminobutyrate-2-ketoglutarate transaminase purified from mouse brain	1974	Schousboe, Wu and Roberts
SUMMARY		
Purification, characterization and kinetic studies of GAD and GABA-T from mouse brain	1976	Wu

Steps along the way	Date	Authors
2. Immunological Studies		
GAD		
Immunochemical studies on glutamic decarboxylase from mouse brain	1973	Matsuda, Wu and Roberts
Immunochemical comparisons of vertebrate glutamic acid decarboxylase	1974	Saito, Wu, Matsuda and Roberts
Immunochemical studies of brain glutamate decarboxylase and GABA-transaminase of six inbred strains of mice	1974	Wong, Schousboe, Saito, Wu and Roberts
GABA-T		
Some immunochemical properties and species specificity of GABA-α-ketoglutarate	1974	Saito, Schousboe, Wu and Roberts
SUMMARY		
Immunochemical studies of glutamate decarboxylase and GABA-α-ketoglutarate transaminase	1976	Saito
3. Immunocytochemical Approaches		
GAD		
Immunohistochemical localization of glutamate decarboxylase in rat cerebellum	1974	Saito, Barber, Wu, Matsuda, Roberts and Vaughn
The fine structural localization of glutamate decarboxylase in synaptic terminals of rodent cerebellum	1974	McLaughlin, Wood, Saito, Barber, Vaughn, Roberts and Wu
The fine structural localization of glutamate decarboxylase in developing axonal processes and presynaptic terminals of rodent cerebellum	1975	McLaughlin, Wood, Saito, Roberts and Wu
Immunocytochemical localization of glutamate decarboxylase in rat spinal cord	1975	McLaughlin, Barber, Saito, Roberts and Wu
Immunocytochemical localization of glutamate decarboxylase in the substantia nigra of the rat	1976	Ribak, Vaughn, Saito and Barber
Immunocytochemical localization of glutamate decarboxylase in rat substantia nigra	1976	Ribak, Vaughn, Saito, Barber and Roberts
Immunocytochemical localization of glutamate decarboxylase (GAD) in the olfactory bulb	1976	Ribak, Vaughn and Saito
Glutamate decarboxylase (GAD) localization in neurons of the olfactory bulb	1977	Ribak, Vaughn, Saito, Barber and Roberts
Immunocytochemical localization of GAD in somata and dendrites of GABAergic neurons following colchicine treatment	1976	Ribak and Vaughn
The immunocytochemical localization of GAD within stellate neurons of rat visual cortex	1977	Ribak

Steps along the way	Date	Authors
Immunocytochemical localization of glutamic acid decarboxylase in neuronal somata following colchicine inhibition of axonal transport	1978	Ribak, Vaughn and Saito
GABAergic terminals are presynaptic to primary afferent terminals in the substantia gelatinosa of the rat spinal cord	1978	Barber, Vaughn, Saito, McLaughlin and Roberts
Immunocytochemical localization of GAD in electron microscopic preparations of rodent CNS	1976	Wood, McLaughlin and Vaughn
Aspinous and sparsely-spinous stellate neurons contain glutamic acid decarboxylase in the visual cortex of rats	1978	Ribak
Immunocytochemical identification of GABA-ergic neurons in rat retina	1978	Vaughn, Barber, Saito, Roberts and Famiglietti
Immunocytochemical localization of glutamic acid decarboxylase (GAD) in the rat corpus striatum	1978	Ribak
GABAergic axon terminals decrease at experimental seizure foci in monkey cerebral cortex	1978	Ribak, Harris, Anderson, Vaughn and Roberts
GABA-T		
See Wood *et al.*		
SUMMARIES		
Light microscopic visualization of GAD and GABA-T in immunocytochemical preparations of rodent CNS	1976	Barber and Saito
Immunochemistry of the GABA system - a novel approach to an old transmitter	1976	Roberts
Immunocytochemical identification of GABA-ergic neurons	1977	Saito, Roberts and Barber
Roles of GABA neurons in information processing in the vertebrate CNS	1978	Roberts

in the various regions of the rat CNS studied to date, which include spinal cord, cerebellum, cortex, hippocampus, olfactory bulb, retina, substantia nigra, and striatum. In Fig. 1 are shown electron micrographs of various types of synaptic junctions formed by presynaptic terminals containing GAD. In almost all instances several, if not all, of these types of GABA synapses are found in close proximity to each other.

In the section that follows, some of the salient findings in the several regions of rat CNS studied to date will be mentioned.

Spinal Cord

Immediately after the transduction event in a particular sensory receptor, there is a surprising amount of information processing, consisting of a coordinated interplay of excitation and inhibition. Two general aspects of sensory inhibition have emerged. Units in receptor systems inhibit their nearest neighbors via interneurons (intrasystem), causing enhancement of contrast and sharpening of input signals; and inhibitory and disinhibitory influences from higher relay centers (intersystem) may impinge on the receptors to set their gain. Thus, even close to the place and time of entry of environmental information, the organism abstracts those aspects which are most essential for adaptation. Subsequently, the neural signals carrying the information from the sensory receptors enter the CNS. Many variable sources of excitatory sensory inputs constantly are competing for the attention of the organism. It would be asking a great deal of a mainline central neural element alone to adjudicate all of the competing claims. Nature has devised ways for helping resolve such conflicts. A great deal of information processing also occurs even at the most peripheral levels of the CNS with the aid of networks of closely-lying indigenous neurons, many of which were presumed to be GABA neurons. Our investigations of rat spinal cord now have confirmed the above suggestions and furnished a morphological basis for the physiological observations.

Light microscopic localization of GAD in the rat lumbosacral spinal cord showed heavy, punctate GAD-positive reaction product in the dorsal horn laminae I-III (McLaughlin *et al.*, 1975 b; Barber *et al.*, 1978). Moderately heavy reaction product was also seen in the deeper dorsal horn laminae IV-VI, the medial aspect of the intermediate gray (lamina VII), and the region around the central canal (lamina X). A moderately light concentration of GAD-positive reaction product was observed in the ventral horn, and punctate deposits of reaction product also were seen on motoneuron cell bodies. The visually observed punctate distribution of GAD-positive reaction product described above corresponded to GAD-positive synaptic terminals visualized by electron microscopy in comparable regions. Many more GAD-positive terminals were observed in dorsal horn laminae I-III than in deeper laminae IV-VI. GAD-containing terminals in the dorsal horn were presynaptic to dendrites, cell bodies, and other axon terminals. The latter were more numerous in laminae II and III. GAD-positive synaptic terminals were presynaptic to large and small dendrites and motoneuron somata in the motor nuclei. In addition, small GAD-

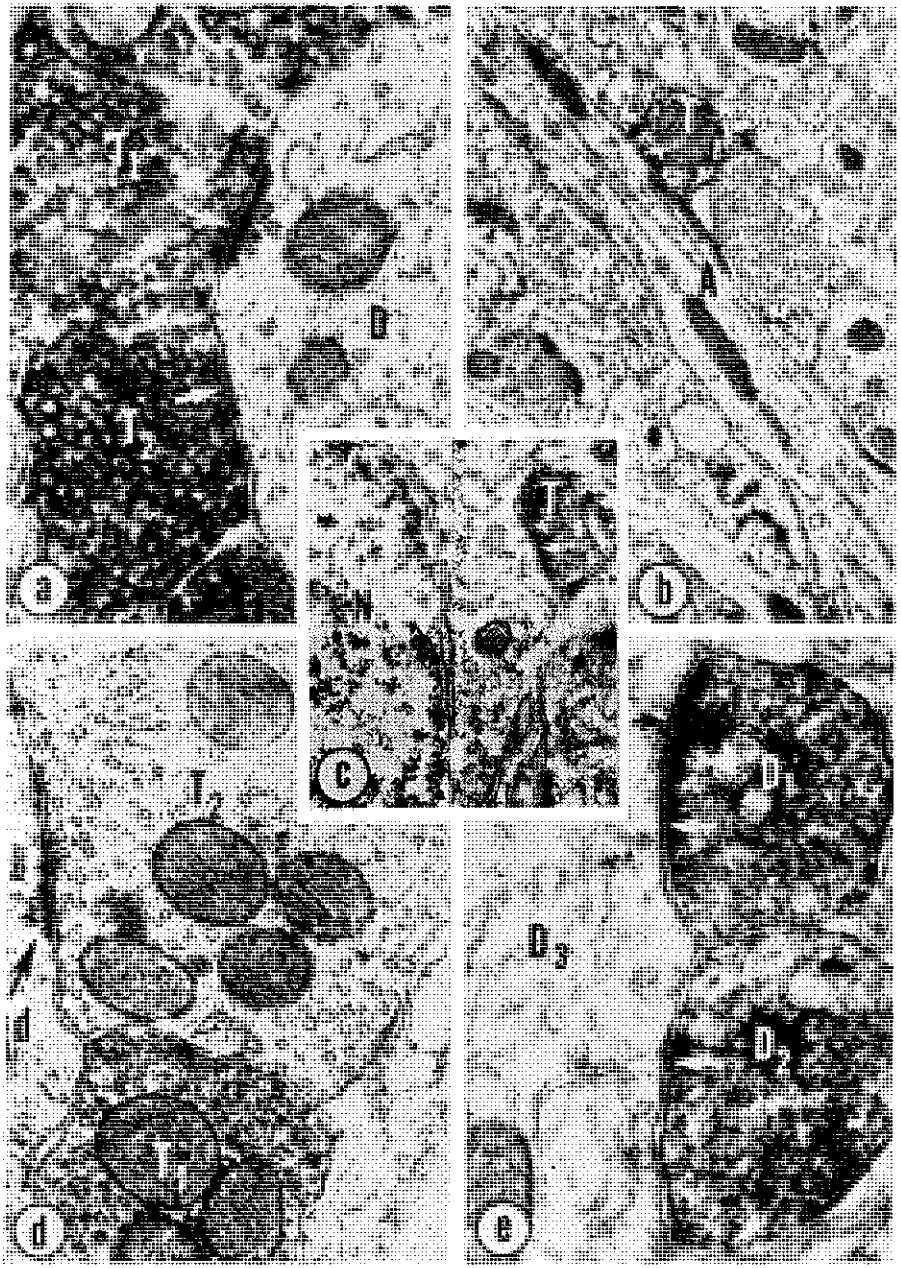

FIG. 1. Electron micrographs of various types of synaptic junctions formed by presynaptic terminals which contain glutamate decarboxylase (GAD), the enzyme that synthesizes the neurotransmitter γ-aminobutyric acid (GABA). All specimens were obtained from rat CNS.

containing terminals also were presynaptic to larger axonal terminals which were in turn presynaptic to motoneuron somata. The observation of GAD-positive terminals presynaptic to dendrites and cell bodies in both dorsal and ventral horns was compatible with the evidence suggesting that GABA terminals may mediate postsynaptic inhibition of spinal interneurons and of motoneurons.

Multiple dorsal rhizotomies were performed unilaterally at lumbar levels L1-L4 in adult rats, and the spinal cords were examined 24-48 hours later (Barber *et al.*, 1978). Large numbers of degenerating terminals, probably entirely from cutaneous afferents, were observed in the ipsilateral substantia gelatinosa, but not contralaterally. However, the distribution of GAD-positive reaction product, most intense within laminae II and III, appeared to be normal on both sides of the cord. Electron microscopically, primary afferent terminals were found in various stages of degeneration on the side of the rhizotomies, and GAD-positive axon terminals were found to be presynaptic to degenerating primary afferent terminals in the substantia gelatinosa. The data furnish a chemomorphological basis for the conclusion that the presynaptic inhibition of primary afferents is mediated by axoaxonal synapses formed between GABA-releasing interneurons and primary sensory neurons. A detailed analysis of the various synaptic relationships of GAD-positive terminals in the dorsal horn of the rat spinal cord has led to reasonable hypotheses about how release of GABA from these terminals could participate in such presynaptically

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(a) Axodendritic synapses in the substantia nigra. Two axon terminals (T_1 and T_2) filled with electron opaque, GAD-positive reaction product are shown to synapse with a dendritic shaft (D) in the pars reticulata. One of the terminals (T_1) forms an asymmetric synaptic junction (arrow), while the other terminal (T_2) forms a symmetric synapse (arrow). ×44,000. (b) An axoaxonal synapse in the cerebral cortex. A GAD-positive axon terminal (T) is shown forming a symmetric synapse (arrow) with an axon initial segment identified by a dense undercoating of the axolemma (arrow heads) and a fasciculation of microtubules (e.g., asterisk). ×20,000. (c) An axosomatic synapse in the dorsal horn of the spinal cord. A probable synaptic junction (arrow) is shown between a GAD-positive terminal (T) and a neuron (N) in the substantia gelatinosa. ×26,000. (d) An axoaxonal synapse in the dorsal horn of the spinal cord. A synaptic junction (white arrow) is shown between the GAD-positive presynaptic terminal (T_1) and another synaptic terminal (T) which is not GAD-positive. In addition, T_2 is the presynaptic component of another synaptic junction (black arrow) with a dendrite (D). ×38,000. (e) Dendrodendritic synapses in the glomerular layer of the olfactory bulb. Two GAD-positive gemmules (D_1, D_2) from dendrites of periglomerular neurons form synapses with a mitral/tufted dendritic shaft (D_3). One gemmule (D_1) appears to form a reciprocal synapse and the other gemmule (D_2) appears to be presynaptic only. ×54,000. Directions of synaptic transmission are indicated by arrows in a-e. Electron micrographs provided by R. P. Barber, B. J. McLaughlin, C. E. Ribak and J. E. Vaughn.

modulated phenomena as primary afferent depolarization, the dorsal root reflex, and primary afferent hyperpolarization. A schematization of some of the relations of GABAergic neurons in the substantia gelatinosa is shown in Fig. 2 and explained in the legend to the figure.

Cerebellum

The cerebellum was a favorable site for investigation of possible substances which may mediate the activity of neurons with inhibitory functions because more extensive correlative neuranatomical and neurophysiological analyses have been made of the cerebellum than of any other structure in the vertebrate brain (Eccles *et al.,* 1967; Llinas, 1969). The overall function of the cerebellar cortex probably is entirely inhibitory. The only output cells of the cerebellar cortex, the Purkinje cells, inbibit monosynaptically in Deiter's and intracerebellar nuclei. Cells that lie entirely in the cerebellar cortex, the basket, stellate and Golgi II cells, are believed to play inhibitory roles within the cerehellum. The basket cells make numerous powerful inhibitory synapses on the somata, axon hillocks, and initial axon segments of the Purkinje cells. The superficial stellate cells form inhibitory synapses on the dendrites of Purkinje cells. The Golgi II cells make inhibitory synapses on the dendrites of the granule cells. Afferent excitatory inputs reach the cerebellum via the climbing and mossy fibers, which excite the dendrites of the Purkinje and granule cells, respectively. The latter are believed to be the only cells with an excitatory function that lie entirely within the cerebellum.

Even the first comprehensive biochemical laminar analyses of the GABA system suggested the possibility that all of the inhibitory cells of the cerebellum (Purkinje, basket, stellate and Golgi) might use GABA as transmitter (Roberts and Kuriyama, 1968). Subsequently, evidence was adduced for the occurrence of both GAD and GABA in high concentrations in the deep cerebellar nuclei, where the Purkinje cell axons terminate (Fonnum et al., 1970; Fonnum and Walberg, 1973 a, b). Electrophysiologic and pharmacologic studies suggested strongly that Purkinje cell terminals might liberate GABA as transmitter (Curtis *et al.,* 1970; Obata *et al.,* 1967; Obata and Takeda, 1969). Application of the immunoperoxidase technique at the light level showed an intense punctate deposition of reaction product around the Purkinje cells and around the neurons of the deep cerebellar nuclei, cells known to receive an inhibitory synaptic input from the Purkinje cells, suggesting the impingement of many nerve terminals containing GAD upon these neuronal surfaces (Saito

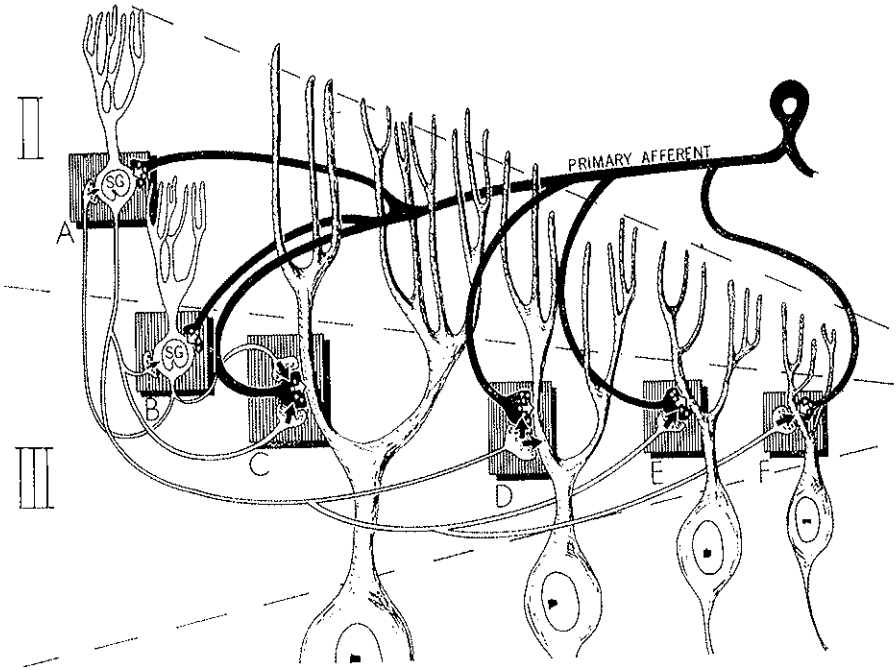

FIG. 2. Schematic representation of possible synaptic pathways within the substantia gelatinosa (Rexed's laminae II and III) which involve both GABAergic and primary afferent axon terminals. GABAergic, substantia gelatinosa neurons (SG) are white, and a primary afferent neuron is black. The single primary afferent neuron represents all of the different neurons of this class whose axons terminate in the substantia gelatinosa. Black arrows indicate the direction of GABAergic synaptic transmission, and white arrows indicate the direction of primary afferent synaptic transmission. In a number of the synaptic complexes observed, a GAD-positive terminal was presynaptic to a primary afferent terminal, and in addition both kinds of terminals (i.e., GAD-positive and primary afferent terminals) were presynaptic to the same dendritic profile (Fig. 2 D). In these cases, the same GABAergic axon terminal could mediate both presynaptic and postsynaptic inhibition. Depolarization of a primary afferent axon terminal by GABA could mean that a subsequent primary afferent volley would result in less transmitter being released. This reduced amount of released primary afferent transmitter would also have to exert its effect upon a postsynaptic dendrite that has been hyperpolarized by the action of a GABAergic axodendritic synapse. Consequently, there would be a further reduction of the effectiveness of the primary afferent transmitter substance in producing an excitatory postsynaptic potential in the second order neuron. It is plausible to assume that inhibitory synaptic complexes such as those established by the GABAergic terminals which make synaptic contacts with both pre- and postsynaptic elements (Fig. 2 D) and those established by several GABAergic presynaptic terminals with the same primary afferent terminal (Fig. 2 C) could form the center of the inhibitory gradient which has been described surrounding primary afferent fibers immediately following an afferent volley. The scheme illustrating possible synaptic pathways involved in a GABAergic modification of primary afferent activity (Fig. 2) is largely based upon data accrued from investigation of rat lumbar spinal cord. However, primary afferent depolarization has been shown to occur at several spinal levels as well as in the cuneate nucleus, and it appears to be mediated by GABA in several species. Therefore, primary afferent depolarization that is mediated by the synaptic transmitter GABA would appear to be a rather general mechanism for the regulation of transmitter release from primary afferent terminals. On this basis, it is reasonable to suggest that synaptic pathways similar to those illustrated in Fig. 2 may be generally distributed throughout the substantia gelatinosa. Thus such pathways may provide repetitive units in which GABAergic interneurons presynaptically modulate the various exteroceptive impulses carried to the substantia gelatinosa by primary afferent fibers. (Fig. 2 taken from R. Barber *et al.*, 1978).

et al., 1974 c). At the electron microscopic level GAD appeared to be highly localized in certain synaptic terminals in close association with the membranes of synaptic vesicles and mitochondria, but not within these organelles (McLaughlin *et al.,* 1974). GAD-positive terminals, presumably largely from Purkinje cells, were seen on the somata and proximal dendrites of neurons in the deep cerebellar nuclei. Similarly, terminals presumably arising from basket, stellate and Golgi type II cells also were strongly positive for tbe enzyme. Thus, all of the cerebellar cortical cells known to be inhibitory were shown probably to use GABA as transmitter. Direct injections of colchicine into the cerebellar cortex, disrupting axoplasmic transport, made it possible to demonstrate that the proximal dendrites and somata of Purkinje and Golgi type II neurons and the somata of basket and stellate cells contained detectable accumulations of GAD-positive reaction product (Ribak and Vaugbn, 1976; Ribak *et al.,* 1978 a). The GAD-positive reaction product was concentrated around the cisternae of the Golgi apparatus and was not seen in nuclei or nucleoli. The Golgi apparatus may be a way station for newly synthesized GAD prior to axoplasmic transport to presynaptic terminals.

In the developing rat cerebellum, GAD was present in growing neurites in close association with small vesicles prior to the time the neurites make protosynaptic contacts; differentiation of these contacts coincided with the sequestration of GAD into synaptic terminals (McLaughlin *et al.,* 1975 a). It appears that the initial signal for GAD synthesis in the developing cerebellum predates the establishment of contacts between pre- and postsynaptic elements of a developing synapse.

Olfactory Bulb

Morphologic and physiologic aspects of the mammalian olfactory bulb bave been studied extensively (Shepherd, 1972). In addition, high levels of GAD and GABA were found in the external plexiform, glomerular, and granule cell layers of tbis laminar structure (Graham, 1973). The mitral and tufted cells, which receive impulses from the olfactory nerves and from extrabulbar sources, are inhibited via reciprocal dendrodendritic synapses on their dendrites in the external plexiform and glomerular layers formed with the indigenous granule and periglomerular cells (Shepherd, 1972; Getchell and Shepherd, 1975). Pbarmacologic studies suggested that both of the latter interneuronal cell types are GABAergic (McLennan, 1971; Nicoll, 1971; Kemp and Powell, 1971). GAD has now been localized in the olfactory bulb by immunocytochemical methods

at the light and electron microscopic levels (Ribak *et al.*, 1977). Light microscopic studies demonstrated GAD-positive puncta throughout all layers of the olfactory bulb with the greatest concentration in the external plexiform layer and in the glomeruli of the glomerular layer. The cytoplasm of many neuronal somata in the granule and glomerular cell layers was GAD-positive, but not the cytoplasm of mitral and tufted cell somata. The GAD-positive staining of presumed granule and periglomerular neuronal somata also extended into their dendrites for many microns. There is evidence that some of the latter also may be dopaminergic (Hökfelt *et al.*, 1975).

Electron microscopic observations confirmed the presence of GAD-positive reaction product within the cytoplasm of granule and periglomerular neurons. Also, in the external plexiform layer, reaction product filled many of the granule cell gemmules which form reciprocal dendrodendritic synapses with mitral cell dendrites. The presence of GAD within granule and periglomerular cells lends support to suggestions based on previous physiologic and pharmacologic studies that these inhibitory interneurons use GABA as their neurotransmitter.

Basal Ganglia

The nigrostriatal-pallidal system is concerned to a considerable extent with processing information related to proprioceptive, vestibular, and visual stimuli in the service of coordinating mechanisms involved in the physical orientation of an organism in its perceived space-time continuum. The caudate nucleus, putamen, and substantia nigra exchange fibers with each other, as do the substantia nigra and globus pallidus. The globus pallidus and substantia nigra receive inputs from the caudate and putamen and appear to have two-way communication with the subthalamic nucleus. There also are thalamic, cortical, and midbrain inputs to the caudate and putamen. Most of the final results of the computations in the basal ganglia are sent out via a fiber system from the globus pallidus to the nuclei of the thalamus and thence largely to the motor cortex. There are some nigrothalamic connections as well. In addition, there probably are connections between the globus pallidus and the midbrain tegmentum through which descending influences may be mediated (Kemp and Powell, 1971). In this regard, it is of considerable interest that facilitation of the gamma motor neurons can be achieved by stimulation of a midbrain region close to termination of the fibers from the globus pallidus, as well as by stimulation of the caudate nucleus. Normal relations within and between the

above structures must involve minimally a coordinated functioning of different groups of intra- and intersystem neurons whose transmitters and/or cybernenes [1] may be GABA, acetylcholine, dopamine, serotonin, norepinephrine and possibly still unidentified excitatory transmitters, one of which may be the polypeptide, substance P (Kanazawa *et al.*, 1977; Fonnum *et al.*, 1978; Roberts, 1976; Fentress, 1976).

Large amounts of GAD-positive reaction products, seen throughout the substantia nigra in light microscopic preparations, appeared to be localized in punctate structures that were apposed to dendrites and somata (Ribak *et al.*, 1976 a, b). Electron microscopic studies revealed that most of the axon terminals in the substantia nigra were filled with GAD-positive reaction product and formed both axodendritic and axosomatic synapses. Many dendrites were extensively surrounded by GAD-positive terminals, which most commonly formed symmetric synaptic junctions, although on the same dendrites some also formed asymmetric synaptic junctions. The results were consistent with biochemical, pharmacologic, and physiologic data which previously had indicated that neurons of the neostriatum and globus pallidus could exert a GABA-mediated, post-synaptic inhibition upon the neurons of the substantia nigra. The GAD-positive somata in the globus pallidus were medium-sized neurons. In the striatum the medium-sized spiny neurons, with round or fusiform shapes, appeared to be GABAergic (Ribak, 1978 b). The pattern of GAD-positive terminals associated with dendrites of pallidal neurons resembled that observed in the substantia nigra. The results from the latter study are consistent with the interpretation of the results of other investigations that have indicated that the striatopallidal and striatoentopeduncular pathways, as well as striatal local circuit neurons and/or collaterals from striatal projection neurons, use GABA as a neurotransmitter (Fonnum *et al.*, 1978).

Cortex

GAD was observed in somata, proximal dendrites and axon terminals of non-pyramidal neurons in the rat visual cortex in all cortical layers, including the immediately subjacent white matter (Ribak, 1978 a). GAD-positive terminals formed symmetric synaptic junctions most commonly with dendritic shafts and somata of pyramidal and stellate neurons and less frequently with dendrite spines and with initial axon segments of pyramidal neurons. Extensive pericellular plexuses were formed by these

[1] Substances which optimize neural function without, themselves, necessarily being directly involved in neurotransmission.

terminals with the somata of pyramidal neurons in layers III and V. Detailed morphological study of the GAD-positive neurons indicate that they are aspinous and sparsely-spinous stellate interneurons with extensive intracortical axonal arborizations. At the light level GAD-positive somata were seen to have GAD-positive terminals upon their surfaces. The localization of GAD within these neurons in combination with physiologic and pharmacologic data indicate that these local circuit neurons mediate GABAergic inhibition and disinhibition in the neocortex and probably play a key role in information processing.

Retina

Dense aggregations of GAD-positive puncta appear to form four separate sublayers within the inner plexiform layer of rat retina, a broad sublayer being adjacent to both the inner nuclear and ganglion cell layers, and two thin sublayers lying in the middle portion of the inner nuclear layer (Vaughn *et al.*, 1978). GAD-positive staining was not observed in any of the other retinal layers. Electron microscopy showed GABAergic terminals from amacrine cells ending on bipolar and amacrine cell processes and on ganglion cell dendrites and somata. GAD-positive terminals were also postsynaptic to bipolar cells and occasionally were observed to form reciprocal synapses with bipolar terminals. It appears from the results that some classes of amacrine cells are the only GABAergic neurons in rat retina. It will be important to determine whether other transmitters, which are believed by some to be dopamine, glycine, and taurine, are found in some amacrine cells and what their relations are to the GABA-ergic amacrines.

Hippocampus

Some of the GAD-positive puncta appeared in light microscopy as peri-cellular baskets around pyramidal and granule cell somata and these puncta corresponded to the known distribution of basket cell axon terminals (Ribak *et al.*, 1978 a). Also GAD-positive reaction product was observed in somata and dendrites. GAD-positive reaction product filled the somata and bipolar dendrites of many horizontal neurons located near the boundary between stratum oriens and the alveus. The majority of the GAD-positive somata in stratum radiatum and stratum lacunosum corresponded to those of horizontal and other short-axon neurons. Granule and pyramidal cells did not appear to contain GAD-positive reaction product either in light or electron microscopic preparations. Some of the GAD-positive axon

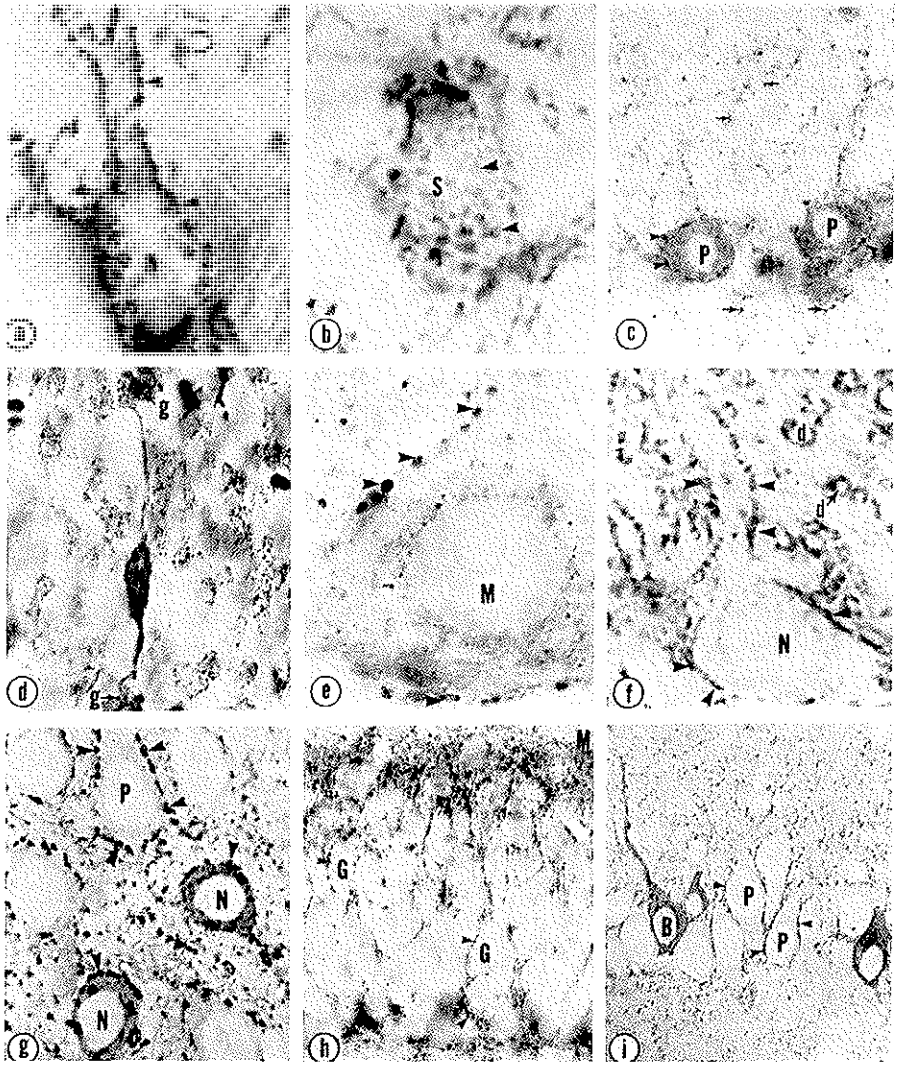

FIG. 3. Photomicrographs of GAD-positive terminals in rat central nervous system. (a) Deep cerebellar nucleus (nucleus interpositus). A neuron (N) is shown studded by round, GAD-positive terminals (arrow heads) which are probably Purkinje cell axonal terminals (×1590). (b) Deep cerebellar nucleus (nucleus interpositus). A grazed neuronal soma (S) showing numerous GAD-positive terminals (arrow heads) on the somal surface (×1590). (c) Cerebellar cortex. The Purkinje cell somata are studded with GAD-positive terminals (arrow heads) which may be basket cell terminals. In the molecular layer (M), there are numerous GAD-positive punctate structures (arrows), and in the granular layer (G), GAD-positive punctate structures (Golgi II axonal terminals) are associated with glomeruli (×660). (d) Granular layer of the cerebellum from tissue treated with colchicine to block axon flow. The Golgi II neuronal soma (N) has GAD-positive cytoplasm and two of its processes

terminals formed symmetrical synaptic junctions with pyramidal and granule cell somata and their dendritic shafts. The GAD-positive terminals forming axosomatic synapses corresponded in location to the endings of basket cells, known indigenous inhibitory interneurons.

GABA NEURONS PARTICIPATE IN NUMEROUS AND COMPLEX RELATIONS

Those of us who are concerned with the GABA story largely are involved in problems of neural inhibition. But it is necessary to remember that neither inhibition nor the GABA system, *per se*, are the main issues. The question is how the GABA neurons, wherever they are demonstrated to be present, participate in information-processing, together with all other neural elements, in such a way that it is possible for particular organisms to respond adaptively to their environments in a manner compatible with survival and successful reproduction. The ubiquity and extent of presynaptic endings of GABA neurons on various structures in the vertebrate nervous system are illustrated by the group of light micrographs of sections of rat CNS immunocytochemically stained for GAD assembled in Fig. 3.

The extensive studding by GAD-positive endings on neuronal postsynaptic surfaces in the nucleus interpositus (Figs. 3 a and b), globus pallidus (Fig. 3 f), cortex (Fig. 3 g), and hippocampus (Figs. 3 h and 3 i) is particularly striking. After examining many such pictures and related electron micrographs, the impression is gained that one is looking at a highly restrained nervous system, the inhibitory neurons acting like reins that serve to keep the neuronal "horses" from running away. Elsewhere evidence has been cited supporting the view that disinhibition may be

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are seen to terminate on glomeruli (G) (×614). (e) Ventral horn of the spinal cord. A single motoneuron (M) exhibits several GAD-positive terminals (arrow heads) on the somal membranes (×1571). (f) Globus pallidus. A neuronal profile (N) is shown which has numerous GAD-positive terminals (arrow heads) studding its surface. Several dendritic cross sectional profiles (D) also are covered by punctate, GAD-positive terminals (×1575). (g) Visual cortex from tissue treated with colchicine. Two neurons (N) have GAD-positive product in their cytoplasm while pyramidal cells (P) in layer V have clear cytoplasm. There are some GAD-positive terminals (arrow heads) on the somal surface of all of the neurons shown here (×940). (h) Dentate gyrus (Ammon's horn). Numerous GAD-positive terminals (arrow heads) stud the profiles of granule cells (G). In the molecular layer (M), there is a heavier investment of GAD-positive terminals around apical dendrites of the granule cells (×630). (i) Ammon's horn, treated with colchicine. A basket cell soma (B) exhibits cytoplasmic GAD-positive product, while pyramidal cell somata (P) do not. GAD-positive punctate structures (arrow heads), presumably basket cell terminals, cover the pyramidal cell somal profiles (×560). (I am indebted to my colleagues, Robert Barber and Charles Ribak, for the preparation of this figure).

one of the major principles of nervous system function (Roberts, 1976; Fentress, 1976; Maynard, 1972). A major tenet of this hypothesis is that in behavioral sequences, innate or learned, preprogrammed circuits are *released* to function at varying rates and in various combinations largely by the *disinhibition* of pacemaker neurons whose activities are under the control of tonically active inhibitory command neurons, many of which may use GABA as a transmitter. According to this view, disinhibition is permissive and excitatory input to pacemaker neurons would have largely a modulatory role.

"Thus, disinhibition, acting in conjunction with intrinsic pacemaker activity and often with modulatory excitatory input, appears to be one of the major organizing principles in nervous system function. Disinhibition may act as a switch, turning on a specific coherent neuronal pattern which is otherwise actively and continuously inhibited, as well as play a role in the organization of sequential and alternating discharges among separate groups of elements" (Maynard, 1972).

Metaphorically, one may consider the pacesetter neurons in various sectors of the CNS to be like the classical pictures of Gulliver when he awoke to find himself restrained by ropes attached to him hy the Lilliputians when he was asleep (Fig. 4).

GABA NEURONS AND FUNCTIONAL COORDINATION

The successful or adaptive operation of a nervous system requires a coordination of neuronal activity which can determine the ability to prevent the too-frequent or too-infrequent firing of preprogrammed circuits of behavioral options spontaneously or maladaptively; and, under a variety of environmental circumstances, internal and external, an organism must maintain within physiologic limits the rates of operation of continuously needed neuronal circuits, such as those required for cardiac function, respiration, and maintenance of blood pressure. When gross malfunctions of the coordination of inhibitory and disinhibitory neuronal systems occur, there may result lethal effects either through generalized seizures or cessation of operation of some vital function; alternatively, some obviously severe neurologic-physiologic dysfunctions may occur.

When there is incoordination between the GABA system and other neurotransmitter systems, for whatever reason, the defect might be restricted to a local brain region, might include several regions, or might be global throughout the CNS. Under relatively simple environmental conditions, the nervous system in such individuals could function in an ap-

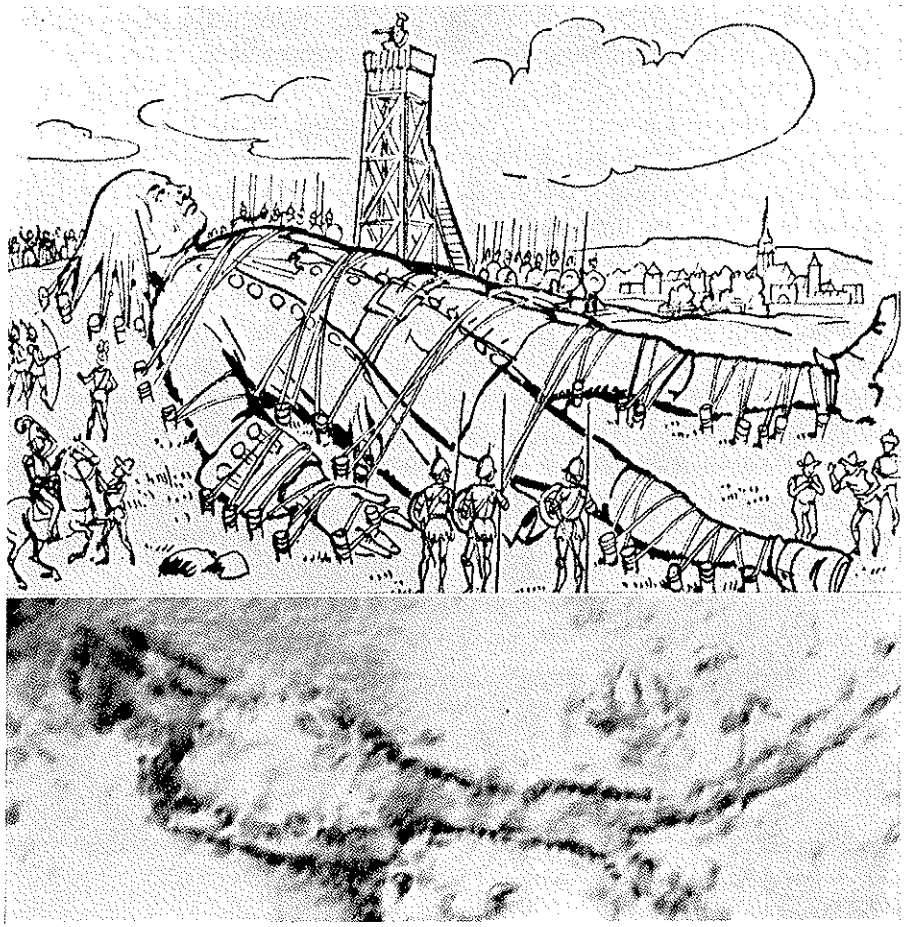


FIG. 4. The inhibited nervous system. An unretouched photograph taken with Nomarski optics of a neuron in the rat nucleus interpositus studded with GAD-positive terminals, presumably Purkinje cell axonal terminals, is placed below a picture of Gulliver, showing him when he awoke to find himself pinioned to the ground. The latter picture is taken from the 1956 edition of volume 7 of *The Book of Knowledge,* The Grolier Society Inc., New York. (Figure prepared by Robert Barber).

parently adequately adaptive manner, which might appear to be in the normal range. As the complexity and intensity of environmental inputs is increased, there would be a correlated increased degree of incoordination. Then, those subsystems in the nervous system that are most poorly controlled will tend to break down under the stress and produce symptoms that are consequent to such a breakdown.

Let us suppose that for some reason in the entire brain or in specific regions, tonically active inhibitory GABA neurons have a considerably lower-than-normal effectiveness on their recipient neurons, which, themselves, are normally effective. As the complexity and intensity of environmental inputs is increased, there would be an increased tendency to release pacesetter neurons. More-than-normal numbers of behavioral options or inappropriate ones would be released (behavior disorders, mania, schizophrenia, etc.); choreic movements, seizures, or spasticity might occur; there might be hypersensitivity to visual, auditory, tactile, olfactory, gustatory, pain stimuli, etc.; and abnormal fluctuations would be observed in autonomic functions. Recent experimental results suggest that GABA neurons may play an important role in control mechanisms in several hypothalamic centers. Thus, if specific hypothalamic regions were affected, greater-than-normal degrees of changes in responses might be observed in emotional reactivity, cardiac and respiratory functions, blood pressure, food and water intake, sweating, galvanic skin response, insulin secretion, liberation of gastric acid, motility of the colon, etc.

Virtually from the beginning of physiologic and pharmacologic observations of the GABA system it has been conjectured that decreases in the efficacy of the GABA system could result in convulsive seizures (Roberts *et al.*, 1960). It certainly seems possible that in schizophrenia (Roberts, 1972; Roberts, 1977; Bird *et al.*, 1977) and Huntington's and Parkinson's diseases (Iversen *et al.*, 1979; McGeer E. G. and McGeer P. L., 1979), as well as in other disorders, deficiencies might exist in the relative numbers of GABA neurons in specific brain areas, or that there might be defects in the structural or functional relationships of GABA neurons with other neurons, which either might inhibit or excite them, or which they, themselves, presumably might inhibit.

Interactions among neurotransmitter systems

GABA neurons obviously are in constant interaction with neurons liberating other transmitters or neuro-modulators as well as with each other. One of the major objectives of current neurobiological research is to delineate the relationships of various neuronal cell types to each other. The work in tracing the complexities of the synaptic relationships of GABA neurons has raised serious questions about the conceptual utility of formulating detailed hypotheses about information processing in the whole brain or in specific regions strictly on the basic of "classical" biochemical analytical approaches, by observing behavioral changes produced

by lesions or pharmacological manipulations, or even by combinations of approaches. Just as intracellular physiologic recordings from neurons must be carried out in morphologically characterized regions before they can reveal secrets of information processing, so now it is necessary to begin to perform chemical studies in more adequately described settings. It seems obvious to me that it is necessary to begin to develop detailed chemomorphologic maps of various brain regions in order to understand how neurons liberating the various putative transmitters, known and still unknown, are related to each other. Further refinement of cytochemical and immunocytochemical techniques, which are still in their infancy, will be required. It will be necessary to develop procedures that can be applied to ultrathin sections and which can visualize unequivocally two or more antigens, peptides, ions, etc. on one electronmicroscopic section, so that three dimensional reconstructions eventually will be possible.

In this connection it seems advisable for conceptual purposes to make a distinction between at least two major classes of neurons in the vertebrate central nervous system. It appears that some neurons, such as those that may release GABA, acetylcholine, glutamate, aspartate, and glycine, probably largely are involved in direct point-to-point information transmittal. In other words, release of transmitter from the presynaptic endings of such neurons affects postsynaptic sites in such a way that in a fraction of a millisecond excitatory or inhibitory information is transmitted, recognized, and the signalling substance removed. There are other groups of neurons, such as those which release catecholamines, serotonin, a variety of peptides, prostaglandins, and possibly other substances. Current evidence suggests to us that in most, but not all, instances these latter neurons may act by liberating their transmitter more generally into whole regions which contain various neuronal elements as well as glial cells and blood vessels, where they may exert relatively long lasting effects on blood flow and capillary permeability as well as more direct metabolic and trophic effects on the cellular elements, possibly setting the gain on the efficacy of individual synapses, on specific types, or on all of the synapses in a given region, etc. The effects may be exerted at many functional loci, often via the cyclic nucleotides, from transmitter synthesis to the postsynaptic control of the length of time of ion channel opening resulting from transmitter impingement and the setting of windows on which of the total range of potential firing frequencies may be employed by given neurons or groups of neurons. Their effects could be exerted by a large variety of cascading molecular mechanisms. Such neurons may furnish the "oil" required for the neuronal machinery to

function smoothly. That is, the effects of the neurons releasing such substances may be analogous to that of squirting oil into inadequately lubricated, but intact machinery, the parts of which will not function properly if sufficient oil is not furnished. However, the oil is not part of the actual machinery. We would like to suggest that in many instances in which they act in the CNS, the catecholamines, serotonin, neurally released peptides, and prostaglandins may serve to optimize regional nervous system function in relation to functional demands without themselves necessarily being involved in specific information transmittal. Roger Guillemin has called such substances "cybernenes". For example, immunocytochemical, isotope labelling, and physiologic experiments are compatible with the suggestion that norepinephrine neurons in rat brain largely perform global, hormone-like functions, although occasionally they also may participate in typical synaptic relationships (Amaral and Sinnamon, 1977; Cimarusti *et al.*, 1978; Hoffer *et al.*, 1978; Pickel *et al.*, 1976). An inappropriate balance between availability and distribution of such cybernenes and activity in neural circuits could result in gross malfunction of the CNS such as may be found in Parkinson's and Huntington's diseases and schizophrenia (Garattini *et al.*, 1978). It is striking that when the substantia nigra is stimulated, physiologically recorded signals in the corpus striatum do not seem to be greatly altered when the nigrostriatal dopamine neurons are destroyed by 6-OH-dopamine or when the action of dopamine is blocked completely by large doses of haloperidol. This suggests that the physiologically relevant signals are carried by fibers of still uncharacterized non-dopaminergic neurons and that the effects of dopamine release from the dopaminergic fibers are not informational in the strictest sense of the word, as suggested above. A phenomenon related to the above is described in many anecdotal accounts about Parkinsonian patients, obviously suffering from a defective functioning of nigrostriatal dopaminergic neurons, who can fully mobilize normal and adaptive physical activity in an emergency but who relapse into the typically inactive Parkinsonian state as soon as the emergency is over. The above and the therapeutic effects of exogenously supplied L-Dopa in Parkinsonism are compatible with the suggestion that the neuronal circuitry in the neostriatum is potentially available and that the dopaminergic neurons furnish dopamine, the "oil", required for the neuronal machinery to function smoothly.

The work described on the immunocytochemical localization of GABA neurons has made it possible to begin to study the biology of an important class of identified local circuit neurons in various parts of the vertebrate

central nervous system. However, this is only one small part of the story. It is necessary to be able to trace the connectivities of other types of inhibitory neurons (i.e., glycinergic) as well as those of the mainline neurons that excite the local circuit neurons directly or through their collaterals and whose activities the latter serve to regulate. Glutamate, aspartate, and possibly some of the neural peptides may be major excitatory transmitters in vertebrate and invertebrate nervous systems and glycine may be a vertebrate inhibitory transmitter (Takeuchi A. and Takeuchi N., 1964; Graham *et al.*, 1967; Johnson, 1972; Curtis and Johnston, 1974; Young *et al.*, 1974; Iversen *et al.*, 1978). It seems to us that eventually it will be necessary to identify the relationships of the neurons liberating these various substances by techniques similar to those developed for GABA neurons. The peptides are amenable to direct immunocytochemical visualization. Currently what is lacking is the knowledge of the enzymes that are rate-limiting in the biosynthesis of glutamate, aspartate, or glycine in the pre-synaptic endings of the neurons that may liberate them. Once these enzymes are identified with certainty, their purification, development of antisera to them, and application of immunocytochemical procedures to CNS tissue for their visualization could be achieved rapidly.

GABA NEURONS AND HUMAN DISEASE

One of the first steps in determining whether or not GABA neurons are involved in a disease process should be to locate and study the GABA neurons and/or their processes at both the histological and ultrastructural levels. Application of the currently available immunocytochemical tools to human material is difficult, and, except for biopsy samples, at the present time probably would have to be restricted to studies at the light microscopic level. The immunocytochemical technique for GAD requires fixation for ultrastructural analysis by rapid and adequate perfusion, a procedure that normally would not be feasible in human material, which usually is obtained only at autopsy several hours after death. Furthermore, the techniques could only be applied to small, specific regions, since they do not lend themselves to broad screening approaches. It would seem to me that it would be minimally informative to study tissue with extensive pathological changes such as the striatum in Huntington's disease or the substantia nigra in advanced Parkinsonism. On the other hand, it might be most interesting to examine regions of the ventrobasal complex of the thalamus in Huntington's chorea, since, in the absence

of grossly observable pathological changes in this region, quantitative cytometric measurements showed there to be a loss of more than 50 per cent of small Golgi type II neurons (presumably GABA neurons) in tbe ventrolateral thalamus by comparison with controls and a decrease in size of those cells remaining (Dom, 1976 Dom *et al.*, 1976). The latter type of neuron appeared unaffected in other thalamic sites. A loss of coordinative neural elements in the ventrolateral thalamus, a key relay station for processing information involved in motor control and muscle tone could be importantly related to the choreic symptoms of the disease. Detailed immunocytochemical study of GABA neurons in this region might give important leads about the primary degenerative cbanges that occur in Huntington's disease.

The largest and best-controlled series of measurements on autopsy tissue obtained from schizophrenic and non-schizophrenic individuals has shown there to be a statistically significant reduction in the GAD level in the nucleus accumbens of the schizophrenic patients (Bird *et al.*, 1977). The latter region could be studied profitably with the immunocytochemical technique for clues about possible neuropathologic correlates. Results of a much smaller series than the latter showed that of six brain regions measured, not including the nucleus accumbens or other limbic structures, only in the case of the thalamus were the values in the schizophrenic brains lower tban those of the normal controls at the 5 per cent level of significance (Roberts, 1977). In this connection, it is of considerable interest that, of the various thalamic regions studied, only the pulvinar showed a significant decrement (40 per cent) in the number of Golgi type II neurons in the brains of catatonic schizophrenic patients in comparison with controls (Dom, 1976). Since the pulvinar plays an important role in coordinating visual and auditory inputs, the cellular defect observed might be associated with the well-known hallucinatory phenomena observed in the above state.

Many studies of the neuropathology of neurological and psychiatric disorders will be helped greatly when the materials and techniques become more generally available not only for the visualization of GABA neurons and their processes, but also for the neurons specializing in the release of other neurotransmitters or cybernenes.

Virtually from the beginning of physiologic and pharmacologic observations of the GABA system it has been conjectured that decreases in the efficacy of this system could result in convulsive seizures (Roberts *et al.*, 1960), and much indirect evidence has been adduced to support this belief (Roberts *et al.*, 1976, Krogsgaard-Larsen *et al.*, 1979; Meldrum,

1975). In order to test the above supposition it is necessary to study the neuronal interrelationships in the CNS of organisms with various types of naturally-occurring and experimentally-induced seizures, with a view to determining whether or not there are decreases in numbers of GABA neurons, whether their relationships to other neurons or to each other are disturbed morphologically, and whether or not the GABA neurons are impaired in their functional activity in such a way that the restraints on the activities of the excitatory pacesetter neurons in a given region of the CNS are weakened so that it has become easier for them to recruit each other into the runaway activities observed in seizure states. The availability of the immunocytochemical techniques and the identification of neuronal types as GABA neurons now has made it feasible to begin to attempt to answer some of the morphologic questions. As discussed in a previous section, employing the immunocytochemical procedure for visualizing GAD, GABAergic neurons in the cortex have been identified as aspinous and sparsely-spinous stellate interneurons with extensive intracortical axonal arborizations forming numerous symmetric synapses (Ribak, 1978 a). A study of sections from sensorimotor cortex obtained from five electrographically proven epileptic monkeys treated with alumina cream has made it possible to determine whether or not a correlation existed between epileptic activity and morphologic effects of the treatment on GABA neurons. Mean numbers of GAD-positive terminals counted in contiguous areas from the bottom of layer VI to the middle of layer V showed in each instance a highly significant reduction in numbers of GAD-positive terminals at the electrographically epileptiform sites of alumina gel application by comparison with the contralateral homotopic cortex (Ribak *et al.*, 1979). Ipsilateral sections further away from tbe alumina gel also showed significant decreases in numbers of GABAergic endings. These results support the idea that a selective loss of inhibitory GABAergic neurons, the aspinous and sparsely-spinous stellate cells, could be responsible for the epileptic activity observed at seizure foci. The latter observation is particularly pertinent since many epilepsies in humans are caused by trauma to a brain region as a result of injuries, tumors, etc. Accidental or experimental trauma may interfere with the normal blood supply to a particular region. From a considerable amount of experimental evidence it appears possible that GABAergic local circuit neurons as well as other types of local circuit neurons may be particularly susceptible to injury as a result of ischemia (Graham *et al.*, 1967; Miyata and Otsuka, 1975). This vulnerability may extend not only to ischemia but also to excessive functional demands and the increases in metabolism that occur in febrile

states. In those instances where a mirror focus may be produced in one hemisphere as a result of discharge in the other, a tremendous bombardment of a particular cortical area via the callosal afferents, in addition to producing plastic changes, could create conditions via direct or indirect impingement onto the local circuit neurons or through metabolic depletion of glucose and oxygen and thus, ATP, in the affected region which would differentially affect the function and possibly even the existence of the GABA interneurons in the region, thus sensitizing the area to epileptiform discharge.

Nature has performed a pertinent human experiment in the form of the hereditary disorders known as the neuronal ceroid-lipofuscinoses (Zeman, 1974). In general, it appears that these disorders are inherited in a Mendelian recessive manner and that the different phenotypes are produced by different mutants. A dominant form of this class of disorders also has been described (Boehme *et al.,* 1971). The reason for bringing up this class of disorders in the present context is that a prominent feature in all of them is the occurrence of seizures. It is interesting that in the dominant form of adult neuronal ceroid-lipofuscinosis in all instances the first sign of the disease is the appearance of *grand mal* seizures (Boehme *et al.,* 1971).

The diagnosis is based on the morphological demonstration of excessive amounts of autofluorescent lipopigments in neuronal perikarya, astrocytes, and certain visceral cells. These disorders are clearly chemically distinguishable from the gangliosidoses, since no accumulation of gangliosides is found, but their biochemical basis still is not known with certainty. Some abnormalities may exist in the metabolism of the linolenic family (n-3) of fatty acids in these disorders that result in the cross-linking of intracellular constituents into pigmented insoluble polymeric material (Pullarkat *et al.,* 1977). The lipopigment accumulation is attended by a diffuse loss of nerve cells which often produces brain atrophy. In preparations specifically employed for observation of pigment, but not in ordinary neurohistological sections, it was determined that the lipopigment is stored specifically in stellate neurons. This eventually results in such cases in the virtually complete destruction of the stellate cells in the cortex while preserving the pyramidal neurons (Braak and Goebel, 1978). From the discussion in preceding sections of this paper and the decrement in GABA content in the brains of patients with this disorder (Zeman, 1971) it may be presumed that many of the cortical stellate cells that are destroyed are GABAergic neurons and that the progressive behavioral deterioration is correlatable with the progressive loss of these neurons. Electron micro-

scopic study of a biopsy sample from a four year old girl with a moderately advanced stage of this disorder disclosed (Williams *et al.*, 1977) "an apparent rarity of type II synapses on the perikarya and axon hillocks of large projection neurons". Type II symmetric synapses are typical of those formed by GABA cortical neurons (Ribak, 1978 a). Type I synapses, typical of cortical excitatory neurons, remain abundant in the neuropil. It was suggested that such a differential loss of inhibitory synapses "might be the basis of the diffuse paroxysmal activity and other dimensions of cerebral dysfunction characteristic of this and similar cases". A synopsis of the clinical findings in the case from which the above biopsy sample was obtained is presented below so that the reader can get a feeling for the types of abnormalities in human behavior that might be encountered with the progressive loss of GABA interneurons.

"The patient walked at 11 months and talked in complete sentences and was toilet trained by the age of 2 years. Somatic growth and head circumference progressed along the fiftieth percentile for the first year. Deterioration in her performance was recognized in the Fall of 1974 at the age of three years. This was manifested by clumsiness, frequent falls, dysarthria, and loss of ability to speak in full sentences. Tremor and myoclonic jerking supervened and she was occasionally incontinent. In January, 1975, an EEG showed spikes, poly-spikes and atypical spike save complexes superimposed on a background of irregular high voltage slow activity. In March, 1975, at the age of four years her gait was ataxic, and she was unable to walk without assistance. Her reflexes were hyperactive. There was no paresis, and the plantars were flexor. Cranial nerve findings were normal and there was no detectable somatosensory deficit. She seemed alert and followed one-step demands but spoke only in a few single poorly articulated words. Multiple EEG's obtained during the hospitalization showed poorly organized delta and theta activity with continuous poly-spike activity. During the subsequent nine months of observation she became increasingly irritable and prone to temper tantrums. She spoke and followed simple verbal commands less consistently. Her tremors increased in amplitude and became more generalized. Single myoclonic jerks interrupted distal extremity movements and there were brief lapses of postural tone. Muscle tone and tendon reflex activity increased and became associated with ankle clonus, but the plantars remained flexor. She could no longer stand alone or walk with assistance at the time that the cerebral biopsy was performed".

In many of the other cases described in the literature *grand mal* seizures were a prominent feature of the symptom complex.

GABA ACTION AND INACTIVATION

A current major pharmacological objective in many laboratories is to develop procedures and substances which will allow manipulation of various aspects of the GABA system *in vivo* in a rational and predictable manner (see Roberts *et al.*, 1976; Krogsgaard-Larsen *et al.*, 1979; Meldrum, 1975; Meldrum, 1978). From what has been discussed previously, it is obvious that it would be of great importance from an experimental point of view, as well as in the development of therapies, to be able specifically either to decrease or increase the effectiveness of GABA neurons at will either in the CNS as a whole or regionally. There minimally are six loci at which it might be possible to influence the activity of the GABA system: (a) the activity of the GABA neurons, themselves; (b) the synthesis of GABA; (c) its release; (d) its postsynaptic effectiveness; (e) the synaptic inactivation of GABA by carrier-mediated transport, and (f) its metabolic destruction by transamination and oxidation of the carbon chain. Enhancement of any of the first four of the above processes alone or together and/or decreases in carrier-mediated transport and catabolic processes presumably should lead to enhanced GABA function, while the converse would be expected to result in decreased effectiveness of this system.

I would like to propose that there might be at least four independent entities involved in the postsynaptic operation of the GABA system: a GABA recognition site, an anion channel or carrier-protein, a GABA removal and transport mechanism, and an anion pump (Meyer and Lux, 1974; Lux, 1971; Llinas *et al.*, 1974). Of course, a variety of membrane-located substances, protein and lipid, as well as other types of molecules, ranging from ions to hormones, also could act as regulators of this assembly. I would imagine that the most precise operation of such a system would consist of a non-covalent coupling of the potentially independent constituents in such a manner at postsynaptic membrane sites that the impingement of a given number of GABA molecules released from presynaptic sites would be followed by configurational changes which would result in the inward movement of chloride ions, the number of which would be determined largely by the time required for removal of GABA from receptor sites. GABA probably is inactivated at synapses by a mechanism that involves binding to unique membrane recognition sites, different from those for the receptor-anionophore, and the subsequent transport out of the synaptic junction by a process that is similar to that for many substances. Substances tbat potently and specifically block the binding of GABA to the GABA-recognition site of the inactivation system,

while not affecting the transport part of the process and possessing no mimetic or antagonistic properties at GABA receptor sites and not, themselves, becoming false transmitters, might be effective amplifiers of GABA action at synapses at which GABA normally is liberated. It is likely that removal of GABA from its receptor recognition site would almost immediately inactivate the mechanisms for Cl^- movement. The closely associated Cl^- pump mechanism would begin its operation as soon as chloride ion activity in its vicinity would be increased by a small, given amount, and would act sufficiently rapidly to maintain the ionic gradient, so that hyperpolarizing inhibition could take place when GABA molecules again would be liberated onto postsynaptic sites.

All of the above postulated components also could have an existence independent of each other in the fluid structures of neuronal membranes. The same anionophore and anion pump systems also might associate with glycine recognition sites, for example, operating as part of the glycine inhibitory mechanism (Defeudis, 1978). The GABA recognition entity, at least in some invertebrate systems, even might associate with Na^+ and K^+ ionophores as well as with the one for Cl^- (Yarowsky and Carpenter, 1978). The GABA removal and transport system appears to be ubiquitously distributed in synaptic and non-synaptic portions of neurons and in glial cells (Sano and Roberts, 1961; Sano and Roberts, 1963; Varon *et al.*, 1965; Weinstein *et al.*, 1965; Iversen and Kelly, 1975; Schousboe *et al.*, 1977) and probably often may not be associated with the other components of the GABA apparatus. One of its functions could be to act as a fail-safe device to ensure that the effects of fortuitous accumulation of non-synaptically liberated GABA or that resulting from an overactivity of GABA neurons would be minimized. This is necessary because in a number of instances it has been shown that GABA can cause increases in Cl^- conductance when applied to non-synaptic regions of neural membranes, or to neurons with no known inhibitory input (Kuffler and Edwards, 1958).

The conditions at postsynaptic sites of operative GABA synapses may be such as to favor the appropriate association of the above components into tightly coordinated units, the degrees of coupling of the individual components being dependent to some extent on the liberation of transmitter or of some specific macromolecular constituents from presynaptic sites. In turn the postsynaptic supramolecular association in membranes through some intracellular representations may help regulate the amounts and rates of production of their individual components, perhaps even at the transcriptional or translational levels. In this connection it is of in-

terest that employing the immunocytochemical technique in the developing rat cerebellum, GAD was found to be present in growing neurites in close association with small vesicles prior to the time the neurites make protosynaptic contacts; differentiation of these contacts coincided with the sequestration of GAD into synaptic terminals (McLaughlin, 1975 a) and predated the establishment of contacts between pre- and postsynaptic elements of developing synapses. One would like to know whether the same is true for GABA postsynaptic receptor-ionophore complexes or whether contact between GAD-containing nerve endings and the postsynaptic membranes must take place prior to the synthesis of the individual components and/or the organization of these complexes. The task now becomes one of developing techniques for the study at a molecular level of the individual components of this system and the properties of their assemblies.

In order to begin to develop congruent quantitative test systems for use in studies of the GABA system we have studied a variety of chemical substances, many used similarly hy others before, employing quantitative conductance measurements in the GABA-responding crayfish stretch receptor neuron (Swagel *et al.*, 1973 a, 1973 b; Hori *et al.*, 1978; Krause *et al.*, 1978). The same substances also were examined using adaptations of previously published procedures with membrane preparations from mouse brain to measure GABA binding to presumed GABA-recogni-

TABLE 2. *Efficacy of Substances in Displacing ^{3}H-GABA from Binding to Receptor-related* (A) *and Transport-related* (B) *Membrane Sites*

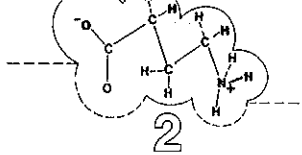
Compound	IC_{50} (μM) A	B
Glycine	* [1]	*
β-Alanine	25	16
GABA	0.13	0.25
DL-β-Hydroxy GABA	4	50
δ-Aminovalerate	4	71
δ-Aminolevulinate	710	2240
ε-Aminocaproate	1590	*
L-α, β-Diaminopropionate	2520	13
L-α, γ-Diaminobutyrate	316	126
L-Ornithine	794	*
L-Lysine	*	3160

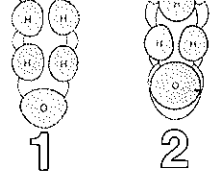
Compound	IC_{50} (μM) A	B
Guanidino acetate	1.4	200
β-Guanidino propionate	1.2	4
γ-Guanidino butyrate	270	32
2-Aminoethane sulfonate (taurine)	126	501
2-Aminoethane phosphonate	*	*
3-Aminopropane sulfonate	0.46	224
3-Aminopropane phosphonate	3200	*
3-Aminopropane sulfinate	0.5	—[2]
Cysteate	*	*
Glycyl-L-histidine	*	*
β-Alanyl-L-histidine (carnosine)	*	*
GABA-L-histidine (homocarnosine)	40	224
Imidazole	*	*
Imidazole carboxylate	*	*
Imidazole-4-acetate	1.3	400
1-Methylimidazole-4-acetate	25	282
Imidazole-4-acetate, isopropyl ester	25	1000
Imidazole propionate	490	195
Isonipecotate (piperidine-4-carboxylate)	0.6	2500
(±) Nipecotate (piperidine-3-carboxylate)	180	13
Isonicotinate	*	*
Isonicotinic hydrazide	*	*
Muscimol	0.03	708
Isoguvacine	0.4	—
THIP[3]	1.6	—
Bicuculline	22	141
Strychnine	22	—
d-Tubocurarine	252	—
Diazepam	252	—
Penicillin G	*	—
Diphenylhydantoin	*	—
Picrotoxin	*	—

[1] $IC_{50} > 3500$ μM.
[2] Determination not performed.
[3] 4,5,6,7-tetrahydroisoxazole - [5,4-c pyridine-3-ol] zwitterion.

tion sites of the receptor-anionophore complex (Enna and Snyder, 1975) and to the GABA binding sites of the GABA transport system (Roberts *et al.*, 1979).

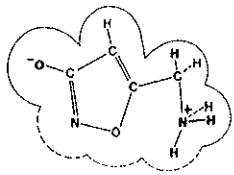
In Table 2 are shown the potencies (IC_{50} values) of GABA and several other substances in displacing ^{3}H-GABA in the receptor-related (A) and transport-related (B) binding assay systems in mouse brain membrane preparations. With the exception of the last group in Table 2, the substances are arranged according to structural similarities. Detailed analyses of the structural relationships within each group of related substances were made as well as examinations of CPK models of GABA and of all of the other substances tested. At the top of Fig. 5 are shown projections of the CPK models of the fully extended GABA molecule, the side, top, and bottom views being depicted. In both the receptor-related (left column) and transported-related (right column) groups, the compounds are shown from top to bottom in order of increasing IC_{50} values, as in the respective test systems, shown in Table 2. Current data and a good deal of previous work has suggested that GABA combines with its receptor recognition site in the extended form as a zwitterion, as represented in Fig. 5 (Olsen *et al.*, 1978 a; Olsen *et al.*, 1978 b; Krogsgaard-Larsen, 1978). The stippled atoms are those that protrude from the surfaces of the molecule and might be the first ones to hit a membrane surface that would be approached by the faces of the molecules. Side 1 and face 1 of the GABA molecule, as depicted at the top of Fig. 5, presumably resemble the part of the GABA molecule that would be recognized by a receptor ionophore recognition site. Indeed, every one of the most active compounds in the receptor-related binding system can be shown from the models to have at least one possible configuration that presents a surface and a charge distribution quite similar to side 1 and face 1 of the GABA molecule, with closely similar distances between a protonated N atom and a negatively charged O atom (see left hand column on Fig. 5 for examples and Table 2 for the related IC_{50} values). The pertinent portions of the molecules which should be compared with GABA (shown in silhouette) are outlined with solid lines. Detailed quantitative structural considerations will be reported elsewhere. Of the 43 substances studied in the receptor-related system, only muscimol was more potent than GABA. In the lower right hand corner of Fig. 5 are shown the top views of the CPK models of GABA and two of the GABA-mimetic substances, muscimol and imidazole-4-acetic acid. Not a single one of the substances tested by us was nearly as potent as GABA, itself, in the transport-related binding system, 16 times the concentration of the next most



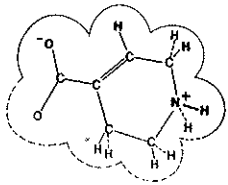


RECEPTOR-RELATED

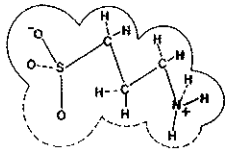
MUSCIMOL



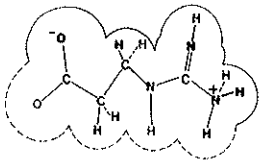
ISOGUVACINE



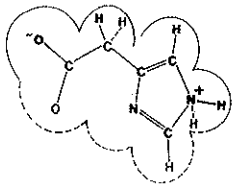
3-AMINOPROPANE SULFONATE



β-GUANIDINO PROPIONATE



IMIDAZOLE-4 ACETATE



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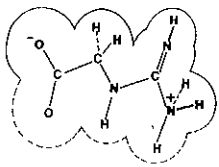
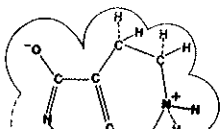
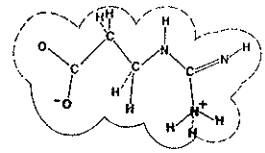


THIP

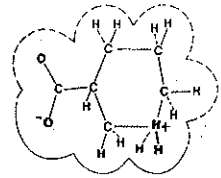


TRANSPORT-RELATED

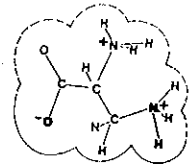
β-GUANIDINO PROPIONATE



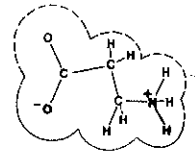
NIPECOTATE



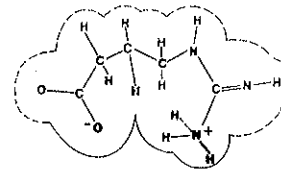
L-α, β-DIAMINO-PROPIONATE



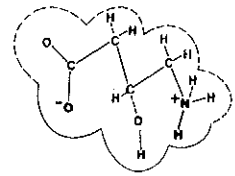
β-ALANINE



γ-GUANIDINO BUTYRATE



β-HYDROXY GABA



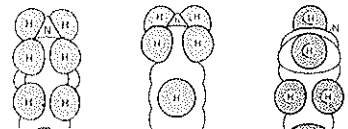


FIG. 5. Models of GABA and receptor-active and transport-active substances, as discussed in text.

potent compound, β-guanidinopropionate, being required to achieve the same effect (IC_{50}) as GABA (Table 2). (±) Nipecotate and L-α, β-diaminopropionate were the most potent blockers of GABA binding to the recognition site of the transport-related system among the substances tested which also possessed very low affinities for the receptor-related sites. The two latter substances were chosen for subsequent study in the crayfish stretch receptor system (see below). CPK models of the most potent transport-site inhibitors showed that each of the substances had at least one permissible configuration that closely resembled side 2 and face 2 of GABA (right hand column of Fig. 5), the charge distributions and distances between the protonated nitrogen and negative oxygen atoms and the shapes of the projecting surfaces being quite similar. Face 2 of the GABA model appears to be the one that attaches to the binding site or sites involved in the transport of GABA. A rough approximation of the relationship of the closeness of fit of the various substances tested to the proposed "active" portions of the GABA molecule may be gained by comparing the solid lines on the tracing of the CPK models with the pertinent part of the silhouette of the side view of the GABA molecule shown in each instance. In both the receptor-site and transport-site paradigms the extent of fit observed to the proposed pertinent molecular sites correlated well with the IC_{50} values (Table 2) obtained by direct measurement. In the case of 3-aminopropane sulfonate, the third oxygen atom of the sulfonate group did not appear to interfere with attachment to the receptor-recognition site, but decreased the affinity for the transport-recognition site to one-thousandth that of GABA, itself.

The fact that correlations such as those above were obtained by assuming single, well-defined conformations of GABA and a number of other flexible molecules without considering the mole fractions of the particular conformations in solution speaks against mechanisms of interaction in which only those molecules that upon collision would possess the correct conformation and orientation would combine with the pertinent membrane binding sites. A number of the conformations depicted in Fig. 5 probably would have a relatively low representation in the total molecular population in solution. The data are more in keeping with the "zipper" model in which it is proposed "that an initial, 'nucleation' complex can be formed by interaction of a single segment of the ligand with its subsite, and this is followed by a series of conformational rearrangements of the partly bound ligand, leading to binding of the remaining segments to their appropriate subsites. The 'zipper' model thus provides a mechanism for the rapid binding of ligands, even when a conformation

of low population is involved" (Burgen *et al.*, 1975). If, in addition, one assumes (Williams, 1977) that membrane-effective ligands, such as GABA, react with relatively flexible portions of mobile receptor-complexes, one can begin to understand how manipulations that may alter the molecular associations in these complexes in membranes can affect greatly all aspects of the interactions between ligand and receptor. In this connection it is of considerable interest that recently it has been found by us that several detergents had no effect on the ability of muscimol or other conformationally restricted GABA agonists such as imidazoleacetic acid, isoguvacine, and THIP in displacing ^{3}H-muscimol in binding to mouse brain membranes, but that treatment of the membrane preparation with the detergents potentiated the efficacy of flexible agonists such as GABA and related substances (Wang *et al.*, 1979).

From a number of previous studies in other laboratories, similar in principle to those above, it has been proposed that GABA may bind to its receptor-recognition sites in an extended conformation and to its transport-recognition sites in a folded conformation (see Defeudis, 1977 for review). Our data suggest another possibility (Table 2 and Fig. 5). When the GABA molecule attaches to the recognition site of the receptor complex in the extended form, the other side of the GABA molecule already is in the configuration that is recognized by the transport binding site. If the two sites are in close juxtaposition, attachment might take place of the GABA recognition site of the transport system to receptor bound GABA, and its removal from the receptor could occur automatically and immediately following the conformational changes that accompany membrane activation by the transmitter and anion channel opening. This would eliminate the physiological uncertainties that might attend processes involving the passive equilibrium dissociation of the transmitter from the active site and diffusion to removal sites and would ensure immediate removal of the transmitter from its recognition site and the closing of the associated ion channel in a manner compatible with the quantal and temporal requirements for precise synaptic function. Preliminary data (Roberts *et al.*, 1979) are consistent with such a hypothesis, but do not prove it, and alternative explanations still are possible.

A definitive test of the above hypothesis might be obtained in study of the effects of GABA uptake blockers or single channel characteristics (noise analysis) in a GABA responding neuron. Some studies with glutamate, probably a major excitatory transmitter at vertebrate and invertebrate synapses (Takeuchi A. and Takeuchi N., 1964; Young *et al.*, 1974), are pertinent to the above discussion. It was found at crustacean neuromuscular

junctions that in concentrations that were ineffective by themselves, aspartate, a potent blocker of glutamate uptake (Baker and Potashner, 1971) greatly enhanced the excitatory potency of glutamate application or of neural excitation produced presumably via presynaptically liberated glutamate (Freeman, 1976; Crawford and McBurney, 1977 a, b). The rates of decay of the quantal currents flowing at the excitatory junctions, attributable to cessation of ion movement consequent to removal of glutamate from receptor sites, were too rapid to be accounted for by diffusion of glutamate from such sites (Crawford and McBurney, 1977 a, b). The effects of aspartate could not be accounted for by increases in presynaptic release of glutamate or in the affinity of glutamate for receptor sites. "A reduction in the rate of exit of transmitter from the region of the receptors can explain all observed actions of aspartate" (Crawford and McBurney, 1977 b). An inspection of CPK models of glutamate and aspartate, as well as of a variety of substances affecting glutamate action on membranes, or mimicking it, suggests to me that glutamate may act on its receptor through attachment of the removal mechansm to the other surface of the same configuration as suggested for the case of GABA (Roberts, in preparation).

In Fig. 6 the log of the reciprocals of the IC_{50} values of several structurally related substances that displace ^{3}H-GABA in receptor-related mouse brain membrane binding assays are plotted *vs* the log of the ratios of the EC_{50} value of GABA to these substances determined by conductance changes in the crayfish stretch receptor system. The binding assay used is believed to be a measure of the affinities of several test substances relative to that of GABA for the GABA recognition site of the receptor-anionophore complex. The crayfish stretch receptor assay is a measure of the efficacies of the substances tested relative to that of GABA in initiating a series of events that begins with association of ligand and membrane, results in initiation of anion movement, and ends in the dissociation of the ligand-receptor complex and cessation of anion movement. The latter comprises a complex series of events, and a linear relationship between the two measurements would have been expected if the GABA-recognition sites in mouse brain and the crayfish stretch receptor neuron were very similar, if the rates of the steps in the sequence of changes in the latter preparation were independent of tbe nature of the effective ligand, and if the rate-limiting step in the sequence were the attachment of ligand. Isonipecotate, 3-aminopropane sulfonate (+) GABOB, THIP, and δ-aminovalerate were somewhat less potent on the crayfish stretch receptor than expected from membrane binding; and guanidino-acetate

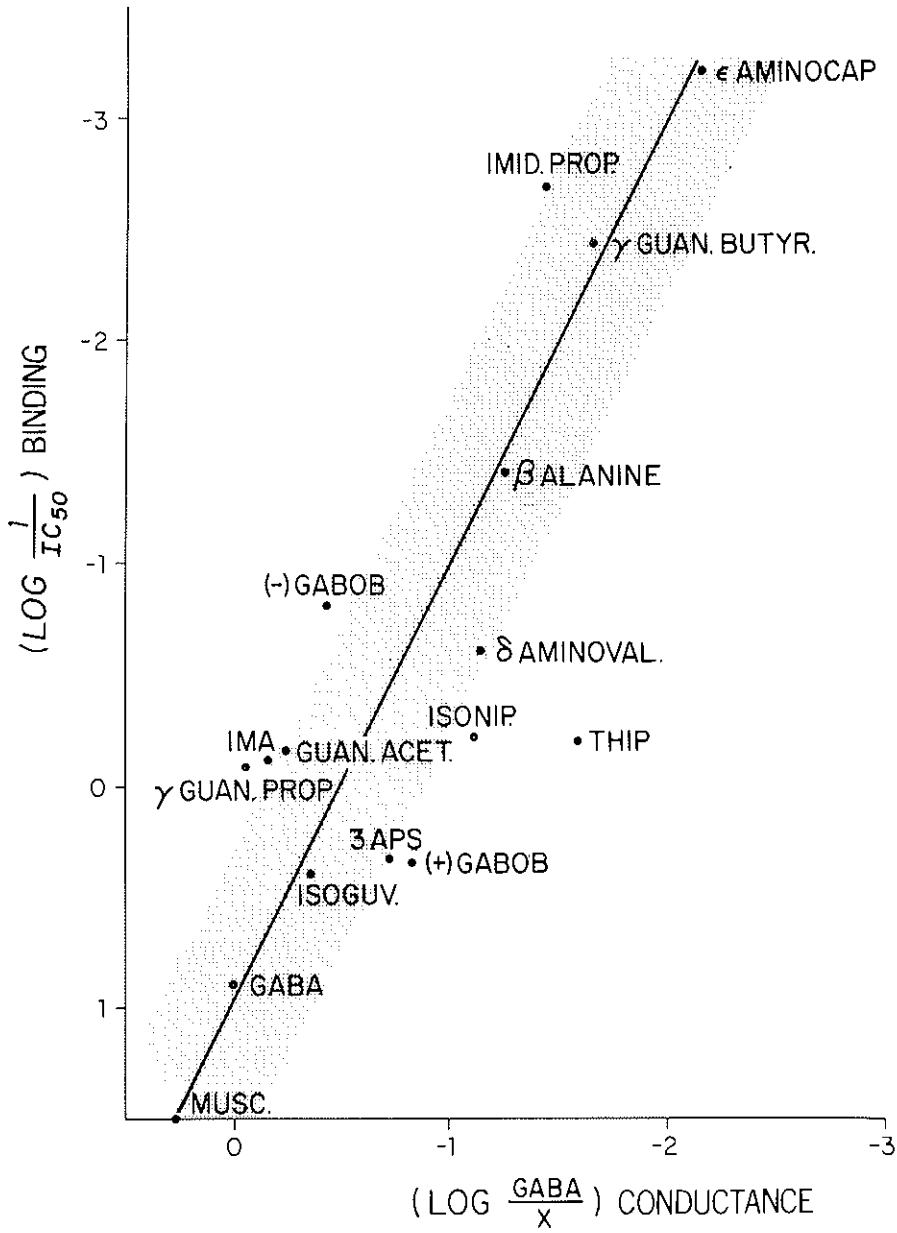


FIG. 6. Binding to mouse brain membranes and conductance effects on the crayfish stretch receptor. See text for details. Abbreviations: APS, 3-aminopropane sulfonate; (±) GABOB, DL-β-hydroxy GABA; IMA, imidazole-4-acetate; THIP, 4,5,6,7-tetrahydroisoxazole-[5,4-c pyridine-3-ol] zwitterion. For other abbreviations see Table 2.

and propionate, imidazoleacetate, (—) GABOB, and imidazole propionate were somewhat more potent. Substances with a relatively high affinity for the GABA receptor in crayfish muscle may be considerably less effective than GABA at the neuromuscular junction in terms of the maximal conductance changes that they can produce (Takeuchi A. and Takeuchi N., 1975). In our experiments GABA and the other substances tested usually produced the same maximal effect on conductance. In both the *in vitro* and *in vivo* test systems muscimol and GABA were the most potent substances examined by us. Further work is in progress to determine the structural and physico-chemical parameters pertinent to determining relative efficacy in the two test systems (Steward and Clarke, 1975). Meanwhile, we now have assurance that there is some quantitative correspondence between our measurements on mouse brain membranes and the crayfish stretch receptor neuron. Further exploration of both types of assays are under way.

A recent study of GABA-induced membrane current noise in crayfish muscle fibers showed the presence of "fast" synaptic channels, and "slow" extrasynaptic channels (Dudel, Finger and Stettmeier, 1977), as has been shown to be the case for acetylcholine and glutamate synapses (Dreyer *et al.*, 1976; Katz and Miledi, 1973; Neher and Sakman, 1976). The effects of bath application of substances to preparations with reactive membranes may take place at both synaptic and extrasynaptic membranes. Iontophoretic studies have shown there to be a high degree of sensitivity to GABA localized to the rostral dendrites of the crayfish stretch receptor neuron, where inhibitory synapses are most numerous (Graelius, 1976). However, GABA can affect non-synaptic sites as well as the latter preparation. An important approach to getting information about the effects of bath applied substances at postsynaptic sites is to examine their influence on neurally evoked inhibitory responses. Maximally useful types of information might be obtained by a combination of the above approaches with the study in suitable preparations of synaptic noise during the action of GABA and GABA agonists, antagonists, and uptake blockers. During the writing of this paper the first report appeared of the study of GABA-induced conductance fluctuations in mammalian neurons (McBurney and Barker, 1978). The neurons, obtained from cultures of dissociated spinal cords of 12 to 14 day old mouse embryos, gave results consistent with the existence of a single population of GABA-induced anion-specific channels.

Studies like those above are beginning to lay the ground work for a meaningful molecular pharmacology of the GABA system.

Conclusion

It has taken almost thirty years of work to move from an unknown ninhydrin-reactive spot on a two-dimensional paper chromatogram of an extract of mouse brain to the establishment of GABA as a major inhibitory transmitter, the visualization of GABA-releasing neurons in nervous system structures, and the establishment of a heginning of a rational pharmacology of the GABA system. Even this relatively modest degree of progress has been possible only because of the participation in these studies of scientists from the several pertinent disciplines in laboratories the world over. The recent coalescence of these separate disciplines into the single one of "neurosciences" has made it possible for us to begin to share techniques, vocabularies, and outlooks. And yet, there is a sense of uneasiness among us. Who can master all of the pertinent facts and technologies, or even keep up with a small portion of the literature? Will we be drowned by the sea of observations before we will be able to recognize the forest for the trees and divine the master plans of nervous system function? All of us know that great generalizations are achieved in individual brains and not by committees. And, yet, which individual has the time and intelligence to assimilate so much and to deal creatively with it?

To date it seems to me that, in spite of some valiant efforts, attempts at achieving synthetic or unified views of nervous system function all have failed because it has not been possible to establish a valid core position from which one can view meaningfully *both* phenomena of major human interest such as memory, consciousness, various aspects of normal and abnormal behavior, aging, etc., and the molecular and submolecular events that constantly are taking place at the level of excitable memhranes.

Too often data about nervous systems are produced and floated out onto the ever-expanding sea of undigested information with the implicit hope that some friendly theoretician will attempt to fit the data into a framework that is relevant to the main business of nervous systems, information processing. In reality, fear of criticism by our colleagues prevents many of us from making our own integrative efforts. Yet there is a courageous one among us today, John C. Eccles, who has contributed so much to our understanding of nervous system function through his many seminal research findings and also keeps daring to attempt to scale the Mount Everest of ultimate understanding (Popper and Eccles, 1977). Let us hope that his example will encourage the rest of us to be brave enough to try.

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DISCUSSION

RAITERI

I would like to comment on some of your speculations which I like very much. The first one is that concerning the possibility that GAD is part of the vesicular membrane and it does not act only to transform glutamate into GABA but also as a pump for the uptake of GABA into the vesicles. Are you implying that inhibitors of GABA uptake can also inhibit this pump?

ROBERTS

Such inhibitors probably would inhibit a coordinated enzyme-vesicle pump because they prevent the action of the enzyme on the substrate. If this occurs, the postulated subsequent events could not happen. The details of such a mechanism would have to be worked out step-by-step, but it would be expected that if the first step in the process were blocked, i.e. the attachment of the glutamate to the enzyme, then all the other steps would not occur.

RAITERI

But do you know any inhibitor of GAD which inhibits GABA uptake? As far as I know, they do not. In this case you should assume that the GABA which is taken up goes into a pool different from that of GABA which is newly synthesized. Well, some experiments that we have done with Dr. Levi do not seem to support the idea that GABA taken up and GABA newly synthesized behave in a different way.

ROBERTS

At this point, I would prefer not to speculate about what is happening *in vivo*. I think our next step is to get vesicles to associate with pure glutamate decarboxylase and see if we can demonstrate such a pumping action. In other words, starting with labeled glutamate on the outside of such vesicles, can a differential accumulation of labeled GABA be sbown on the inside as compared to that found on the outside? I would aim for such a relatively simple experiment because I cannot think of experimental approaches to the more complex phenomena at the moment.

RAITERI

May I put another question? This is a very naive question since I am not an expert in morphology. I would like to know what is the dimension of a GABAergic synapse, and is it tight enough to allow the two sides of the GABA molecule to be bound simultaneously?

ROBERTS

No. The synaptic gap is just about 200-300 Å. The GABA molecule is much too small to bridge the gap.

ECCLES

I greatly admired the pictures and work that Dr. Roberts has given. As a physiologist one has to postulate many things, but he finds the real pictures of what we were postulating—that is delightful! I particularly wanted to ask a question about the receptors of the surface of cells where you had evidence that there could be both GABA and non-GABA inhibitory synapses.

ROBERTS

Yes, in the spinal motoneuron.

ECCLES

Well, I wonder whether you looked at the dorsal column nuclei, cuneate and gracile, because there we got physiological evidence of two kinds of inhibitory transmitters on some cells. That would be a very good check, to test again the physiological versus the beautiful biochemical work you have done.

ROBERTS

These regions are scheduled to be studied or are now being studied. A great difficulty is that we do not know the rate-limiting enzyme for transmitter glycine biosynthesis. If we were able to do for glycinergic endings and for glutaminergic endings what we can do for the GABAergic endings, we would be able to create montages showing where the glycinergic, GABAergic, and glutaminergic endings are in given neuroanatomic settings and to see how they relate to each other.

ECCLES

In the cuneate nuclei, there are some cells that seem to be entirely GABA-inhibited, some which are entirely glycine, and some which are both. This would make a very nice picture for you if you try your beautiful techniques on that.

MECHANISMS OF GABA RELEASE AND REUPTAKE IN PRESYNAPTIC NERVE ENDINGS

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I. INTRODUCTION

The present article summarizes our studies on the presynaptic mechanisms of GABA uptake and release and presents a model for the GABA fluxes at nerve endings based on such studies.

Some of the main features of GABA transport in hrain subcellular particles were described, as early as 13 years ago by the group of Roberts (Weinstein *et al.*, 1965; Kuriyama *et al.*, 1969). In the late sixties and early seventies the topic has been resumed by Iversen and his group (Iversen and Neal, 1968; Iversen and Johnston, 1971) and then by many others (Henn and Hamberger, 1971; Bowery and Brown, 1972; Martin and Smith, 1972; Bond, 1973; Balcar and Johnston, 1973; Levi and Raiteri, 1973; Schon and Kelly, 1974). Kinetic studies conducted in those years on the accumulation of radioactive GABA by synaptosomes and brain "minislices" suggested the existence of a specific high affinity uptake system for GABA, characterized by an apparent Km lower than 50 μM and by an absolute sodium dependence. In analogy with preceding theories on the inactivation of other neurotransmitters such as catecholamines (Iversen, 1971; Axelrod, 1973) the theory was proposed that this specific transport system is responsible for the rapid inactivation of the GABA released in synapse (Iversen and Johnston, 1971; Iversen and Kelly, 1975). The discovery of this transport system and of its specific

localization in GABA-ergic nerve endings provided one of the first tools for the localization of GABA-ergic pathways in the brain.

In 1973 (Levi and Raiteri, 1973) we described the existence, in synaptosomal preparations, of a second transport system for GABA characterized by a lower affinity and a higher capacity, and detectable at relatively high GABA concentrations. According to the current theories, that transport system, comparable to other systems described for non neurotransmitter amino acids, was not related to any specific synaptic function and possibly subserved a "metabolic" role.

In summary, until 1974 the presynaptic fluxes of GABA were described in a rather simple way. Schematically, one could distinguish between a depolarization-induced, calcium-dependent release process (Levy *et al.*, 1973), which was poorly understood in terms of mechanism of release and origin of the transmitter released, and an inactivation process during which reuptake of GABA occurred through a specific, sodium-dependent high affinity uptake system.

This simple picture started to appear incomplete in 1974 when we demonstrated that the accumulation of radioactive GABA observed in many *in vitro* studies had to be largely attributed to a homoexchange process, rather than to a process of *net,* high affinity uptake (Levi and Raiteri, 1974; Raiteri *et al.*, 1975). A similar conclusion was reached independently by Simon *et al.*, 1974 and later confirmed by other authors (Sellström *et al.*, 1976; Storm-Mathisen *et al.*, 1976; Ryan and Roskoski, 1977).

The studies conducted since then in our laboratory brought us to the formulation of a new more complex picture of GABA presynaptic fluxes (Levi and Raiteri, 1978) which is presented diagramatically in Fig. 1. According to the model shown, the GABA transport system of nerve endings is susceptible to fine modulation by changes in the direction of cationic fluxes similar to those occurring *in vivo* during depolarization and repolarization. In resting conditions, that is in the absence of net ionic fluxes, the carrier-mediated transport of GABA would consist of a homoexchange process, which does not lead to any net inward or outward transport of the amino acid. In the presence of the cationic fluxes characteristic of physiological depolarization, which, by themselves, cause some release of GABA not mediated hy the membrane carrier, the stoichiometry of GABA carrier mediated homoexchange would be shifted in the direction of net outward transport. In other words, extracellular GABA would trigger a release of GABA greater than the concomitant influx of the amino acid. When the cationic fluxes are reversed, as it occurs during

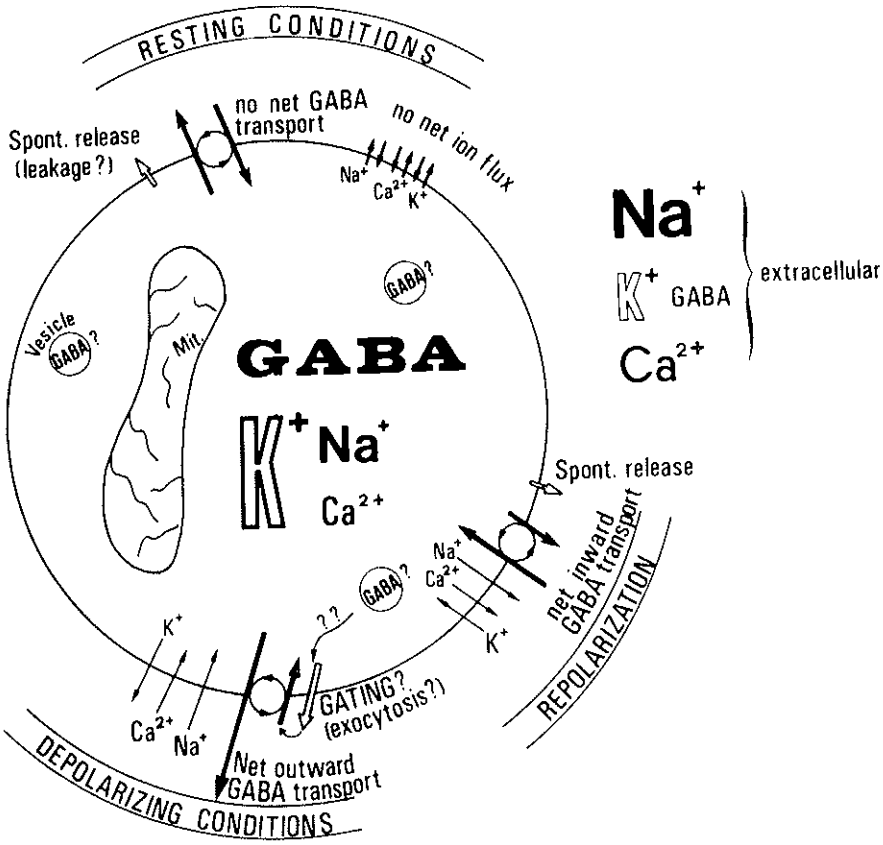

FIG. 1. Schematic representation of GABA and cation fluxes in a GABA-ergic nerve ending at rest, during depolarization and during repolarization.

repolarization, also the stoichiometry of GABA homoexchange would be reversed and influx would prevail over efflux, with a resulting net uptake.

In the following sections of this article we shall examine these various phases in more detail, and summarize the experimental evidence in favour of this interpretation.

II. GABA FLUXES IN RESTING CONDITIONS

In resting conditions, no net ion flux is present, and the major cations (Na^+, Ca^{2+} and K^+) have a physiological distribution between intracellular and extracellular spaces. When purified synaptosomes prelaheled with trace amounts of radioactive GABA are superfused in these condi-

tions, in the absence of extracellular GABA, a modest "spontaneous" release takes place, by a still unknown mechanism, which however, seems independent of the membrane carrier (Simon *et al.*, 1974; Levi *et al.*, 1976a). If extracellular unlabeled (or differently labeled) GABA is present, even at concentrations in the range of the high affinity uptake system, the labeled GABA contained in the synaptosomes is released. This GABA-stimulated GABA release has the following characteristics: 1) Is concentration dependent and saturable (Levi and Raiteri, 1974; Raiteri *et al.*, 1975), and has an apparent Vmax and Km similar to those of the high affinity uptake (Simon *et al.*, 1974). 2) Is totally sodium-dependent (Simon *et al.*, 1974; Raiteri *et al.*, 1975). 3) Is elicited only by GABA and GABA analogs sharing the GABA transport system (Raiteri *et al.*, 1975). 4) Is highly temperature sensitive, and is abolished at 0° (Simon *et al.*, 1974). 5) Is blocked by pretreating the synaptosomes with the GABA carrier blocker L-diaminobutyric acid (DAB) (Levi *et al.*, 1976a). 6) Is quantitatively similar (when the concentration of exogenous GABA is sufficiently low) to the simultaneous accumulation of radioactive GABA observed in double label experiments or in separate uptake experiments (Simon *et al.*, 1974; Raiteri *et al.*, 1975). The calculations made to reach this conclusion were originally based on the assumption that exogenous GABA does not exchange preferentially with the radioactive GABA used to prelabel the synaptosomes. In fact, if this were the case, the rates of GABA-stimulated GABA release would have been overestimated. Subsequent experiments showed that this assumption was essentially correct. In one set of experiments (Levi *et al.*, 1976b) (Table 1, first two columns) we compared the ability of exogenous unlabeled GABA to release ^{3}H-GABA previously taken up by the synaptosomes, and ^{14}C-GABA previously synthesized from ^{14}C-glutamate in the same synaptosomes. It can be seen that the pattern of release was similar, the ^{3}H-GABA taken up being released only slightly more than the ^{14}C-GABA synthesized (difference not statistically significant). In another set of experiments (Table 1, right column) we used the GABA analog γ-amino-β-hydroxybutyric acid (OH-GABA) to stimulate the release of GABA by heteroexchange from synaptosomes prelabeled with trace amounts of radioactive GABA, and we compared the specific radioactivity of the GABA released to that present in synaptosomes (Levi *et al.*, 1978). Also in this case we noted only a modest, although statistically significant, preferential release of the labeled GABA as compared to the endogenous unlabeled GABA.

In conclusion, the characteristics of the GABA-stimulated GABA

TABLE 1. *Comparison between the release of exogenous and endogenous GABA in synaptosomes superfused with unlabeled GABA or OH-GABA*

Type of sample analyzed	homoexchange with unlabeled GABA (percent of total radioactivity)		heteroexchange with OH-GABA (relative specific radioactivity)
	^{3}H-GABA taken up	^{14}C-GABA synthesized from ^{14}C-glu	^{14}C-GABA taken up
Synaptosomes, start of superfusion	100	100	100
Effluent (prestimulation)	2.7 ± 0.3	2.4 ± 0.4	104 ± 9
Effluent (stimulation)	20.5 ± 3.3	16.2 ± 2.9	121 ± 11 *
Synaptosomes, end of superfusion	70.0 ± 4.0	74.4 ± 3.5	91 ± 10
Number of experiments	7	7	9

* Significantly higher than in the prestimulation medium ($p < 0.02$) and in synaptosomes at the end of superfusion ($p < 0.01$). The relative specific radioactivity of synaptosomes superfused throughout the experiment with standard, OH-GABA-free medium did not differ significantly from that at the start of superfusion.

In the experiment reported in the first 2 columns (Levi *et al.*, 1976 b), synaptosomes were incubated for 30 min with 3.3 μCi/ml of [U]^{14}C-L-glutamate. During the last 5 min of incubation, 1 μM ^{3}H-GABA was added to the incubation mixture. Aliquots of the suspension were superfused for 10 min with standard medium, and then with a medium containing 100 μM unlabeled GABA (Experimental details as in Fig. 2). The radioactivity present as ^{3}H- or ^{14}C-GABA was measured after high voltage electrophoretic separation in extracts of synaptosomes at the start and at the end of superfusion, and in pooled fractions of the superfusion effluent, before (min 7-10) and during stimulation (min 11-14). The radioactivity present in synaptosomes as ^{3}H-GABA or ^{14}C-GABA, at the start of superfusion, was taken as 100, and the results are expressed as percentages of this radioactivity.

In the experiment reported in the third column, synaptosomes were prelabeled for 10 min in a medium containing 0.5 μM ^{14}C-GABA, superfused with standard medium for 10 min and then with new medium containing 100 μM OH-GABA. GABA concentration and radioactivity were measured in synaptosomes at the start and at the end of superfusion, and in pooled effluent fractions before (min 6-10) and during (min 12-16) stimulation, after ion exchange chromatography. The GABA specific radioactivities are expressed as relative values, taking the specific radioactivity of synaptosomes at the start of superfusion as 100.

release are practically identical to those of the high affinity uptake, and justify the statement that, under resting conditions, the accumulation of radioactive GABA observed in isolated nerve endings is largely due to a homoexchange process whereby the GABA entering the synaptosomes drives, by countertransport, the outward flux of an equivalent amount of endogenous intraterminal GABA.

III. GABA FLUXES IN THE PRESENCE OF ALTERED CATIONIC FLUXES

1. *Cationic fluxes characteristic of physiological depolarization*

Physiological depolarization is accompanied by an influx of Na^+ and Ca^{2+} and by an efflux of K^+. According to the model presented in Fig. 1, these fluxes determine two types of GABA release: a carrier-independent release, observable in the absence of extracellular GABA, and a carrier-mediated release, superimposed on the first, and triggered by extracellular GABA itself through a homoexchange process characterized by a stoichiometry in favor of net outward transport (Levi and Raiteri, 1978).

Let us now examine the experiments that led us to this conclusion. We studied the release of GABA from superfused synaptosomes in a number of conditions altering the fluxes of one or more cations in the same direction as physiological depolarization. The following conditions were used: ouabain, or a K^+-free medium, which inhibits the Na^+-K^+ ATPase (Schwartz *et al.*, 1972) and causes influx of Na^+, efflux of K^+ (Abdel-Latif, 1973; Archibald and White, 1974; Blaustein and Goldring, 1975; Goddard and Robinson, 1976) and possibly influx of Ca^{2+} (Stahl and Swanson, 1969; Swanson *et al.*, 1974; Goddard and Robinson, 1976); veratridine, which causes influx of Na^+ and Ca^{2+} and efflux of K^+ (Blaustein and Goldring, 1975; Blaustein, 1975; Goddard and Robinson, 1976; Li and White, 1977); the ionophore A23187, which produces a passive downhill Ca^{2+} flux (Pressman, 1976); and a pretreatment with a low Na^+ medium, followed by exposure to a medium with a standard Na^+ concentration (Levi and Raiteri, 1978; Levi *et al.*, 1978), which determines a rapid Na^+ influx (Li and White, 1977). All these conditions stimulated the release of ^{3}H-GABA from synaptosomes, in the absence of extracellular GABA (Levi *et al.*, 1976a; Levi and Raiteri, 1978; Levi *et al.*, 1978). This release did not appear to be mediated by the membrane transport system. In fact, pretreating the synaptosomes with the GABA carrier blocker DAB did not produce any decrease in the

release of GABA, as it would be expected if GABA exit were mediated by the membrane carrier (Levi *et al.*, 1976a). When the effect of the same releasing agents was tested in the presence of a small concentration of extracellular GABA, which, by itself, exchanges with intrasynaptosomal GABA on a 1:1 basis but does not cause *net* GABA release, the release of ^{3}H-GABA was increased much more than what would be accounted for by the sum of the release due to GABA alone plus that due to each of the agents tested alone (Fig. 2). These data suggest that, in the conditions tested, the presence of even small amounts of extracellular GABA triggers a *net* release of intrasynaptosomal GABA, superimposed on that elicited by the various agents tested alone. The possibility that this "supra-additive" release of radioactive GABA was due to an acceleration of the rate of the 1:1 GABA homoexchange was excluded by the fact that the simultaneous influx of GABA was either unaffected or depressed in the conditions studied. In order to exclude the additional possihility that the "supra-additive" release of radioactive GABA was due to an increase in the specific radioactivity, and not in the actual amount of GABA released, we measured chemically the GABA released into the superfusion medium, in some of the conditions analyzed in the previous experiments. The only variation that had to be introduced in the experimental protocol was that of inducing GABA exchange with a GABA analog, rather than with GABA itself. The substance chosen was OH-GABA, which is transported by tbe GABA transport system (Iversen and Johnston, 1971; Raiteri *et al.*, 1975), can be separated chromatographically from GABA and, as shown in Fig. 3, causes a "supra-additive" release of GABA similar to that elicited by GABA itself. The data on the chemical determination of the GABA released are shown in Table 2. It is clear that a potentiation of the net release of GABA was present when the synaptosomes were exposed to OH-GABA together with veratridine, A23187, or a sudden increase in the inward Na^+ gradient.

The experiments presented so far do not help in elucidating the mechanism responsible for the potentiation of GABA release. It seemed worthwhile to determine whether the extra release of GABA ohserved in the presence of extracellular GABA was carrier-mediated, as GABA homoexchange, or was independent of the GABA carrier, as the release induced by the various agents in the absence of extracellular GABA. For this purpose, we measured the "supra-additive" release of GABA after pretreating the synaptosomes with DAB, which, as previously mentioned, causes a long lasting inhihition of GABA carrier-mediated transport (Levi

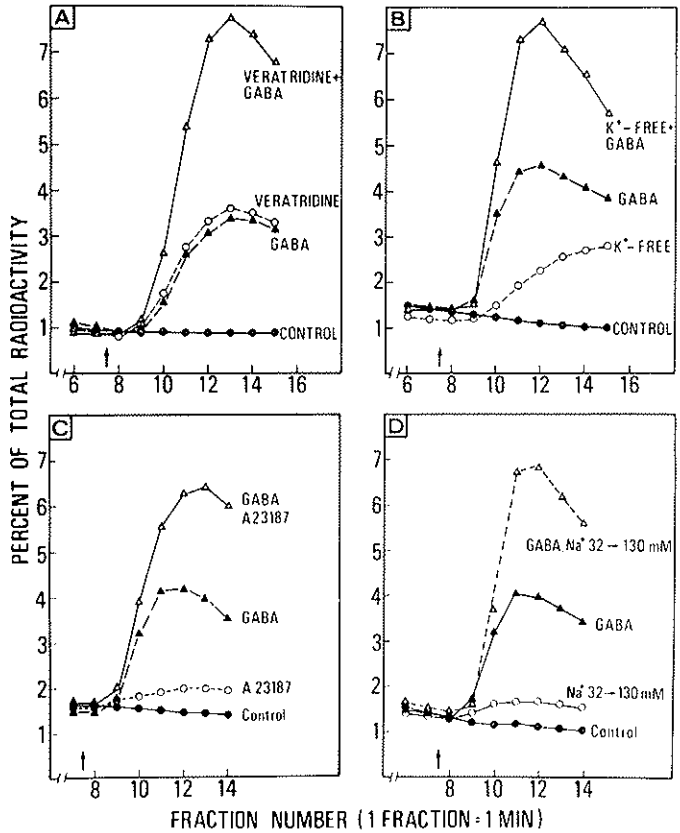

FIG. 2. "Supra-additive" release of ^{3}H-GABA elicited by unlabeled GABA in various conditions. Except for the experiments of Panel D (see below), purified rat brain synaptosomes resuspended in 0.32 M glucose were diluted 1:10 in a standard medium (128 mM NaCl, 5 mM KCl, 2.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM Na_2HPO_4 and 20 mM HEPES buffer at pH 7.35), incubated for 20 min at 37°C (the last 10 min in the presence of 0.5 μM ^{3}H-GABA) and then superfused in 8 parallel superfusion chambers (Raiteri and Levi, 1978) as follows: Panel A: superfusion with standard (glucose containing) medium for 7.5 min and then with a medium containing either 10 μM veratridine or 10 μM GABA or both these compounds. Panel B: superfusion with standard medium for 7.5 min and then with a K-free medium, or with a GABA containing (10 μM) medium, or with a K-free, GABA containing medium. Panel C: superfusion with standard medium for 7.5 min, and then with a medium containing either 19 μM A23187, or 10 μM GABA or both these compounds. Panel D: control synaptosomes were prelabeled as described above and superfused either with standard medium or (starting from min 7.5) with a medium containing 10 μM GABA. Other synaptosomes from the same preparation were prelabeled with ^{3}H-GABA in a medium containing 32 mM NaCl (the missing NaCl was replaced by sucrose) and superfused for 7.5 min with the same low Na medium. Then the superfusion was continued with standard medium, containing 10 μM GABA or no GABA. Aminooxyacetic acid (10 μM) was present in all the media to prevent ^{3}H-GABA metabolism. The radioactivity released in each 1 min fraction is expressed as a percentage of the total radioactivity recovered (total fractions plus synaptosomes at the end of superfusion). The data were obtained from Levi *et al.*, 1976 c, and Levi and Raiteri, 1978.

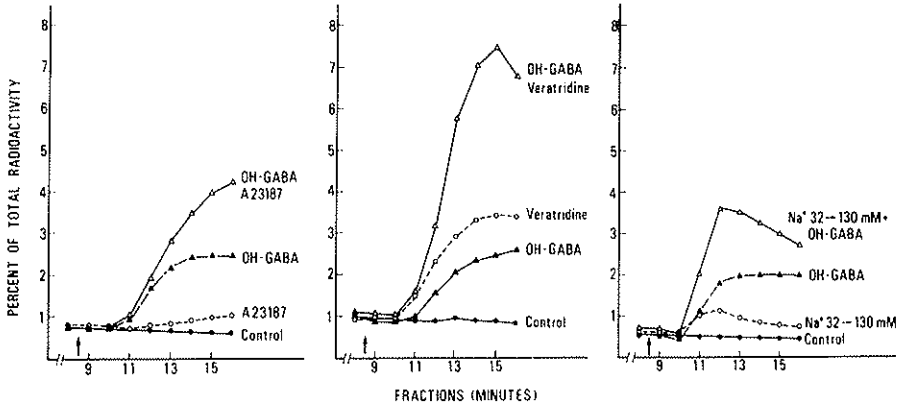

FIG. 3. "Supra-additive" release of ^{3}H-GABA elicited by OH-GABA in various conditions. Experimental conditions were as in the experiments reported in Fig. 2, except for the presence of 100 μM OH-GABA (instead of 10 μM GABA) in the superfusion media. Left panel: see panel C of Fig. 2. Central panel: see panel A of Fig. 2. Right panel: see panel D of Fig. 2. Data from Levi and Raiteri, 1978 and unpublished.

TABLE 2. *Net release of GABA from synaptosomes*

GABA releasing agent	nmol/mg protein released *	supra-additive release index **	N.
OH-GABA, 100 μM	0.84 ± 0.18		8 (10)
Veratridine, 10 μM	1.41 ± 0.18		3 (5)
Veratridine, 10 μM + OH-GABA, 100 μM	4.57 ± 0.20	2.03	3 (5)
A23187, 19 μM	0.04 ± 0.02		3 (3)
A23187, 19 μM + OH-GABA, 100 μM	1.54 ± 0.38	1.75	4 (7)
Na^+, 32 mM → 130 mM	0.22		2 (2)
Na^+, 32 mM → 130 mM + OH-GABA, 100 μM	1.82	1.72	2 (2)

* Over control, during 5 min of stimulation.

** Ratio between release observed and release expected on a purely additive basis. N = number of experiments and of determinations (in parentheses).

Synaptosomes were treated as described in the legend for Fig. 3. The concentration of GABA was measured by ion exchange chromatography followed by reaction with o-phtalaldehyde, in pooled effluent fractions (min 12-16 of superfusion).

et al., 1976 a). The pretreatment with DAB should inhibit the "supra-additive" release of GABA, if this were carrier-mediated. Fig. 4 shows the results obtained in experiments with ouabain. The data indicate that the release of ^{3}H-GABA elicited by the drug alone was unaffected by the DAB pretreatment, while that elicited by GABA alone, or by GABA plus ouabain was strongly inhibited. The inset shows the inhibition of the "supra-additive" component of GABA release. Similar results were

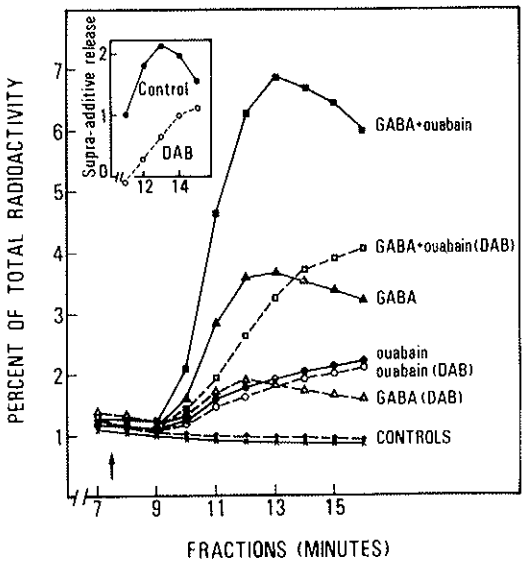

FIG. 4. Effect of pretreatment with DAB on the "supra-additive" release of ^{3}H-GABA. Synaptosomes were prelabeled with 0.33 μM ^{3}H-GABA (controls) or with 1 μM ^{3}H-GABA in the presence of 200 μM DAB. Four aliquots of each of the two synaptosomal suspensions were then superfused in parallel with standard DAB-free medium. After 7.5 min the superfusion was continued either with standard medium (spontaneous release) or with media containing 100 μM ouabain or 10 μM GABA or both these compounds. Dashed lines: synaptosomes pretreated with DAB. Solid lines: control synaptosomes. Inset: Inhibition by DAB of the "supra-additive" component of ^{3}H-GABA release. Other experimental details as in the legend for Fig. 2. Data from Levi and Raiteri, 1978.

obtained in experiments in which the "supra-additive" release of ^{3}H-GABA was elicited by the ionophore A23187 and extracellular GABA (Levi and Raiteri, 1978). In conclusion, the data support the view that the "supra-additive" release of GABA is largely mediated by the GABA transport system. Thus, the phenomenon of "supra-additive" release appears to be due to a change in the stoichiometry of GABA homoexchange, resulting in an efflux/influx ratio greater than unity (net efflux).

According to the data presented, an increase in the efflux/influx ratio of GABA can be obtained in the presence of either a net influx of Ca^{2+} (as in the case of A23187), or of Na^+ (as in the case of veratridine, Na^+-K^+-ATPase inhibitors and a sudden favorable Na^+ gradient). In the latter three cases the same effects were observed, whether or not Ca^{2+} was present in the medium (Levi *et al.*, 1978); which suggests that

Ca^{2+} influx, although capable by itself of altering the stoichiometry of GABA homoexchange in favor of efflux, is no longer effective when a major influx of Na^+ occurs concomitantly. At present, it is difficult to say whether changes in K^+ fluxes contribute to the effects observed. Veratridine and Na^+-K^+-ATPase inhibitors cause a drop of intrasynaptosomal K^+ (Blaustein and Goldring, 1975; Goddard and Robinson, 1976) which, in view of the inhibitory effect of high K^+ concentrations on GABA influx (Martin, 1976), and efflux (Levi *et al.*, 1978), may facilitate carrier-mediated efflux.

2. *Cationic fluxes characteristic of repolarization*

If the stoichiometry of GABA homoexchange is shifted in the direction of net outward transport by the cationic fluxes characteristic of depolarization, an opposite change should be observed when the cationic fluxes are reversed, as during repolarization. The lack of appropriate tools has limited our studies on this aspect of the problem. For example, the ionophore A23187 cannot be used to produce a pure downhill Ca^{2+} efflux, since it has been shown that, in the absence of extracellular Ca^{2+}, A23187 causes a substantial increase in the influx of Na^+ (Flatman and Lew, 1977), which, in our case, would counteract any effect of Ca^{2+} efflux on GABA transport. Therefore, we confined our study to the effect of a net Na^+ efflux on GABA homoexchange. Na^+ efflux was produced by first superfusing the synaptosomes in a standard medium, and then exposing them to a Na^+-poor medium (Li and White, 1977). Synaptosomes maintained throughout the experiment in a Na^+-poor medium served as controls. The influx of radioactive GABA as tested during the first five minutes after the medium change was identical in the two groups of synaptosomes, while the GABA-stimulated ^{3}H-GABA release was substantially lower in synaptosomes loosing Na^+ than in controls (Levi and Raiteri, 1978). Thus during Na^+ efflux, the stoichiometry of GABA homoexchange was shifted towards an efflux/influx ratio lower than unity, that is to a condition capable of producing net uptake.

IV. Behavior of other putative neurotransmitters

It seemed interesting to investigate whether the behavior exhibited by GABA was shared by other putative neurotransmitters. As discussed in detail elsewhere, homoexchange at the synaptosomal plasma membrane

does not seem to be of major importance in the case of catecholamines, at least in conditions in which the cytoplasmic content of the amines is not artificially increased (Levi and Raiteri, 1976; Raiteri and Levi, 1978). In keeping with this view, no "supra-additive" release of catecholamines could be evidenced in conditions in which a "supra-additive" release of GABA was present (Levi *et al.*, 1976c and unpublished observations). On the other hand, our still incomplete data on glutamate seem to support the possibility that other putative transmitter amino acids may behave similarly to GABA. Purified rat brain synaptosomes exhibit a concentration and Na^+ dependent homoexchange of glutamate (Levi *et al.*, 1976b) and exogenous aspartate causes ^{14}C-glutamate release from synaptosomes (Fig. 5). The latter observation is consistent with the finding that

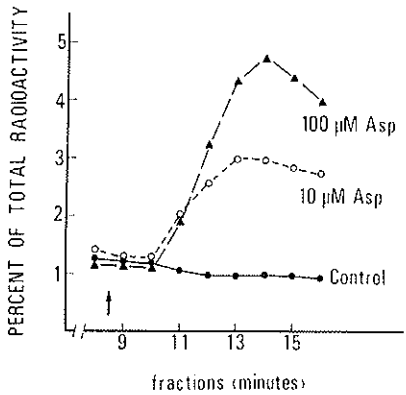

FIG. 5. Heteroexchange between exogenous aspartate and synaptosomal ^{14}C-glutamate. Purified synaptosomes were prelabelled for 5 min with 5 µM ^{14}C-L-glutamic acid and then superfused with standard medium for 8.5 min. The superfusion medium was then replaced with new medium containing 10 or 100 µM L-aspartic acid. For other details, see legend for Fig. 2.

glutamate and aspartate utilize the same transport system (Snyder *et al.*, 1973). Table 3 shows some preliminary data on the net release of glutamate in one of the conditions in which a "supra-additive" release of GABA had been observed. The behavior of glutamate appears to be similar to that of GABA, in that the release observed in the presence of aspartate plus the ionophore A23187 was greater than the sum of the release elicited by aspartate alone by heteroexchange plus that due to A23187 alone.

In conclusion, the evidence available so far suggests that glutamate fluxes at the level of central nerve endings are regulated by mechanisms similar to those controlling the fluxes of GABA.

TABLE 3. *Net release of glutamate from synaptosomes*

Glutamate releasing agent	nmol/mg protein released *		"supra-additive" release index **	
Aspartate, 10 μM	4.1	3.9		
A23187, 19 μM	0.13	0.05		
A23187, 19 μM + Aspartate, 10 μM	6.3	5.9	1.49	1.49

* Over control, during 5 min of stimulation.

** Ratios between release observed and release expected on a purely additive basis. Data from two separate experiments are presented.

Synaptosomes were prelabeled for 5 min with 10 μM ^{14}C-L-glutalate, superfused with standard medium for 10 min and then with a medium containing the compounds indicated in the first column. The concentration of glutamate was measured by ion exchange chromatography followed by reaction with o-phtalaldehyde, in pooled effluent fractions (min 12-16 of superfusion). Other experimental details as in the legend for Fig. 2.

V. CONCLUSION

We have presented the experimental evidence supporting the scheme of GABA presynaptic fluxes shown in Fig. 1 and briefly described in the introduction. It is reasonable to assume that the *capability of response* of nerve endings revealed by our *in vitro* studies is shared by GABA-ergic nerve terminals in the living brain. If the experimental conditions utilized in this study represent a good model of the events occurring in nerve endings *in situ* during physiological depolarization and repolarization, the GABA fluxes at the level of presynaptic nerve endings can be viewed as follows. The depolarization-induced release of GABA would have two components: a carrier independent, Ca^{2+}-dependent component (whose molecular mechanism is still unknown), and a carrier-mediated component, triggered by the very appearance of extracellular GABA in the synapse, through a homoexchange mechanism characterized by a stoichiometry in favor of net outward transport. In turn, net reuptake would become possible during the restoration phase following depolarization. In this phase, the inversion of the cationic fluxes would allow an inversion of the GABA flux ratio. In resting conditions, no net transport of GABA would be possible.

As previously mentioned, this mechanism may apply to other neurotransmitter amino acids, but not to biogenic amines. The differences

between the characteristics of biogenic amine transport and those of amino acids may be partly due to the fact that amino acids, but not biogenic amines, are largely "free" in the cytoplasm, and readily available to the plasma membrane for transport.

The existence of a release mechanism for amino acids which largely draws from the cytoplasmic pool, and is driven by the same cationic fluxes accompanying depolarization appears compatible with the intrasynaptosomal distribution of amino acids, and energetically economical.

From a pharmacological point of view, the data presented and the mechanism of GABA transport proposed underline the difficulty of developing drugs acting *specifically* as inbibitors of GABA reuptake. In fact if a compound inhibits uptake by competing witb GABA for the carrier, and is transported by the carrier, it will cause a release of GABA by heteroexchange. Such compound may be progressively accumulated in the nerve endings; and thus inhibit competitively also GABA carrier-mediated release. On the other hand, any blocker of the GABA carrier not transported by the carrier, will inhibit not only GABA uptake, but also the component of GABA release mediated by the membrane carrier, and triggered by the concomitant carrier-mediated entry of GABA into the nerve endings.

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DISCUSSION

FAMBROUGH

Following what you said about inhibitors of uptake, would you not expect blockers of uptake which are transported to give detectable post-synaptic responses? The existence of pure blockers together with continued transport would be evidence against GABA-mediated efflux, I think.

LEVI

Well, you can have two cases. One is that of a pure (not transported) blocker of transport, which will block the uptake, and by this mechanism increase the concentration of GABA in the synapse, and block the carrier-mediated component of release, and by this mechanism decrease the amount of GABA released. I do not know which comes first, nor which effect would be functionally predominant *in vivo*. The second case is that of a transported inhibitor of GABA uptake, which should release GABA by exchange and inhibit GABA reuptake. Now, this compound might be expected to have some GABA-mimicking action, at least initially, that is before being itself accumulated into the nerve endings in an amount sufficient to compete with GABA at the inner side of the membrane and thus limit carrier-mediated release. This aspect of the problem has to be studied in detail before drawing any conclusion.

ROBERTS

When we were first studying the GABA uptake system, our impression was that virtually every type of neural membrane preparation we made from mouse brain showed the binding typical of the uptake system. I got a feeling that this system was acting something like a biochemical vacuum cleaner. In other words, if GABA were liberated into a synaptic gap, it would be sucked out so rapidly that, under normal conditions of operation, membrane-effective concentrations would not remain for long periods. However, the finding you are describing might be related to a kind of super-additive release that one might see in a type of spreading depression. If there were a large GABA release, its extracellular presence might increase GABA release further and in this manner greatly amplify the post-synaptic effect and produce a prolonged depression of neural activity. I wonder whether the extension of

your analysis might be related more to spreading depression than to normal brain function. With regard to excitatory transmitters, such super-additive release might lead to over-stimulation and seizures rather than to normal excitatory transmission.

LEVI

Thank you for this comment. The phenomena described were observed *in vitro*, and we have suggested that they may exist also *in vivo*. Now, the difficult gap is: in what conditions do they exist *in vivo*? Dr. Robert's comment was very pertinent to this point and he made some good suggestions. As to the first part of the comment, I agree that neural tissue is able to suck GABA from the medium, particularly if the medium has a fairly high concentration of GABA, and particularly if one uses a fairly intact preparation of nervous tissue, like a classical thick slice. Now, in any preparation that is not as thick and as integral as a big slice, the "vacuum-cleaner" phenomenon is actually not seen as much—so that the slice is able to concentrate GABA much more than synaptosomes, or much more than the finely chopped slices of the type used by Iversen and then by many others after him. Now, the thick slice is a very complex piece of tissue, and you cannot attribute what you see to any structure in the slice. Synaptosomal preparations have, among others, the advantage that you can attribute uptake to a well defined structure.

ECCLES

You showed that in the absence of sodium in the medium there is no GABA release. Did you try graded concentrations of sodium, less than normal? and at what rate?

LEVI

There are two different aspects. The "spontaneous" release was unchanged at any concentration of sodium, from zero to normal. The GABA-stimulated GABA release showed a strict sodium-dependence, and increased as the external sodium concentration increased.

ECCLES

I think that is an important thing because Barker and Nicol were misled on pre-synaptic inhibition by using only zero sodium and normal sodium. When Nicol tried graded sodium, it was recognized that sodium was not the ion. You have to do the grading.

HAMPRECHT

When an action potential arrives at the nerve ending, the sodium concentration in the synaptic cleft should be lowered, but you need high sodium concentration for re-uptake. How do you reconcile these things?

LEVI

What I am stressing here is the importance of the fluxes of ions, rather than of the concentrations established either intracellularly or extracellularly. So, GABA reuptake would be possible in the presence of sodium efflux, and GABA release through the carrier-mediated process in the presence of sodium influx. Viewed in these terms, a decrease in the extracellular sodium concentration consequent to the previous influx of sodium may not be very important. It is difficult to determine how deep this fall in sodium concentration following the influx of sodium may be. If one considers a thin fluid layer close to the membrane, it may be quite significant, while if one considers the whole synaptic cleft, it may be almost negligible.

ECCLES

About sodium influx and the release of the transmitter at the neuromuscular junction, Katz and Miledi have shown that the impulse propagating to the terminal is not necessary for release. All that is necessary is to have calcium influx. They have done that of course by having electrotonic depolarization to the terminals in the TTX-treated preparations. So I am wondering how important the sodium influx is. With *in vivo* work all the emphasis is put on calcium.

LEVI

In the model I have shown, I would say that calcium is necessary for the first step of GABA release, which is not mediated by the membrane carrier. Calcium, by itself, is also able to modify the stoichiometry of the carrier-mediated GABA homoexchange in the direction of net outward transport, but I think that this effect may be obscured in the presence of a major influx of sodium. So, although calcium is able to do the whole thing, in the presence of a sodium flux which is probably many times larger than that of calcium the effect on transport that is seen is mainly due to sodium rather than to calcium; but calcium is still necessary for the first stimulus-secretion coupling.

CALISSANO

When you mentioned the effect of GABA on GABA release, I was thinking of the newly discovered property known as negative cooperativity of some ligand-receptor interaction whereby increasing the concentration of the ligand decreases its affinity for the receptor. I wonder if you can elaborate somewhat on these analogies.

LEVI

It is difficult to elaborate right away, but I think that saturable homoexchange is the easiest explanation. Trying to follow your suggestion, one may perhaps speculate that, by increasing GABA, one actually decreases the affinity of a pool of GABA bound intracellularly, and thus causes a release of GABA out of the nerve endings.

CALISSANO

I think it is a little more difficult to visualize it that way, because you need extracellular GABA not only to act immediately on something that is ready there to be triggered but to enter first and to act somewhere inside the cell or the nerve ending.

HÖKFELT

Did I understand you correctly that one difference between catecholamine and GABA neurons was that in the former we have storage vesicles which concentrate and store the transmitter? What is the present status of the vesicular storage of GABA—perhaps you or Dr. Roberts could comment on that.

ROBERTS

Because of the great solubility of GABA and the probable absence of high affinity intrasynaptic binding substances, fractionation procedures in aqueous media invariably lead to great leakage of GABA from subcellular fractions prepared in the usual way. The presence or absence of vesicular stores of GABA is difficult to demonstrate. If the kind of mechanism that I am envisioning should occur, then you would not expect much GABA to be *taken up* by the vesicles. Instead, they would be *loaded* by cooperative interaction of GAD and the vesicular membrane prior to the presynaptic release of their load via exocytosis, in the manner usually suggested. The failure to show vesicular pools of GABA has been a difficulty. GABA, with no net charge, certainly cannot be bound by intrasynaptic polyanions, such as ATP, in the manner known to take place for catecholamines.

THE ROLE OF PROSTAGLANDINS AND CYCLIC NUCLEOTIDES IN TREMORS AND CONVULSIONS

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Experiments in our and other laboratories have shown that exogenous prostaglandins (PGs) of E_1 and E_2 type are able to antagonize the convulsions induced by Pentamethylentetrazole (PMT) or by electroshock (ECS) (Horton and Main, 1967; Paoletti *et al.*, 1978). These data have been recently confirmed by Rosenkrantz (1978), who has also found that low concentrations of PGE_1 and PGE_2 administered intracerebroventricularly inhibit the tonic hindlimb extension and death induced by transcorneal maximal ECS. On the contrary, similar doses of $PGF_{2\alpha}$ increase tonic extension and mortality in mice (Rosenkrantz, 1978).

These results prompted us to investigate the possible formation and role of PGs in the brain after administration of convulsive or tremorogenic drugs, and how PGs may interfere with the biochemical and functional effects of such drugs.

Prostaglandins in brain

The first direct demonstration that PGs are present in CNS has been given by Samuelsson (1964), who found $PGF_{2\alpha}$ in ox brain. Later, various PGs of the E and F series have been found in brain of different species, with $PGF_{2\alpha}$ as the most common compound, followed by PGE_2. The quantitative values are vastly different, because they often simply reflect the endogenous biosynthesis occurring during animal sacrifice and tissue manipulation. In addition, until recently, the identification of PGs was not highly specific (Fumagalli *et al.*, 1977; Holmes and Horton,

1968 a; Ambache *et al.*, 1966; Holmes and Horton, 1968 h; Wolfe *et al.*, 1967).

In our laboratory, a specific analytical technique, coupled with particular precautions in animal sacrifice and tissue handling, has been developed. The artifacts due to post-mortem PGs formation from endogenous precursors have been minimized by using focused microwave radiations for animal sacrifice (Wolfe *et al.*, 1976 a; Nicosia and Galli, 1975). In addition, a sequence of steps has been developed for the purification of PGs and of a non cyclized metabolite of arachidonic acid (12-OH-arachidonic acid, HETE) which has been found to be present in mammalian hrain (Sautebin *et al.*, 1978) (Fig. 1). This procedure includes the addition to tissue homogenates of deuterated standards and a mass fragmentographic analysis of the prepurified compounds.

Wolfe and coworkers (1976 b) have recently found that slices and homogenates of guinea-pig brain cortex and rat cortical homogenates are ahle to form thromboxane, detected as its stable metabolite thromhoxane B_2, from endogenous precursors. In guinea-pig brain the production of TXB_2 is enhanced by norepinephrine and blocked by indomethacin. The catabolism of PGs in mammalian cortex is rather low (Wolfe *et al.*, 1976 a), but the presence of 15-DH-dehydrogenase and Δ^{13} reductase activity has been described in pig hrain (Änggard *et al.*, 1971), whereas PG-dehydrogenase have been shown by Siggins to be present almost exclusively in the cerebellar cortex of the rat, particularly in the Purkinje cell layer (Siggins *et al.*, 1971 a). These findings suggest that the ability to inactivate PGs may be present in some specific neuronal pathway, where PGs may play a role in the occurring hiochemical events.

Brain prostaglandins and cyclic nucleotides

A distinct influence of the PGs of the E series on CNS, and on the hypothalamic-pituitary axis, has been demonstrated *in vivo* and *in vitro*. A significant increase of total brain levels of cAMP in mice and rats, has been reported after intravenous injection of PGEs, but not after $PGF_{2\alpha}$. The elevation of these cyclic nucleotide levels was long lasting, and higher in the cerebral cortex and in the talamus, and lowest in cerehellum and brain stem (Wellmann and Schwabe, 1973). The increase of cAMP contents correlates with sedation. In *in vitro* experiments, PGEs but not PGFs, stimulate cAMP formation in rat brain (Berti *et al.*, 1972 a; Berti *et al.*, 1972 b), in murine neuroblastoma cells (Gilmans and Nirenberg, 1971) and in cultured cells of fetal rat brain (Gilman and Schrier,

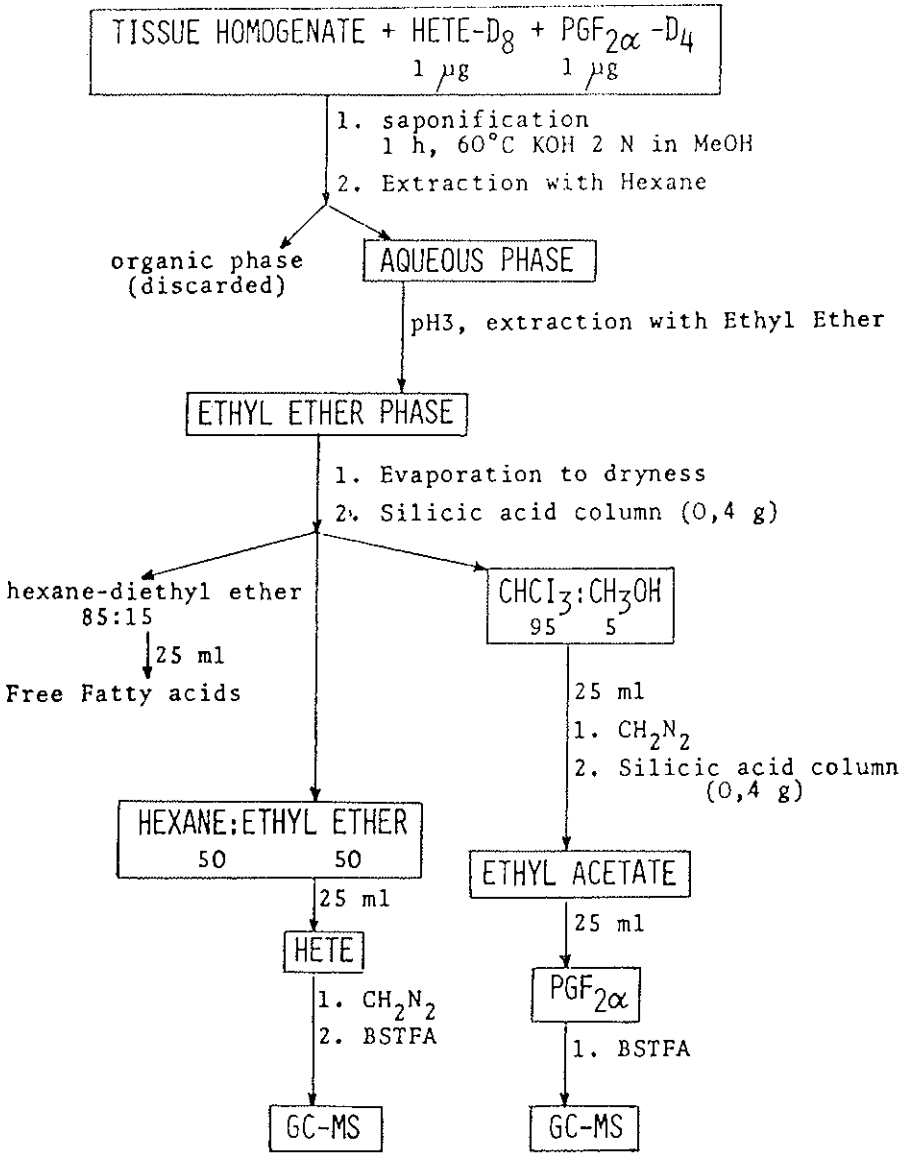
TISSUE HOMOGENATE + HETE-D8 + PGF2α -D4
1 μg 1 μg
1. saponification
1 h, 60°C KOH 2 N in MeOH
2. Extraction with Hexane
organic phase
(discarded)
AQUEOUS PHASE
pH3, extraction with Ethyl Ether
ETHYL ETHER PHASE
1. Evaporation to dryness
2. Silicic acid column (0,4 g)
hexane-diethyl ether
85:15
25 ml
Free Fatty acids
CHCl3:CH3OH
95 5
25 ml
1. CH2N2
2. Silicic acid column
(0,4 g)
HEXANE:ETHYL ETHER
50 50
25 ml
HETE
1. CH2N2
2. BSTFA
GC-MS
ETHYL ACETATE
25 ml
PGF2α
1. BSTFA
GC-MS

FIG. 1

1972). PGE_2 and norepinephrine are synergistic in stimulating cAMP formation in rat cortex (Berti *et al.*, 1972 b) and, at birth, cAMP formation is stimulated by PGE_2 but not by catecholamines (Fumagalli *et al.*, unpublished data), indicating that the two agonists act on different receptors. In addition, α and β blockers partially prevent cAMP elevation elicited by norepinephrine but not that by PGE_2 (Paoletti *et al.*, 1974).

Further interest in the relation between PGs and the cAMP system has been provided by Collier and Roy, reporting that opiates antagonize the production of cAMP stimulated by PGEs in rat brain homogenates, heroin being more and methadone less active than morphine (Collier and Roy, 1974). Naloxone counteracts morphine on this biochemical parameter (Traber *et al.*, 1975 a).

The stimulating action of PGs on cAMP formation in cultured cells is antagonized not only by morphine, but also by adrenergic and cholinergic agonists via the α adrenergic and muscarinic receptors (Traber *et al.*, 1975 b and 1975 c). All these antagonists of the PGE_1 - increased cAMP formation enhance the cellular levels of cGMP (Traber and Hamprecht, 1976).

Also enkephalins mimic morphine in increasing cGMP and in antagonizing the PGs evoked cAMP increase in cultured cells (Iversen and Dingledine, 1976). No data are available on the effects of PGs on cGMP metabolism in CNS; however, an *in vitro* increase of cerebellar cGMP is constantly present when tremorogenic agents are administered to the rat, and endogenous PGs are released (Berti *et al.*, 1976).

Exogenous PGE_2, at doses which prevent the increase of cerebellar cGMP, also antagonize the induction of convulsion. On the other hand, the existence of a strict correlation between the increasing cerebellar cGMP and the onset of convulsion, i.e. induced by PMT, has been demonstrated (Folco *et al.*, 1976). The variation of cGMP always precedes the onset of convulsions, whereas cAMP variations in brain invariably follow the appearance of convulsions, and they may only reflect the state of brain anoxia induced by seizures (Sattin, 1971).

Variations of endogenous prostaglandins

The regulatory role of PGs on the intracellular concentration of cyclic nucleotides in CNS is well established. PGs of the E type stimulate *in vitro* the accumulation of cAMP in culture neural tissue and in slices of cerebral cortex (Berti *et al.*, 1972 b; Gilman and Nirenberg, 1971).

On the contrary, the PGs of F type are completely ineffective in this respect, even if $PGF_{2\alpha}$ is the major PG present in the brain. In addition to this, neurophysiological studies indicated that the microiontophoretic administration of PGE_1 and PGE_2 consistently antagonizes the reduction in discharge rate of rat Purkinje cells caused by norepinephrine, but not by cAMP (Siggins *et al.*, 1971 a; Siggins *et al.*, 1971 b). These investigations, placing the actions of PGs at the level of cAMP formation, indicate a possible regulatory role for this compound in central transmission.

From the behavioural point of view, PGEs exert a sedative-tranquillizing action (Horton, 1964). PGE_1 also shows marked anticonvulsive properties. Animals are protected against seizures produced by PMT, strichnine, but not by picrotoxine, whereas PGFs are completely inactive (Horton, 1972).

A fully convulsant dose of PMT (100 mg/kg i.p.) has a profound effect on cortical PGs as well as on cyclic nucleotides. The $PGF_{2\alpha}$ levels are increased 10-12 times, and PGE_2 values are also increased, even if considerably less, with a maximal increase of four folds during the convulsive phase (Table 1). The animals, in this experiment, were killed

TABLE 1. *Effect of a convulsant dose of pentamethylenetetrazole (PMT) on rat cortical cAMP, cGMP, PGE_2, $PGF_{2\alpha}$*

Treatment	cAMP pmoles/mg prot. X±S. E.	cGMP pmoles/mg prot. X±S. E.	PGE_2 pgrams/mg prot. X±S. E.	$PGF_{2\alpha}$ pgrams/mg prot. X±S. E.
Controls	7.9±1.2 (8)	0.24±0.05 (8)	148±22 (12)	126±17 (13)
PMT 100 mg/kg i.p.	18.2±1.0* (8)	0.69±0.05* (8)	579±82** (4)	1425±200** (4)

Rats were sacrificed 90" after treatment.
* vs. controls $P < 0.01$.
** vs. controls $P < 0.001$.

90 seconds after treatment with PMT, a time at which seizures are fully present, and cortical cyclic nucleotides are also considerably increased.

When a subconvulsive dose of PMT is administered (50 mg/kg i.p.), only $PGF_{2\alpha}$ levels but not E_2 or cyclic nucleotides are increased in brain cortex (Table 2). The selective increase of $PGF_{2\alpha}$ in rat brain after the administration of convulsive agents may be a factor in the facilitation of the convulsive responses. When $PGF_{2\alpha}$ is injected intravenously in young

TABLE 2. *Effect of a subconvulsant dose of pentamethylenetetrazole (PMT) on rat cortical cAMP, cGMP, PGE_2, $PGF_{2\alpha}$*

Treatment	cAMP pmoles/mg prot. X±S.E.	cGMP pmoles/mg prot. X±S.E.	PGE_2 pgrams/mg prot. X±S.E.	$PGF_{2\alpha}$ pgrams/mg prot. X±S.E.
Controls	7.9±1.2 (8)	0.24±0.05 (8)	148±22 (12)	126±17 (13)
PMT 50 mg/kg i.p.	8.3±1.1. (8)	0.23±0.04 (8)	203±31 (15)	396±49 * (15)

Rats were sacrificed 90" after treatment.
In brackets the number of animals used.
* vs. controls $p < 0.01$.

chicks, which are devoid of blood-brain barrier, it causes in extreme extension of the limbs with dorsiflection of the neck, which is blocked only by deep anesthesia (Horton et al., 1967). In addition, $PGF_{2\alpha}$ injected intravenously in mice potentiates the effects of subthreshold convulsant doses of PMT.

INTERACTION BETWEEN PGE_2 AND CEREBELLAR cGMP

It is known that high concentrations of cGMP are present in the rat cerebellum, and that these levels can be increased by electroshock, PMT treatment, and even by harmaline (Mao *et al.*, 1975), an alcaloid of Peganum Harmala known to produce a high frequency tremor in mammals (Mato *et al.*, 1975) through a selective stimulation of the olivo-cerebellar system (Llinas and Volkind, 1973).

The administration of scalar doses of PMT induces an increase of cGMP which is already present with subconvulsive doses of the drug (50 mg/kg i.p.), and it is above the concentration of 35 pmoles/mg proteins when convulsive doses are used (Berti *et al.*, 1976). cAMP increases only after the administration of fully convulsive doses. The administration of PGE_2 (1 mg/kg) before the convulsive agent, completely prevents the increase of cGMP stimulated by PMT, without affecting the control levels (Table 3).

This is in striking contrast with the effects of chlordiazepoxide (CDP), which antagonizes the convulsions induced by PMT and reduces the levels of cGMP in cerebellum not only after stimulation, but also in control animals.

In conclusion, PGE_1 specifically and completely inhibits the response of cGMP to PMT, whereas CDP has a much less specific effect. PGE_2 appears as a good candidate as a physiological modulator of the neuronal pathways eliciting cGMP accumulation in Purkinje cells.

The administration of harmaline to normal rats, also induces increase

TABLE 3. *Cerebellar cyclic nucleotides: effect of CDP and PGE_2 on PMT-induced convulsions*

Treatment [a]	n	cGMP (pmoles/mg pr. ± S.E.)	cAMP (pmoles/mg pr. ± SE.)
Controls	16	8.6 ± 1.7	10.3 ± 1.2
PMT	16	57.3 ± 3.4[b]	27.9 ± 4.2[b]
CDP	8	2.8 ± 0.4[b]	8.1 ± 0.8
PGE_2	8	7.3 ± 1.1	10.1 ± 1.5
PMT + CDP	8	16.7 ± 1.8[b]	9.4 ± 0.9
PMT + PGE_2	8	9.7 ± 1.8	10.5 ± 2.0

[a] CDP, 15 mg/kg i.p. 60 min before PMT; PGE_2, 1 mg/kg i.p. 3.5 min. before PMT; PMT, 100 mg/kg i.p. 1.5 min before sacrifice.

[b] $p < 0.01$.

of cGMP at tremorogenic doses. The administration of PGE_2 fully prevents the tremors and tbe rise in cGMP (Table 4).

This experiment is of interest because the tremors induced by harmaline are related to a stimulation of the climbing fibers impinging into the Purkinje cells, where the cGMP accumulates. In fact, harmaline fails to induce tremors and the related increase of cerebellar cGMP in newborn rats, before the full formation of synapses between climbing fibers and Purkinje cells (Henderson and Wolley, 1970; Spano *et al.*, 1975), or in adult rats injected with 3-acetyl-pyridine, which causes a selective degeneration of the climbing fibers (Guidotti *et al.*, 1975). It may be therefore deduced that PGE_2 acts as at the synaptic link between climbing fibers and Purkinje cells. The putative stimulatory neurotransmitter involved at this level appears to be glutamic acid (Mao *et al.*, 1974; Young *et al.*, 1974) which increases, also *in vitro,* cGMP in slices of rat cerebellum (Ferrendelli *et al.*, 1974).

TABLE 4. A) *Inhibiting effect of PGE_2 on increased cerebellar cGMP induced by harmaline*

Treatment [a]	cGMP (pmoles/mg pr. ± SE.)
Conntrols	8.6 ± 1.0
PGE_2	6.4 ± 0.5
Harmaline	35.6 ± 2.6[b]
PGE_2 + Harmaline	9.6 ± 1.3

[a] PGE_2 1 mg/kg i.p. 5 min before sacrifice; harmaline HCl, 40 mg/kg i.p. 3 min before sacrifice.
[b] $p < 0.001$.

B) *Rat cerebellar cyclic nucleotides: effect of harmaline (20 mg/kg i.p.) and 16 (S)-16-methyl PGE_2 (200 microg/kg i.p.)*

Treatment	cGMP (pmoles/mg pr. ± SE.)
Controls	8.3 ± 0.3
16(S)-16-Me PGE_2	6.2 ± 0.9
Harmaline	22.2 ± 0.8 *
Harmaline + 16(S)-16-Me PGE_2	6.7 ± 0.6

— Harmaline was given 5' before sacrifice by microwave radiation (4").
— 16(S)-16-Methyl PGE_2 was given 7' before sacrifice by microwave radiation (4").
— Each value represents the mean of 8 experiments.
— Comparison with corresponding control value.
* $p < 0.05$.

The high doses of PGs requested to inhibit the effects of harmaline or PMT are explained by the rapid peripheral inactivation of PGE_2. When a more stable analogue (16S,16-methyl-PGE_2) is used, a complete protection is obtained with much lower doses (Berti *et al.*, 1978), and, as shown by Rosenkrantz, very low doses are sufficient when PGE_2 is injected intracerebrally (Rosenkrantz, 1978).

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DISCUSSION

FAMBROUGH

Could you remind us when the Purkinje cells become innervated and when significant output from cerebellum begins in the mouse?

PAOLETTI

I have no personal experience of that, but in discussion with our colleagues working in neurophysiology and particularly with Dr. Bloom, I had the information that the functional activity of the Purkinje cells can be recorded in rats after the age of 8 days.

ROBERTS

It seems that in order to have any changes in prostaglandins, neural activity must take place. Does one need excitation followed by cationic flux, an increased phospholipid turnover in membranes, and a consequent liberation of arachidonic acid, etc.? In other words, is there a whole cascade of events that follows the opening of cation channels?

PAOLETTI

I think the liberation of arachidonic acid is controlled mainly or uniquely in the brain by phospholipase A_2 or C. We do not know yet what are the physiological factors controlling these enzymes. Of course bradikinin is a good candidate; adrenaline is another candidate. And also adenosine in the cortex has been found to stimulate the phospholipase A_2. But you are certainly right: until there is a complete maturation of the Purkinje cell system, there is no increase of prostaglandins in the cerebellum. Mechanical manipulations of the brain, including use of microelectrodes, are likely to release prostaglandins in the brain. What is important here is what kind of prostaglandins are released and if these are likely to be different according to oxidation-reduction potential of the cells. So I think it is no wonder that some of the target cells may respond differently because of the different types of prostaglandins which are produced locally by the manipulations.

NIRENBERG

How are different drugs supposed to control prostaglandin formation?

PAOLETTI

This has been seen from pharmacological experiments that angiotensin administration stimulates quite heavily the formation of thromboxane in the brain.

RAITERI

Can you tell us something about the subcellular localization of prostaglandin synthesis? Is any storage of prostaglandin possible?

PAOLETTI

Prostaglandins are formed mainly in synaptosomes—this is the evidence from many laboratories. Secondly, the prostaglandins are not stored—not in the central nervous tissues nor in peripheral tissues. They act locally and then they are released and the prostaglandin metabolites are released with the effluent blood.

HAMPRECHT

In the slides prior to those dealing with the age of the animals, I saw that the base levels of cyclic GMP changed tremendously from one experiment to the other; sometimes they were below one picomole, sometimes they were 8 picomoles per milligram protein. Do you have any explanation for that?

PAOLETTI

It depends on the tissue. The level of CGMP in the cerebellum is between 10 and 15 picomoles and it is fairly constant. The level in the cortex is in the range of 1 to 2 picomoles. That is why the cerebellum is so much easier to be used to measure drug effects on CGMP concentrations.

TERENIUS

We know that the prostaglandins are part of a family of very unstable compounds and maybe there are still others to be detected than those we know at the present time. Now you emphasized the use of microwave irradiation as a tool for freezing out the system you believe is present in the normal animal. My question is simple. Do you really think that giving so much energy to the animal is going to preserve the natural situation?

PAOLETTI

At least the results are quite reproducible and the effect of previous treatment can be easily detected in that way. Of course I am not sure that the amounts of free fatty acid which were found in these animals are the same amounts which are present in the normal living rat, but they are so low that they are likely to be close to the physiological levels. Arachidonic acid is never in a free form in the brain; the only exception is when there is anoxia or a convulsive state.

TERENIUS

Could I make an additional comment? Have you done comparable studies with for instance freeze blowing techniques?

PAOLETTI

We did.

PURVES

What do you take to be the link between an increase of cyclic nucleotides and the generation of uncontrolled action potentials?

PAOLETTI

I have not done any neurophysiological work myself, but I may mention the time relation between increase of cyclic GMP and the onset of convulsions or tremors. When the concentration of about 50 picomoles is reached convulsions start almost instantaneously, just a matter of a few seconds.

PURVES

My question was rather how the level of cyclic nucleotides is related to the generation of action potentials.

PAOLETTI

We plan experiments in the area of neurophysiological experiments with Dr. Bloom because we think it is interesting to check this with these methods. But they have not been done so far.

NELSON

A comment in connection with Dr. Purves' question, there may be some actions of prostaglandins that are not directly dependent on cyclic nucleotide changes. In experiments with neuroblastoma X glioma hybrids synapsing with muscle cells, $PGF_{2\alpha}$ could be shown to cause release of acetylcholine. There was also a facilitation of action potential that elicited acetylcholine release. These effects were quite prompt and might or might not involve cyclic nucleotide changes.

PAOLETTI

We know that the increase of cyclic AMP affects in turn the activation of phospholipids in some systems. When convulsions or tremors are induced, an increase in cyclic AMP in different brain areas takes place, and in turn there is continuous activation of the release of arachidonic acid and the formation of cyclic nucleotides.

NEUROTRANSMITTERS, COTRANSMITTERS, NEUROMODULATORS AND TROPHIC FACTORS IN THE AUTONOMIC NERVOUS SYSTEM

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I. Introduction

Over the past 20 years, investigations on the autonomic nervous system have concentrated on the mechanisms of synthesis, storage, release, receptor activation and inactivation of the two classical autonomic neurotransmitters, acetylcholine (ACh) and noradrenaline (NA). More recently several new concepts have emerged. Firstly, new transmitters have been considered. While there were early hints of the presence of autonomic nerves other than those of the two classical components (see for example Langley, 1898; McSwiney and Robson, 1929; Ambache, 1951), clear evidence for the existence of non-adrenergic, non-cholinergic nerves supplying smooth muscle appeared in the early 1960's (Burnstock *et al.*, 1963; Martinson and Muren, 1963). The presence of these nerves is now established in a wide variety of visceral and vascular organs (see Burnstock, 1969; Campbell, 1970; Furness and Costa, 1973; Cook and Burnstock, 1976). Evidence will be presented that the non-adrenergic, non-cholinergic fibres to the smooth muscle of the gastrointestinal tract, lung, bladder and some blood vessels are purinergic (i.e. release ATP as the principal transmitter). The presence of polypeptides, 5-hydroxytryptamine (5-HT) and γ-aminobutyric acid (GABA), in enteric neurones will be described and the claims that these are principal transmitters will be assessed. Secondly, the possibility that some nerves store and release more than one transmitter will be considered, a concept that challenges Dale's Principle (Burnstock, 1976). Thirdly, the concept that transmitters or co-transmitters may act on presynaptic

receptors leading to modulation of the nerve-mediated release of transmitter will be described. Finally there will be a brief account of recent discoveries about the release of trophic factors from both nerves and effector tissues that are involved in their interaction during the development of autonomic neuroeffector junctions.

II. New neurotransmitters

A) *Evidence that non-adrenergic, non-cholinergic efferent inhibitory fibres supplying smooth muscle are purinergic*

In experiments carried out to identify the transmitter in non-adrenergic, non-cholinergic inhibitory nerves supplying the gastrointestinal smooth muscle and bladder, ATP has emerged as the most likely contender (see Burnstock, 1972, 1975 a, 1979). Other substances have been explored, including catecholamines, 5-HT, adenosine 3′,5′ monophosphate (cAMP), histamine, prostaglandins, various amino acids such as alanine, arginine, histidine, glycine, glutamic acid and GABA and the polypeptides, enkephalin, neurotensin, vasoactive intestinal peptide (VIP), somatostatin, bradykinin and Substance P. However, these substances have been rejected as contenders by most workers for various reasons; either they were inactive, they did not mimic the nerve-mediated responses, specific blocking drugs for these substances did not affect the nerve-mediated response, or their action was by stimulation of nerves and not by direct action on smooth muscle.

To establish a substance as a neurotransmitter at least five criteria need to be satisfied. The substance must be shown to (1) be synthesised and stored in nerve terminals; (2) be released during nerve stimulation; (3) produce postjunctional responses that mimic responses to nerve stimulation; (4) be inactivated by specific enzymes and/or an high affinity uptake system; (5) produce responses that are blocked or potentiated by drugs which similarly affect the responses to nerve stimulation. A schematic representation of synthesis, storage, release and inactivation of ATP at a purinergic neuromuscular junction is depicted in Fig. 1.

1. *Synthesis and Storage*

Both ATP and the enzyme systems that synthesize ATP occur ubiquitously in cells, so that it is not contentious that non-adrenergic, non-cholinergic nerves are able to produce and store ATP. Tritium-labelled adenosine was taken up by preparations of stomach and intestine and

rapidly converted mostly to ^{3}H-ATP. Most of the label was found to be stored as ^{3}H-ATP in nerves.

Electronmicroscopic studies of axon profiles in preparations innervated by non-adrenergic, non-cholinergic nerves led to the proposal that they were characterized by a predominance of "large opaque vesicles" (LOV). These vesicles differ from the small number of "large granular vesicles"

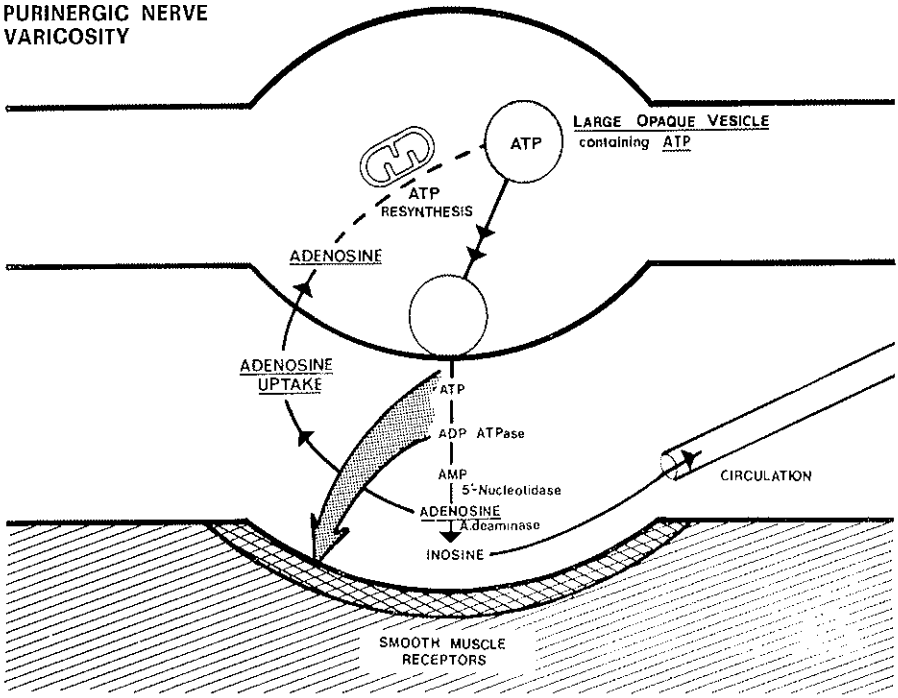

FIG. 1. A schematic representation of synthesis, storage, release and inactivation of ATP at a purinergic neuromuscular junction. From Burnstock G. (1972).

(LGV) found in both adrenergic and cholinergic nerves in that they are larger (80 to 200 nm cf. 60 to 120 nm in diameter), have a less prominent halo between the granular core and vesicle membrane, and are unaffected by 6-hydroxydopamine, which destroys adrenergic nerve terminals. Preliminary studies in our laboratory suggest that after exposure of intestine to low concentrations of ^{3}H-adenosine for short periods (45 secs) there is selective accumulation of silver grains over nerve profiles containing LOV. Indirect support for the view that ATP is contained in LOV is that the unicellular parasite *Trypanosoma cruzi,* responsible for Chagas' disease, is

unable to synthesize its own adenine and the LOV in nerve profiles are damaged in the intestine of infected patients.

A fluorescence histochemical method for localising quinacrine was introduced by Olson *et al.* (1976). Quinacrine has been shown to bind to ATP (Irvin J. L. and Irvin E. M., 1954) and it gives positive staining of adrenal medullary cells, megakaryocytes and blood platelets, known to contain high levels of ATP. Microsomal fractions obtained by differential and sucrose-density gradient centrifugation of homogenates of purinergically-innervated preparations (taenia coli and bladder) preloaded with ^{3}H-adenosine and ^{14}C-quinacrine showed peaks of ^{14}C which corresponded to peaks of ^{3}H-ATP (Cocks, Stitzel, Edgar and Burnstock, in preparation). Until more information is available about the specificity of quinacrine binding to ATP (for example, it is known to bind to regions of DNA rich in adenine and thymine), quinacrine staining alone cannot be used as a marker for purinergic nerves. However, it can be used to corroborate pharmacological evidence: for example quinacrine-positive nerve cell bodies and varicose fibres have been demonstrated in tissues where pharmacological evidence for purinergic transmission has been presented, but are absent from the iris, which contains abundant adrenergic and cholinergic, but no purinergic nerves.

2. *Release*

There was early evidence for release of ATP during stimulation of purinergic nerves. Thus, venous efflux of adenosine and inosine, breakdown products of adenine nucleotides, occurred from the stomach of both guinea-pigs and toads on stimulation of the vagus nerves, and it was shown that the release of nucleosides was due to stimulation of non-adrenergic inhibitory fibres in the vagus nerves rather than of cholinergic fibres. Also, radioactive compounds were released from guinea-pig taenia coli previously incubated in ^{3}H-adenosine upon stimulation of the intramural nerves; and this release was blocked by tetrodotoxin.

The possibility has been considered that the purine nucleotides or nucleosides released are not neurotransmitter substances but arise from other sources. The nerve membrane during propagation of an action potential is an unlikely source since the amount of nucleosides collected during stimulation of non-adrenergic inhibitory nerves was 1000-fold greater than that released as a direct result of the process of axon membrane activation during impulse propagation. The possibility that ATP is released as a result of antidromic stimulation of enteric sensory nerves

was ruled out when it was shown that the non-adrenergic inhibitory response to stimulation of the vagal nerves supplying the rabbit stomach was abolished after degeneration of the efferent, but not the afferent, component. The problem of whether the ATP released comes secondarily from muscle has been resolved recently in our laboratory: while there was a 2-6 fold increase in ATP release from the guinea-pig taenia coli or bladder during isometric responses to non-cholinergic, non-adrenergic nerve stimulation, there was no significant release of ATP during comparable responses elicited by direct muscle stimulation (Burnstock *et al.*, 1978 a, 1978 b). Release from muscle was also considered unlikely, since stimulation of portions of Auerbach's plexus from turkey gizzard (heavily innervated by non-adrenergic inhibitory nerves), dissected free of the underlying muscle, still resulted in efflux of purine nucleotides, in this case mostly AMP.

3. *Responses which mimic those to nerve stimulation*

The form and time course of the response to exogenously applied ATP closely mimics that to non-adrenergic inhibitory nerve stimulation (Fig. 2). Typically, the relaxations of the gut produced by ATP and nerve stimulation rapidly reach a maximum that declines quickly; this is in contrast to the relaxations to NA and sympathetic nerve stimulation, which reach a maximum more slowly and are maintained for a longer time. The possibility that ATP might be causing relaxation by initiating action potentials in non-adrenergic inhibitory nerves was negated by the finding that tetrodotoxin, which abolished the response to nerve stimulation, did not affect the relaxation produced by ATP.

The transmitter released by non-adrenergic inhibitory nerves produces inhibitory junction potentials (IJP's), and ATP also causes rapid hyperpolarisation of smooth muscle cells; both are due to a specific increase in K^+ conductivity. The finding that in the taenia coli both ATP release (Burnstock *et al.*, 1978 a) and amplitude of IJP's (Holman and Weinrich, 1975) are Ca^{++} dependent, provide further evidence that ATP is the transmitter producing inhibitory effects in the tissue.

The discovery that ATP is a potent inducer of prostaglandin synthesis (Needleman *et al.*, 1974) led Burnstock *et al.* (1975) to suggest that ATP released from purinergic nerves may be linked with prostaglandins in peristalsis. The authors showed that the prostaglandin synthesis inhibitor, indomethacin, blocked the "rebound contractions" that follow the inhibitory

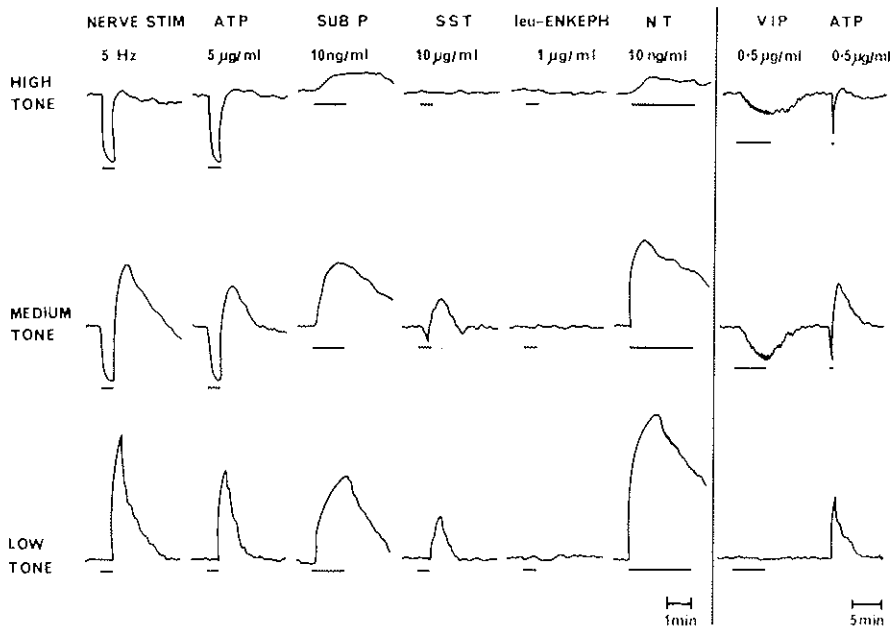

FIG. 2. Comparison of the responses of smooth muscle of the guinea-pig taenia coli to: intramural nerve stimulation [NERVE STIM: 0.2ms duration pulses delivered at 5Hz and supramaximal voltage (40 v)]; ATP (5µg/ml); Substance P (SUB P: 10ng/ml); somatostatin (SST: 10µg/ml); leu-enkephalin (leu-ENKEPH: 1µg/ml) and neurotensin (NT: 10ng/ml) in high, medium and low tone preparations. In another preparation, the inhibitory response to VIP (0.5µg/ml) is compared with that to ATP (0.5µg/ml). Atropine (1µg/ml) and guanethidine (1µg/ml) were present throughout. From Cocks T. and Burnstock G. (1979).

responses of the guinea-pig taenia coli to purinergic nerve stimulation and ATP.

Burnstock (1978a) has recently suggested a basis for distinguishing two types of purinergic receptor according to four criteria: relative potencies of agonists, competitive antagonists, changes in levels of cAMP and induction of prostaglandin synthesis. Thus P_1 purinoceptors are most sensitive to adenosine, are competitively blocked by methylxanthines and occupation leads to changes in cAMP accumulation; while P_2 purinoceptors are most sensitive to ATP, are blocked (although not competitively) by high concentrations of quinidine, 2-substituted imidazolines and 2'2-pyridylisatogen and by low concentrations of apamin (see below) and occupation leads to production of prostaglandin. Different locations for these receptors are apparent. For example, P_2 purinoceptors mediate the responses of smooth muscle to ATP released from purinergic nerves,

while P_1 purinoceptors mediate the presynaptic actions of adenosine (ATP is rapidly broken down to adenosine before it is effective) on adrenergic, cholinergic and purinergic nerve terminals (see Section on Neuromodulation).

4. *Inactivation*

The rapid recovery of smooth muscle after application of ATP or stimulation of non-adrenergic, non-cholinergic nerves and the absence of long-lasting action despite continued stimulation indicates an efficient inactivation mechanism. By analogy with other neuroeffector systems, if the non-adrenergic inhibitory nerves act on the gut by releasing ATP, the action of ATP would be terminated by uptake into nerves or smooth muscle and/or breakdown of ATP by enzymes into compounds with greatly reduced potency.

When ATP is added to a perfusion fluid recycled through the vasculature of the stomach, very little ATP is recovered, but the perfusate contains substantially increased amounts of adenosine and inosine as well as some ADP and AMP. While there is no direct evidence for the enzymic breakdown of ATP released from nerves, the gut is known to contain high levels of 5′-nucleotidase and adenosine deaminase. Mg^{++}-activated ATPase localisation has been described in micropinocytotic vesicles in smooth muscle membranes closely adjacent (20 nm) to non-adrenergic, non-cholinergic nerve profiles in the intestine.

An uptake mechanism for adenosine, but not nucleotides, has been demonstrated and this suggests that ATP released from nerves may be broken down to adenosine before uptake occurs, in a manner comparable to ACh which is broken down to choline. This view is supported by the finding that when the adenosine moiety of ATP is labelled with tritium and the phosphate moiety labelled with ^{32}P, the rate of uptake of ^{3}H into taenia coli is considerably greater than that of ^{32}P. Further support comes from studies of adenosine overflow; stimulation at low physiological frequencies (5Hz) shows little overflow, while stimulation at 30 Hz leads to substantial overflow, this difference disappears in the presence of low concentrations of the adenosine uptake inhibitor dipyridamole.

5. *Drugs*

Several groups of drugs block the responses to either adenine nucleotides or nucleosides in a wide variety of preparations. These include anti-malarial drugs such as mepacrine, quinine and quinidine, and methyl-

xanthines such as caffeine, theophylline and aminophylline. With the development of the P_1, P_2 purinoceptor hypothesis (see above) some of the apparently contradictory data have been clarified. It has been shown that the methylxanthines are competitive antagonists to adenosine and AMP, while several new agents which block responses of intestine to ATP have been found. These include the 2-substituted imidazoline compounds such as antazoline and phentolamine (Satchell *et al.*, 1973) and 2'-2-pyridylisatogen (Hooper *et al.*, 1974; Kazic and Milosavljevic, 1976). Unfortunately, these drugs are not specific for ATP. In a recent report, however, it has been claimed that very low concentrations (10^{-8}M) of apamin, a constituent of bee venom, selectively block both the inhibitory junction potentials in response to purinergic nerve stimulation in the intestine and the hyperpolarisations produced hy ATP (Vladimirova and Shuba, 1978).

B) *Other putative transmitters*

It seems unlikely that all non-adrenergic, non-cholinergic nerves in the autonomic nervous system correspond to those shown to have effects on muscle. Up to 9 morphologically distinguishable neurones in the enteric plexuses have been demonstrated (Cook and Burnstock, 1976), and some of these may represent sensory nerves, interneurones, or nerves supplying some blood vessels, endocrine cells or mucosal epithelial cells.

1. *Immunohistochemical localisation of polypeptides in autonomic nerves and their actions on smooth muscle*

Recent immunohistocbemical studies have demonstrated tbat some biologically active polypeptides are localized within enteric neurones of the gastrointestinal tract and urogenital system (see Schultzberg *et al.*, 1978); these include Substance P (Hökfelt *et al.*, 1977; Mroz and Leeman, 1977; Nilsson *et al.*, 1975; Pearse and Polak, 1975; Sundler *et al.*, 1977; Uddman *et al.*, 1978), somatostatin (Costa *et al.*, 1977; Hökfelt *et al.*, 1975 a; Hökfelt *et al.*, 1975 b), enkephalin (Elde *et al.*, 1976; Johansson *et al.*, 1978; Polak *et al.*, 1977) and VIP (Bryant *et al.*, 1976; Fuxe *et al.*, 1977; Larsson, 1977; Larsson *et al.*, 1976). Other vasoactive polypeptides such as neurotensin have also been found in the gut (Carraway and Leeman, 1976; Leeman *et al.*, 1977), although not localised histochemically within enteric neurones (Orci *et al.*, 1976).

On the basis of these studies it has been claimed that tbe non-adrenergic, non-cholinergic nerves supplying intestinal smooth muscle are

"peptidergic" rather than "purinergic" (Bloom and Polak, 1978; Humphrey and Fischer, 1978). However, while the responses of the intestine to ATP mimic closely the rapid responses to intramural nerve stimulation in all preparations whether the tone is low, medium or high, Substance P, somatostatin, enkephalin and VIP do not produce mimicking responses (see Fig. 2 and Cocks and Burnstock, 1979). Substance P always causes contraction, enkephalin and somatostatin are inactive, while VIP produces a very slow relaxation. It is unlikely that the contractile responses to the peptides tested are produced by excitatory prostaglandins, which mask any inhibitory effects, since no inhibitory responses to peptides were unmasked following inhibition of prostaglandin synthesis (see also Bury and Mashford, 1977).

Since Substance P, enkephalin, somatostatin and VIP are clearly not the transmitters released from the non-adrenergic, non-cholinergic inhibitory nerves supplying the intestinal musculature, other possible roles for these intraneuronal polypeptides should be considered.

There is strong evidence that Substance P is a neurotransmitter in primary afferent fibres in the spinal cord and skin (see Hökfelt *et al.*, 1977; Otsuka and Konishi, 1976; Lembeck, 1953) so that it is possible that it is also localised in sensory fibres in the gut (see also Hökfelt *et al.*, 1977). However, Suhstance P is known to have a variety of peripheral actions including contraction of intestinal longitudinal muscle (Franco and Costa, 1978; Pernow, 1960; Von Euler and Gaddum, 1931) and dilatation of blood vessels (Von Euler and Gaddum, 1931).

The endogenous opiate ligands, enkephalins, have potent inhibitory actions on electrically-stimulated contractions of both the mouse vas deferens and the guinea-pig ileum (Hughes *et al.*, 1975 a *et* b). Also, both enkephalins and morphine have been shown to inhibit neuronal firing in Auerbach's plexus (Dingledine and Goldstein, 1976; North and Williams, 1976) by causing membrane hyperpolarization (North and Tonini, 1976). Therefore, it seems likely that enkephalins are released from interneurones involved in modulation of nerve-mediated responses of the intestine. Morphine has a depressant effect on the inhibitory response to non-adrenergic intramural nerve stimulation in the guinea-pig taenia coli, while naloxone, a potent opiate antagonist (Klee, 1977), reduces this effect (Shimo and Ishii, 1978). It has also been suggested that neurones which display somatostatin immunoreactivity are interneurones within the enteric nervous system (Costa *et al.*, 1977). This is supported by the finding that somatostatin inhibits the release of ACh from nerves within Auerbach's plexus of the small intestine (Guillemin, 1976), inhibits gastric

motility in response to distension (Stadaas *et al.*, 1978) and that no somatostatin immunoreactive fibres were found in either the longitudinal or circular muscle of the ileum (Costa *et al.*, 1977).

Intrinsic VIP-containing neurones found throughout the gut may also be interneurones, since they have been shown to innervate nerve cells in both Meissner's and Auerbach's plexuses (Fuxe *et al.*, 1977). VIP-positive nerve fibres have been observed within the circular muscle layer and muscularis mucosa of the rat stomach, duodenum and colon, but these appear to be en route to the mucosal epithelium (Fuxe *et al.*, 1977); VIP is known to increase intestinal secretion (Gaginella and O'Dorisio, 1979). Release of VIP from gastrointestinal tract during stimulation by vagal nerves and ACh has recently been demonstrated (Fahrenkrug *et al.*, 1978; Edwards *et al.*, 1978). The role of the very slow inhibitory response of the taenia coli to VIP is not clear, since it does not mimic the nerve-mediated response. Similar slow relaxations to VIP have been reported in the gall bladder, trachea and stomach (Piper *et al.*, 1970) and canine small intestine (Kachelhoffer *et al.*, 1976) and oesophagus (Uddman *et al.*, 1978).

2. *Uptake of 5-hydroxytryptamine (5HT) and γ-aminobutyric acid (GABA) into enteric neurones*

There are several lines of evidence that suggest that neurones containing an indoleamine related to 5-HT may be present in the myenteric plexus, although some authors have pointed out the difficulty in demonstrating the presence of endogenous 5-HT in these neurones with conventional histofluorescence and have questioned whether they normally store and release aromatic amines (Ahlman and Enerbach, 1974; Dubois and Jacobowitz, 1974; Furness and Costa, 1978).

1) Certain enteric neurones take up 5-HT (Gershon and Altman, 1971; Robinson and Gershon, 1971). This uptake is unaffected by chemical sympathectomy (Gershon *et al.*, 1976) and precedes the development of the adrenergic innervation of the gut in ontogeny (Rothman *et al.*, 1976). Therefore the neurones responsible for 5-HT uptake are not adrenergic. In more recent experiments it was shown that after surgical or chemical sympathectomy, about 11% of the nerve cell bodies in the submucous plexus of the guinea-pig and 0.4% of those in the myenteric plexus showed fluorescence for aromatic L-amino acid decarboxylase after injection of L-dopa, dopamine or 6-hydroxytryptamine and the inhibition of monamine oxidase (Furness and Costa, 1978). Varicose intrinsic axons

which also take up amines are found amongst the cell bodies of both the submucous and myenteric plexuses; others appear to supply arterioles in the submucosa and the lamina propria of the mucosa.

2) Some intestinal neurones have been shown to synthesize 5-HT after incubation in L-tryptophan (Kuhar *et al.*, 1972). Further, intestine cultured for 3 weeks continues to convert L-(^{3}H) tryptophan to 5-(^{3}H) HT and contains neurones exhibiting fluorescence with the special characteristics of 5-HT, indicating that these neurones are intramural (Dreyfus *et al.*, 1977 a *et* b).

3) A 5-HT binding protein has been isolated from enteric neurones (Jonakait *et al.*, 1977), and more recently a specific antibody to tryptophan hydroxylase, the enzyme catalysing the first step in the synthesis of 5-HT from tryptophan, has been localised immunohistochemically in enteric neurones of mice, rats and guinea-pigs (Gershon *et al.*, 1977). These are intrinsic to the gut, since they survive for up to 3 weeks in organotypic tissue culture preparations.

It is not yet clear what role these neurones play in the gut. They may be responsible for the atropine-resistant excitatory responses of the gastrointestinal tract (Campbell, 1966; Ambache and Freeman, 1968; Furness, 1970; Costa and Furness, 1976; Takewaki *et al.*, 1977; Semba and Mizonish, 1978). They might be interneurones in view of the antagonistic actions of methysergide on postsynaptic potentials recorded in neurones of the submucous plexus of guinea-pig intestine (Hirst and McKirdy, 1975). The suggestion has also been made that dilatation of intestinal arterioles might be mediated by 5-HT released from nerves (Furness and Costa, 1978; Biber *et al.*, 1971; Biber, 1973). Finally, in view of the recent evidence that 5-HT is contained together with Substance P in certain neurones in the lower medulla oblongata of the rat (Hökfelt *et al.*, 1978a), it is possible that this might also occur in the gut, although the functional implications are not known.

GABA is a neurotransmitter in the peripheral system of certain invertebrates and is thought to be a major transmitter in the vertebrate central nervous system (Curtis and Johnston, 1974; Iversen, 1972; Roberts *et al.*, 1976). Recent experiments in our laboratory suggest that GABA may also be a neurotransmitter in the vertebrate peripheral autonomic nervous system (Jessen *et al.*, 1979). A small population of neurones in the myenteric plexus of the guinea-pig has been shown to possess high affinity uptake sites for GABA (Jessen *et al.*, 1979). These neurones are small with long processes that penetrate the muscle coat. When the

cerebellar cortex, superior cervical ganglion and the myenteric plexus were incubated in the presence of ^{3}H-glutamic acid, the amount of ^{3}H-GABA synthesized and accumulated by the plexus was closer to that of the cerebellar cortex, which contains GABAergic neurones than the superior cervical ganglion, where there is no evidence for GABAergic neurones. The myenteric plexus also produced homocarnosine, a dipeptide of unknown function found in brain. The known complexities of GABA metabolism and compartmentation are such that a cautious interpretation of these results is necessary, but these results do suggest that GABA or even homocarnosine may have a role in neuronal transmission in the gut.

III. Co-transmitters

The concept that each nerve cell makes and releases only one transmitter (widely known as Dale's Principle) has been re-examined in recent years (Burnstock, 1976, 1978 c; Schultzberg *et al.*, 1978; Burn and Rand, 1965; Brownstein *et al.*, 1974; Cottrell, 1976; Bunge *et al.*, 1978; Hökfelt *et al.*, 1978 b).

Two essentially opposing views have been put forward for the mechanism whereby new neurotransmitter systems evolve. One view is that neurones have a common phylo-histogenetic origin and that they differentiate into various chemical types in the course of evolution due to functional specialisation (Burnstock, 1969; Lentz, 1968; Burnstock, 1975 b). The opposing view is that there were multiple origins of nerve cells which meant wide early diversity of neurotransmitters and reduction of the number of transmitters during evolution as a result of selection (see Sakharov, 1974). Probably both mechanisms have operated during the course of evolution, but either way it is unlikely that a new nerve type with structurally distinct intra-axonal vesicles, new biochemical machinery for synthesis and breakdown of transmitter and specific postsynaptic receptors appeared suddenly during the course of evolution. It is more likely that there was a gradual evolutionary transition from one neurotransmitter to another. If this is true, then one might begin to look more carefully over a wide range of animals for nerves showing gradations of transmitter mixtures.

A) *Acetylcholine and noradrenaline*

There is compelling evidence that under certain conditions *in vitro* a single sympathetic neurone may at different times release NA, ACh, or a mixture of these two transmitter substances (O'Lague *et al.*, 1974; Purves

et al., 1974; Patterson and Chun, 1974; Burdon and Bunge, 1975; Patterson *et al.,* 1976; Johnson *et al.,* 1976; Hill *et al.,* 1976; Furshpan *et al.,* 1976; Reichardt and Patterson, 1977).

In a particularly elegant experiment Furshpan and his colleagues (1976) carried out electrophysiological studies on single isolated sympathetic neurones grown on previously dissociated heart cells from newborn rats (Fig. 3 a). They showed that some neurones inhibited, some excited, and others first inhibited and then excited the cardiac myocytes (Fig. 3 b). There is pharmacological and electronmicroscopical evidence for storage and release of ACh by the first group, catecholamines by the second, and both ACh and catecholamines by the third. It seems likely that this represents a true reflection of events that occur *in vivo,* during perinatal development (Hill and Hendry, 1977).

On the basis of these and other results it appears that a population of sympathetic cells which have the potential to synthesize both NA and ACh is present at birth. These multipotential cells require nerve growth factor (NGF) to survive and respond with an increased production of both choline acetyltransferase and tyrosine hydroxylase, the enzymes that synthesize ACh and NA. At this stage they have a viable amine uptake pump. Under the influence of conditioning factors, most of the cells appear to differentiate into either cholinergic or adrenergic neurones. The cells differentiating into cholinergic neurones gradually lose their ability to synthesize tyrosine hydroxylase, their ability to take up catecholamines is reduced and they become unresponsive to NGF; while those differentiating into adrenergic neurones lose their ability to synthesize choline acetyltransferase. However, it is possible that some sympathetic neurones supplying some organs in some animals retain the ability to produce and release both ACh and NA (Burnstock, 1978 c; Holmgren and Nilsson, 1976; Burn, 1977).

In the adult animal, there are both structural and pharmacological indications that the distinction between adrenergic and cholinergic neurones in some organs of some species is not as rigid as previously supposed. For example, some nerves characterised as adrenergic because of a predominance of small granular vesicles stain heavily for acetylcholinesterase and all ganglion cell bodies in the superior cervical ganglion contain this enzyme; studies of guanethidine sympathectomy indicate a weak amine uptake mechanism in adult sympathetic cholinergic neurones; cholinergic agonists and antagonists may mimic or reduce responses to "adrenergic" nerve stimulation; denervated nictitating membrane appears to be rein-

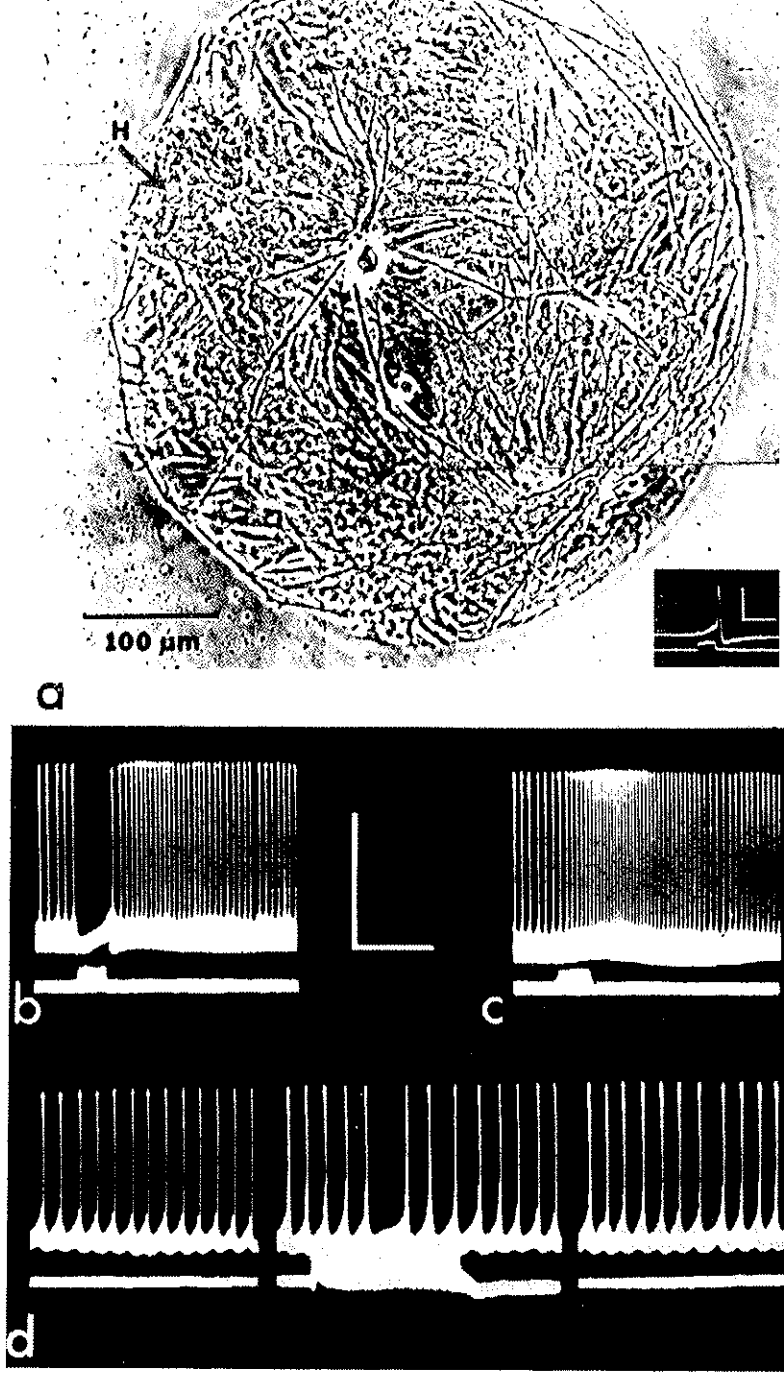

FIG. 3. (a) A microculture containing a solitary neurone from superior cervical ganglion of new-born rat in culture for 19 days, growing on previously dissociated new-born rat heart cells. Arrow at H indicates cluster of myocytes. Inset shows impulse in this neurone, scales are 50 mV, 20 msec for y and x axis respectively. (b) Train of neuronal impulses (deflection of lower trace) produced inhibition and then excitation of spontaneous myocyte activity (upper trace, scales 50 mV and 30 msec). (c) Inhibition was blocked by atropine (0.1 µM). (d) Excitation was blocked by propanalol [0.6 µM, atropine still

nervated by pre-ganglionic cholinergic fibres that then develop adrenergic features; cholinergic vasodilation of uterine arteries appears in late pregnancy, when adrenergic vasoconstriction is diminished; some sympathetic nerves in lower vertebrates appear to exhibit a combination of pharmacological and morphological characteristics of adrenergic and cholinergic nerves, both of which are abolished by 6-hydroxydopamine.

B) *ATP, noradrenaline and acetylcholine*

ATP, the transmitter proposed for purinergic nerves in the vertebrate gastrointestinal tract and probably other organs is released together with catecholamines from adrenal medullary vesicles in perfused adrenal glands (Douglas and Poisner, 1966; Douglas, 1968; Stevens *et al.*, 1972), and it seems likely that some ATP is released together with NA from adrenergic nerves (Geffen and Livett, 1971; Su *et al.*, 1971; Burnstock *et al.*, 1978 c). There are "receptors" for ATP as well as NA in smooth muscle cells (Drury and Szent-Gyorgyi, 1929; Burnstock *et al.*, 1970). Langer and Pinto (1976) have suggested that the substantial residual non-cholinergic, non-adrenergic response of the cat nictitating membrane following depletion of NA may be due to the release of ATP remaining in the adrenergic nerves; ATP behaves as an agonist on normal and reserpine-treated nictitating membrane. More recently Westfall, Stitzel and Rowe (1978) have claimed that ATP is released together with NA from adrenergic nerves in the vas deferens. Hoyes, Barber and Martin (1975) have suggested, on the basis of mixed vesicle populations in axon profiles in the bladder, that a second transmitter is released together with ACh during transmission. ATP is located with ACh in synaptic vesicles of cholinergic nerves supplying the electric organ of torpedine rays (Whittaker *et al.*, 1972; Bohan *et al.*, 1973; Zimmerman and Whittaker, 1974; Dowdall *et al.*, 1974; Israel *et al.*, 1975), and it has been shown that ATP is released together with ACh from the phrenic nerves in the rat diaphragm (Silinsky and Hubbard, 1973; Silinsky, 1975). Following injection into the striate cortex, ^{3}H-adenosine or its derivatives are taken up by certain cortical neurones, transported by fast axonal flow and released from the terminals into the synaptic cleft (Schubert and Kreutzberg, 1974). Whether the adenine compound is released in addition to the principal transmitter in these neurones as the authors imply, or whether it itself is the principal transmitter (Burnstock, 1972; Shimizu, 1973; Kuroda and McIlwain, 1974; Phillis *et al.*, 1975), has not yet been determined.

C. *Polypeptides and noradrenaline*

Somatostatin may be a co-transmitter with NA in the inferior mesenteric ganglion (Hökfelt *et al.*, 1977) and enkephalin is contained with NA in neurones of the superior cervical ganglion (Hökfelt, personal communication) and earlier Von Euler (1963) showed that splenic nerves contain vesicular Substance P.

IV. Presynaptic neuromodulation

Presynaptic neuromodulation, defined as the regulation of release of transmitter from nerves by the action of neurohumoral agents on prejunctional receptors (see Fig. 4), is a relatively recent concept of physiological importance (see Hedqvist and Fredholm, 1976; Langer *et al.*, 1975; Stjärne, 1975; Story *et al.*, 1975; Westfall, 1977).

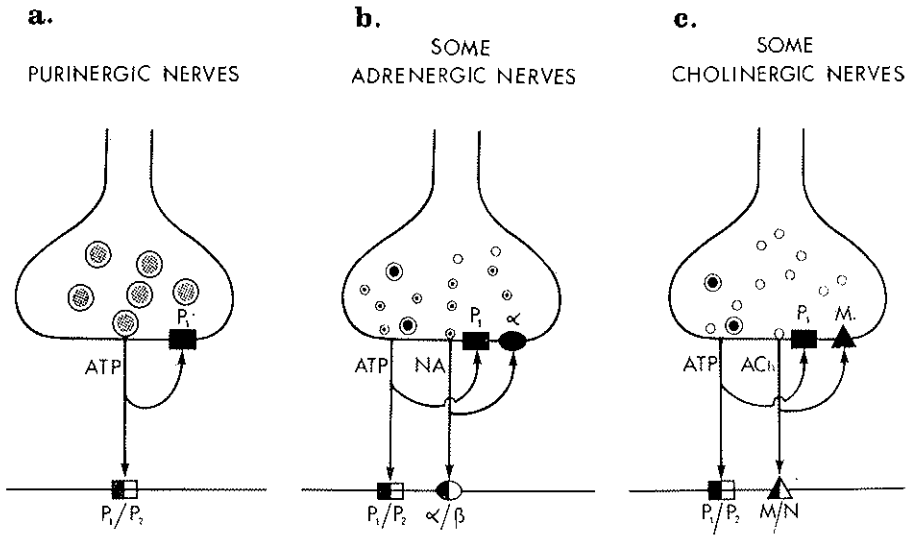

Fig. 4. Schematic representation of presynaptic neuromodulation of transmitter release. (a) Reduction of ATP release by activation of presynaptic P_1 purinoceptors. (b) Reduction of NA release by activation of presynaptic α-adrenoceptors and P_1 purinoceptors. (c) Reduction of ACh release by activation of presynaptic muscarinic and P_1 purinoceptors.

A. *Feedback modulation of noradrenaline release from adrenergic nerves*

Inhibition of transmitter output by the negative feedback of NA mediated through prejunctional α-adrenoceptors has been demonstrated (Fig. 4 b and Enero *et al.*, 1972; Häggendahl, 1970; Kirpekar *et al.*,

1973; Langer, 1974; Rand *et al.*, 1975; Starke, 1972). Positive feedback via presynaptic β-receptors on adrenergic nerve terminals has also been claimed (Story *et al.*, 1975).

B) *Modulation of adrenergic activity by acetylcholine, ATP and adenosine*

It has been known for some years that high concentrations of ACh have an inhibitory effect on responses to sympathetic nerve stimulation of rat mesenteric artery (Malik and Ling, 1969) and rabbit ear artery (Burn and Rand, 1960; Rand and Varma, 1970). This is due to release of NA produced by the action of ACh on prejunctional muscarinic receptors (Hedqvist and Fredholm, 1976; Story *et al.*, 1975; Löffelholz and Muscholl, 1969). Facilitatory effects of low concentrations of ACh on adrenergic transmission have also been described (Story *et al.*, 1975). The ultrastructural arrangement of perivascular nerve terminals is consistent with these interactions since there is frequently close apposition of adrenergic and cholinergic nerve varicosities, which are often enclosed within the same Schwann cell sheath (Graham *et al.*, 1968; Iwayama *et al.*, 1970; Nelson and Rennels, 1970; Nielson *et al.*, 1971; Edvinsson *et al.*, 1973; Burnstock and Costa, 1975). Pharmacological findings add further support (Story *et al.*, 1975; Löffelholz and Muscholl, 1969; Ehinger *et al.*, 1970; Vizi and Knoll, 1971). For example, evidence has been presented that ACh released by stimulation of intrinsic cholinergic nerves in the rabbit atria (Story *et al.*, 1975), and in a variety of blood vessels (Vanhoutte, 1974; Van Hee and Vanhoutte, 1976) can lead to decrease in release of NA during adrenergic transmission.

Adenine nucleotides and nucleosides have also been shown to inhibit NA release from adrenergic nerves in isolated canine subcutaneous adipose tissue, rat vas deferens, rabbit kidney, guinea pig vas deferens and probably terminal ileum and dog saphenous vein (Fig. 4 b and Kazic and Milosavljevic, 1976; Von Euler, 1963; Fredholm, 1974; Clanachan and Paton, 1977; Amsler and Pick, 1920; Verhaeghe *et al.*, 1977).

ATP induction of prostaglandin synthesis has been described (Needleman *et al.*, 1974), and the possibility has been raised earlier in this article that purinergic nerves are linked with prostaglandin in physiological regulatory mechanisms. Thus it is of some interest that prostaglandins of the E series can also act as neuromodulators, reducing release of NA from adrenergic nerves (Langer, 1974; Stjärne, 1972; Hedqvist, 1976; Sakato and Shimo, 1976).

C) *Modulation of cholinergic nerve activity by catecholamines, ATP and adenosine*

NA released from sympathetic nerve terminals reduces the release of ACh from cholinergic nerves in the gut, thereby inhibiting gastrointestinal motility (Vizi and Knoll, 1971; Paton and Vizi, 1969; Vizi, 1973).

There is considerable evidence for a prejunctional site of action for adenosine and ATP on cholinergic terminals (see Fig. 4 c). Adenosine reduces ACh release from phrenic nerves in the diaphragm (Ginsborg and Hirst, 1971), from frog motor nerves in sartorius muscle (Ribeiro and Walker, 1975) and from excitatory nerves in the intestine (Hayashi *et al.*, 1976; Vizi and Knoll, 1976). Further, theophylline, which blocks adenosine action, enhances the release of ACh from both intestine and cortical slices (Sawynok and Jhamandas, 1976; Phillis and Edstrom, 1976). It has been proposed that depression of spontaneous activity of neurones in cerebral and cerebellar cortices by ATP is largely presynaptic (Vizi and Knoll, 1976; Phillis and Edstrom, 1976). Vizi and Knoll (1976), have considered the possibilities that the source of adenine nucleotides involved in prejunctional inhibition of ACh release hoth in the intestine and cerebral cortex, is either purinergic nerves, or cholinergic nerves which have been claimed to release ATP together with ACh (Whittaker *et al.*, 1972; Silinsky and Huhbard, 1973).

V. Trophic factors

There is growing recognition that in addition to neurotransmitters, nerves release trophic factors that influence the development of muscle; and also that trophic factors released from effector cells influence the development of nerves. While most studies of this kind have employed motor nerve-skeletal muscle models (Gutmann, 1976; Vrbova *et al.*, 1978), work on the autonomic neuroeffector system has also been carried out (Burnstock and Costa, 1975; Burnstock, 1978 b).

A) *Influence of neuronal trophic factors on the development of smooth muscle*

The behaviour of single smooth muscle cells in culture is shown in Figure 5. *Undifferentiated* smooth muscle cells (e.g. from 10-day embryo chicken gizzard) divide and proliferate in culture until a confluent monolayer is formed within 36 hours (Campbell *et al.*, 1974). In contrast, *differentiated* smooth muscle cells from more mature animals (e.g. from

newborn guinea-pig vas deferens, taenia coli and ureter) dedifferentiate before proliferation takes place (Fig. 5 and Campbell *et al.*, 1974). As soon as a confluent monolayer is formed redifferentiation occurs, as indicated by appearance of myosin immunofluorescence and thick filaments (Gröschel-Stewart *et al.*, 1975). The cells then

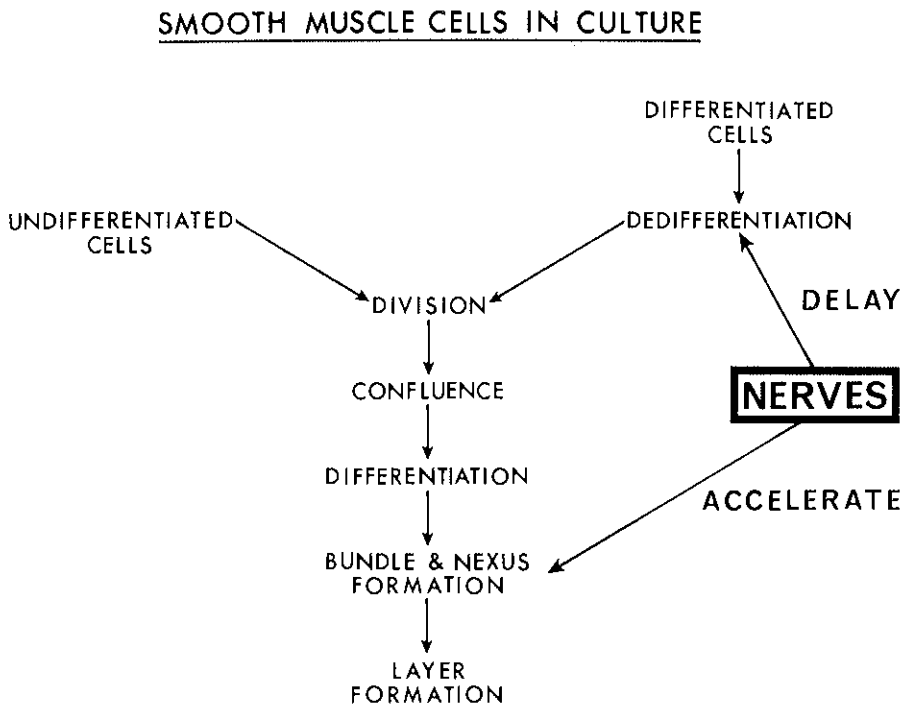

FIG. 5. Diagrammatic representation of the behaviour of enzymatically dispersed undifferentiated smooth muscle cells (e.g. from 10 day embryo chicken gizzard) and differentiated smooth muscle cells (e.g. newborn guinea-pig vas deferens). From Burnstock G., 1978 b.

aggregate into either clumps or chains. Spontaneous contractions develop which become synchronous as gap junctions (nexuses) form low resistance pathways between neighbouring cells. This sequence of changes in smooth muscle cells in culture is similar to that described during normal development *in vivo* (Yamaucbi and Burnstock, 1969) and in anterior eye chamber transplants of smooth muscle (Campbell *et al.*, 1971).

The presence of sympathetic nerves (but not necessarily long-lasting associations) delays this process by 2 to 7 days. This effect is not mimicked by NA, ACh or spinal cord extract, but is mimicked by sym-

pathetic chain extract and dibutyryl cyclic AMP (together with theophylline to prevent cyclic AMP breakdown) (Chamley and Campbell, 1975). These results could therefore be explained by the release of a trophic substance from sympathetic nerves which acts on adenylate cyclase receptors in the smooth muscle cells, resulting in the production of cAMP, which promotes differentiation, delaying dedifferentiation and proliferation. A comparable result has been ohtained with cultured vascular smooth muscle cells (Stout *et al.*, 1975; Chamley *et al.*, 1977). However, Bevan (1975) has shown that sympathetic denervation of the ear artery in the young rabbit results in a reduction in the number of dividing cells compared with the control side, suggesting that the presence of nerves enhances proliferation. Further factors may therefore be involved in the regulation of smooth muscle proliferation *in vivo*.

Muscle effector bundle and nexus formation occur in culture in the absence of sympathetic nerves, but are accelerated by their presence (Chamley *et al.*, 1974). In small clumps of muscle supplied by nerves, foci of synchronous contraction appear much earlier (3 to 5 days) than in similar clumps without nerves (8-12 days). An increase of approximately 50% in gap junctions was seen in the muscle clumps associated with nerves. The mechanism by which nerves influence muscle development in this way is not known, but cAMP has been shown to increase the formation of gap junctions in culture preparations (Sheridan, personal communication). Muscle effector bundle formation in anterior eye chamber transplants occurs at about the same time that varicose adrenergic nerves penetrate into the muscle layer (Malmfors *et al.*, 1971; Burnstock *et al.*, 1971), suggesting that in this situation too, nerves might influence muscle differentiation and aggregation.

B) *Influence of effector cell trophic factors on development of autonomic nerves*

The first report of the influence of explants of autonomic effector organs on the growth of nerves from sympathetic ganglia *in vitro* was by Levi-Montalcini *et al.* (1954). These authors showed that during the first 16 hours in culture, the number of nerve fibres originating from the sympathetic ganglion (but not the spinal cord) was consistently higher on the side facing either mouse heart or sarcoma explants. Since then a number of similar experiments have been carried out (see Fig. 6 and Chamley *et al.*, 1974; Bueker *et al.*, 1960; Silberstein *et al.*, 1971; Charlwood *et al.*, 1972; Johnson *et al.*, 1972; Chamley *et al.*, 1973 a; Chamley and Dowel, 1975; Ebendal and Jacobson, 1977).

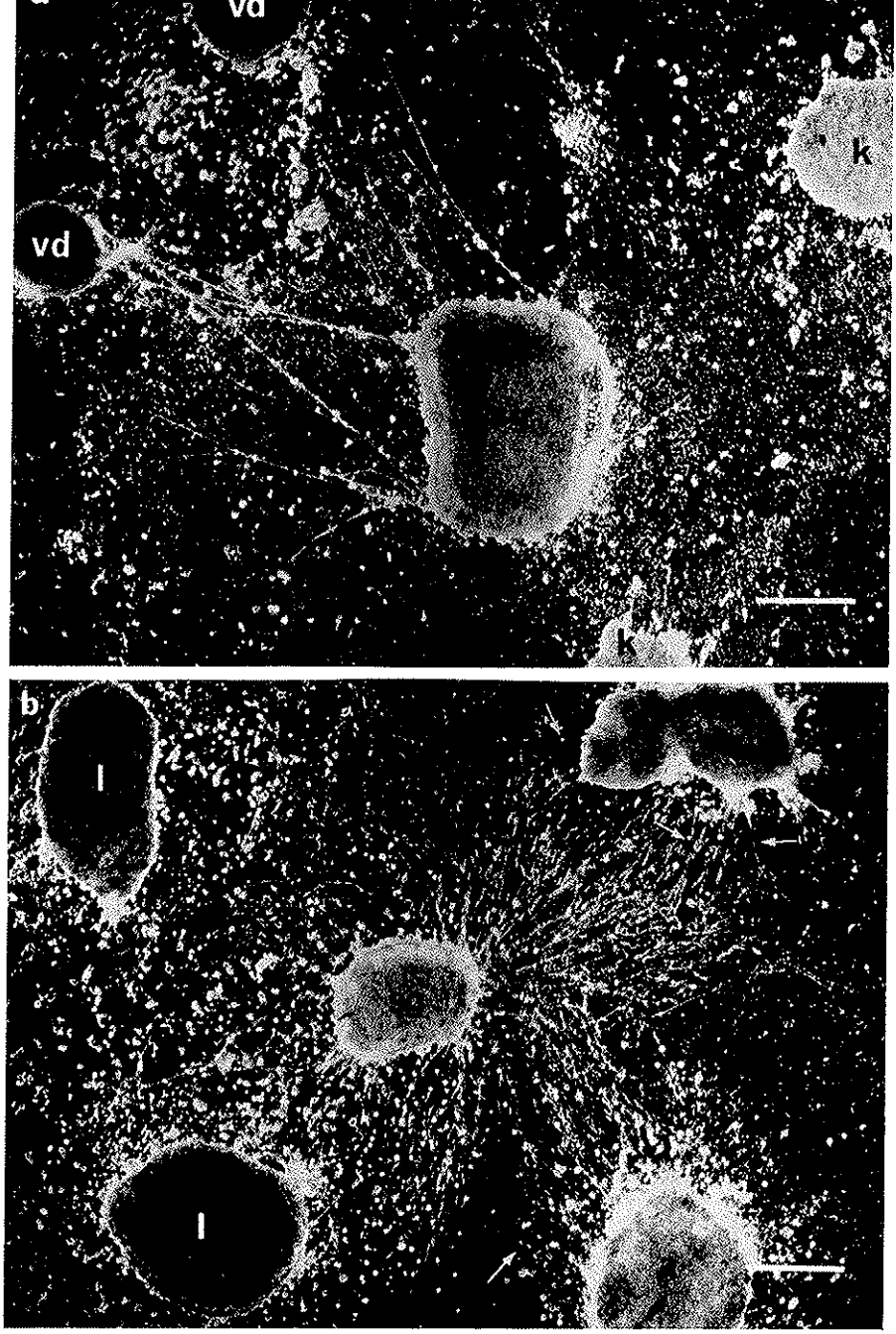

FIG. 6. (a) Nerve fiber growth from a sympathetic ganglion explant (sg) to explants of vas deferens (vd) and kidney (k). 5-Day-old rat, 1 unit NGF/ml. 5 days *in vitro*. At all stages of growth, nerve fibers grow to vas deferens explants in marked preference to kidney explants. Scale 400 μ (x 28). (b) Nerve fiber growth from a sympathetic ganglion explant (sg) to explants of lung (l) and atrium (a). 5-Day-old rat, 5 days *in vitro*. At all times a greater number of nerve fibers grew on the side of the sympathetic ganglion explant nearer the atrium than lung explants. Scale 400 μ (x 28). From Chamley J., Goller I. and Burnstock G., 1973.

"Attraction" of sympathetic nerves is evident soon after the nerve fibres emerge from ganglion explants and it was therefore suggested that it might involve a chemical substance released from the smooth muscle explants (Chamley *et al.,* 1973 b). There are indications from a number of different sources that this substance is NGF: a) there is increased growth of sympathetic or sensory nerves in the presence of NGF-producing mouse sarcomas (Levi-Montalcini *et al.,* 1954; Bueker, 1948); b) sensory nerves grow towards the tip of capillary tubes containing a solution of NGF, but not to tubes containing saline (Charlwood *et al.,* 1972); c) there are higher levels of NGF in densely innervated tissues than in sparsely-innervated tissues (Levi Montalcini and Angeletti, 1961; Johnson *et al.,* 1971), and densely innervated tissues stimulate greater nerve growth (Chamley *et al.,* 1973 b).

Whether or not a chemical factor is produced, there is still much debate about the mechanism of "attraction". Our own experiments suggest that production of growth factor in the effector organ explant leads to preferential growth on tbe side of the nerve explant facing the effector: and that once contact is established by a few nerve fibres there is a "reinforcement" phase, in which other fibres follow the path established by the initial fibres.

Development and growth of the sympathetic nervous system *in vivo* also appears to be under the influence of NGF (Levi Montalcini and Angeletti, 1968; Zaimis and Knight, 1972; Hendry, 1976; Coughlin *et al.,* 1977). NGF is present in sympathetically innervated tissues during early embryogenesis and is taken up by adrenergic nerve terminals and transported by retrograde axonal flow to the cell body, where it exerts its major actions (Hendry *et al.,* 1974). It increases the size of immature neurones, the survival of differentiated neurones, the rate of growth of adrenergic axons and the content of NA by induction of the biosynthetic enzymes tyrosine hydroxylase and dopamine-β-hydroxylase (Levi Montalcini and Booker, 1960; Thoenen *et al.,* 1971; Hendry, 1977). NGF appears to affect cholinergic as well as adrenergic synthetic enzymes in immature sympathetic neurones, since high doses of NGF in 2-day old rats result in increases in endogenous choline acetyltransferase as well as tyrosine hydroxylase activity (Hill and Hendry, 1977).

At an early stage of postnatal life, most sympatbetic neurones receive functional preganglionic innervation. About the same time there is a rapid rise in tyrosine hydroxylase and dopamine-β-hydroxylase activity. This rise is prevented by preganglionic section or ganglionic blockade, indicating that maturation of the adrenergic neurone depends in part on

transynaptic influences resulting from preganglionic nerve activity (Black, 1973). The presence of pre-synaptic fibres is also required for normal increase in monoamine oxidase in immature sympathetic neurones, while preganglionic activity is without effect on this enzyme in the adult (Black *et al.*, 1972). In addition it has been shown that retrograde regulation by target organs also plays an important role. For example, unilateral removal of the salivary glands and iris prevented the normal developmental increase in tyrosine hydroxylase activity in the ipsilateral superior cervical ganglion, a depression which persisted for at least 6 months (Hendry and Campbell, 1976; Dibner *et al.*, 1977). Failure in development may be due to the absence of NGF, since its addition reverses the effect and leads to an increase in tyrosine hydroxylase activity (Hendry, 1976; Hendry and Campbell, 1976; Hendry and Iverson, 1973; Paravicini *et al.*, 1975).

VI. Summary

Four new concepts about the organisation of the autonomic nervous system have emerged in the past few years.

1) The existence of autonomic nerves which are neither of the two classical types, adrenergic nor cholinergic has been established. There is evidence that the non-adrenergic, non-cholinergic inhibitory fibres that form junctions with smooth muscle are purinergic, i.e., release a purine nucleotide or related substance as the principle transmitter; and that neurones shown with immunohistochemical methods to contain the polypeptides Substance P, somatostatin, vasoactive intestinal peptide or enkephalin may be interneurones, sensory neurones, or may supply blood vessels, mucosal epithelial cells and possibly endocrine cells. There is also evidence for uptake and synthesis of 5-hydroxytryptamine and γ-aminobutyric acid in some enteric neurones.

2) It seems possible that some autonomic nerves store and release more than one transmitter; for example, noradrenaline has been claimed to be associated with acetylcholine or somatostatin or ATP.

3) Neuromodulation of the release of the principal transmitter via presynaptic adrenergic, cholinergic or purinergic receptors has been described.

4) Trophic factors involved in the mechanisms of long-term interaction between autonomic nerves and effector cells during development have been discussed.

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DISCUSSION

LEVI-MONTALCINI

Could you tell us something about the morphology and topography of purinergic nerve cells?

BURNSTOCK

Quite a lot is known now; wherever they have been found, they are intramural. For example, in the gut, bladder, lung and probably in the heart, and even in the portal vein. This explains why in 1967, Hughes and Vane found that nicotine and other ganglion stimulants produced non-adrenergic, non-cholinergic inhibitory responses in isolated preparations of portal vein and that hexamethonium blocked these responses. Intramural purinergic neurones seem to be part of the parasympathetic pathway; they are controlled by preganglionic cholinergic fibres in the parasympathetic nerves.

ECCLES

Just a word about Dale's principle—and I coined the word "transmitter"—because we realized, say, that there were nerve fibers that mostly were using noradrenaline—we could use adrenaline as well—and so on, so maybe it only says that any nerve cell at all of its terminals produces the same transmitters, and so your new findings may fall within that scope because it just means you have got purinergic as well as adrenergic fibers.

BURNSTOCK

Yes. It is just that most people have come to regard it as "one nerve - one transmitter". I know you defined it more carefully when you credited this to Dale—in fact he was supposed to be a bit annoyed with you for calling it "Dale's Principle".

ECCLES

Embarrassed, let us put it that way—he was a very good friend and I talked to him about it and he used to kind of look abashed ... I think it was like that.

BURNSTOCK

The thing is that however careful one is, it still is taken by most people to mean "one nerve - one transmitter", even though this might be a distortion of the original definition.

ECCLES

It would be one cell, not one nerve fiber. The nerve cell may be able to make more than one transmitter, but the nerve cell cannot despatch one transmitter down some and another transmitter down other branches. That is, I suppose, the basic idea.

CALISSANO

In the experiments of induction of neurite retraction, did you check at the electron microscope what are the intracellular structures first affected by this drug?

BURNSTOCK

Yes, we did ask this question, but we never really solved the mechanism. We did do electronmicroscopy and found that there were curious accumulations of dark lysosome-like bodies and that there was damage to those terminals, but we did not learn from this about the real mechanism. However, we do know, as has been mentioned already, that swollen mitochondria in the cell bodies are the first morphological sign of damage (there are no damaged mitochondria in the terminals). My guess is that this is because there are lots of vesicles in the terminals and that the guanethidine gets into the vesicles, so that the cytoplasmic concentration is relatively low compared to that in the cell body where there are not so many vesicles. Exactly why axons retract, I still do not know but I suspect it is part of the system we heard about in your talk.

CALISSANO

Also, did you try to counteract this effect by increasing the concentration of NGF?

BURNSTOCK

Well, this has been shown but we have not repeated it or followed it up.

LEVI

Do you have any suggestion why nature should use a high energy compound as a transmitter and is there any evidence that other high energy compounds may be utilized as transmitters in other systems?

BURNSTOCK

That is a good question. I know it bothers people; in fact I must say that many people have intuitive reservations about this hypothesis, I think largely because we have all been taught the well established role of ATP as an energy source inside cells, so that it is unsettling to think of ATP having an extracellular role too. However, biochemists tend to be more sympathetic when you say to them, "Well, why have you got these highly potent ecto-enzymes for breakdown of ATP?". Regarding your comment about ATP being a high energy compound, is energy really wasted in a significant way by extracellular breakdown of this relatively small amount of nerve-released ATP compared to the substantial levels of ATP being metabolised intracellularly and how does the energy released compare with breakdown of other transmitters? We do not know enough about this yet.

ROBERTS

Do you think it is possible that it splits when it hits the receptor?

BURNSTOCK

We are not sure exactly where it splits; it looks as though the ATPases are associated with the nerve side and the 5′-nucleotidase with the muscle side. It breaks down to adenosine but it is hard to know where the first split is. We really need to do quite a lot more work on breakdown. If you are also asking, could the energy released from ATP breakdown be utilized for some pre- or post-synaptic process, I do not know.

HAMPRECHT

I wonder about the specificity of the quinacrine stain. What is the explanation, in your mind?

BURNSTOCK

It is a question of concentration. Under the conditions used in our experiments, quinacrine appears to be selective for sites with very high levels of

ATP, such as platelets, adrenal medullary cells or purinergic nerves since other cells including adrenergic nerves (which also contain ATP) do not stain. However, when you look at sympathetic neurones in culture, there is something about these conditions that leads to positive staining of most cells. So positive staining appears to depend on the conditions of the reaction and the concentrations of ATP present under given circumstances. What we are doing now is labelling the quinacrine with ^{14}C for autoradiography and also binding the quinacrine to a heavy metal like platinum, which should then make it possible to localise quinacrine at the electronmicroscopic level. My colleagues Bob Stitzel and Tom Cocks have recently spun down homogenates of purinergically-innervated preparations which had been pre-incubated in both ^{3}H-adenosine and ^{14}C-quinacrine. They isolated a heavy fraction in this way which was higher than the large granular vesicle fraction from adrenergic nerves, persisted after sympathectomy, and contained the peaks for both ^{14}C and tritium. Finally, there is a very good correlation for the presence of quinacrine-positive nerves in all organs where there is physiological and pharmacological evidence for the presence of purinergic nerves.

HAMPRECHT

Do you find a defined stoichiometry between quinacrine and ATP?

BURNSTOCK

We have not done this.

KOSTERLITZ

I have been interested in the electromicroscopic appearance of the nerve terminals in the myenteric plexus of the guinea-pig ileum. There are small vesicles and large granules and it is possible that the latter may contain enkephalins although there is so far no evidence either for or against this view.

BURNSTOCK

Great.

FILOGAMO

According to you, undifferentiated cells and dedifferentiated cells are the same?

BURNSTOCK

No, they are not strictly all the same; that is, undifferentiated cells could be cells early in embryological development or cells that have dedifferentiated from mature cells. However, either way, they are capable of division. That was the sense in which I used the terms.

PURVES

With respect to co-transmission, do you think that there is a good reason for co-transmission, or do you tend to regard this as noise in the system?

BURNSTOCK

No. You see, I come from an evolutionary background, so to speak, and it seems to me that we are often inclined to oversimplify when examining complicated systems. I do not believe that evolution works like that; many ways are introduced, some are eliminated by natural selection. Now, every cell contains all the genetic machinery for every cell type, and there is no doubt now that developing sympathetic nerves have the machinery, and indeed produce, the synthesizing enzymes for both acetylcholine and noradrenaline. It may well be that most of these nerves are programmed to become either adrenergic or cholinergic in the adult; but there is evidence to suggest that it may have been a selective advantage to retain both transmitters in some sympathetic nerves. So I do not think it is just an odd anomaly or "noise" as you put it—the co-existence of transmitters may easily turn out to be very important in some systems, in some species.

PURVES

Do you have any cues as to what the advantage might be?

BURNSTOCK

Well one way of getting differential responses from one nerve fibre is to use one transmitter but two kinds of postjunctional receptors. Another way would be to employ two (or maybe more) different transmitters.

ROBERTS

Is it possible that noradrenaline might be an amplifier of acetylcholine so that with increasing intensity of stimulation there would be an increased release of noradrenaline which would increasingly amplify the postsynaptic effects of a given number of quanta of acetylcholine?

HISTOCHEMICAL CHARACTERIZATION OF NEURON POPULATIONS: IMMUNOCYTOCHEMICAL STUDIES ON TRANSMITTER RELATED SUBSTANCES

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INTRODUCTION

The possibilities to entangle the complex built up of the nervous system have markedly increased during the last decades. The classical neuroanatomical techniques (Nauta and Gygax) have been supplemented by a number of ingenious techniques utilizing certain properties of the living neuron, such as the uptake mechanisms located at the cell membrane and intraaxonal transport. These techniques include the anterograde tracing of fibre pathways after administration of certain amino acids and their uptake into cell bodies as well as the retrograde (and anterograde) tracing technique using peroxidase as a marker (see Cowan and Cuenod, 1975). Other attempts to characterize neuronal populations are based on histochemical approaches. Almost 30 years ago Koelle and Friedenwald (1949) developed a technique for visualizing acetylcholinesterase, the enzyme inactivating acetylcholine. In the beginning of the 60'ies Falck, Hillarp and coworkers introduced a fluorescence technique which allowed visualization of catecholamines and 5-hydroxytryptamine in tissue sections after treatment with formaldehyde vapours (Eränkö, 1955; Falck, 1962; Falck *et al.*, 1962; Carlsson *et al.*, 1962). This tech-

nique and its recent modifications have lead to an extensive knowledge of the distribution and characteristics of monoamine neurons in the peripheral and central nervous system. Monoamine neurons have also been identified at the ultrastructural level using cytochemical techniques. They include simple osmium fixation eventually preceeded by glutaraldehyde fixation (Lever and Esterhuizen, 1961; De Robertis and Pellegrino de Iraldi, 1961 a *et* b; Richardson, 1962), potassium permanganate fixation (Richardson, 1966; Hökfelt, 1968) and autoradiography (Wolfe *et al.*, 1962; Aghajanian and Bloom, 1966 a *et* b). Thus, a number of principally different histochemical techniques have been employed in studies on monoamine neurons. The latter technique, i.e. autoradiography, however, represents a general approach which should be possible to use for studies on every neuron system having at its cell membrane a specific uptake mechanism for its transmitter. This technique was employed in *attempts* to identify certain neuron populations utilizing amino acids as their transmitters. The uptake patterns of both γ-aminobutyric acid (GABA) and glycine were studied (Ehinger, 1970; Hökfelt and Ljungdahl, 1970 *et* 1971; Bloom and Iversen, 1971; Matus and Dennison, 1971). Surprisingly it was found that these amino acids did not only accumulate in neurons but also in glial cells (see Hösli L. and Hösli E., 1978) and it was early suggested that glial uptake may represent an important mechanism of inactivation of these substances after their action at the receptors (Iversen and Kelly, 1975).

In the present article, however, we would like to deal with a different approach, immunocytochemistry. The possibility to combine immunological and histochemical principles were recognized more than 30 years ago by Coons and his collaborators who introduced the so called direct and indirect immunofluorescence technique (see Coons, 1958). This approach has a general applicability since principally every substance can be visualized against which an antiserum can be raised. Since not only large proteins have antigenic properties, but since also smaller molecules can be rendered antigenic by conjugation to larger carrier proteins, a wide research field has been opened up. These unique possibilities were early recognized by Gibb *et al.* (1967) who demonstrated that the enzyme dopamine-β-hydroxylase (DBH) has antigenic properties. Subsequently Geffen and collaborators (1969) used antisera to this enzyme to visualize peripheral adrenergic neurons and adrenal gland cells with the indirect immunofluorescence technique.

During the last years immunocytochemistry has been extensively used to trace not only catecholamine systems but also a large variety of other

neuron populations. The rapid advancement in this field is due to the important discoveries and work in the protein and peptide biochemical field, by which numerous proteins have been purified and by which a large number of small peptides have been isolated and structurally characterized. This has allowed the production of synthetic peptides in sufficient amounts to be used for production of antisera. In the following we would like to describe some of the results obtained with this technique using antisera raised to three different types of antigens: large proteins such as catecholamine synthesizing enzymes, small peptides conjugated to large proteins, such as bovine serum albumin and a very small molecule such as 5-hydroxytryptamine, also conjugated to a carrier protein. Using such antisera on series of consecutive sections evidence has been obtained for coexistence of biogenic amines and peptides in the same neuron, as will be discussed in the last paragraph. Firstly, however, we would like to discuss some methodological aspects.

On the methodology of immunocytochemistry

The original description of the immunofluorescence technique of Coons and collaborators dealt with two approaches. With the direct technique the primary antiserum was directly labeled with a fluorescent dye, e.g. fluorescein isothiocyanate, whereas with the indirect technique incubation with the specific antiserum (raised e.g. in rabbits) is followed by a second labeled antiserum (raised e.g. in sheep against rabbit γ-globulin). The latter modification offers certain advantages, such as a higher sensitivity as well as the possibility to use the same labeled antiserum for several primary antisera (provided, of course, that all the primary antisera were raised in the same species). Furthermore, the specific antiserum can be used in higher dilutions thus conserving precious antiserum. Damage to the specific antiserum caused by the labeling procedure is also avoided. More recently several modifications of the original technique have been introduced. They include labeling with peroxidase instead of fluorescent dyes with the advantage of making both light and electron microscopic analysis possible (Nakane and Pierce, 1967). The unlabeled antibody enzyme method (peroxidase-antiperoxidase method) of Sternberger *et al.* (1970) is characterized by a very high sensitivity, i.e. the primary antiserum can be used in very high dilutions. These techniques and further modifications and principles of immunocytochemistry have been discussed extensively in a monograph by Sternberger (1974).

The immunocytochemical techniques are generally considered as sensitive and comparatively specific. It has, however, to be pointed out that several problems exist. Apart from technical questions such as sensitivity and problems of penetration of the antisera into the tissue, the most serious problem is the specificity of the technique. This problem has been discussed by Nairn (1969) and consists of several issues. Here we would only like to discuss two aspects. The first important issue is the purity of the antigen. This may (hopefully) be a minor problem when raising antisera to small peptides which have been structurally characterized and are available in synthetic form. It should, however, be emphasized that a rabbit will produce many different antibodies against a conjugate of small peptide and a larger carrier protein. Thus, we have a heterospecific antiserum. New possibilities to obtain monospecific antisera have opened up by using hybrid cell lines, a method introduced by Köhler and Milstein (1975). A second major problem is the possibility of the existence of cross-reacting peptide or proteins. This is of particular significance when studying antisera raised to small peptides. Thus, although the antisera may have been checked with regard to crossreactivity to numerous well-known, structurally characterized peptides, it cannot be excluded that in the tissue section hitherto unknown peptides exist with an aminoacid sequence similar to that present in the peptide, against which the antiserum was raised. We have therefore consequently tried to use expressions such as "substance P-like immunoreactivity", "substance P immunoreactive", etc. to indicate such possibilities. In fact there are already examples of such crossreacting peptides. Thus, the gastrin-like immunoreactivity described by Vanderhaegen *et al.* (1975) seems to represent cholecystokinin-like peptides (Dockray, 1976; Straus *et al.*, 1977; Dockray *et al.*, 1978; Robberecht *et al.*, 1978). Furthermore, TRH-like immunoreactivity has been observed in most parts of the central nervous system (Brownstein *et al.*, 1974; Jackson and Reichlin, 1974; Winokur and Utiger, 1974; Hökfelt *et al.*, 1975 b *et* c) but genuine TRH has been claimed to be present only in the hypothalamus (Youngblood *et al.*, 1978).

In the following we will mainly concentrate on findings obtained in our own laboratory. They are based on the indirect immunofluorescence technique. Briefly, the experimental animals were perfused with ice-cold formalin, rinsed for at least 24 hours in buffer containing 5% sucrose. The tissues were then cut on a cryostat (Dittes, West Germany) and the sections were incubated with the primary antiserum diluted 1:20-1:500, mostly at +4°C over night. After rinsing the second incubation was per-

formed with fluoresceinisothiocyanate (FITC) labeled antibodies at +37°C temperature for 30 min. After mounting the sections were examined in a Zeiss fluorescence microscope.

Mostly untreated animals have been used but in certain situations experimental procedures have to be carried out. During our analysis of central peptide systems it has become obvious that it is often difficult to visualize peptide stores within cell somata. This may be due to several reasons, such as low levels of peptides in this part of the neuron or perhaps the presence of mainly precursor molecules not crossreacting with the antiserum. This problem has partly been overcome by pretreating the animals with colchicine or vinblastine, two mitosis inhibitors. It is assumed that these drugs arrest axonal transport via an effect on microtubules and in this way prevent outflow into the axon of peptides produced in the cell body, resulting in an accumulation within the cytoplasm (see Dahlström, 1968; Kreutzberg, 1969).

Catecholamine neurons

The extensive knowledge of the distribution of catecholamine systems obtained with formaldehyde fluorescence studies, has been confirmed and extended by the immunocytochemical approach and little conflicting data have been reported. Particularly noteworthy are the extensive mapping of central DBH containing neurons by Swanson and Hartman (1975) and the elegant ultrastructural investigations by Pickel and collaborators (1976). We would like to point to a few issues, where immunocytochemistry has offered certain additional information.

A major advantage seems to be that immunohistochemistry, carried out with all four enzymes in the catecholamine synthesis (Fig. 1), offers possibilities to differentiate between the catecholamine neurons. Thus, an adrenaline neuron should have all four enzymes, whereas a noradrenaline neuron should lack the last enzyme, phenylethanolamine-N-methyltransferase (PNMT), and dopamine neurons should only contain the first two enzymes, tyrosine hydroxylase (TH) and dopadecarboxylase (DDC). By incubating consecutive sections with the various antisera, a careful comparison of the results should indicate what amine a neuron is able to produce. This principle is illustrated with some micrographs of consecutive sections of the adrenal gland incubated with antisera to the four enzymes (Figs. 2 A-D; 3 A-D). This issue is important, since with the Falck-Hillarp formaldehyde fluorescence technique it is not possible to differentiate between the three catecholamines. This is due to the fact that they all

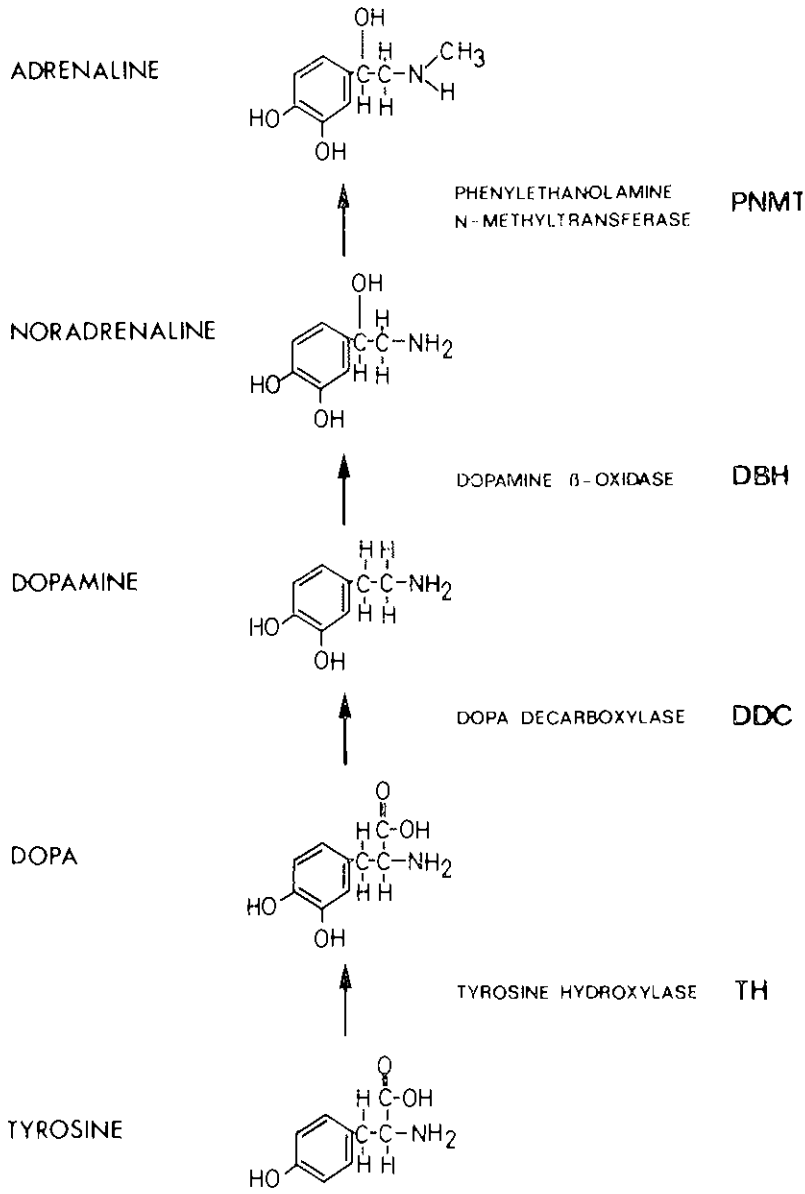

FIG. 1. Schematic illustration of the catecholamine synthesis. Adrenaline is derived from tyrosine via dihydroxyphenylalanine (DOPA), dopamine and noradrenaline. Each step is controlled by a specific enzyme (From Hökfelt *et al.*, 1973b).

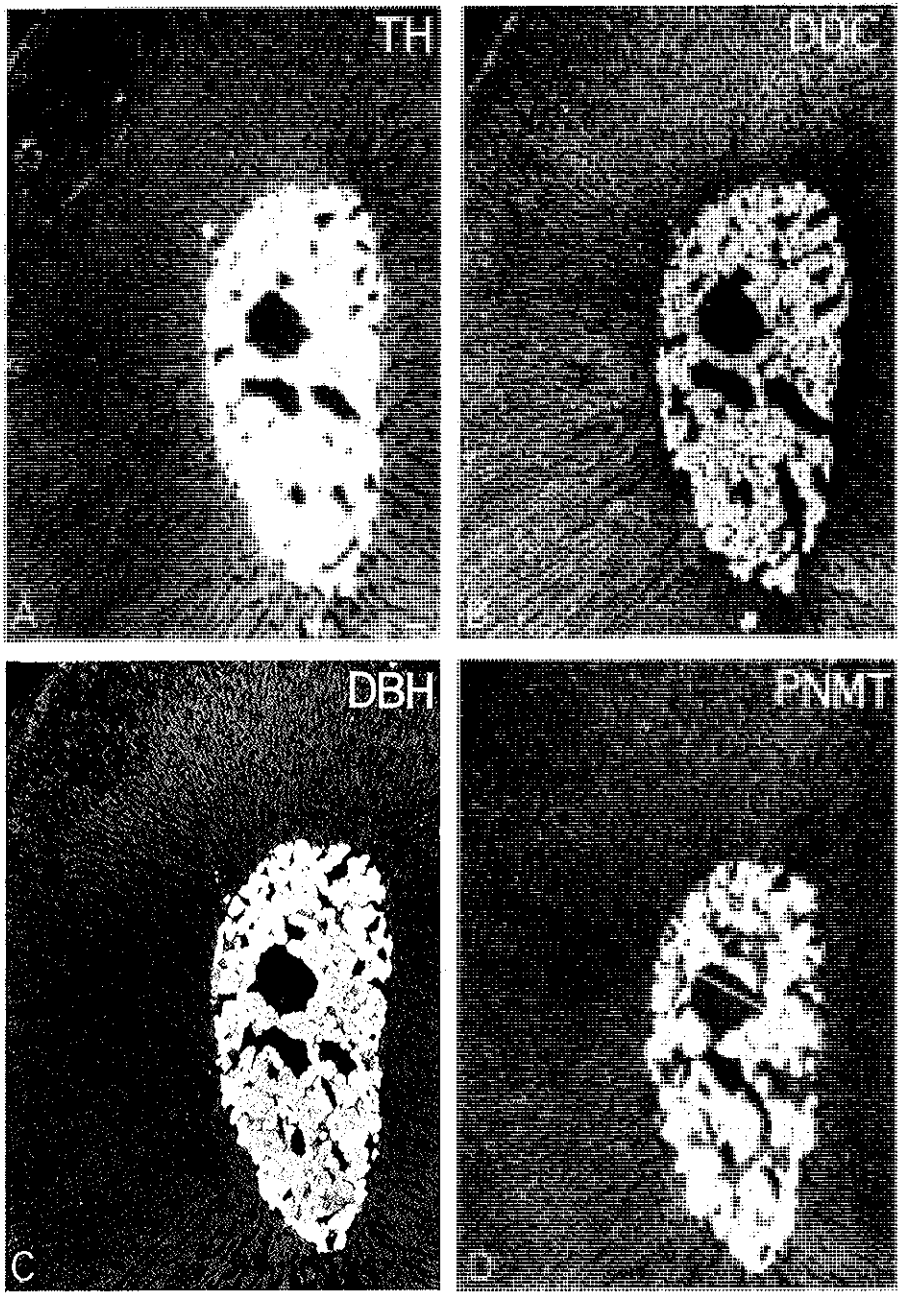

FIG. 2A-D. Immunofluorescence micrographs of consecutive sections of the rat adrenal gland after incubation with antiserum to tyrosine hydroxylase (TH) (A), dopadecarboxylase (DDC) (B), dopamine β-hydroxylase (DBH) (C) and phenylethanolamine N-methyltransferase (PNMT) (D). The medullary gland cells are immunoreactive whereas the cortex only exhibit background fluorescence. Note PNMT negative cells at asterisk in D (*c.f.* Fig. 3D). Bar indicates 50 μm.

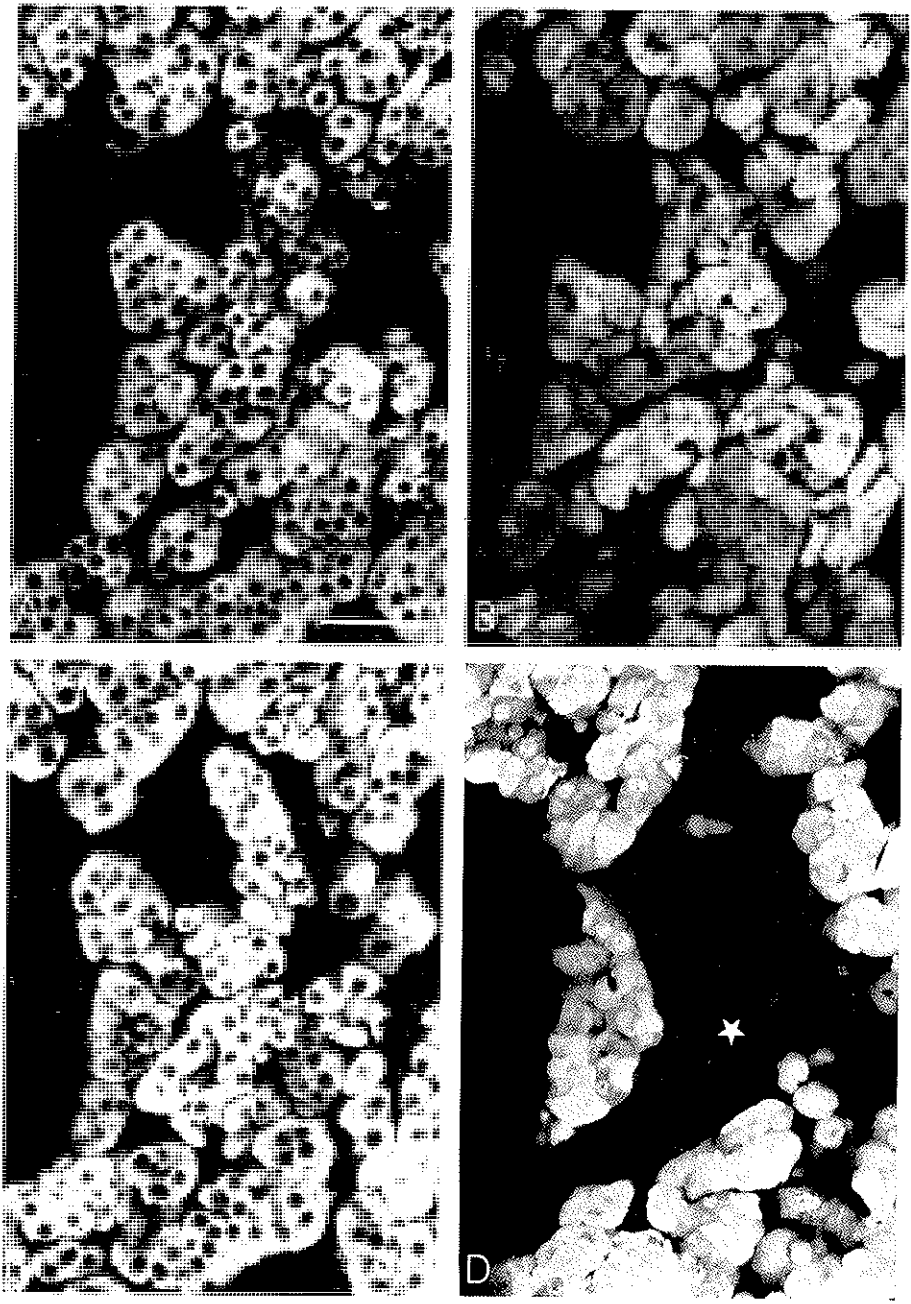

FIG. 3A-D. Higher magnifications of Fig. 2A-D, respectively. The approximate area shown is indicated by rectangle in Fig. 2D. Note that a group of cells are PNMT negative (asterisk in 3D) but that the cells at the corresponding place are TH (A), DDC (B) and DBH (C) positive. These PNM negative cells represent noradrenaline producing cells whereas the remaining gland cells are PNMT positive and produce adrenaline. Note that there is a tendency that the PNMT negative cells have a stronger DBH immunoreactivity than the adjacent PNMT positive cells (*c.f.* C and D). Bar indicates 50 μm.

have the same excitation and emission spectra (see Jonsson, 1971). A further advantage may be that the enzymes, in contrast to the amines themselves, occur in comparatively high concentrations in the cell somata. This may therefore facilitate identification of catecholamine cells. In fact it seems to us that with the immunohistochemical approach more catecholamine cells have been observed than with the formaldehyde fluorescence technique or its modifications.

Using an antiserum to the last enzyme in the catecholamine synthesis it has recently been discovered that there exist in the brain and spinal cord systems containing PNMT immunoreactivity (see Hökfelt *et al.*, 1973b *et* 1974). Their cell bodies are located in the so called A1 and A2 regions (see Dahlström and Fuxe, 1964) in the lower medulla oblongata. They give rise to descending projection to the spinal cord, mainly to the sympathetic lateral column, as well as to ascending projections to the periventricular areas, particular in the hypothalamus, but also to deeper limbic areas as well as to amygdala. It is likely that these systems produce adrenaline, which thus may be a third catecholamine transmittor candidate in the central nervous system. These findings extend earlier biochemical results demonstrating both adrenaline and PNMT in the brain (Euler, 1946; Vogt, 1954; Gunne, 1962, McGeer and McGeer, 1964; Barchas *et al.*, 1969; Pohorecky *et al.*, 1969) and are also in agreement with more recent studies (Goldstein *et al.*, 1974; Koslow and Schlumpf, 1974; Saavedra *et al.*, 1974; Goldstein *et al.*, 1975; Lew *et al.*, 1977; Goldstein *et al.*, 1978).

The same approach using antisera to the enzymes involved in the catecholamine synthesis has been used to study e.g. intrabulbar dopamine neurons (Hökfelt *et al.*, 1975; Halász *et al.*, 1977), hypothalamic catecholamine neurons (Hökfelt *et al.*, 1976) and recently evidence has also heen obtained for the existence of dopamine neurons in the lower brain stem located in the medial part of the A2 group in the nucleus commissuralis (Hökfelt *et al.*, 1979). In the peripheral nervous system possibilities have opened up with this approach to differentiate between the catecholamines in the principal ganglion cells and in the small intensely fluorescent (SIF) cells (Elfvin *et al.*, 1975; Rybarczyk *et al.*, 1976).

Peptide neurons

The structural characterization of several small biologically active peptides such as the three hypothalamic hormones LHRH, TRH and somatostatin by Guillemin, Schally and their collaborators rapidly led to the

production of antisera against these peptides. The first immunohistochemical studies using such antisera were published in 1973 particularly by Barry and collaborators on LHRH (Barry *et al.*, 1973; Calas *et al.*, 1973; Leonardelli *et al.*, 1973). In fact, the carrier protein neurophysin present in neurosecretory cells was visualized by immunohistochemistry already in 1971 by Livett *et al.* (1971) and later by Zimmermann *et al.* (1973). In the last years a huge number of studies have been published in this area and some of the work has been reviewed in some recent articles (see Hökfelt *et al.*, 1978; Elde and Hökfelt, 1978). Several of these peptides are present not only in the hypothalamus but also in extrahypothalamic brain regions and the spinal cord as well as in the peripheral nervous system. Thus, e.g. after incubation with antiserum to substance P, an undecapeptide originally discovered by Euler and Gaddum (1931) and structurally characterized by Chang *et al.* (1971), more than 30 cell groups have been observed in the central nervous system (Fig. 4 A, C) with nerve terminal networks in most brain areas (Cuello and Kanazawa, 1978; Ljungdahl *et al.*, 1978). Substance P immunoreactive neurons have also been observed in the gastrointestinal wall as well as in primary sensory neurons (see Hökfelt *et al.*, 1978 a; Schultzberg *et al.*, 1978 a). It is therefore obvious that such peptides are present in many different types of neurons and that they may exert a transmitter or modulator role at many levels in the nervous system. The evidence for a transmitter role of peptides is at present studied in many laboratories and some aspects of this have recently been reviewed by Otsuka and Takahashi (1977). Another extensive peptide system contains enkephalin-like immunoreactivity (Fig. 4 B). Enkephalin was originally discovered by Hughes, Kosterlitz and collaborators (Hughes *et al.*, 1975) and is assumed to represent an endogenous ligand for the opiate receptor. More recently it has become obvious that also other opioid peptides, particularly β-endorphin are present in the brain (Lazarus *et al.*, 1976), probably in different systems (Bloom *et al.*, 1978; Johansson *et al.*, 1978). Particularly, the enkephalin systems are widespread and may have a similar extent as the substance P systems (Bloom *et al.*, 1978; Elde *et al.*, 1976; Hökfelt *et al.*, 1977 a; Simantov *et al.*, 1977; Watson *et al.*, 1977). They are also present in the periphery (Elde *et al.*, 1976; Schultzberg *et al.*, 1978 a *et* b *et* 1979; Alumets *et al.*, 1978).

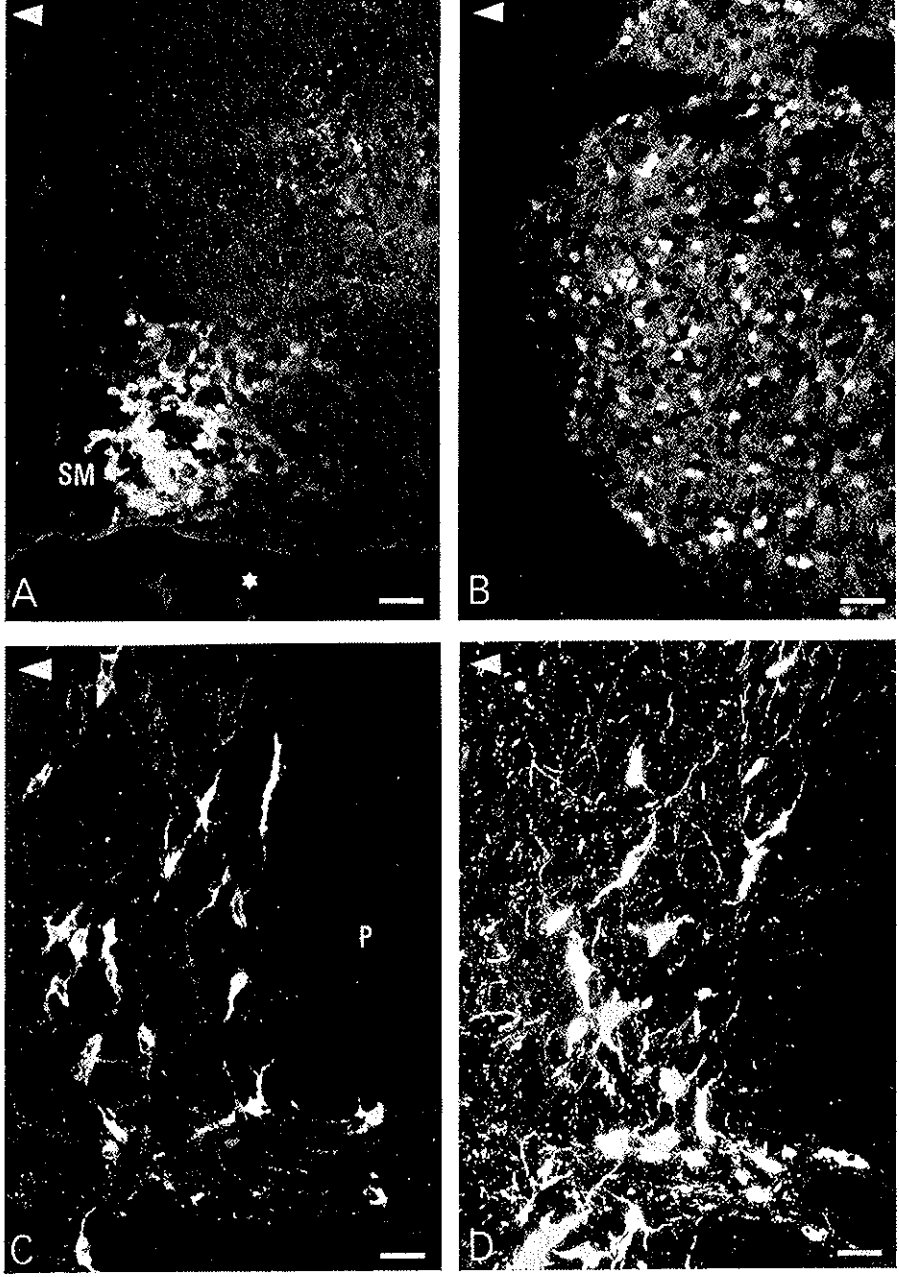

FIG. 4A-D. Immunofluorescence micrographs of the habenula (A), nucleus caudatus putamen (B), and the nucleus raphe magnus (C, D) after incubation with antiserum to substance P (A, C), methionine-enkephalin (B) and 5-hydroxytryptamine (D). C and D are consecutive sections. Numerous small substance P immunoreactive cells are seen in the medial habenula partly intermingling with the stria medullaris (SM) (A). Numerous enkephalin immunoreactive cells and a dense nerve terminal network is present in the neostriatum (B). In the raphe magnus nucleus substance P and 5-hydroxytryptamine immunoreactive cells can be seen with a very similar distribution (C, D). Asterisk in A indicates ventricles. P in C indicates pyramidal tract. Arrow heads point dorsally. Bars indicate 50 μm.

5-Hydroxytryptamine neurons

It has since long been known that antisera can be raised against even such small molecules as 5-hydroxytryptamine, i.e. the transmitter itself (Spector *et al.*, 1973). However, only recently could it be demonstrated that such an antiserum also can be used to visualize such a small molecule histochemically (Steinbusch *et al.*, 1978). In preliminary studies using an antisera raised to 5-HT coupled to bovine serum albumin extensive networks of immunoreactive nerve terminals and cell bodies have been demonstrated (Steinbusch *et al.*, 1978; Hökfelt *et al.*, 1978 c). The distribution of the cell groups agree well with the original description by Dahlström and Fuxe (1964) obtained with the formaldehyde fluorescence method. Although this antiserum crossreacts to a few percent with catecholamines there is no evidence from the immunohistochemical studies of staining of catecholamine neurons in the central nervous system. However, crossreaction with other indolamines cannot he excluded. The findings of Steinbusch *et al.* (1978) have opened up new, exciting possibilities to characterize neurons on a transmitter histochemical basis.

Coexistence of peptide and amine

It has generally been assumed that one neuron produces, stores and releases only one transmitter, a rule often referred to as Dale's principle (see Burnstock, 1976). This impression has also in general been obtained when comparing histochemical and biochemical data on the distribution patterns in mammals of "classical transmitters", such as acetylcholine, biogenic monoamines and certain amino acids. More recently evidence has accumulated that a neuron may contain several substances, which are known as putative transmitters and some of this material has recently been critically analysed by Burnstock (1976). The mere occurrence of two transmitter suspects in one neuron is, of course, not sufficient proof for a one neuron—two transmitters model, but such findings may represent a start for further investigation of this problem. The existence of a peptide and an amine within the same neuron is also of interest in relation to Pearse's (1969) so called APUD (Amine Precursor and/or amine Uptake Decarboxylation) concept, according to which peptide hormones and biogenic amines may coexist in the same endocrine cells, possibly even in the same storage vesicles. The findings described below indicate that such coexistence may occur also in peripheral and central neurons. Although the number of such cases so far discovered is small, coexistence of

a biologically active peptide and an amine may be a frequent phenomenon. Perhaps it is sooner the rule than an exception.

In the peripheral nervous system somatostatin-like immunoreactivity has been observed in principal ganglion cells of certain sympathetic ganglia (Hökfelt *et al.*, 1977 b). Thus, e.g. in the inferior and superior mesenteric and in the coeliac ganglia more than half of all neurons seem to produce both noradrenalin and a somatostatin-like peptide. In the central nervous system substance P-like immunoreactivity was found in a large proportion, but probably not all, of the 5-hydroxytryptamine cells of the raphe nuclei and adjacent areas of the medulla oblongata (Fig. 4 D) (Chan-Palay *et al.*, 1978; Hökfelt *et al.*, 1978 c). Since both somatostatin (Renaud *et al.*, 1975) and substance P (Otsuka and Takahashi, 1977) may act as modulators or transmitters in the brain, the two mentioned cases may represent examples of coexistence of two transmitters in one neuron. It must, however, be emphasized that our findings must be interpreted with caution and a number of questions still must be raised. (1) The possibility exists that the antisera cross-react with other structurally similar peptides, perhaps even with a sequence in much larger molecules, which have little to do with the putative transmitter peptides. (2) The occurrence of such peptides in the cell *somata* tells us little about a possible transmitter role. It is therefore important to demonstrate coexistence of the substances also in nerve endings. This is technically a difficult problem, but in the case of "substance P - 5-hydroxytryptamine neurons" indirect evidence supports the view of coexistence also in nerve endings. Thus, treatment with 5,7- (or 5,6)-dihydroxytryptamine, agents assumed to comparatively selectively destroy 5-hydroxytryptamine (and in the case of 5,7-DHT, in addition noradrenaline) neurons (Baumgarten *et al.*, 1971; Baumgarten *et al.*, 1973; Daly *et al.*, 1973 *et* 1974), causes a disappearance of most SP immunoreactivity in nerve endings in the ventral horns of the spinal cord, together with a disappearance of the 5-HT nerve terminals (Hökfelt *et al.*, 1978 c). This strongly suggests that SP and 5-HT is present in the same nerve terminals. (3) Even if the two substances are present in the same nerve terminals, this, of course, does not give evidence for a release of 5-HT and substance P and, even if they are released, their transmitter or modulator action has to he demonstrated. Thus, further experiments certainly are needed to satisfy the transmitter criteria for the coexisting 5-hydroxytryptamine and substance P-like peptide.

Using antisera raised to methionine-enkephalin, enkephalin-like immunoreactivity has been demonstrated in many peripheral nerves probably with a central origin, in certain peripheral noradrenergic ganglion cells

and also in the adrenal medulla of several species (Schultzberg *et al.*, 1978 b *et* 1979). Both in guinea pig (Fig. 5 A), cat and cow a large proportion of the medullary gland cells contain enkephalin-like immunoreactivity. In the untreated rat, however, only few positive cells can be seen, whereas after sectioning of the splanchnic nerve numerous strongly fluorescent enkephalin immunoreactive cells are seen (Fig. 5 C). On consecutive sections the medullary catecholamine cells can be shown after incubation with antisera to DBH (Fig. 5 B, D). Interestingly enkephalin-like immunoreactivity can also be seen in nerve fibres in the adrenal medulla (Fig. 5 A) and these fibres disappear after the nerve sectioning described above. Whether these enkephalin immunoreactive fibres belong to a population separate from the "classical" cholinergic nerves of the splanchnic nerve or whether an enkephalin-like peptide coexists with acetylcholine in the same neuron can at the present time not be decided.

SUMMARY

In the present brief article some attempts to characterize neuron populations on a transmitter histochemical basis are discussed. Emphasis is focused on immunohistochemistry representing a very general approach, since principally any substance can be traced against which an antiserum can be raised. The studies can be carried out both at the light and electron microscopic level. In our studies the indirect immunofluorescence technique has mainly been used and examples with antisera raised to antigens of different type and molecular weight are discussed. Using antisera to large molecules such as the enzymes in the catecholamine synthesis, tyrosine hydroxylase, dopa-decarboxylase, dopamine-β-hydroxylase and phenylethanolamine-N-methyltransferase it has been possible to map out and differentiate between dopamine, noradrenaline and adrenaline neurons. Peptides such as substance P and enkephalin are much smaller molecules, which have to be conjugated to larger carrier proteins, e.g. bovine serum albumin, prior to the immunization. Neurons containing such small peptides are widely distributed both in the peripheral and central nervous system. It is also possible to localize even such small molecules as 5-hydroxytryptamine with immunocytochemistry. Using antibodies to this indoleamine conjugated to bovine serum albumin, this indoleamine can now be mapped throughout the central nervous system with a high degree of sensitivity.

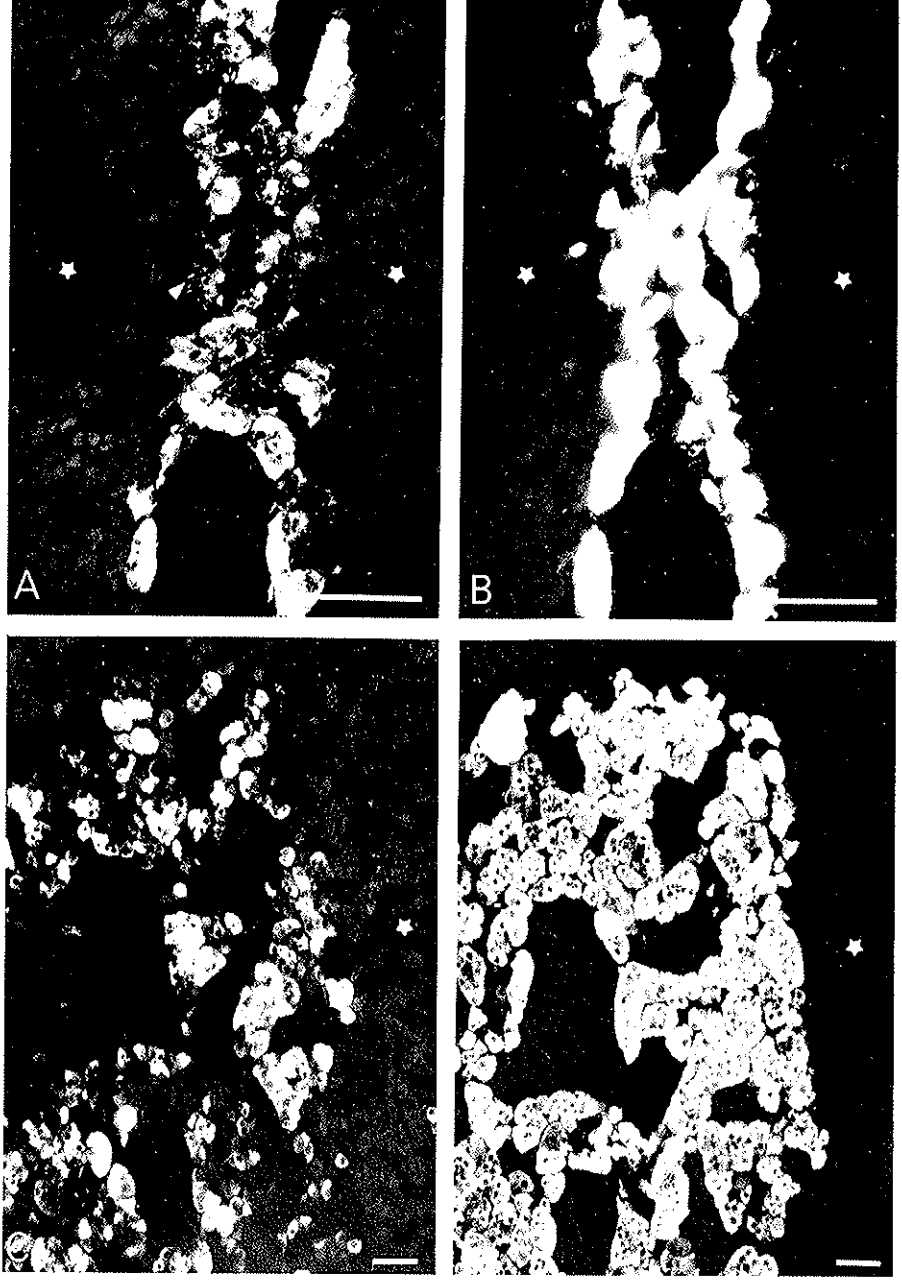

FIG. 5A-D. Immunofluorescence micrographs of the guinea pig (A, B) and rat (C, D) adrenal gland after incubation with antiserum to methionine-enkephalin (A, C) and dopamine β-hydroxylase (DBH) (B, D). In C and D the splanchnic nerve has been sectioned 7 days before sacrifice. A and B, on one hand, and C and D, on the other hand, are consecutive sections. Note enkephalin immunoreactive gland cells and nerve terminals (arrow heads) in the guinea pig medulla (A). Many gland cells are enkephalin immunoreactive in the rat medulla after sectioning of the splanchnic nerve. Asterisks in A-D indicate cortex. Bars indicate 50 μm.

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DISCUSSION

BURNSTOCK

You have been careful about saying whether polypeptides are neurotransmitters or not. But supposing they were not, could you speculate as to what else they might be?

HÖKFELT

Well, they could act as a number of other types of factors. The question of trophic factors has been brought up. I know that in Stockholm Dr. Uvnäs and Dr. Uvnäs-Waltensten are very excited because they have found both gastrin-like and possibly insulin-like immunoreactivity nerves, which are present in the sciatic nerve. Of course, such substances would be more likely candidates for some sort of trophic influence on muscle cells. So I think that is another possibility which we have to consider.

NIRENBERG

Does the antisera that you used against enkephalon also react with β-endorphine? Does it cross-react? Is it enkephalon-like material or endorphine?

HÖKFELT

I would say enkephalin-like material. They cross-react to a different degree with endorphins. We have some antisera raised against α- and β-endorphin, and with these we do not see anything in the adrenal gland, although with the same antisera we see neurons in the central nervous system in places very similar to where Bloom and others see the endorphin-positive cells. As I see it, the amounts of enkephalin-like material in the adrenal glands must be rather high, particularly in species such as cat and cow. If it would be more an endorphin-like substance, I would believe that we should have seen it.

KOSTERLITZ

I want to follow up Dr. Nirenherg's question: did you get any β-endorphin reactivity in the cells of rat adrenal medulla before cutting the splanchnic nerves when they showed little enkephalin reactivity?

HÖKFELT

No.

KOSTERLITZ

This finding appears to support the view now held by more and more people that β-endorphin is not the precursor of enkephalin.

HÖKFELT

I think this could be actually taken as support for that.

HAMPRECHT

Are the enkephalin-positive neurons or cells in the adrenal medulla positive for ACTH?

HÖKFELT

We have not checked that. I will go home and do that right away.*

FAMBROUGH

If you had a substance totally confined to synaptic vesicles, would you expect any positive reaction, using your immunochemical assay?

HÖKFELT

I would say yes. I think that there could be a diffusion of the peptides out from the storage sites into the cytoplasm. If we do not fix the tissue properly, we will lose them, since they are very small molecules like enkephalins. But even with a good fixation, a redistribution may occur intraneuronally. At the electron microscopic level the peptides seem to be present mainly in so-called large granular vesicles. The small synaptic vesicles appear "empty". The problem here may be that the antibodies are too large molecules and do not penetrate into the synaptic vesicles, or the peptides may have leaked out as discussed above, or the peptides may, in fact, not be present in the classical type of synaptic vesicles.

RAITERI

I would like to know something about the specificity of the antiserum against serotonin. Does it cross-react with tryptamine or with other indoleamines?

* They are not (added in proofs).

HÖKFELT

It has been checked rather carefully by Steinbusch and his colleagues. I do not have the table with me now. It cross-reacts with catecholamines and serotonin analogues. If I remember correctly, the antiserum cross-reacts with dopamine and 5-methoxytryptamine to about 2%. This is not sufficient to see any of the catecholamine systems in the brain; so we are rather convinced that we are not looking at catecholamine systems. But we cannot exclude that there are indoleamine systems, which we do not know about. Dr. Steinbusch and his colleagues are making antisera to other indoleamines to test this possibility.

GREENE

In the adrenals, was there any evidence that the PNMT-containing cells were more likely to contain peptides than the non-PNMT-containing cells?

HÖKFELT

In the rat, those cell groups which were strongest enkephalin-positive were PNMT negative, i.e., they were noradrenaline cells. But as you saw, after cutting the splanchnic nerve, almost all cells got positive. So also adrenaline cells probably contain the enkephalin-like peptide, although perhaps in lower amounts or differently bound.

CHAGAS

Do your polypeptide antibodies react with bigger molecules?

HÖKFELT

The question is, if one could imagine that there are larger proteins which contain a sequence of one of these small peptides. Of course, this is a question which bothers me very much. This is why we prefer to talk about "like immunoreactivity". It is not possible to exclude the possibility that our antisera cross-react with analogues to the peptides, against which they were raised. It may be, as indicated by Professor Chagas, that the antisera cross-react with large protein molecules, which have nothing to do with the events at synapses. It is interesting to note that antisera to leu-enkephalin, which to a large extent is part of the β-endorphin molecule, do not cross-react with β-endorphin to a significant extent. Thus, antisera raised to fragments of large protein molecules may not necessarily cross-react with the large molecules.

STUDIES ON THE MECHANISM OF RELEASE OF BIOGENIC AMINES FROM CEREBRAL NERVE ENDINGS

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INTRODUCTION

It has been amply demonstrated that in the peripheral nervous system noradrenaline (NA) is released by depolarizing stimuli through a mechanism of exocytosis whereby the amine is directly transferred from the storage vesicles into the synapse (Douglas, 1968; Rubin, 1970). It is also known that large part of the transmitter released is recaptured by presynaptic nerve endings through a specific carrier-mediated transport system (Iversen, 1967).

The existence of a reuptake system for NA similar to that present in peripheral nerve endings has been demonstrated also in the CNS, where analogous but distinct Na^+-dependent reuptake systems exist for dopamine (DA) and 5-hydroxytryptamine (5HT) (Iversen, 1975; Ross, 1976; Horn, 1976; Fuller and Wong, 1977). On the other hand, the mechanism of the depolarization-induced release of biogenic amines from central nerve endings has been less thoroughly investigated. It has been suggested that the Ca^{2+}-dependent stimulus-coupled release of biogenic amines from central nerve endings may also occur by exocytosis (Raiteri *et al.*, 1975b; 1977a; Holz, 1975; Colburn *et al.*, 1976). However, the demonstration

of a direct extracellular transfer of the amine from the storage vesicles has not been provided.

A large number of recent studies has been devoted to the elucidation of the homeostatic mechanism controlling the amount of neuro-transmitter released in the synapse. Also in this case, pioneering observations have been made in the peripheral noradrenergic system, where it has been shown that the amount of NA released by depolarization is modulated hy a negative feedback mechanism, whereby the amine present in excess in the synapse depresses its further release by activating specific presynaptic α-receptors (Enero *et al.*, 1972; Starke, 1972, 1977; Langer, 1977). Evidence for the existence of such a mechanism also in central noradrenergic synapses has been recently provided (Dismukes and Mulder, 1976; Langer, 1977; Starke, 1977). Although the problem has been investigated also in the case of DA release (Farnebo and Hamberger, 1971; Westfall *et al.*, 1976; Dismukes and Mulder, 1977; Raiteri *et al.*, 1978a), the presence of presynaptic receptors controlling directly the release of this amine is still a matter of controversy.

The release of biogenic amines can be elicited by stimuli other than depolarization. For example, release of catecholamines can be induced by drugs such as tyramine and other phenylethylamines, by reserpine, by changes in the ionic environment, etc. However, the mechanism by which the amines exit from the nerve endings following these stimuli have been rarely analyzed.

In our studies on biogenic amine release from isolated central nerve endings we have attempted to clarify some of the above mentioned unsolved problems. In particular we shall provide evidence for the existence of a carrier-mediated release of catecholamines, which may operate during stimuli other than depolarization. We shall describe experiments compatible with a direct extracellular transfer of catecholamines from central storage vesicles, during depolarization. Finally, we shall show that the presynaptic mechanism controlling DA release differs from that modulating the release of NA.

Carrier-mediated release of catecholamines

The first question that we wanted to answer was the following: can catecholamines exit from central nerve endings by a carrier-mediated process? It is known that the carrier-mediated uptake of catecholamines strictly depends on the inward downhill Na^+ gradient and can be inhibited by specific drugs (Iversen, 1975; Horn, 1976; Ross, 1976). If the amine

transport system were symmetrical at the two sides of the membrane, an inversion of the Na^+ gradient should stimulate the exit of any amine available in the cytoplasm through the carrier working in the inside-outside direction and the uptake inhibitors may prevent this release.

Fig. 1 shows that when rat striatal synaptosomes, prelabeled with

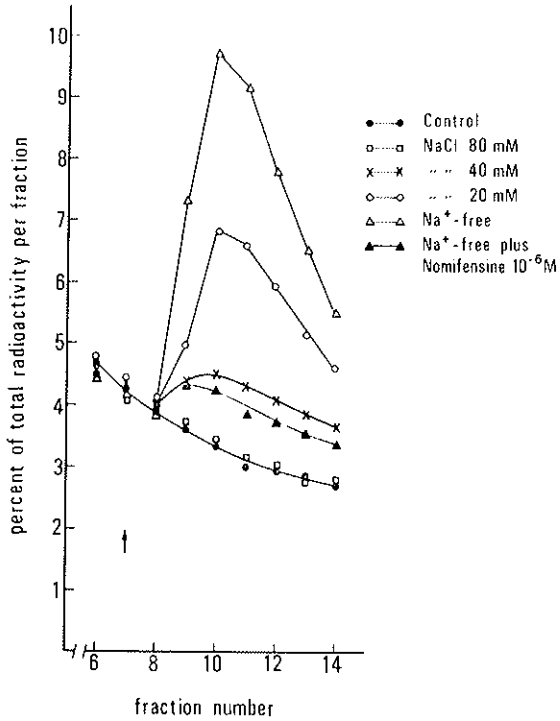

FIG. 1. Nomifensine-sensitive increase of ^{3}H-DA release from striatal synaptosomes superfused with media lacking Na^+.

Crude synaptosomal fractions were washed once with 0.32 M sucrose, resuspended in 0.32 M glucose at a protein concentration of about 6-8 mg/ml, diluted 1:10 in Krebs-Ringer medium and prelabeled with 0.1 μM ^{3}H-DA for 10 min at 37°. Then aliquots of the suspension corresponding to 300-400 μg of protein were plated on 0.65 μm Millipore filters laying at the bottom of several parallel superfusion chambers (Raiteri *et al.*, 1974), washed with 20 ml of medium and superfused with glucose-containing oxygenated medium at 37°, at a rate of about 0.5 ml/min. Fractions were collected every min. When indicated by the arrow, the superfusion medium containing 128 mM NaCl was replaced with new media containing 80, 40, or 20 mM NaCl, or with a Na^+-free medium, or with Na^+-free medium containing 1 μM nomifensine (sucrose replaced the NaCl omitted). The incubation and superfusion media contained 12.5 μM nialamide and 1 mM ascorbic acid. The radioactivity of each fraction is expressed as percentage of the total radioactivity recovered (total fractions plus filter). Each curve is the average of 2-4 experiments run in triplicate. Data from Raiteri *et al.* (1978a).

^{3}H-DA in the presence of physiological concentrations of Na ions, were superfused (Raiteri *et al.*, 1974) with a Na^+-free medium (a condition in which the Na^+ gradient across the nerve ending membrane is reversed), a large release of ^{3}H-DA was observed. This release was almost totally abolished when the DA uptake inhibitor nomifensine (Hunt *et al.*, 1974) was present in the Na^+-free medium. The figure also shows that DA release was consistently increased when NaCl concentration was 40 mM; in this condition, the release of NA and that of 5HT could not be stimulated (Raiteri *et al.*, 1978a) which suggests that the dopaminergic transport system is particularly sensitive to alterations of the Na^+ gradient.

It is likely that, in the presence of an outward downhill Na^+ gradient, the relatively small amount of free DA present in the cytoplasm can be rapidly transported out of the nerve ending; the removal of cytoplasmic DA would alter the equilibrium between cytoplasmic and vesicular pools, so that vesicular DA would be continuously released into the cytoplasm and transported extracellularly through the carrier.

One might object that the radioactive DA taken up by the synaptosomes during the prelabeling period is localized in an intrasynaptosomal pool particularly susceptible to being released through the carrier. In order to exclude this possibility, we analyzed the effect of the Na^+-free medium on the release of ^{3}H-DA newly synthesized from radioactive tyrosine. Fig. 2 shows that the release of radioactive DA synthesized from tyrosine was stimulated by Na^+ deprivation (panel a) similarly to that of exogenous radioactive DA preaccumlated by the nerve endings (panel b). In both cases nomifensine antagonized the effect of Na^+ deprivation.

Similar results were obtained in the noradrenergic system. The NA uptake blocker desipramine (DMI) strongly inhibited the release of ^{3}H-NA induced by a Na^+-free medium in hypothalamic synaptosomes (Raiteri *et al.*, 1977a) and in heart slices (Paton, 1973a).

The Na^+ gradient across the nerve endings membrane can be modified not only by reducing the extracellular concentration of Na^+, but also by increasing its intracellular concentration, for example by the Na^+-K^+-ATPase inhibitor ouabain (Archibald and White, 1974). As previously reported, superfusing striatal synaptosomes, prelabeled with ^{3}H-DA, with ouabain led to an increase in the release of DA that could be prevented by the carrier blocker nomifensine (Raiteri *et al.*, 1978a).

The results presented so far indicate that, in conditions of altered Na^+ gradient, an outward transport of catecholamines is possible. Carrier-mediated release may also be possible in the presence of a normal Na^+

gradient, provided that the cytoplasmic concentration of the amine is sufficiently high. An increase in the cytoplasmic concentration of biogenic amines can be induced by some well known drugs. For example, we know from classical pharmacology that several sympathomimetic amines, like tyramine, act indirectly by displacing NA from its storage sites (Burn and Rand, 1958). Similarly, amphetamine seems to act indirectly by releasing DA from dopaminergic nerve terminals (Besson *et al.*, 1969;

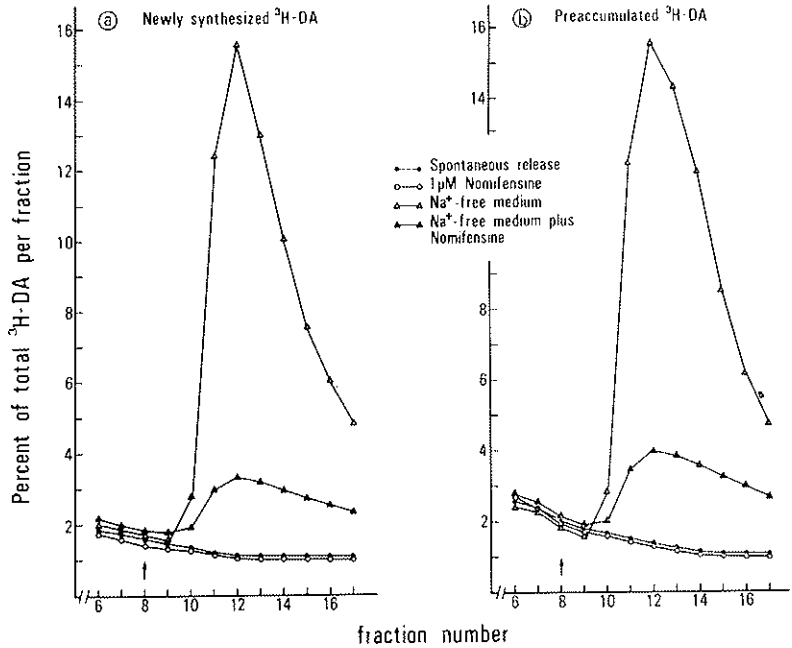

FIG. 2. The release of both newly synthesized and preaccumulated ^{3}H-DA is stimulated by Na^+-deprivation through a nomifensine-sensitive process. Panel a: after a 10 min equilibration at 37° in a rotary waterbath, synaptosomes were plated on Millipore filters (about 700 μg protein per filter) in several parallel superfusion chambers, superfused 10 min with standard medium containing 0.3 μM ^{3}H-tyrosine, washed twice with 15 ml of standard medium and superfused with the same medium for 8 min. When indicated by the arrow, the medium in some of the chambers was substituted either with a Na^+-free medium (with or without 1 μM nomifensine) or with standard medium (with or without nomifensine). Fractions were collected every min into a protective solution and the radioactivity present as ^{3}H-DA in each fraction and in the filters at the end of superfusion was measured as previously described (Raiteri *et al.*, 1978b). Panel b: after a 10 min equilibration at 37°, synaptosomes were prelabeled with 0.1 μM ^{3}H-DA for 10 min. After washing and superfusion with standard medium for 8 min, the synaptosomes were treated with different media and the fractions collected were analyzed for ^{3}H-DA as described in Panel a. In all the experiments of Fig. 2 the incubation and superfusion media did not contain monoamineoxidase inhibitors. The curves presented are the average of 3 experiments in triplicate.

Costa and Garattini, 1970; Raiteri *et al.*, 1975a; 1978a). However, the mechanism by which the pharmacologically active agents, NA or DA, cross the nerve ending plasma membrane, following tyramine or amphetamine treatment, is not known. The release caused by these drugs is not Ca^{2+}-dependent and therefore should not occur by exocytosis. On the other hand, exit by passive diffusion is unlikely, due to the physicochemical characteristics of the catecholamines. If exit is not by exocytosis, the transmitter must be released from the storage vesicles into the cytoplasm, before crossing the plasma membrane. We have investigated the possibility that cytoplasmic NA or DA leave the nerve terminals utilizing the membrane carrier.

In the experiments described in Fig. 3 the release of radioactive NA from hypothalamic synaptosomes was stimulated with p-tyramine or

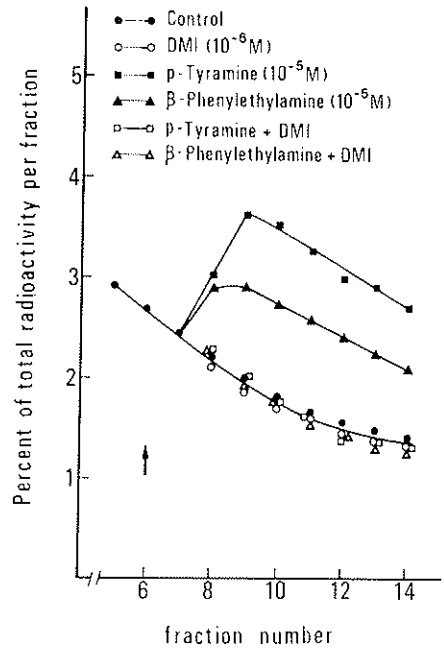

FIG. 3. Phenylethylamines accelerate ^{3}H-NA release through a process which is inhibited by desipramine.
Crude hypothalamic synaptosomes were labeled with 0.1 μM ^{3}H-NA for 10 min and then superfused with standard medium. When indicated by the arrow, the release of ^{3}H-NA was stimulated by adding to the superfusion medium tyramine or β-phenylethylamine. The effect of desipramine (DMI) on the phenylethylamine-induced release was tested by adding the drug together with each of the two phenylethylamines to the superfusion fluid. Other experimental details as in the legend for Fig. 1. Each curve is the average of 2 triplicate experiments performed on different days. Data from Raiteri *et al.* (1977a).

β-phenyletbylamine. The stimulation of release, which quantitatively depends on the structural characteristics of the two phenyletbylamines (Raiteri *et al.*, 1977b), was totally abolished when the NA uptake inhibitor DMI was present in the superfusion fluid, together with the releasing drugs. DMI itself did not alter the basal release of NA. DMI

may be thought to inhibit the phenylethylamine-induced release by preventing the phenylethylamines from entering the nerve endings through the membrane carrier. However, this explanation may hold for phenylethylamines carrying phenolic hydroxyl groups (like tyramine), whereas non-phenolic phenylethylamines are known to be poor substrates for the NA carrier and to enter nerve endings more easily by diffusion (Ross *et al.*, 1968; Raiteri *et al.*, 1977a). In view of this consideration, the inhibition by DMI of the release of NA induced by β-phenylethylamine can be interpreted as largely due to inhibition of the carrier-mediated efflux of the NA displaced from its storage sites by the non-phenolic amine.

It seemed interesting to analyze whether also the release of DA, elicited by amphetamine and related phenylethylamines, was susceptible of inhibition by the blocker of the DA carrier nomifensine. Striatal synaptosomes, prelabeled with tritiated DA, were superfused with various phenylethylamines (Fig. 4). d-Amphetamine, β-phenylethylamine and octopamine stimulated ^{3}H-DA release with different potency and nomifensine

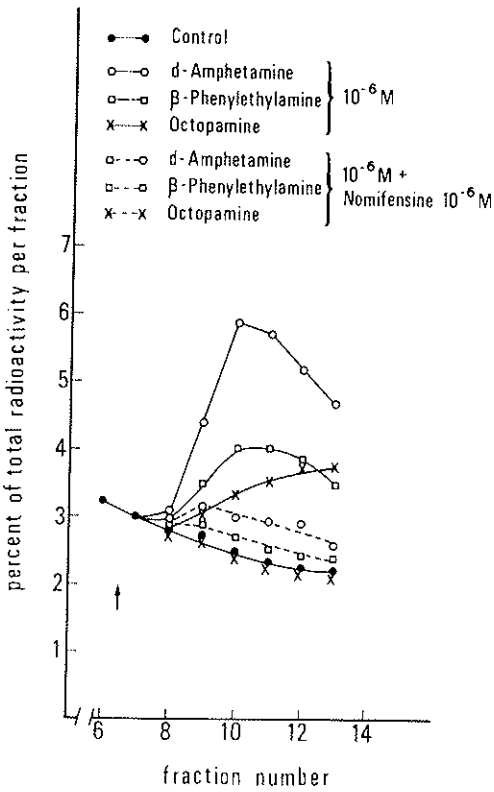

FIG. 4. Nomifensine inhibits ^{3}H-DA release elicited by phenylethylamines. Crude striatal synaptosomes were labeled with 0.1 μM ^{3}H-DA for 10 min and then superfused with standard medium. When indicated by the arrow the medium was substituted with new media containing d-amphetamine, β-phenylethylamine or octopamine, with or without nomifensine. Other experimental details as in the legend for Fig. 1 . Each curve is the average of 2 triplicate experiments. Data from Raiteri *et al.* (1978a).

counteracted their releasing effect. It should be noted that amphetamine is a non-phenolic amine, even less polar than β-phenylethylamine, and therefore it should enter dopaminergic nerve endings largely by diffusion. Thus the results of Fig. 4 suggest that nomifensine can inhibit the release elicited by the phenylethylamines by preventing the exit through the carrier of the DA displaced from its storage sites by the releasing agents.

The amphetamine-induced DA release was studied in more detail. In particular, we compared the effects of the drug on the release of ^{3}H-DA previously taken up with the effects on the release of the amine newly synthesized from labeled tyrosine. Fig. 5 shows that "taken up" and "newly synthesized" DA behaved similarly towards amphetamine and that the release elicited by the drug occurred through a mechanism sensitive to the DA carrier inhibitor nomifensine.

It can be concluded that phenylethylamines stimulate a carrier-mediated release of NA and DA; therefore, a release through the carrier can occur not only following manipulations of the Na^+ gradient, but also

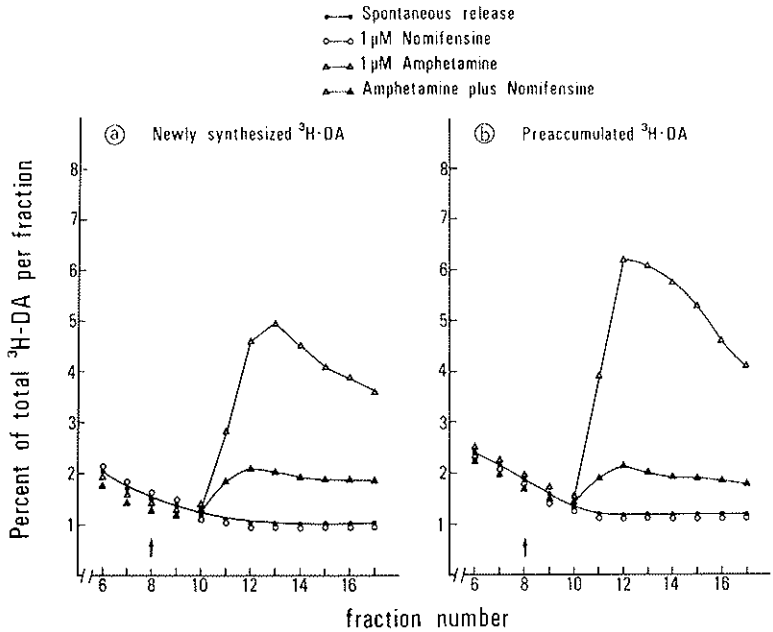

FIG. 5. The release of both newly synthesized and preaccumulated ^{3}H-DA is stimulated by d-amphetamine through a process sensitive to nomifensine. Experimental details as in the legend for Fig. 2. When indicated by the arrow (panel a and b) the standard medium was substituted with media containing either d-amphetamine or nomifensine or both. The curves presented are the average of 3 experiments run in triplicate on different days.

when the Na^+ gradient is normal and the cytoplasmic concentration of the amine is increased.

It may be interesting to note that several phenylethylamines are normal brain constituents and that their concentration can increase under various pharmacological and pathological conditions affecting their metabolic pathways (Fisher and Baldessarini, 1971; Axelrod and Saavedra, 1974; Boulton, 1974; Willner *et al.*, 1974). The fact that catecholamine carrier blockers are able to inhibit the phenylethylamine-induced release of DA and NA raises the possibility that the carrier-mediated release of the major catecholamines, stimulated by the endogenous phenylethylamines, represents an as yet unexplored target of the action of those neuroactive drugs whose effects are attributed only to their ability to inhibit catecholamine reuptake.

TYRAMINE SYMPATHOMIMETIC EFFECT AND TACHYPHYLAXIS: A NEW INTERPRETATION

The finding that catecholamines can exit from nerve endings utilizing the membrane carrier and the fact that some phenylethylamines, including tyramine, can themselves be transported by the catecholamine carrier (Ross, 1976; Horn, 1976) suggested to us a new interpretation of some pharmacological properties of tyramine which is schematized in Fig. 6.

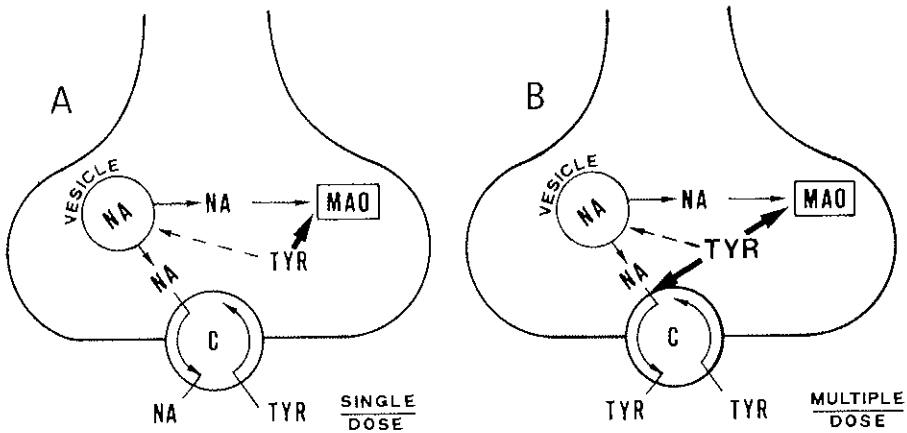

FIG. 6. Interpretation of the indirect sympathomimetic effect and tachyphylaxis of tyramine on the basis of transport mechanisms at the nerve ending membrane.
C = noradrenaline carrier. TYR = tyramine. MAO = monoamineoxidase.

Panel A concerns the indirect sympathomimetic effect of tyramine. Tyramine enters the nerve ending through the NA carrier (C) and displaces NA from the storage vesicles. We propose that cytoplasmic NA leaves the nerve terminals utilizing the NA carrier and that the carrier-mediated release is accelerated, through a process of exchange, by the extracellular tyramine entering the nerve ending. The acceleration of the exit of NA would be one of the reasons why the amine can escape deamination by monoamineoxidases (MAO) and reach intact the receptors. Another reason may be that tyramine itself is a substrate for MAO and, since fairly large doses of tyramine are required to elicit the sympathomimetic effects (for instance to increase blood pressure), NA is probably protected from deamination by tyramine which competes with NA for the enzymes.

Panel B illustrates our interpretation of the phenomenon of tyramine tachyphylaxis. It is known that repeated administrations of tyramine are accompanied by a progressive reduction and, eventually, a disappearance of the pharmacological response. Our interpretation of tachyphylaxis is the following: repeated administrations of tyramine lead to a progressive accumulation of the drug in the cytoplasm; the cytoplasmic tyramine will compete with NA for the exit through the carrier and the extracellular tyramine will exchange with progressively more tyramine and less NA, up to the extinction of the sympathomimetic effect.

Several interpretations were previously proposed for the phenomenon of tyramine tachyphylaxis. Some of these, including the classical assumption of a limited tyramine-sensitive pool of NA, have not received experimental support. One more recent interpretation originated from the finding that tyramine can be transformed into octopamine in noradrenergic nerve endings; octopamine would replace NA into the storage vesicles and tyramine, when repeatedly administered, would release inactive octopamine and NA in a progressively increasing ratio (Poch and Kopin, 1966). Although octopamine synthesis may contribute to the onset of tyramine tachypbylaxis in some experimental conditions, our interpretation appears susceptible of more general application. For example, it could explain the tachyphylaxis to tyramine obtained in reserpinized preparations (Burn and Rand, 1958; Axelrod *et al.,* 1962), that is in conditions in which octopamine formation should be excluded. Moreover, tacbyphylaxis was reported to occur also with phenylethylamine or tryptamine derivatives wbich are believed to act indirectly, as tyramine, by releasing NA, but are not β-hydroxylated to inactive compounds. Tacbyphylaxis to dopamine in rat vas deferens preparations (de Graaf *et al.,* 1977) and to serotonin in cat spleen strips (Innes, 1962) could be explained according to our

model. Both dopamine and serotonin can be transported by the NA carrier, can release NA from noradrenergic nerve endings and can undergo exchange processes at the NA carrier (Iversen, 1975; Ross, 1976; Paton, 1973b; Raiteri *et al.*, 1977b).

Exocytosis in central catecholaminergic nerve endings

Up to now we have confined our attention to conditions eliciting a release of catecholamines susceptible to inhibition by specific blockers of the membrane carrier. On the other hand, carrier blockers would not be expected to interfere with the depolarization-induced Ca^{2+}-dependent release of catecholamines, if this release occurred directly from the vesicles through an exocytotic process. We have therefore analyzed the release of DA and NA induced by various Ca^{2+}-dependent stimuli.

The release of DA from striatal synaptosomes, prelabeled with the exogenous radioactive amine, was stimulated with two depolarizing agents (high KCl and veratridine) and with the calcium ionophore A23187 (Holz, 1975). No changes in the release pattern were observed when the DA carrier blocker nomifensine was present in the medium (Fig. 7).

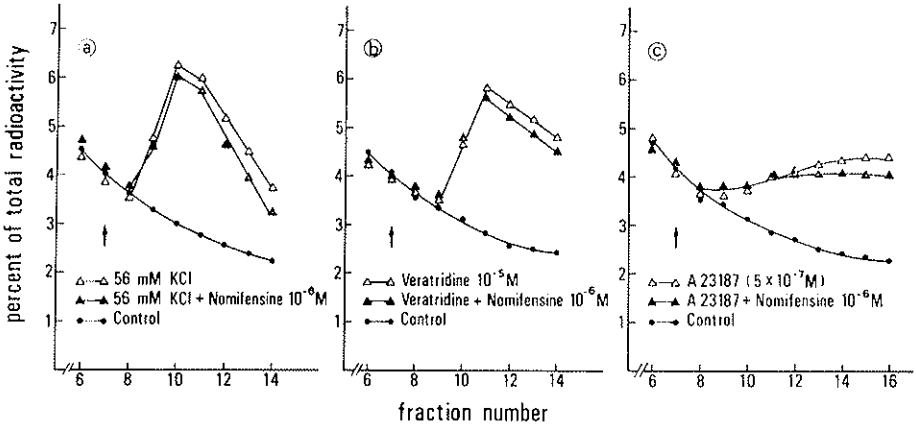

Fig. 7. The Ca^{2+}-dependent release of ^{3}H-DA is not affected by the DA carrier blocker nomifensine.

Striatal synaptosomes were prelabeled with 0.1 μM ^{3}H-DA for 10 min and superfused with standard medium. When indicated by the arrow the synaptosomes were treated with media containing high K^+ (panel a), veratridine (panel b) or the calcium ionophore A23187 (panel c), both in the presence and in the absence of nomifensine. Other experimental details as in the legend for Fig. 1. The curves presented are the averages of 8 duplicate experiments (panel a and b) and 4 triplicate experiments (panel c). Data from Raiteri *et al.* (1978a).

Identical results were obtained when the release of DA synthesized from tyrosine was stimulated with high K^+: Fig. 8 illustrates a set of experiments in which the behavior of the newly synthesized DA (panel a) was compared with that of the preaccumulated exogenous amine (panel b).

Also in central noradrenergic nerve endings the release of the trans-

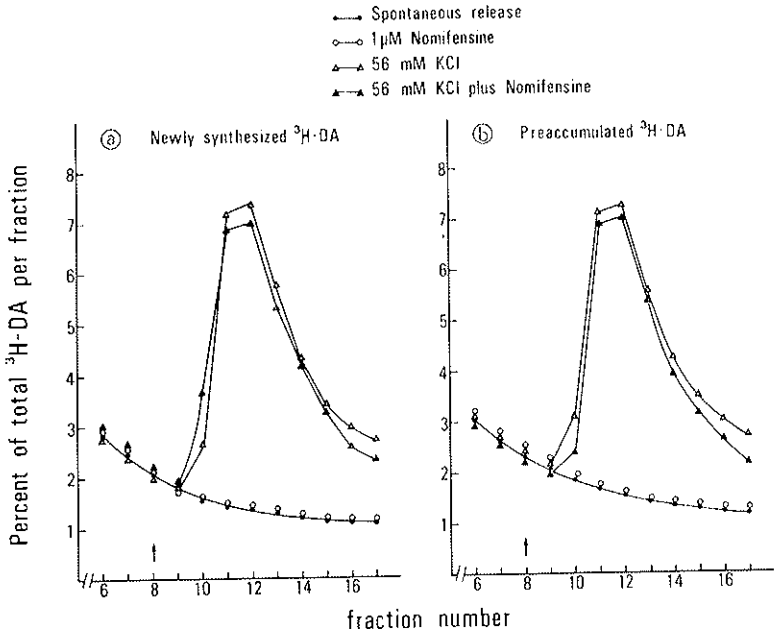

FIG. 8. The depolarization-induced release of both newly synthesized and preaccumulated ^{3}H-DA is insensitive to nomifensine.
Experimental details as in the legend for Fig. 2. When indicated by the arrows the standard medium was substituted with a medium containing 56 mM KCl, both in the presence and in the absence of nomifensine. The curves presented are averages of 3 experiments run in triplicate on different days.

mitter induced by Ca^{2+}-dependent stimuli was unaffected by a blockade of the NA carrier system. Fig. 9 shows that the NA carrier blocker desipramine did not inhibit the depolarization-induced release of NA from hypothalamic synaptosomes, nor the release elicited by the calcium ionophore A23187.

These results provide the first demonstration tbat, in central nerve endings, the release of catecholamines induced by depolarization occurs

independently of the membrane carrier, which gives further support to the hypothesis that catecholamines can be released also from central nerve endings by a mechanism of exocytosis.

PRESYNAPTIC AUTORECEPTORS CONTROLLING CATECHOLAMINE RELEASE

The existence of a feedback control of neurotransmitter release through the activation of specific presynaptic autoreceptors, although demonstrated only for NA (Enero *et al.*, 1972; Starke, 1972; Dismukes and Mulder, 1976; Langer, 1977; Starke, 1977), has been generalized to several neurotransmitters and in particular to DA (Farnebo and Hamberger, 1971; Westfall *et al.*, 1976; Starke, 1977). However, the data of the literature concerning this amine are very contradictory and therefore the existence of a direct control of DA release through presynaptic autoreceptors is not unanimously accepted.

We have approached the problem concerning the existence of a pre-

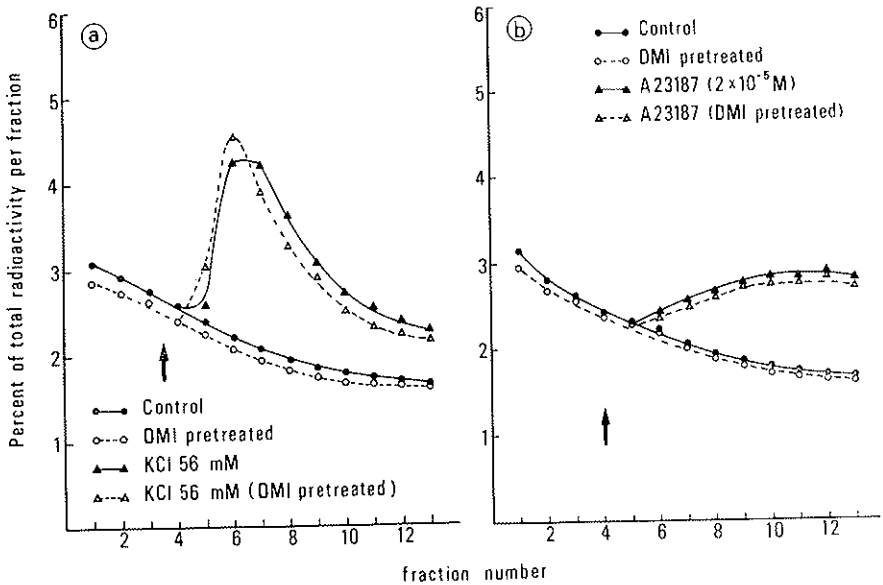

FIG. 9. The Ca^{2+}-dependent release of ^{3}H-NA is not affected by the NA carrier blocker desipramine.
Crude hypothalamic synaptosomes were prelabeled with 0.1 μM ^{3}H-NA and then superfused for 10 min with standard medium or with medium containing 10 μM desipramine (DMI). After washing with standard medium, the particles were superfused for 6 min with the same medium which was then replaced (see arrow) with media containing high K^+ (panel a) or the calcium ionophore A23187 (panel b). Each curve is the average of 3-4 experiments performed in duplicate or in triplicate on different days. Data from Raiteri *et al.* (1977a).

synaptic control of DA release utilizing superfused synaptosomes, an experimental condition which should be particularly suitable for the following reasons: *a*) synaptosomes are a relatively pure presynaptic preparation; *b*) the transmitter released is rapidly removed by the flow of superfusion fluid before a concentration capable of causing autoinhibition of release is reached. Therefore, presynaptic receptors are completely available to the exogenous agonists or antagonists added to the fluid.

The validity of this procedure is supported by experiments on the presynaptic control of NA release. The results reported by Mulder *et al.* (1978) with superfused synaptosomes, together with those obtained in our laboratory (unpublished), show that NA (in the presence of desipramine to prevent its uptake) inhibited the depolarization-induced release of preaccumulated radioactive NA. The inhibitory effect of NA was counteracted by α-antagonist drugs, in keeping with the accepted theory that NA autoreceptors are of the α-type.

By analogy with the noradrenergic system, both peripheral and central, where NA agonists effectively depress the depolarization-induced release of the exogenous radioactive preaccumulated NA, our first attempt was to ascertain whether DA agonists (like apomorphine) or DA itself could inhibit the release of ^{3}H-DA previously taken up by striatal synaptosomes. The effect of apomorphine was tested in a variety of experimental conditions (depolarization with different concentrations of KCl or veratridine, in the presence of varying concentrations of Ca ions, pretreatment with apomorphine or simultaneous addition of the drug with the depolarizing agents, etc.). In none of these conditions did the DA agonist inhibit consistently the release of ^{3}H-DA (Raiteri *et al.*, 1978a, b). Also when DA was used as an agonist (in the presence of nomifensine to prevent DA uptake and the consequent displacement of ^{3}H-DA), no inhibitory effect was observed (Raiteri *et al.*, 1978b). The DA agonist haloperidol increased only the spontaneous release of DA, but decreased that elicited by depolarization, after correction for the effect on spontaneous release (Raiteri *et al.*, 1978a). These results are in keeping with those previously obtained by Seeman and Lee (1975) and subsequently by Dismukes and Mulder (1977) utilizing electrically stimulated brain slices.

These data seem to indicate that the release of the DA previously taken up by dopaminergic nerve endings is not modulated by presynaptic receptors, in contrast to what has been demonstrated in the case of NA release. It should be noted, however, that the data do not exclude the existence of a presynaptic regulatory mechanism for the endogenously synthesized DA. Since newly synthesized DA is known to be preferentially

released by depolarizing stimuli (Besson *et al.*, 1971), the release of newly synthesized DA could in fact be particularly susceptible to a feedback control mechanism.

In order to test this possibility, we had to circumvent the difficulty that DA agonists inhibit the synthesis of DA in synaptosomes (Christiansen and Squires, 1974; Iversen *et al.*, 1976) and can therefore decrease the release of the amine indirectly. In other words, any effect of DA agonists on DA synthesis had to be prevented. This was obtained by superfusing the synaptosomes with the catecholamine synthesis blocker α-methyl-p-tyrosine, just after the labeling period with the synthesis precursor tyrosine. Now, if DA agonists had an effect on DA release independent of that on synthesis, the release should be inhibited also when DA synthesis is blocked. However, Fig. 10 shows that apomorphine did not affect the release of newly synthesized DA elicited by high K^+ or veratridine.

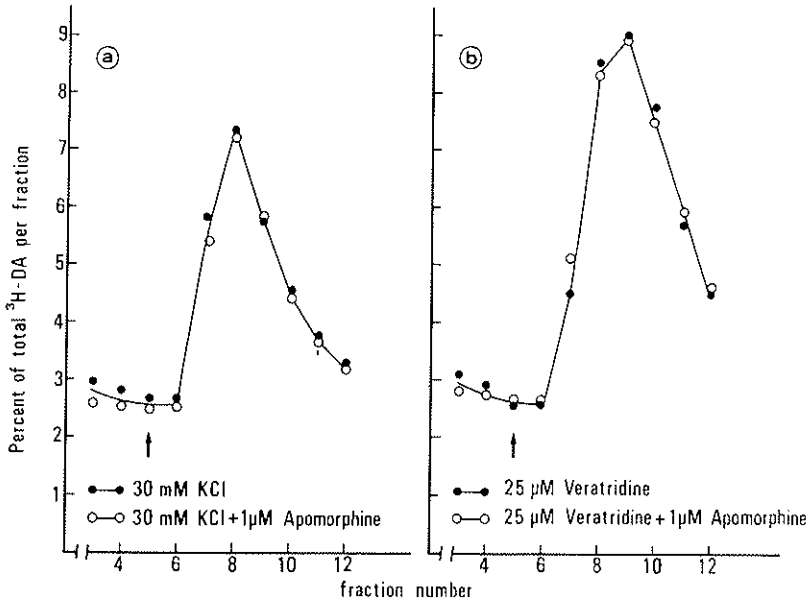

FIG. 10. The depolarization-induced release of newly synthesized ^{3}H-DA from striatal synaptosomes is unaffected by apomorphine.
After a 10 min equilibration at 37° in a rotary waterbath, synaptosomes were superfused 10 min with 0.3 μM ^{3}H-tyrosine in standard medium, washed with medium containing 0.1 mM α-methyl-p-tyrosine (2×15 ml) and then superfused with standard medium containing α-methyl-p-tyrosine, with or without 1 μM apomorphine. After 5 min (see arrow) 30 mM KCl (panel a) or 25 μM veratridine (panel b) was added as a depolarizing agent. Other experimental details as in the legend for Fig. 2. Each curve is the average of 3 experiments in sextuplicate run on different days. Data from Raiteri *et al.* (1978b).

In conclusion, DA agonists appear to be unable to decrease directly the depolarization-induced release of DA, either taken up or newly synthesized.

Data of the literature (Kehr *et al.*, 1972; Christiansen and Squires, 1974; Iversen *et al.*, 1976; Westfall *et al.*, 1976; Grabowska and Andén, 1976) seem to indicate that the above mentioned inhibition of DA synthesis by apomorphine takes place through an activation of presynaptic receptors; the inhibition is thought to occur at the tyrosine hydroxylation step of DA synthesis. The results shown in Table 1 are in keeping with this view. When striatal synaptosomes were incubated with tritiated tyrosine, as a precursor of DA synthesis, apomorphine strongly inhibited DA formation. In contrast, apomorphine did not affect significantly DA synthesis when DOPA was used as a precursor.

TABLE 1. *^{3}H-DA synthesis from ^{3}H-tyrosine, but not from ^{3}H-DOPA, is inhibited by apomorphine in striatal synaptosomes*

Precursor	Drug	Relative amount of ^{3}H-DA synthesized		
		Synaptosomes	Supernatant	Total
^{3}H-Tyrosine	—	92±4	8±4	100
^{3}H-Tyrosine	Apomorphine	38±5	2±1	40±5
^{3}H-Tyrosine	Nomifensine	79±1	25±4	104±4
^{3}H-Tyrosine	Apomorphine+ Nomifensine	37±1	9±2	46±2
^{3}H-DOPA	—	91±3	9±3	100
^{3}H-DOPA	Apomorphine	85±17	7±1	92±18
^{3}H-DOPA	Nomifensine	67±14	32±8	99±5
^{3}H-DOPA	Apomorphine+ Nomifensine	72±9	27±3	98±10

Crude striatal synaptosomes were incubated at 37° in a Krebs-Ringer medium, at a protein concentration of 1 mg/ml. After 10 min of equilibration, 0.3 μM ^{3}H-tyrosine (sp. act. 13.3 Ci/mmole) or 0.1 μM ^{3}H-DOPA (sp. act. 28 Ci/mmole) was added and the incubation was continued for 20 min. Apomorphine (1 μM) was added to the synaptosomes together with the radioactive precursor. Nomifensine, a DA reuptake inhibitor (Hunt *et al.*, 1974), was present in some cases in order to prevent: *a*) the possible end-product inhibition of the synthesis due to the DA spontaneously released and recaptured by the nerve endings; *b*) the possible entry of apomorphine into the nerve endings through the DA carrier. After incubation, the synaptosomes were centrifuged and the ^{3}H-DA formed was measured in the pellet and in the supernatant as described (Raiteri *et al.*, 1978b). The results are averages of 3-4 experiments and of 5-14 determinations. The values shown were calculated taking as 100 the total control synthesis of ^{3}H-DA either from ^{3}H-tyrosine or from ^{3}H-DOPA.

All together, the data available so far suggest that the homeostatic mechanism controlling the synaptic concentration of DA differs substantially from that operating at noradrenergic synapses. As shown schematically in Fig. 11, the depolarization-evoked Ca^{2+}-dependent release of DA would not be directly inhibited through the activation of presynaptic

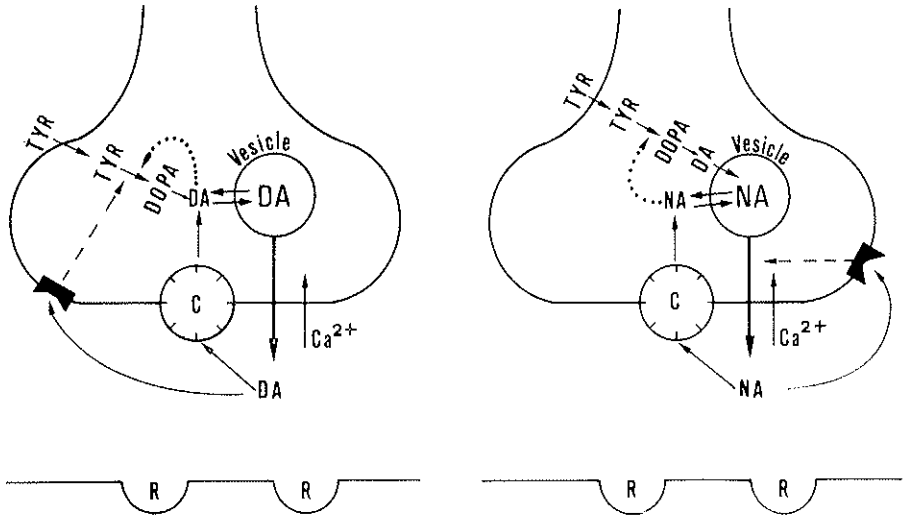

FIG. 11. The presynaptic control of NA release is direct; the control of DA release is secondary to a feed-back inhibition of synthesis.
Dashed arrows = inhibition of synthesis (DA) or release (NA) mediated by autoreceptors. Dotted arrows = end-product inhibition of DA and NA synthesis. C = membrane carrier. R = postsynaptic receptors.

receptors. The regulation of DA release seems to occur indirectly, through a control of tyrosine hydroxylation possibly mediated by presynaptic receptors, although a direct effect of DA agonists on the rate-limiting enzyme should not be excluded. In the case of NA, as demonstrated by several authors (Enero *et al.*, 1972; Starke, 1972; Langer, 1977; Dismukes and Mulder, 1976; Starke, 1977) presynaptic receptors directly modulate the release of the amine and any effect on synthesis is secondary to that on release.

This view is in keeping with the observation that the effects on NA synthesis caused by α-active drugs require an intact impulse flow (Grabowska and Andén, 1976), whereas DA agonists and antagonists modify DA synthesis even in the absence of impulse flow (Kehr *et al.*, 1972;

Christiansen and Squires, 1974; Iversen *et al.*, 1976; Westfall *et al.*, 1976).

Although it may seem that, whether the mechanism controlling amine release is direct or indirect, the ultimate result in terms of synaptic availability of the neurotransmitter is the same, the presence of one or the other mechanism may not be indifferent. For example, the administration of DOPA, a DA precursor which bypasses the tyrosine hydroxylation step, should lead in the dopaminergic nerve ending (see also the results of Table 1) to synthesis and release of DA free of any presynaptic control. A situation of this type may exist in Parkinson patients treated with L-DOPA. In this case, the lack of a direct control of release may allow the viable dopaminergic nerve endings to compensate, with a higher than normal release of DA, for the decreased number of functioning nerve endings.

ACKNOWLEDGEMENTS

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DISCUSSION

DE MELLO

Do you have an idea of how haloperidol causes an increase in the spontaneous release of dopamine in your system?

RAITERI

Well, one possibility would be that haloperidol enters the nerve endings through the dopamine carrier and releases dopamine. However, I think that this is not the case because benztropine, which is an inhibitor of dopamine uptake, does not inhibit the haloperidol-induced release. So my idea is that haloperidol enters the nerve ending by diffusion and, once entered by diffusion, in some way displaces dopamine from its storage sites.

CHAGAS

Is the tachyphylaxis, as shown in your model, dose dependent?

RAITERI

Well certainly. In fact, pharmacologically, tachyphylaxis can be obtained with various doses of tyramine. Tachyphylaxis can be reached after administration of one milligram per kilogram of tyramine for maybe twenty times, but it can be obtained after only ten administrations provided that the dose of tyramine is increased. But I think that the model may still be valid also in this case because I think that following repeated administrations, tyramine increases in the cytoplasm and then competes with noradrenaline for the exit through the carrier. This fact, this competition, may occur even if the concentration of noradrenaline in the nerve endings is not very much decreased.

CHAGAS

There is a limitation of the carrier.

RAITERI

Yes, there is a limitation in the carrier.

CHAGAS

Have you done any experiments on this?

RAITERI

No, we have done no experiments on this. This is a speculative model based on several experimental data, and in particular the following: (a) tyramine can be transported by the carrier; (b) tyramine cannot be stored in the vesicles and it can be accumulated in the cytoplasm, of course if you give large doses; (c) an exchange between noradrenaline and phenylethylamines has been demonstrated and (d) carrier blocker desipramine blocks these movements through the carrier. So these are the experimental data on which we built this model. Of course there may be explanations for tachyphylaxis, as you know. I think the oldest explanation was that in noradrenergic nerve endings there would be a limited tyramine-sensitive pool of noradrenaline and when this pool is empty, tachyphylaxis is reached. But this hypothesis has not received experimental support. I think that provided you give enough tyramine, practically all the noradrenaline contained in the nerve endings can be released. So I would not be in favor of a limited tyramine-sensitive pool of noradrenaline.

DOPAMINE DEPENDENT MODULATION OF cAMP LEVEL IN THE CHICK RETINA

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Abbreviations used

cAMP	Adenosine 3′, 5′-cyclic monophosphate
cGMP	Guanosine 3′, 5′-cyclic monophosphate
IBMX	3-isobutyl-1-methyl xantine
RO-20-1724	4-(3-butoxy-4-methoxybenzyl)-2-imidozolidinone
TCA	Trichloroacetic acid
CMF	Ca^{++} Mg^{++} free medium
BME	Basal medium of Eagle
Hepes	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid

ABSTRACT

The adenosine 3′, 5′-cyclic monophosphate (cAMP) level of chick embryonic retina changes during the course of development. In retinas from 6 to 15 day-old embryos the cAMP level is approximately 7 pmoles/mg protein. A sharp 3-fold increase is observed between the 16th and 18th embryonic day and remains constant thereafter. A dopamine-dependent increase in cAMP of the chick retina is already present in 7 day-old

embryos, and hy the 8th embryonic day maximal response is attained. Glutamate promotes a 2-fold stimulation. Carbachol, γ-aminobutyric acid and glycine do not cause any significant change in the level of cAMP of the embryonic tissue.

Guanosine 3′, 5′-cyclic monophosphate also accumulates during development. Its concentration is approximately 0,5 pmoles/mg protein from the 8th to the 14th embryonic day, then increases gradually until the 19th day of development when the level observed is approximately of 14 pmoles/mg protein.

Introduction

A variety of compounds have been implicated as possible neurotransmitters in the retina (Curtis and Johnston, 1974). Dopamine, localized predominantly in amacrine-like cells located in the junction of the inner nuclear and inner plexiform layers, is the most abundant catecholamine of this tissue (Ehinger and Flack, 1969; Haggendal and Malmfors, 1965; Nichols *et al.*, 1967). Several lines of evidence indicate that dopamine may be involved in the transmission of the visual information and suggest that it plays an inhibitory role in this tissue (Haggendal and Malmfors, 1965; Ames and Pollen, 1969; Straschill and Perwein, 1975).

The presence of a dopamine-sensitive adenylate cyclase in several parts of the brain has raised the hypothesis that dopamine modulates neurotransmission through the activation of that enzyme (Clement-Cormier *et al.*, 1974; Iversen, 1975). Likewise, in the retina, an adenylate cyclase activity sensitive to dopamine has been reported by Brown and Makman (1972) and further corroborated by other investigators (Bucher and Schorderet, 1975).

The retina is part of the central nervous system where relatively few cell types are organized in order to receive and partly transform the visual information from the outside world (Stell, 1972). This tissue is easily accessihle and can be obtained free from surrounding structures. Its morphological differentiation has been well documented thus providing an excellent model for the study of CNS differentiation, where biochemical and morphological parameters can be well correlated during retina ontogeny (Coulombre, 1955).

In the present study the appearance of a dopamine-sensitive adenylate cyclase during the ontogeny of chick retina was observed. Changes in the basal level of cAMP were also investigated in a tentative approach to

correlate the levels of this nucleotide with the formation of functional synaptic contact between dopamine containing neurons and post synaptic cells sensitive to this neurotransmitter.

Materials and methods

cAMP (Schwarz Mann, Orangeburg, New York), protein kinase, glycine, carbachol and GABA (Sigma Chemical Co., Saint Louis, Missouri), Basal Medium of Eagle (BME) (GIBCO, Grand Island, New York), dopamine (Calbiochem, Los Angeles, California), IBMX (Aldrich Chem. Co., Milwaukee, Wisconsin), RO 20-1724 (Hoffman La-Roche, Basel, Switzerland), Bovine serum albumin (Armour Pharmaceutical Co., Chicago, Illinois), pargyline (Regis Chem. Co., Chicago, Illinois), cGMP antiserum and (^{125}I)-succinyl-cGMP tyrosine methyl esther (Collaborative Research, Inc., Waltham, Massachusetts), (^{3}H)-cAMP (New England Nuclear Inc., Boston, Massachusetts) and (^{3}H)-cGMP (Amersham/Searle, Arlington Heights, Illinois) were used throughout the study. All other reagents were of analytical grade.

Fertilized white Leghorn eggs were obtained from Truslow Farms Inc., Chestertown, Maryland. Retina dissections were performed according to a published procedure (Moscona *et al.*, 1968) and embryos staged according to Hamburger and Hamilton (1951).

Incubation and assay procedures: Retinas were dissected in Ca^{++}, Mg^{++} free medium (CMF) and immediately transferred to cold CMF. After 3 to 5 min the CMF was discarded and the retinas resuspended in 2 ml of BME at 37°C containing 20 mM Hepes pH 7.3, 0,5 mM IBMX or RO 20-1724, 0.1 mM pargyline and 0.1 mM ascorbate.

After 10 min of preincubation at 37°C dopamine or other test compounds were added in a 100 µl volume to the final concentration for each experiment. The retinas were further incubated with gentle agitation for 5 min and the reaction stopped by the addition of 1 ml of 15% TCA. For the determination of the basal levels of cAMP and cGMP, the retinas were dissected in CMF as above and immediately transferred to 5% TCA.

The precipitated material was cooled down in an ice cold bath and briefly sonicated (2 sec) in a Raytheon sonic oscillator (Model DF 101). The suspension was sedimented by centrifugation at 17,000 rpm for 15 min in a Sorvall centrifuge (Model TC2B) and the supernatant was used for the purification of the cyclic nucleotides according to a published procedure (Matsuzawa and Nirenberg, 1975). cAMP was assayed by the

method of Gilman (1970) and cGMP by a modification of the radio-immunoassay method described by Steiner *et al.* (1972). The pellet was dissolved in 0.1 N NaOH and protein was assayed by the method of Lowry *et al.* (1951).

Results

The existence of a dopamine-sensitive adenylate cyclase in the retina of different species has been reported by Brown and Makman, 1972; Bucher and Schorderet, 1975; Makman *et al.*, 1975. Experiments were designed to see whether or not a dopamine-dependent increase in cAMP was also present in the chick embryonic retina. Fig. 1 shows the dose

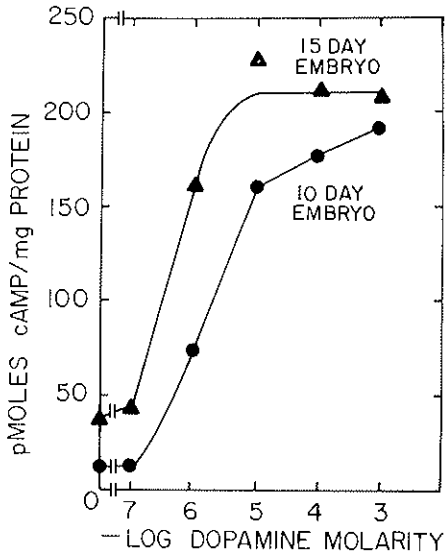

Fig. 1. Dopamine-dependent stimulation of cAMP level of chick embryonic retina. Effect of increasing concentrations of dopamine. Each point represents the mean value from at least 2 experiments. The values found in each individual experiment were within 10% of the values shown.

response curves obtained with retinas from 10 and 15 day-old embryos respectively. In both cases the cAMP level is stimulated by the amine. A concentration of 10^{-5}M produces maximal response. The retinal cGMP level is not affected by dopamine (unpublished observation). In one experiment using retina from 16 day-old embryo, 10^{-4}M dopamine promoted an elevation of cAMP content from 39.5 to 443 pmoles per mg of protein. Fluphenazine (10^{-5}M), a potent antagonist of Dopamine receptor reduced the effect observed with dopamine to 140 pmoles per mg of protein.

A more detailed study of the developmental appearance of the dopamine-dependent increase of cAMP in the embryonic retina is shown in Fig. 2. In retinas from 7 day-old embryos, 10^{-4}M dopamine produces a stimulation of cAMP formation that is equivalent to a five-fold increase above the basal level. By the 8th developmental day the maximal response to dopamine is obtained with a 15-to 20-fold increase in the levels of this cyclic nucleotide which remains fairly constant until the 16th embryonic day (last stage of development studied in this experiment). The basal

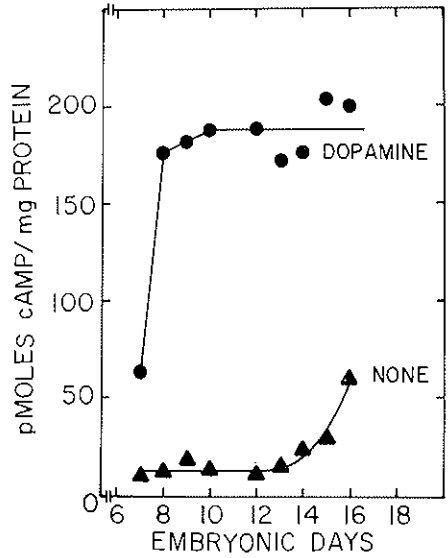

FIG. 2. Dopamine stimulation of cAMP levels in chick embryonic retina. The data represents mean values from at least 4 experiments. The values found in different experiments were within 10% of the values shown.

level of cAMP under these experimental conditions, i.e., in presence of a phosphodiesterase inhibitor, is approximately 10 pmoles per mg protein increasing to 70 pmoles per mg protein on the 16th embryonic day. Therefore, the net increase in cAMP content promoted by dopamine in retinas from 15 and 16 day-old embryos is relatively less than that measured in young embryos, although the maximal response is the same.

The basal levels of both cAMP and cGMP as a function of the developmental stage of the chick embryonic retina are shown in Fig. 3 A and 3 B respectively. The cAMP level in the retina of 7 day-old embryo is approximately 7 pmoles per mg protein. No significant change occurs until the 15th day of development. However, a sharp 3-fold increase in the cAMP content of the retina is observed between the 16th and 18th embryonic day. Similarly, the amount of cGMP in the retina does not

show any appreciable change in 8 to 15 day-old embryos, when the level is approximately 0.5 pmoles per mg protein. As opposed to a sharp increase in the cAMP levels, a gradual increase in cGMP content is observed after the 16th embryonic day, reaching levels as high as 14 pmoles per mg protein by the 19th day of development (Fig. 3 B).

Since the observed shift in the basal level of cAMP between the 16th and 18th days of development could be mediated by the maturation of dopaminergic circuitry in the retina, the experiment shown in Table 1 was performed to test such possibility. Fertilized eggs in a stage equivalent to 15 day-old embryos were injected with 625 μg of fluphenazine and returned to the incubator where they remained until the 18th day of development. The embryos were then inspected for their develop-

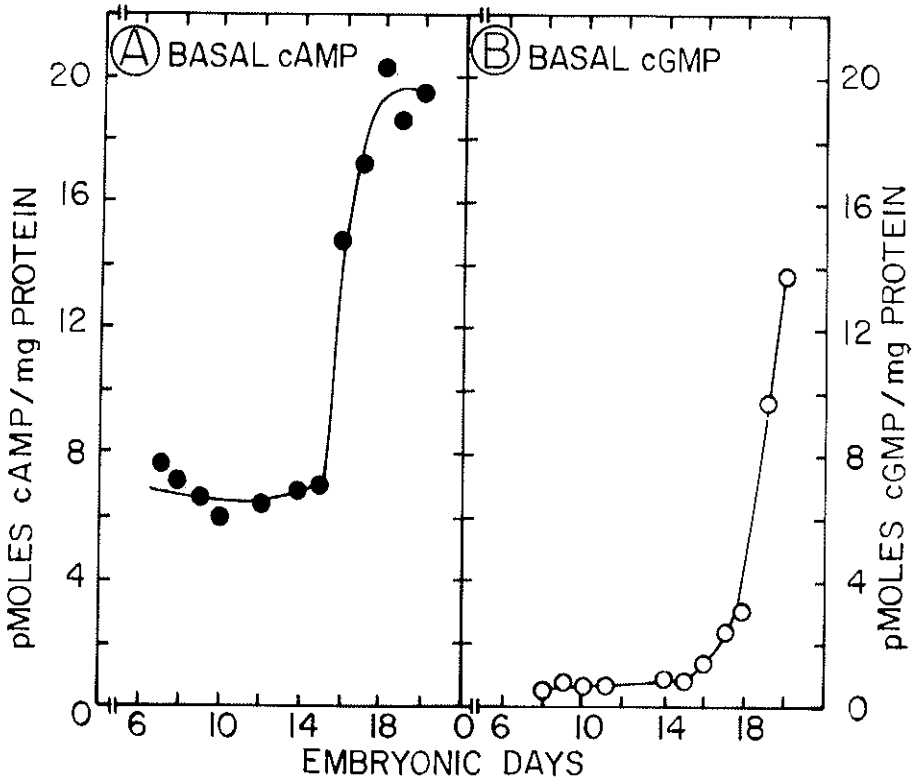

FIG. 3. Developmental profile of the basal levels of cAMP and cGMP in the chick embryonic retina. The data of Fig. 3A represents the mean value from at least 4 different experiments. For Fig. 3B each point represents the mean value from 2 experiments. In both cases the values found in different experiments were within 5 to 10% of the values shown.

mental characteristics and no difference was seen between control and fluphenazine treated embryos. The retinas were dissected and the basal level of cAMP was determined.

As can be seen in Table 1, the treatment of the embryos by fluphenazine fully prevented the rise of the cAMP contents of the retina normally observed in the 18 day-old embryos.

The possibility that the observed increase in retinal cAMP upon dopamine stimulation could be mediated hy the release in this tissue of other putative neurotransmitters led us to examine the effect of different

TABLE 1. *Effect of Fluphenazine on the basal level of cAMP of the chick embryonic retina*

Treatment on day 15	Embryo age when cAMP was determined (days)	pMoles cAMP mg prot^{-1}	% of control
—	15	11.80	100
Fluphenazine (625 µg/egg)	18	11.06	93,7
Control	18	25.60	217

Fertilized eggs in a stage equivalent to 15 days of development were injected with 625 µg of fluphenazine (Anatensol) and returned to the incubator where they remained until the 18th day of development. Then the embryos were staged and the retinas removed for cAMP determination as described in methods. Each point represents the average of two independent experiments in which at least 4 retinas were pooled for each determination. The values found in each experiment were within 10% of the values shown.

compounds upon the cAMP content of the retina. Tissues from 15 day-old embryos were used in those experiments, although 16 and 17 day-old retinas gave similar results. As can be seen in Table 2, dopamine is the only compound to produce a marked elevation of cAMP in the embryonic retina.

The high concentrations of glutamate, carbachol, GABA and glycine used in these experiments were chosen to assure saturating concentrations of these compounds in relation to the eventual presence of their respective receptors.

Extending our studies, to the post-hatching period, we have observed a decrease in the relative sensitivity of retinal tissue to dopamine, when

TABLE 2. *Effect of several putative neurotransmitters of the retina on the cAMP level of chick embryonic tissue*

Addition	mM	pMOLS cAMP/mg Protein
Control	—	39.00 ± 3.74 (7)
Dopamine	0.1	220.00 ± 17.60 (5)
Glutamate	5.0	63.00 ± 5.50 (3)
Carbachol	1.0	52.00 ± 4.13 (3)
Gaba	2.0	48.00 ± 6.90 (4)
Glycine	2.0	44.00 ± 4.15

In all experiments retinas from 15 day-old embryos were used. Identical results were observed with tissue from 16 and 17 day-old embryos.
The values represent the average ± standard error of the number of experiments shown in the parentheses. For glycine the values refer to two different experiments.

TABLE 3. *The effect of Dopamine and Fluphenazine on light and dark adapted retinas*

Animal Treatment (From day 2 to 8-post Hatch)	Additions in the incubation medium	pMoles cAMP/retina Light adapted	Dark adapted
Control	None	178.0 ± 7.0 (2)	136.9 ± 19.1 (3)
	Dopamine (10^{-4}M)	259 ± 44 (2)	516.0 ± 77.4 (3)
Fluphenazine (180 μg/animal/day)	None	681.0 ± 78.0 (2)	148.2 ± 2.5 (3)
	Dopamine (10^{-4}M)	709.0 ± 46.0 (2)	736.0 ± 5.3 (4)

Two day-old chicks were daily injected with either saline or Fluphenazine (180 μg/animal) for 6 days. One group of animals was kept under constant illumination during the treatment while the other was kept in the dark. The retinas were dissected out, washed twice in CMF and incubated in BME for 10 min, then dopamine was added and the retinas were further incubated for 5 min. TCA was then added to 5% final concentration, and cAMP was measured as indicated in "Methods".

compared to embryonic stages. The experiment shown in Table 3 was conducted to assess the possihility that the lower level attained by cAMP upon dopamine stimulation in retinas of 8 day-old chicks, could he due to a desensitization of the dopaminergic system of the retina.

Two groups of animals were injected daily with fluphenazine (180 μg/animal) for a period of 6 days. One group was kept in the dark during the treatment while the other was continuously exposed to light. The retinas were dissected out, washed twice in CMF and incubated in BME at 37°C for 10 min (see Methods). Dopamine (10^{-4}M) was added to the incubation medium as indicated in the Table and the retinas were further incubated for 5 min. TCA was added to a 5% final concentration and cAMP was measured as described in "Methods".

The addition of dopamine in the retinas of light adapted chicks promoted a 45% increase in retinal cAMP above control values. However, the treatment of light adapted chicks with fluphenazine was sufficient to promote a 4 fold increase in the cAMP content of the retinas incuhated in BME with no additions. In the last case, when dopamine was added to the incubation medium, no further increase in the level of cAMP was observed. In both cases, the cAMP level of fluphenazine-treated animals was 4 fold higher than in retinas of non treated chicks.

As opposed to the light adapted animals, the chicks deprived of light during fluphenazine treatment showed a different pattern of response to dopamine. The basal level of their retinal cAMP content was the same as those of animals which were exposed to light. However, the retinas from dark adapted chicks responded to dopamine increasing their cAMP level by a factor of 3.8 as compared to 1.45 in the light adapted retinas. The cAMP content of dark adapted retinas of fluphenazine treated animals, after incubation in control medium, did not differ from the basal level observed in retinas of non treated animals. However, the addition of 10^{-4}M dopamine to dark adapted retinas of fluphenazine-treated chicks promoted an elevation of cAMP content comparable to that observed in retinas of light adapted chicks.

Discussion

Dopamine-induced modifications of electrical activity of ganglion cells have been reported in isolated rabhit retina (Ames and Pollen, 1969). Electrophysiological studies in the cat retina have shown that spontaneous activity of the majority of retinal ganglion neurons is suppressed by iontophoretic application of this amine (Straschill and Perwein, 1975).

During morphological differentiation of chick retina, ganglion cells withdraw from the cell cycle between the 3rd and 8th embryonic day (Fujita and Horii, 1963; Kahn, 1973). Synaptic contacts however are not seen in the tissue until the 13th day of development (Hughes and La Velle, 1974; Meller, 1964; Sheffield and Fischman, 1970).

Our results extend to an avian species the findings of Makman *et al.* (1975) and Lolley *et al.* (1974) on dopamine dependent adenylate cyclase development in the rat and mouse retina respectively. They also provide additional evidence for a functional role of this neurotransmitter in this tissue. The dopamine dependent increase of cAMP content in the retina is already present in 7 day-old embryos. By the 8th day of development, a stage still immature in terms of synaptogenesis, the response to dopamine is maximal and does not change throughout the developmental period studied. Coyle and Campochiaro (1976) have shown that dopamine-sensitive adenylate cyclase is present in newborn rat striatum homogenates and that the developmental rise in the activity of dopamine-sensitive adenylate cyclase precedes the development of the presynaptic component of the striatum activity.

Our results, although not conclusive, constitute evidence that a cell type sensitive to dopamine appears between the 7th and 8th embryonic day, suggesting that some ganglion cells may be responsible for this response. The possibility that Muller cells could be involved in the sensitivity of the retina to dopamine should also be considered, since Coulombre (1955) has reported the presence of these cells in retina of 3 day-old embryos.

The fact that dopamine can stimulate the increase of retinal cAMP before synaptic contacts are formed indicate a direct effect of the amine rather than an effect mediated by synaptic communication. The lack of response to other neurotransmitters studied in this work strengthens this suggestion.

Contrary to what was observed with the differentiation of dopamine-dependent adenylate cyclase system, the basal level of cAMP is low from the 6th to the 15th embryonic day increasing 3 fold between the 16th and 18th days of development. The levels of cAMP attained by this stage is only 11% of those observed upon maximal stimulation by dopamine, possibly reflecting a steady state concentration of this cyclic nucleotide. However, the values observed for the basal level of cAMP, may not necessarily reflect the actual values of the retina *in vivo*. The experimental manipulation of the tissue could, to some extent, affect the metabolism of this nucleotide.

Since the cAMP phosphodiesterase of chick retina reportedly does not show any significant change during the course of embryonic differentiation (Chader *et al.*, 1974), the shift ohserved in the basal level of cAMP after the 15th day of development suggests that these changes are due to the formation of synaptic contacts between dopamine-containing neurons (probably amacrine cells) and cells with a dopamine-dependent adenylate cyclase system (probably ganglion neurons). The results shown in Table 1 are consistent with this hypothesis. Fluphenazine, a specific blocker of dopamine receptor, injected into fertilized eggs prior to the stage when cAMP content is shifted to a higher level, fully prevented the increase in the cAMP content of the retina normally observed on the 18th day of development. This effect seems to be specific since no anatomic alteration has been noticed within the period studied. Reinforcing this suggestion is the fact that the cyclic AMP content measured in retinas of up to 15 day-old embryos in presence of phosphodiesterase inhibitor does not differ significantly from those measured in the steady state, i.e., in absence of phosphodiesterase inhibitor (7 pmoles/mg protein as compared to 10 pmoles/mg protein in presence of either IBMX or RO 20-1724). However, after the 15th embryonic day (time of appearance of the first synaptic contacts in this tissue), the presence of phosphodiesterase inhibitors alone causes an increase in the basal level of cAMP, reaching 40% of the dopamine stimulated levels. Thus, the inhibitors alter the steady state of these cells favoring a higher cAMP content prohably due to adenylate cyclase activity alone, activated by the endogenous dopamine output from impinging synaptic contacts onto the receptive cell.

cGMP has been implicated as playing a role in the photoreceptor function of bovine and frog retinas (Virmaux *et al.*, 1976; Fletcher and Chader, 1976). Extraordinarily high guanylate cyclase activity is found in dark adapted rod outer segments (Pannbacker, 1973). The morphological differentiation of the photoreceptor organelle sensitive to light begins in retinas of 15 day-old chick embryos reaching a highly differentiated state by the 19th embryonic day (Mason and Bighouse, 1975). The results reported above, concerning the developmental appearance of cGMP in the chick retina, is in agreement with the morphological differentiation of the photoreceptor outer segment and supports the idea that this nucleotide is predominantly localized in this organelle.

The lack of effect of dopamine upon cGMP level of the retina (unpublished results), reflects a high degree of specificity with which this amine interacts with the adenylate cyclase system of this tissue.

The lower level attained by cAMP upon dopamine stimulation of retinas from 8 day-old chicken, in fact may reflect a process of desensitization of the post-synaptic component of the dopaminergic system of this tissue. Similar observation has been reported in the rat retina by Makman *et al.* (1975). In our case the desensitized state can be reverted to a sensitive one, comparable to that observed in embryonic stages. The fact that light deprivation as well as chronic treatment by fluphenazine were efficient in promoting the resensitization of the retinal tissue to dopamine suggests that in both cases dopamine interaction with its receptor was hampered.

Further work is required to characterize the cell types involved in the dopamine sensitivity of chick retina and also to characterize the role of the dopaminergic system of the retina in the process of dark adaptation.

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DISCUSSION

HAMPRECHT

In respect to your last remark, did you do dose-response curves to see whether the sensitivity really changed or whether maximal response changes?

DE MELLO

No, I just did maximal response measurements.

NIRENBERG

Why should in the dark the levels of cyclic AMP be low relative to the light?

DE MELLO

I think that the high level of cAMP observed in the light adapted retinas, after fluphenazine treatment could be explained by an increased release of dopamine as opposed to the dark adapted tissue.

The fact that the dark adapted retinas do not show an increase in the cAMP level during the 15 minutes assay period suggests that the dopamine receptors are not beeing exposed to saturating concentrations of dopamine, which is likely to be the case in the light adapted tissue.

PAOLETTI

Dr. Spahr in my laboratory has carried out similar experiments on fetal retina with very similar results. In addition he has found that LSD is able to activate the dopamine sensitive adenylate cyclase in the retina. This of course may have something to do with the clinical observation that LSD induces purely visual hallucinations.

HÖKFELT

I know that Dr. Ehinger in Lund has been very interested in the possible existence of serotonin cells in the amacrine cells. Have you possibly looked for an effect of serotonin on cyclic AMP?

DE MELLO

No, I have not.

BERLUCCHI

Do you have any behavior correlates of the effects of fluphenazine on the retina?

DE MELLO

As far as I can say by the simple inspection of the chicks, I could not detect any significant behavioral differences between control and fluphenazine-treated animals. In fact the chicks are quite resistant to this drug. One hundred fold less fluphenazine injected into mice, for instance, kills the animals in 15 minutes.

The lack of effect of fluphenazine on the chicks' behavior in our experiments, may be explained by this relative resistance to this drug.

BERLUCCHI

Do you have any evidence that the retinal effect of fluphenazine has some consequences for visually guided behavior?

DE MELLO

Again, by simply observing the animals I could not detect changes in their capability of looking for food or even in their capability of orienting themselves in the cage where they were kept. I do not believe these chicks had their vision accuracy significantly influenced by fluphenazine treatment.

BURNSTOCK

I do not want to overstress purinergic nerves, but of course adenosine is a very potent inducer of cyclic AMP. Have you considered that at all?

DE MELLO

Yes, I have looked for a possible effect of adenosine upon the cAMP level of embryonic retina in early development stages, when dopamine response is already present. No significant increase or decrease in the cAMP level was observed. I must however extend this study to retinas of older embryos in order to rule out the possibility of an adenosine-cyclase coupled system in this tissue.

GREENE

How do your findings compare with the appearance of dopamine in the retina?

DE MELLO

I do not know yet. I could detect measurable quantities of dopamine in the post-hatch period. In the embryonic tissue however the amount of dopamine seems to be too small to be detected by the method of assay I employed in those analyses. I must look for an improvement in the method of dopamine assay in order to look into this problem in detail.

HAMPRECHT

How quickly does this response change if you switch from light to dark or the reverse?

DE MELLO

These experiments were carried out in a six-day period. We do know however, that 24 hours treatment by fluphenazine is sufficient to elicit retinal hypersensitivity to dopamine. Concerning the light effect I am not sure yet about the temporal requirement of darkness to promote the observed increased response to dopamine. However, a period of darkness shorter than 12 hours does not lead the retinas to the hypersensitive state.

HAMPRECHT

But you have not tried application of light in the range of minutes?

DE MELLO

No, I have not.

ENKEPHALINS: RECEPTORS, BIOSYNTHESIS AND RELEASE

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There are many problems which have to be considered in the investigation of new putative neurotransmitters or neuromodulators. Three particularly important aspects appear to be the nature of their receptors, their biosynthesis and their release.

Receptors of the opioid peptides

When the problem of multiple receptors arises, several experimental approaches can be used. The first method is that of multiple parallel assays which is indirect but can give reliable information (Lord, Waterfield, Hughes and Kosterlitz, 1977). The second depends on the specificity of antagonists, a method which has been so useful for characterization of the α- and β-adrenoceptors and their variants and also of the muscarinic and nicotinic acetylcholine receptors. Lord *et al.* (1977) used four parallel assays, the guinea-pig ileum and mouse vas deferens as pharmacological models and the inhibition of (^{3}H)-naloxone or (^{3}H)-naltrexone binding and (^{3}H)-leucine-enkephalin binding in brain homogenates. The results of these parallel assays led to the conclusion that in the brain there are at least two types of receptors for the opioid peptides, namely the μ-receptors which represent the preferential binding sites for naloxone, naltrexone or morphine, and the δ-receptors which represent the preferential binding sites for the enkephalins. Of the two pharmacological models, the guinea-pig ileum has mainly μ-receptors and the mouse vas deferens mainly δ-receptors. This interpretation was strongly supported by the fact that, in the mouse vas deferens, the opioid peptides are difficult to antagonise by

naloxone in that they require a concentration about 10 times higher than that needed for morphine. In the guinea-pig ileum the enkephalins are less potent than in the mouse vas deferens but they do not require more naloxone for antagonism than morphine. This observation also indicated that the enkephalins can interact with the μ-receptors for which they have a lower affinity than for the δ-receptors.

Another approach is the determination of the maximum number of binding sites for a given ligand (Gillan, Kosterlitz and Paterson, unpublished observations). There is apparently only one type of binding site for D-Ala^2-D-Leu^5-enkephalin in guinea-pig brain homogenate. At this site, the ligand has a K_D of 1.27 ± 0.16 nM ($n=5$) and the number of binding sites corresponds to 7.4 ± 0.31 pmol/g wet wt. ($n=5$). Under the same conditions, only one type of binding site was detected for D-Ala^2-leucine-enkephalin amide or D-Ala^2-methionine-enkephalin amide. The corresponding K_D values were: 2.52 ± 0.48 and 1.96 ± 0.17 nM ($n=5$) and the numbers of binding sites were 12.4 ± 0.93 and 12.8 ± 2.22 pmol/g wet wt. ($n=5$). Etorphine was found to have a similar number of binding sites (15.4 ± 2.4 pmol/g wet wt. [$n=3$]) but a higher affinity (0.37 ± 0.03 nM [$n=3$]) than the D-Ala^2-enkephalin amide. However, with both morphine and dihydromorphine high and low affinity binding sites were found; the total numbers of binding sites were 3.7 ± 0.59 and 4.3 ± 0.36 pmol/g wet wt. ($n=4$), respectively.

Fragments of β-endorphin

As far as the long-chain peptides are concerned, β-endorphin has been shown to be equipotent in depressing the contractions of the guinea-pig ileum and those of the mouse vas deferens and is also equiactive in inhibiting the binding of the three ligands, (^{3}H)-naltrexone, (^{3}H)-dihydromorphine and (^{3}H)-leucine-enkephalin in homogenates of guinea-pig brain (Kosterlitz and Hughes, 1978).

When fragments of β-endorphin were assayed in a similar manner, it was found that in the guinea-pig ileum and for the inhibition of (^{3}H)-naloxone and (^{3}H)-dihydromorphine binding in homogenates of guinea-pig brain, β-endorphin shows the highest potency, to be followed by methionine-enkephalin, LPH_{61-87}, LPH_{61-79} and LPH_{61-77}, in this order. In the mouse vas deferens and for the inhibition of (^{3}H)-leucine-enkephalin binding, methionine-enkephalin is more potent than any of the other

fragments, including β-endorphin (LPH_{61-91}). It may be of physiological significance that the long-chain peptide LPH_{61-91} has similar potencies in different assay systems whereas the short-chain peptide is more differentiated as far as its affinity to different receptors is concerned (Kosterlitz and Hughes, 1978).

Enkephalin analogues

Since the biological half-time of the two naturally occurring enkephalins is very short, many attempts have been made to design stable analogues with strong antinociceptive activity. It is therefore important to know which alterations in the molecule are permissible without concomitant changes in the pattern of pharmacological activity (Kosterlitz, McKnight, Waterfield, Gillan and Paterson, 1978). The replacement of Gly^2 by D-Ala in leucine-enkephalin increases the potencies in both guinea-pig ileum and mouse vas deferens by factors of 14 and 7, respectively, without altering significantly the affinities to the (^{3}H)-naltrexone and (^{3}H)-leucine-enkephalin binding sites. This effect is most likely due to a decrease in the enzymatic degradation of the peptide in the pharmacological models since the binding assays were carried out at 0-4°C. Replacement of L-Leu by D-Leu increased activity in the mouse vas deferens without a major change in the affinity to the binding sites; on the other hand, the activity in the guinea-pig ileum was reduced somewhat. The pharmacological pattern was still of the type characteristic of leucine-enkephalin, perhaps even to an exaggerated extent: the peptide was much more potent in the mouse vas deferens than in the guinea-pig ileum and the affinity for the (^{3}H)-leucine-enkephalin binding site was much higher than that for the (^{3}H)-naloxone binding site. This peptide has been shown by Baxter, Goff, Miller and Saunders (1977) to have antinociceptive activity after injection into the cerebral ventricles. When the C-terminal leucine was replaced by amides of proline, the most important change was an increase in the activity in the guinea-pig ileum; there was also an increase in the affinity for the (^{3}H)-naltrexone binding site with a simultaneous loss in affinity for the (^{3}H)-leucine-enkephalin binding site. Both compounds have antinociceptive activity after intravenous and subcutaneous injection as shown by Székely, Rónai, Dunai-Kovács, Miglécz, Bertzétri, Bajusz and Gráf (1977). Another analogue, which is a powerful antinociceptive agent and shows activity even after oral administration as demonstrated by Roemer, Buescher, Hill, Pless, Bauer, Cardinaux, Closse, Hauser and Huguenin (1977), is Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol.

This compound shows a further shift in relative activities in favour of the guinea-pig ileum and the (^{3}H)-naltrexone binding sites. Its pharmacological pattern is very different from that of methionine-enkephalin and has become more similar to that of morphine which, however, has a much lower overall activity than the enkephalin analogue.

Alterations at the two terminal amino acid residues have considerable effects on the pharmacological pattern of the enkephalins. When the C-terminal leucine of D-Ala^2-leucine-enkephalin is decarboxylated, the relative potency is increased in the guinea-pig ileum and markedly decreased in the mouse vas deferens while the affinity for the naltrexone binding site is improved and that for the enkephalin binding site diminished. When now a methyl group is introduced in the amino group of tyrosine, the potencies are decreased in all four assay systems but not to the same extent, those for the mouse vas deferens and the leucine-enkephalin binding site being particularly affected. In other words, the free carboxylic group at the C-terminus and the primary amino group at the N-terminus would appear to be essential for the maintenance of an enkephalin-like pharmacological pattern. When the leucine residue is removed, the resulting tetrapeptide is less active than the parent compound; the loss in activity is again much more pronounced for the mouse vas deferens and the leucine-enkephalin binding site, showing the importance of Leu^5 or Met^5 for the enkephalin-like properties of the pentapeptides (Kosterlitz *et al.*, 1978).

Antagonist action of naloxone against different enkephalin analogues

It has been stressed (Lord *et al.*, 1977) that the low effectiveness of naloxone against the action of the naturally occurring opioid peptides in the mouse vas deferens is strong supporting evidence for the view that the δ-receptors of the mouse vas deferens are different from the μ-receptors, with which the classical opiates interact. However, the opioid peptides can also interact with μ-receptors which appear to be preponderant in the guinea-pig ileum where naloxome is equally effective against the opioid peptides and morphine. If this concept is correct, then naloxone should be a weaker antagonist in the mouse vas deferens against enkephalin analogues which retain their enkephalin-like pharmacological pattern, as for instance Tyr-D-Ala-Gly-Phe-D-Leu ($K_e = 32$ nM), than against enkephalin analogues which are more morphine-like, as for

instance NCH_3Tyr-D-Ala-Gly-$NH(CH_2)_2$Ph (K_e=6.3 nM) or Tyr-D-Ala-Gly-NCH_3Phe-Met(O)-ol (K_e=5.7 nM). The values obtained so far are compatible with this concept.

Receptors and function

Little is known about the functions that are mediated by the various receptors. However, it has been shown that, after injection into the cerebral ventricles of rats, D-Ala^2-D-Leu^5-enkephalin (Wellcome) which has a high affinity to δ-receptors has only 1% of the antinociceptive activity of Tyr-D-Ala^2-Gly-MePhe-Met(O)-ol (Sandoz) (Bläsig and Herz, 1978), whose affinity to the μ-receptors is as high as that of the Wellcome analogue to the δ-receptor. As a corollary, the affinity of the Sandoz compound to the δ-receptors is as low as that of the Wellcome compound to the μ-receptors (Kosterlitz *et al.*, 1978). It is therefore possible that the μ-receptors are more important for antinociceptive effects than the δ-receptors. β-Endorphin may owe its high antinociceptive potency to the fact that it binds equally well to μ-receptors and δ-receptors. This interpretation would, at least to some extent, explain the difficulty experienced by many observers to show that naloxone has a hyperalgesic effect in normal animals.

While this hypothesis is attractive, a note of caution has to be sounded. We know so little of the pharmacokinetics of opioid peptides after intraventricular injection that differences in the rate of diffusion to the areas responsible for antinociceptive action or of catabolism cannot be excluded. As far as penetration of the blood-brain barrier after intravenous or oral administration is concerned, no reliable measurements are available. Observations by von Graffenried, del Pozo, Roubicek, Krebs, Pöldinger, Burmeister and Kerp (1978) and von Graffenried, del Pozo and Roubicek (1978) with Tyr-D-Ala-Gly-MePhe-Met(O)-ol in human volunteers have shown that after intramuscular injection a number of side-effects occur, which may be at least partly due to a peripheral action of the analogue. Von Graffenried, del Pozo and Roubicek (1978) have postulated that "low doses of the analogue have access primarily to peripheral receptors with muscular, gastrointestinal and vascular symptoms predominating. Central clinical effects might only appear in higher doses because of the low ability of the drug to penetrate into the brain. For morphine, however, central effects predominate and limit the application of higher doses which might be necessary to stimulate peripheral receptors". If this interpretation is correct, it is intriguing that relatively

large doses of naloxone were required to reverse the peripheral side-effects of the Sandoz analogue, an observation which could indicate that the peripheral actions were mediated by δ-receptors.

Biosynthesis of enkephalins

This paper will not deal with the problem of the biosynthesis of the long-chain peptide, β-endorphin. This is derived from β-lipotropin which in turn has a common precursor of 31,000 daltons with ACTH (Mains, Eipper and Ling, 1977).

Since the sequence of methionine-enkephalin is present in β-endorphin, it was originally proposed that β-endorphin may be the precursor of methionine-enkephalin. There are, however, observations which do not support this view. First, immunohistochemical investigations have so far failed to demonstrate co-existence of β-endorphin and methionine-enkephalin in the same neurone (Watson, Akil, Richard and Barchas, 1978). Second, in spite of several attempts, the leucine^5-analogue of β-endorphin has not been found, at least not in the pituitary (Seidah, Gianoulakis, Crine, Lis, Benjannet, Routhier and Chrétien, 1978).

Several groups have attacked the problem of enkephalin biosynthesis by studying the incorporation of radio-isotopically labelled amino acids into the enkephalins after *in vivo* administration. From the first study of this type, Clouet and Ratner (1976) concluded that there was a rapid incorporation into the enkephalins of rat brain 15-30 min after intracisternal administration of (^{3}H)-glycine and that the turnover time was less than 3 h. In contrast, Sosa, McKnight, Hughes and Kosterlitz (1977) reported that maximum incorporation of (^{3}H)-tyrosine into enkephalins of rat brain occurred in less than one hour but remained constant for 16 h after intracisternal administration. More recently, Yang, Hong, Fratta and Costa (1978) reported that the maximum incorporation of (^{3}H)-glycine into rat striatal enkephalins after intracerebroventricular administration was reached after 15 min while the incorporation of (^{3}H)-tyrosine into whole brain minus striatum and cerebellum was reached within 30 min and remained constant for at least 2 h. These methods are somewhat limited in their usefulness because the degree of labelling of enkephalins is usually small and variable. To overcome these problems, we have recently turned our attention to the use of two *in vitro* systems for the study of enkephalin biosynthesis: the myenteric plexus-longitudinal muscle from the guinea-pig ileum and striatal slices from guinea-pig brain (Sosa *et al.*, 1977; Hughes, Kosterlitz and McKnight, 1978).

After an initial "labelling period" in Krebs-bicarbonate solution at 36°C, saturated with 5% CO_2 in oxygen, containing the appropriate labelled amino acid (L-[2,3,5,6-^{3}H]-Tyr, 80 Ci/mmol, L-[4,5-^{3}H]-Leu, 20 Ci/mmol or L-[^{35}S]-Met, 800-1200 Ci/mmol; Radiochemical Centre, Amersham), incubation was continued in fresh medium lacking the labelled amino acid but containing a high concentration (1 μg/ml) of a mixture of cold amino acids (Ala, Arg, Asp, Cys, Glu, Gly, His, Ileu, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val). At the end of the appropriate post-labelling incubation time, tissues were homogenised in 15-20 ml of ice-cold 0.1 M HCl containing synthetic carrier enkephalins (20-40 μg of each of methionine- and leucine-enkephalin). The homogenates were centrifuged at 50,000 *g* for 20 min and the enkephalins were isolated from the supernatants by progressive chromatographic purification by adsorption onto Amberlite XAD-2, eluting with methanol, cation exchange on HC-Pellionex-SCX, desalting the enkephalin-containing fraction from the cation exchange column by a second XAD-2 adsorption step and anion exchange on AE-Pellionex-SAX (Sosa *et al.*, 1977). Finally, resolution of two enkephalins was achieved by thin layer chromatography on silica gel plates developed with ethyl acetate/pyridine/water/acetic acid (100:43:25:11) containing either ethane-1,2-dithiol 0.01% (v/v) or dithiothreitol 0.01% (w/v) to minimize oxidation of the sulphur of methionine. The spots corresponding to the enkephalins were located for elution with 1 ml of 80% (v/v) methanol and subsequent scintillation counting by spraying the plates with ninhydrin-cadmium acetate reagent.

Using these methods, reproducible values for incorporation of labelled amino acids into enkephalins have been obtained in both tissues (Sosa *et al.*, 1977; Hughes *et al.*, 1978). In preliminary experiments with the myenteric plexus, it was found that (^{3}H)-Tyr was incorporated into both methionine- and leucine-enkephalin while (^{35}S)-Met was incorporated only into methionine-enkephalin and (^{3}H)-Leu only into leucine-enkephalin. With regard to the time course of incorporation it was found that the incorporation of (^{3}H)-Tyr into methionine-enkephalin is always at least twice that into leucine-enkephalin, which probably only reflects the higher content of methionine-enkephalin in both the myenteric plexus and the striatum. Tbe rate of incorporation increases linearly in both tissues after an apparent lag phase. This lag is more obvious with the striatum, which may be a result of the shorter labelling time in this tissue, or may be due to an impaired condition of the slices, compared to the more robust myenteric plexus preparation. Since there is no lag in the incorporation of (^{3}H)-Tyr into the tissue proteins, it is likely

that this lag is due to a delay in the formation of the as yet unknown precursors or the conversion of the precursors to the enkephalins.

It is probable that the incorporation of (^{3}H)-Tyr into enkephalin involves ribosomal synthesis since both puromycin and cycloheximide (both 0.1 mM) will inhibit incorporation by more than 90% in the myenteric plexus if they are present throughout the incubations, but hardly affect incorporation at all if they are present only during the post-labelling incubation period. Similar experiments with the striatal slices have shown that incorporation of (^{3}H)-Tyr can be blocked if cycloheximide is present during the labelling period of 30 min and also the first 2 h of a 5 h-incubation (the "lag phase"), but not at all if the protein synthesis inhibitor is present only during the latter 3 h of the 5 h-incubation. Thus, the enkephalins of guinea-pig ileum and striatum can be synthesized locally from a precursor or precursor also produced locally and that the synthetic pathway involves ribosomal assembly of the precursor peptide and conversion to enkephalin from this precursor. Since the rate of incorporation of (^{3}H)-Tyr into enkephalins remains constant for a long time, particularly in the myenteric plexus, it may be that there is a large pool of rapidly synthesized precursor which is only comparatively slowly converted to enkephalin.

Release of the enkephalins

Direct evidence for the release of the enkephalins is difficult to obtain because tissue peptidases rapidly degrade the pentapeptides. It has been shown that the K^+-induced release from rat brain synaptosomes (Smith, Hughes, Kosterlitz and Sosa, 1976), rabbit striatal synaptosomes (Henderson, Hughes and Kosterlitz, 1978) and from brain slices (Iversen, Iversen, Bloom, Vargo and Guillemin, 1978), is calcium-dependent and that veratridine-induced release from guinea-pig striatal slices is blocked by tetrodotoxin. In these experiments, the preparations were exposed to the depolarizing effect of veratridine for varying times and the amount of enkephalin released during these exposures, estimated by bioassay on the mouse vas deferens, ranged from 1-6% of the total tissue contents.

The use of the myenteric plexus-longitudinal muscle preparation of guinea-pig ileum was expected to yield data for release of enkephalins by more physiological means, i.e. after field stimulation of intramural nerves at various frequencies. Because in our laboratory no consistent results could be obtained by estimation of enkephalins in the superfusate, the following indirect method was used (Hughes *et al.*, 1978). The rationale

was that, in the presence of a protein synthesis inhibitor, any enkephalin mobilized from the tissue stores by nerve stimulation will not be replaced by *de nouveau* synthesis, and a measure of the tissue content at the end of the stimulation period compared to an unstimulated control, will provide an indication of the amount of enkephalin released. Preparations of myenteric plexus-longitudinal muscle were set up as described in Krehs solution containing 1 μg/ml of each of the amino acids listed in the section on biosynthesis and also 20 μg/ml ascorbic acid. After 30 min preincuhation, cycloheximide (0.1 mM) was added and the incubation was continued for a further 30 min when field stimulation (0.5 ms, supramaximal voltage) was started. Stimulation at all frequencies was continuous for periods of 0.5 to 4 h and during this time the bathing fluid was replaced by overflow every 20 min. At the end of the stimulation period, tissues were homogenised in 0.1 M HCl and centrifuged and enkephalins were extracted from the supernatants by adsorption onto XAD-2 and elution with methanol. The total tissue contents of enkephalins were measured by bracket assay on the mouse vas deferens. Stimulation at 10 Hz reduced the content by 18% in 30 min and by 46% in 1 h; but continuing stimulation at this frequency for up to 2 h produced no further decrease. Stimulation at 1 Hz for 2 and 4 h reduced the content by 5 and 14%, respectively, while with 0.1 Hz no reduction was observed even after 4 h. These reductions in enkephalin content were due to loss from the tissue stores after stimulation-evoked release by the finding that tetrodotoxin (0.3 μM) blocked the decrease caused by stimulation at 10 Hz for 1 h. From these results it follows that the amount of enkephalin released per pulse during stimulation at 1 and 10 Hz is about 4-5 fmol/g (fractional release of the order of 10^{-5}). This estimate may be too low since 0.1 mM cycloheximide may not block synthesis completely and since the store of precursor may be relatively large. In experiments without cycloheximide it was possible to assess whether *de nouveau* synthesis of enkephalin could offset the loss due to release. The difference between corresponding values for enkephalin contents obtained in the presence and in the absnce of cycloheximide would give an indication of tbe turnover of enkephalins during stimulation at a given frequency. Thus, the maximum turnover occurs after stimulation at 1 Hz for 2 h. Since there is an increase in content after stimulation in the absence of cycloheximide of about 340 pmol/g and in the corresponding experiment with cycloheximide present the content was decreased by 28.8 pmol/g, it follows that the turnover during 2 h was about 370 pmol enkephalin/g tissue. The increase in the enkephalin content

after stimulation at 1 Hz for 2 h in the absence of cycloheximide indicates that the increased enkephalin synthesis overcompensates for the loss due to release. On the other hand, stimulation at 10 Hz for the same duration produces a reduction (36%), i.e. only 6% less than that observed in the presence of cycloheximide. These findings suggest that the maximum rate of enkephalin synthesis cannot match the amount of peptide released.

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DISCUSSION

FAMBROUGH

You mentioned the rapid breakdown of enkephalins in your preparation after release. Do you know anything about the mechanism of breakdown?

KOSTERLITZ

That is a very interesting point. The early investigations were done by Dr. Rance and his colleagues, who thought that aminopeptidases were mainly responsible for the degradation at the N-terminal. They knew that carboxypeptidase would act at the C-terminal but to a lesser extent. Recent work by the group of Dr. Schwartz in Paris has shown that a membrane-bound enkephalinase may be a more specific enzyme, cleaving the enkephalins between Gly^3 and Phe^4.

HÖKFELT

Of course the question of the localization of leu- and met-enkephalin is an interesting prohlem. In one of your first slides on the rabbit brain you had different ratios in different regions, but it is always to the advantage of met-enkephalin. Have you any place where you have the reversed situation? Where you have more leu-enkephalin than met-enkephalin?

KOSTERLITZ

No. There was a paper by Simantov and Snyder in which they had looked at calf brains and thought that they had found a preponderance of leucine enkephalin in that species. However, these findings have not been confirmed in other laboratories. Clearly, this is one of the important problems: why are there two enkephalins, and are they always in the same neuron?

ROBERTS

Are there any data available that can distinguish whether or not the enkephalin-containing neurons are acting directly on the primary afferents in the spinal cord or on GABA neurons, which regulate the activity of primary afferents hy presynaptic inhibition?

KOSTERLITZ

No, I do not think that the situation is clear cut. On the contrary, it is very complex and we should be very careful in finally accepting any hypothesis. The presynaptic inhibition of primary afferents by enkephalin is a most attractive hypothesis but it may be an oversimplification.

In dissociated mouse cell cultures of spinal cord and dorsal root ganglion cells, we have good evidence that an opiate, etorphine, acts in a naloxone sensitive manner to reduce transmitter output from dorsal root ganglion cells at their excitatory synaptic contact with spinal cord cells.

NELSON

In the tissue culture system not working with enkephalins but with endorphins I think we have positive evidence for a direct effect on a spinal cord dorsal root ganglion.

KOSTERLITZ

There have been some very interesting experiments on cultured neurons. Jeffery Barker sent me some most intriguing prints showing the presence of enkephalins by immunohistochemical methods.

ECCLES

I was also going to raise the same question as Dr. Roberts raised about the presynaptic action of enkephalins. I am delighted to see this old presynaptic idea being used more and more, but what is known about the enkephalin action, say, in the hypothalamus, where it is so concentrated? Is anything known about the synaptic action of enkephalin there?

KOSTERLITZ

As far as I know, there is nothing known but maybe some of the other enkephalin people can help in this respect.

HÖKFELT

At a conference recently Dr. Virginia Pickel showed some electron microscopic micrographs demonstrating that in some areas of the brain there are clear axo-somatic or axo-dendritic contacts, so enkephalins probably could act at many different levels.

KOSTERLITZ

Alan North has shown by intracellular recording that enkephalins cause a hyperpolarization of the ganglion cells of the myenteric plexus. This effect on the soma is small and may be caused by an action of the enkephalins on axo-dendritic junctions.

ECCLES

I am coming back to this story. Are you proposing that in the control of pain the whole action is in the transmission of the first stage of relay in the spinal cord?

KOSTERLITZ

Oh no, definitely not. In several laboratories it has been shown that opiates and enkephalins reduce neuronal firing in other parts of the pain pathways. There was no time to deal with these aspects of the action of opioid peptides. Another problem that I did not deal with is the role of enkephalins and endorphins in the control of endocrine function. They stimulate release of prolactin, growth hormone and anti-diuretic hormone and inhibit that of luteinizing hormone.

ENDORPHINS AND THE ENDOGENOUS CONTROL OF PAIN

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Pain may be regarded as one of the senses. Its role as a warning signal cannot be over-estimated. But unlike the otber senses, vision, hearing, etc., pain is normally experienced at long intervals. Prolonged pain is a consequence only of disease processes or injury. Another unique property of pain is its aversive character. Pain as a sensation will therefore strongly affect the individual, both because of its unexpected character and its potentially harmful consequences. It might therefore be expected that pain controlling systems, if they exist only show weak tonic activity and that they will be triggered only when pain becomes protracted.

Recent work has demonstrated the existence of pain-controlling substances, the endorphins. These substances were detected by their similarity to morphine (Goldstein, 1976; Hughes and Kosterlitz, 1977; Snyder and Simantov, 1977; Terenius, 1978). Since the pharmacology of morphine has been studied since classical times, tbis has given a back-ground knowledge which has been instrumental in the rapid development of knowledge on endorphins and their properties. Particularly significant is the observation tbat a drug like naloxone is an efficient and apparently pure antagonist of morphine and endorphins. On the otber hand, it seems likely that endorphin systems constitute only one component in the body's adaptation to pain (Liebeskind and Paul, 1977). Tbis paper will analyse some aspects of pain control and the role of endorphins in this regard.

On the nature of pain

Pain may be defined in relation to the stimulus and the response. A similar pain stimulus may he experienced very differently depending on

the circumstances. If pain is associated with a potentially harmful event, responses will be extensive. This is particularly true of pain in the clinical context. Although pain may then be the main verbal complaint, feelings of anxiety and fear may be the more unbearable. To characterize pain within a sensory system we therefore need different components as is graphically illustrated in Fig. 1. Experimentally induced pain will hardly reach the emotional centers and therefore it is hard to reproduce clinical pain in an experimental model. It should also be emphasized that in

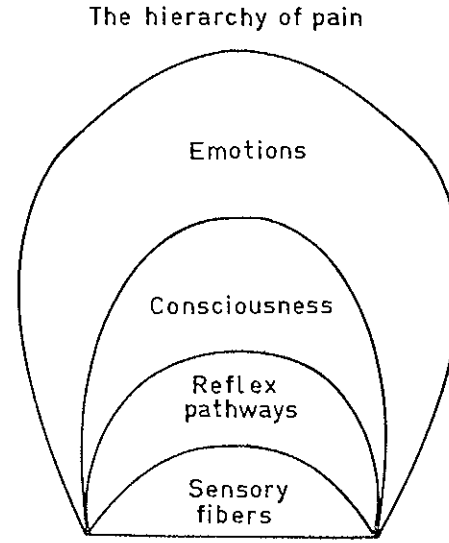

FIG. 1. Graphical presentation of the components of pain.

pain models which are ethically acceptable in humans, morphine is hardly active at all (Jaffé, 1975). In other words, morphine affects the reaction to pain rather than sensory thresholds.

It is now generally appreciated that there are descending control mechanisms which may inhibit or facilitate the processing of sensory messages and their relaying to higher centers (Livingstone, 1959; Towe, 1973). Such mechanisms can account for "attention" (Hernandez-Peón *et al.*, 1956). Pain has been postulated to be under centrifugal inhibitory control (Melzack and Wall, 1965). This inhibitory control was thought to be triggered by changes in the relative proportion of firing of large- and small-diameter fibers. Even if the hypothesis did not went very much further than to the conceptual stage, the detection of the endorphin systems has enlivened an interest in such mechanisms.

ENDORPHINS, GENERAL CHARACTERISTICS (for further details and bibliography, see Terenius, 1978)

From a structural and probably mechanistic point of view, there seem to be (at least) two separate endorphin systems. They show similarities in their connection with opioid receptors and in being antagonised by naloxone. The system with enkephalins, two pentapeptides, is widely distributed in the CNS with high fiber densities in the pain pathways and in the limbic areas. The other system has β-endorphin, a peptide containing 31 amino acids, as the active species. It shows a rather diffuse localization to fibers originating in the hypothalamus and projecting into thalamic areas. In addition, β-endorphin is found in the pituitary where it probably is the dominating endorphin species. While the enkephalins are readily inactivated in nerve tissue or in body fluids, β-endorphin is remarkedly stable within the brain and has a plasma half-life of 10-20 min. It would therefore seem likely that β-endorphin, produced within or outside the brain, might have a neurohormonal, regulatory character. The enkephalins, on the other hand, would rather act as neurotransmitters or short-range neurohormones. Supporting this role of the enkephalins is the recent demonstration of a specific enkephalin-degrading enzyme in synaptic elements of rat brain (Malfroy *et al.*, 1978). In summary then, the two endorphin systems may be complementary, the β-endorphin system accounting for modulation of the general pain sensitivity, the enkephalins giving local amplification upon suitable triggering.

Essentially two approaches may be used to study endorphin mechanisms. One is to use the narcotic antagonist, naloxone, to inhibit endorphin-mediated processes. The other is to measure the levels or turn-over of endorphins. Unfortunately, the experimental techniques are still at infancy. A method developed in my laboratory (Terenius and Wahlström, 1975) utilizes the measurement of endorphin levels in human lumbar cerebrospinal fluid (CSF) as an index of CNS endorphin activity. Although this approach may be criticized on theoretical grounds, actual measurements have shown its applicability (see further below).

ENDORPHINS AND THE MODULATION OF PAIN

Like many other physiological mechanisms, pain sensitivity shows a circadian rhythm. Thus, sensory and pain thresholds are significant lower in the afternoon than in the morning (Rogers and Vilkin, 1978). Recent experiments suggest that endorphins at least partially mediate this varia-

tion in pain sensitivity. Davis and co-workers (1978) showed that naloxone causes hyperalgesia in human normal subjects in the morning while it is not significantly active in the afternoon. Somatosensory evoked potentials were also reduced in the morning hy naloxone while this phenomenon was absent in the afternoon. Extensive experiments hy Frederickson *et al.* (1977) point to the same direction. Mice showed a marked diurnal variation in pain sensitivity in the hot-plate assay with relative indifference to pain in the beginning of the active period and increasing sensitivity against the end of this period. The variation was largely eliminated by naloxone injections. In more recent studies (Frederickson *et al.*, 1978) it was found that the variation in pain sensitivity is probably not related to the pituitary endorphin system since the phenomenon exists also in hypophysectomized animals.

The experiments already cited deal with experimental pain. The effects observed are fairly mild indicating that baseline activity in endorphin systems is low, and not increased markedly by the test situation. Probably more relevant to the supposed role of endorphins in pain control are observations in patients with chronic pain. Such patients experience considerably more pain in evening hours (Glynn and Lloyd, 1976). These authors also made the observation that the subjective feeling of pain is greatly influenced by social and psychological factors. Female patients at home recorded higher increases in pain scores in the morning that patients who went to work. An association between pain suffering and personality traits was also observed. Patients classified as introverts in the Eysenck Personality Inventory, reported stronger pain increases in the early day than extroverts. Pain responsiveness is therefore not an isolated characteristic of a particular individual. Actually we seem to face a more general psychophysiological quality.

In the patient with chronic pain, pain causes and reactions to pain are interwoven. In cases with acute pain the reaction will be more acute and probably more exaggerated. A study carried out by Levine and associates (1978) shows that injection of naloxone will significantly worsen the development of reported pain in the post-operative phase after molar extraction. Experiments, recently performed in my laboratory, indicate that endorphins may play a role in relieving labour pain. Naloxone given during the period of parturition in rats strongly reduced the number of surviving offspring (Hetta and Terenius, 1979). It seems probable that naloxone here interferes with the maternal-offspring interaction, perhaps following a more painful and stressful parturition process. One may

speculate that particularly in mammals, where parturition is a stressful event, pain relieving processes are advantageous and in fact, may have a considerable survival value in evolutionary terms.

Biochemical assessment of endorphin activity in man

Due to the subtle nature of endorphin action and the difficulties in assessing pain sensitivity objectively, there is a need for objective analytical methods. We have therefore developed a procedure for tbe measurement of endorphins in CSF (Table 1). The measured levels of "endorphin Fraction I" have been empirically found to correlate to several clinical variables (Terenius and Wahlström, 1978). An extensive study has been made in a series of patients with chronic pain. In this series, there was a negative correlation between endorphin levels and the sensitivity to pain and a positive correlation to pain tolerance limits (von Knorring *et al.*,

Table 1. *Principle of assay procedure for CSF endorphins* (Terenius and Wahlström, 1975)

4 ml CSF
↓
Ultrafiltration
↓
Chromatography on Sephadex G10
↓
Fractions tested in receptorassay or radioimmunoassay

1978a). There was also a correlation to another psychophysical criterion, visually evoked potentials (von Knorring *et al.*, 1978b). Patients who responded with increased potentials on increasing the visual stimulus intensity had significantly lower Fraction I levels than those who did not show this response. Evoked responses have been studied quite extensively in various contexts, and it is quite well established that psychiatric disorders such as endogenous depression and schizophrenia are accompanied by low responsiveness to various sensory stimuli (e.g., Jones and Callaway, 1970; Shagass *et al.*, 1978). In line with these findings is our observation that there is a significant positive correlation between Fraction I levels and tbe depressive symptomatology (Almay *et al.*, 1978). The correlation between Fraction I levels and the psychophysical variables are sum-

marized in Table 2. It should also be mentioned that Fraction I levels tend to be high in schizophrenia, particularly in the acute phase and in untreated cases (Lindström *et al.*, 1978, and unpublished observations). The results indicate that in fact, CSF endorphin levels correlate to a number of variables relating to the sensory adaptation to the environment and also link these phenomena together. Thus, the well-known link between depressive disorders and insensitivity to pain may have a correspondence in a common biochemical denominator.

TABLE 2. *Relation between CSF endorphin Fraction I and various psychophysical variables in patients with chronic pain* (Almay *et al.*, 1978; von Knorring *et al.*, 1978a, b)

Variable	Relationship
Pain sensitivity	Decreased criterion with increasing levels
Pain tolerance	Increased criterion with increasing levels
Evoked potentials	Decreased criterion with increasing levels
Depression scores	Increased criterion with increasing levels

CONCLUSIONS

The role of endorphins in various physiological systems is gradually being clarified. There is ample evidence to suggest that it is extensive and certainly not confined to pain and the body's response thereto. Perhaps we should regard the endorphins as more general modulators of sensory processing. Such a concept may be of considerable heuristic value and provide a rational analytical approach to complex perceptive and psychophysiological phenomena.

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DISCUSSION

TERENIUS

Dr. Kosterlitz has admirably summarized some of the excitement we had in the early history of the endorphins. I would just like to correct his memory on one point, that neither of us initially liked the idea that they were peptides. I remember Hans Kosterlitz writing a number of formulae of compounds similar to morphine in the early summer of 1974. I was coming from a different field—from the steroid field—so I could think of anything like a steroid or an alkaloid but not like a peptide, so we found ourselves suddenly in a field in which we had no experience whatsoever. John Hughes was more brave than I was at that point so he went on with the chemistry; I decided I would do something else.

ROBERTS

Have you looked at the CSF levels of your fraction I in amputees who have phantom pain and those who do not?

TERENIUS

Up to now we have not had any phantom limb patients. I can mention that there is a paper in the literature (R. Melzack and J. D. Loeser, "Pain", *4*, 195-210, 1978) showing that probably one very important factor in phantom limb pain is a deficiency in sensory input. These authors have based this statement on the use of various electrophysiological techniques.

ECCLES

What do they mean by deficiency in the primary afferent system in phantom limb pain?

TERENIUS

It seems as if the loss in sensory input gives rise to secondary changes in the firing pattern of spinal and brain cells. The authors attribute this to the subtle balance between pain-generating and pain-modulating mechanisms.

ECCLES

Yes, it probably could work by presynaptic inhibition.

GIACOBINI

In your last slide you showed some correlation with depressive symptomatology. Was that related to chronic pain, or do you have data in depressive symptomatology in depressed patients? Endogenous depression or exogenous depression?

TERENIUS

Let me answer the last question first: we have data for endogenous depression where the levels tend to be very high. I can also say there is extensive evidence for a connection between psychogenic pain syndromes and depression (see for instance, R. A. Sternbach, "Pain Patients", Academic Press, New York, 1974).

We have very little positive information on the chemistry of Fraction I endorphin: It is not identical to the known peptides—we have investigated that in several possible chromatographic systems. We also know that they do not cross-react with antibodies to endorphin or to the enkephalins. Maybe I should also say that there are at least two reports in the literature for the presence of enkephalin-like material (H. Akil *et al.*, "Science", *201,* 463-465, 1978; Y. Sarne *et al.*, "Brain Research", *151,* 399-403, 1978) as measured by antibodies. We have tried to reproduce these results with our antibodies, but so far with little success. I believe that again we are coming back to the problem of antibody specificity. We have in fact used antibodies with higher affinity than those described by others. We know, however, that in occasional patients there is enkephalin which is measureable—I mean enkephalin defined by retention volumes on chromatography, by immuno assay and ratio receptor assay. So I think we are 99.99% sure that they may exist but in most of the patients I have been talking about here the levels are beyond the detection limits of our assays.

BERLUCCHI

You have shown a relation between your fraction on endorphin and the amplitude of light above the patient. Are you implying that these endorphins are involved in the control of any afferent input besides the pain?

TERENIUS

Well, these are the only two things we know about, pain and light. We have not looked at auditory or somatosensory evoked potentials. So I cannot answer your question.

BERLUCCHI

But could it be simply due to some change in the pupil?

Since morphine has a strong pupilloconstriction effect, is it possible that endorphins act on the amplitude of light-evoked cerebral potentials by controlling the size of the pupil?

TERENIUS

No, I would not think so.

BERLUCCHI

You have checked for those? You know the amplitude of visually evoked potential is very sensitive to the size of the pupil.

TERENIUS

Yes, well, pupilar measurements have been made and there was nothing.

PURVES

One way, I suppose, of determining the normal role of enkephalins and endorphins would be to look at their clinical effects; I gather that all of these agents, or most of them, have strong analgesic effects. Can you describe what other effects, if any, they are known to have?

TERENIUS

The clinical evidence, as already mentioned by Hans Kosterlitz (B. von Graffenried *et al.*, "Nature", *272*, 729-730, 1978) of studies of enkephalin analogue in man are rather surprising, actually. This is by the way a morphine-like enkephalin. Among the unexpected effects were those on the heart rate, there were vascular reactions, even diarrhea, which is very usual for a morphine-like compound as you know the patients complained of heavy legs, etc. Most of these effects may be peripheral. One interpretation is that in the human being the peripheral ganglionic system is very much more im-

portant than in other species. These were doses which did not produce any analgesia and these side effects may prohibit clinical application. I can also mention that at the Psychiatric Clinic at the Ulleråker Hospital in Uppsala the compound was tested in two addicts and they seemed to like the drug—I mean they felt it to be morphine-like. The suhjects were under abstinence under the experiment.

GREENE

Dr. Kosterlitz mentioned that these peptides were generally very unstable—that they broke down very rapidly. Are you a bit surprised then to find them in detectable levels in the CSF? Secondly, if that is the case, could it be that the differences you see are not in the synthesis and release, but rather are due to differences in breakdown between patients with and without pain?

TERENIUS

If you add enkephalin to CSF, it is quite stable if the CSF is blood-free. So once in the CSF, enkephalins may stay long enough for a considerable build-up.

GREENE

But it has to get there in the first place, and presumably it was released under conditions in which it could have been broken down before it got into the CSF.

TERENIUS

The problem of CSF analysis is a very big one in general; what does it mean that you have a high level in CSF? Would that be dependent on a large spill over, or is it dependent on a high release rate? This is very difficult to say in the first place. However, since we see an increase in Fraction I levels on stimulation produced analgesia this may indicate that when we have activation of these systems we have high levels.

ECCLES

I think that these enkephalins—if I am right, Dr. Kosterlitz—are in high concentration around the third ventricle, and therefore they can move rapidly into the CSF by diffusion. The movement is not ordinary diffusion, because in the pulsating hrain diffusion goes much faster. Pulsation can aid

diffusion channels into the CSF, where it would move quite fast from the areas where it is liberated. So that may be part of the answer to your problem.

TERENIUS

No, we have not looked into it.

KOSTERLITZ

I would like to raise a problem which has been fascinating me for some time now. Lars Terenius talked quite clearly about pain controlling mechanisms. On the other hand, people in the pharmaceutical industry are not so much interested in these mechanisms but in drugs which lead to more or less complete analgesia. Pain control and analgesia are not necessarily the same, because true analgesia does not normally occur physiologically except in exceptional circumstances, namely those described in soldiers on the battlefield, who apparently are analgesic to their injuries even if they have been very severe. I know also of people in sports who do not notice injuries until the particular event is finished. I wonder what Lars thinks about this: whether there is any evidence that these particular events, on the battlefield, etc., involve the enkephalin-endorphin system or whether this type of analgesia may be mediated by non-opiate pain controlling mechanisms. I appreciate that this is speculative but I would like to know what his personal feeling is in this matter.

TERENIUS

I think it is quite likely that to some extent the so called battlefield pain relief is mediated by endorphins and I think the work I mentioned on acute pain (J. D. Level *et al.*, "Nature", *272*, 826-827, 1978) supports that idea. You may also know that there is a clinical condition called congenital analgesia, which is a very rare disease, where patients do not feel pain, and I know at least one case where the patients got the pain sensitivity back on the injection of naloxone—at least some pain sensitivity. So, Hans Kosterlitz emphasized, analgesia is not a natural phenomenon because it is extremely dangerous—we would hurt ourselves by biting our tongues, we would break our legs, etc. We should therefore talk about pain modulation or relief but not of analgesia in relation to endorphins. Moreover, there may be non-opioid pain mechanisms. I think the best evidence for this comes from work with electric stimulation for pain relief. So-called conventional TNS (transcutaneous nerve stimulation) of high frequency and low intensity may give adequate

pain relief in the clinical situation without changing Fraction I levels or being affected by naloxone injection. On the other hand, in electroacupuncture, where one has low frequency stimulation and gets muscle contractions, the pain relief is reversed by naloxone and accompanied by an increase in Fraction I (B. Sjölund *et al.*, "Acta Physiol. Scand.", *100*, 382-384, 1977; B. Sjölund and M. Eriksson, "Brain Res.", in press). Thus our interpretation is that there has to be different mechanism elicited. I think one of the very interesting things for future research is to find out how we can mobilize these systems without using all the technical equipment, for instance by biofeedback training (self) hypnosis, etc.

ECCLES

I want to comment on a problem that has interested me a lot. When in an acute emotional involvement, as in battle or in games, or even accidents, you have an immense lot of happenings that may distract attention from pain, from injuries received. We are apt to think that the brain is purely a machine converting certain kinds of stimuli into pain automatically. I think that is a mistake. What we are doing in consciousness is selecting all the time from all that the brain is receiving, selecting according to interest and attention. There is a lot of work now on attention and how the mind selects from the brain performance. This may be an explanation of many of these findings. In acute emotional involvement your attention is elsewhere and although it may be that the brain is receiving all the pain signals, they are not attended to in the mind and therefore you have the equivalent of analgesia. We at least sbould be aware of this possibility and not think that we have to block everything going into the brain. In order to be converted into pain, it has got to get into the mind!

FILOGAMO

This brings a correlation with the comment of Dr. Eccles. Is it any difference with regard to psychogenic and organic pain as far as the CNS endorphin? Can you see differences in both cases? You mentioned psychogenic pain.

TERENIUS

Yes, there is a difference between the two categories of patients. In the neurogenic pain syndroms the Fraction I levels tend to be low. In psychogenic pain, levels are close to normal or even supernormal. I should have men-

tioned perhaps that in these patients there was no evidence for any psychotic dimension of the symptoms.

FILOGAMO

How was this psychogenic pain elicited?

TERENIUS

Well, how do you know? You have to rely upon the patient's own description of his suffering and if this is highly emotionally colored if it is a patient with a typical hysteric or neurotic background. Besides, a psychiatric rating with the CPRS scale was used. We have also gone into personality variables using different scales and there are some weak correlations. And may I make a short comment to you, Dr. Eccles? Even if we have to accept that many phenomena like hypnosis may involve factors which are hidden for us at the moment, I still believe that even such complex phenomena finally will be found to be related to the activation of one or probably several bioactive molecules and I will be trying myself to work on their identification.

BURNSTOCK

I would like to follow up Lloyd Greene's comment, which I agree is a bit worrying. If it is a physiological mechanism, then you would expect the transmitter to be either broken down locally or taken up again; therefore you would not expect to be able to collect much of the transmitter, at least under physiological conditions. What about measuring breakdown products instead? What is known of uptake?

TERENIUS

Well we are reasoning exactly the way you are doing. We are setting up techniques for looking into breakdown products of enkephalin at the moment. We have not come so far yet. So I believe that is a very good suggestion.

BURNSTOCK

What about uptake? Perhaps Lars knows about it? Is there uptake of the transmitter itself or its breakdown product? Is anything known?

TERENIUS

I do not know anything. Maybe Dr. Kosterlitz does.

KOSTERLITZ

We looked for uptake of enkephalins in synaptosomes but did not find any evidence for such a phenomenon.

BURNSTOCK

That's a pity—if you had an uptake inhibitor present you would get a better measure of breakdown.

KOSTERLITZ

I think the enkephalin system resembles acetylcholine rather than catecholamines, in the sense that inactivation is brought about mainly by enzymatic degradation.

ECCLES

Thank you all very much for this discussion. The chairman's last word of course is that this question I will be raising on Friday and then we will talk more about whether you as a reductionist have the last word.

HYBRID CELLS AS MODEL FOR STUDYING OPIOID ACTION

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ABSTRACT

Neuroblastoma x glioma hybrid cells have been shown to display many neuronal properties. Among these is their susceptibility to opioids. These compounds prevent the increase in the cellular level of cyclic AMP that is elicited by prostaglandin E_1 (PGE_1). Besides these short-term effects, opioids cause long-term effects in the hybrid cells, i.e., an increased maximal response to PGE_1. The acute and chronic effects on the hybrids may be considered as biochemical correlates of opiate analgesia and tolerance, respectively. Recent results show the importance of calcium and sodium ions for the short- and of Ca^{2+} for the long-term regulation of the cyclic-AMP-level in the hybrid cells.

INTRODUCTION

The complex construction of the brain is a serious obstacle to studies of the molecular events underlying the activity of this organ. A biochemist engaged in such efforts would like to investigate the different cell types of the brain first separately and then in stepwise recombination. This would most likely provide insight into the factors and events which are required to organize the constituing cell types into such an effective instrument of information storage, retrieval and processing. In recent years progress is gaining momentum in the field of cell separation. Till the time has arrived that various cell types will be at hand in quantities sufficient for biochemical studies, model systems will have to be employed

for studying basic mechanisms of nervous activity in a paradigmatic way. Permanent cell lines derived from tumors of the nervous system are such models. One of these model systems is the neuroblastoma x glioma hybrid line 108CC15 (Hamprecht, 1974, 1976, 1977).

These cells are considered as model systems for neurons because they express many properties characteristic of neurons (Hamprecht, 1974, 1976, 1977) (Table 1). To neurohormones they respond by changing their membrane potential (Hamprecht 1974, 1976, 1977) and/or their intracellular concentration of cyclic AMP (Hamprecht, 1974, 1976, 1977). It would be important to know if, e.g., the α-adrenergic receptor de-

TABLE 1. *Neuronal properties of neuroblastoma X glioma hybrid cells*
The references for those properties that are not provided with a reference are given in Hamprecht, 1977.

Property
Extension of neurite-like processes
Excitable membranes
Cholineacetyltransferase
Dopamine-β-hydroxylase
Clear vesicles
Dense-core vesicles
Uptake systems for neurotransmitters (4)
Formation of functional synapses
Receptors for neurohormones

pressing the level of cyclic AMP (Traber *et al.*, 1975) is identical with that lowering the membrane potential of these cells (Traber *et al.*, 1975 a, b, c). Generally speaking, the question has to be answered if a receptor is constructed to directly regulate only one effector system (e.g., adenylate cyclase *or* a certain ion channel) or several (e.g., adenylate cyclase *and* a certain ion channel). This fundamental problem has many implications. The properties of the hybrid cells permit us to seek for an answer by using this model system. The principal advantages (Brandt *et al.*, 1978 a) of working with the hybrid cells are: i) in comparison to brain they represent a rather homogenous population of cells; ii) in contrast to normal neurons, they proliferate and can be grown in large quantities, either in a culture or as solid tumors in nude mice (Heumann *et al.*, 1977); iii) cell stocks can be stored at very low temperatures and can be reintroduced into

culture at any time desired. In comparison to other cell lines with neuronal properties, such as mouse neuroblastoma cells, the hybrid cells have the following advantages: *a*) they are large cells which can be successfully penetrated by a microelectrode; *b*) due to the stabile membranes of the cells electrophysiological investigation is possible for extended periods of time; *c*) many neuronal properties are found in one cell line; therefore it is possible to carry out many kinds of studies on one cell line; consequently one can understand this one cell line well instead of many other cell lines only superficially; *d*) the neuronal properties are strongly expressed in the hybrids.

The purpose of this account is to demonstrate in a paradigmatic way the use of this permanent cell line in studying the mechanism of opioid action.

Short-term effects of opioids

The hormones that regulate, most likely via specific receptors, the level of cyclic AMP in the hybrid cells can be divided into two classes (Table 2). Characteristic representatives (Table 2) of the hormones that respectively elevate the level of cyclic AMP or prevent this elevation (incubation time 10 min) are prostaglandin E_1 (PGE_1) and the opioids. Thus the interference of an opioid with the action of PGE_1 can be used as an assay system for effects of opioids. The relationship between opioid and cyclic AMP, including that in the hybrid cells, has been reviewed recently (Klee, 1977; Hamprecht, 1978).

The inhibition by morphine and its congeners of the action of PGE_1 is stereospecific and it can be blocked by the specific opiate antagonist naloxone. A positive correlation was found between the potency of an opiate in exerting this biochemical effect and the affinity for the opiate

Table 2. *Hormones regulating cyclic AMP levels in neuroblastoma X glioma hybrid cells 108CC15*

The literature references for those hormones that are not provided with a reference are given in (Hamprecht, 1977).

Level is increased by	Level is decreased by
PGE_1	Opioids
Adenosine	Somatostatin (9)
Secretin (8)	Acetylcholine (muscarinic receptors)
Glucagon (8)	Noradrenaline (α-receptors)

receptor (Sharma *et al.*, 1975). Several orders of magnitude more potent than opiates in the hybrid cell system are the opioid peptides enkephalins (Brandt *et al.*, 1976 a; Klee and Nirenberg, 1976) and endorphins (Brandt *et al.*, 1977; Goldstein *et al.*, 1977). This sequence of potencies is the reverse of that found in binding studies on brain cell membranes (Wahlström *et al.*, 1977). This indicates that different kinds of opioid receptors (Lord *et al.*, 1977) may be involved. Curtailing of the pentapeptide chain of the enkephalins at either end or substitution of the terminal amino group by a hydrogen renders completely inactive material. Unexpectedly, however, on substitution of the terminal amino group by an arginyl residue the activity drops only by a factor of 10. Replacement by hydrogen of the phenolic hydroxyl group lowers the activity by 3 orders of magnitude. Elongation of the peptide chain at the carboxyl terminal is well tolerated (Wahlström *et al.*, 1977). In agreement with this is the still high activity of Leu-enkephalinamide or -methylester (Agarwal *et al.*, 1977). These data demonstrate that the negative charge of the carboxylate ion is not required for activity.

Somatostatin has been considered as a partial agonist/antagonist at the opioid receptor. In the hybrid cells this tetradecapeptide with no resemblance to opioids affects the influence of PGE_1 like the opioids do. However, opioid receptors were shown not to be involved in this activity (Traber *et al.*, 1977). Thus, at least in these cells opioid and somatostatin receptors must be different species. Also acetylcholine (Traber *et al.*, 1975 b) and noradrenaline (Traber *et al.*, 1975 a) exert, via muscarinic and α-adrenergic receptors, respectively, effects analogous to those of the opioids and somatostatin (see Table 2). This could suggest that all these hormones act by the same mechanism. There are several ways in which they could prevent the increase in the level of cyclic AMP elicited by PGE_1 (Brandt *et al.*, 1978 a). The use of one of them, the inhibition of adenylate cyclase, has been demonstrated (Sharma *et al.*, 1975; Traber, 1976). Preliminary evidence for a concurrent activation of cyclic AMP phosphodiesterase has been presented (Brandt *et al.*, 1978 a). The possibility has been considered that the hormones listed in Table 2, after combination with their corresponding receptor, produce a structural change in the plasma membrane, which in turn, causes a change in the permeability for a certain ion and tbus a possible change in membrane potential and intracellular concentration of the ion. This then might influence the activity of the cyclic AMP enzyme system and thus alter the level of the nucleotide (Hamprecht, 1977; Brandt *et al.*, 1978 a). Ca^{2+} ions have been known to functionally antagonize morphine in the generation of

analgesia (Kaneto, 1971) and tolerance (Weger and Amsler, 1936). Therefore, the effect of Ca^{2+} on the regulation of the cyclic AMP level has been studied in the hybrid cells.

It turns out that the response of the cells to the stimulating hormones (Tahle 2) is strongly dependent on the presence of external Ca^{2+} ions (Brandt *et al.*, 1978 a; Brandt, 1976). Even the basal activity of cyclic AMP generation relies on the presence of Ca^{2+}. If cellular phosphodiesterase activity is blocked by the inhibitor Ro20-1724 (Sheppard and Wiggan, 1971), the cyclic AMP level rises more quickly in the presence than in the absence of external Ca^{2+} (Brandt *et al.*, 1978 b). Since this happens in the absence of a stimulatory hormone (Table 2), the conclusion is that even the basal activity of cyclic AMP formation is dependent on Ca^{2+}. The influence of Ca^{2+} is counteracted by a chelator of Ca^{2+} ions (Brandt *et al.*, 1978 a), hy the Ca^{2+}-antagonists La^{3+} and Co^{2+} (Table 3) but not by Mg^{2+}, and by prenylamine (Segontin), an inhibitor of Ca^{2+}-influx into hybrid cells (Kürzinger, 1978) (Table 4). A comparable situation is the increase in the level of cyclic AMP evoked in adrenal cells by ACTH, since it depends on external Ca^{2+} ions (Sayers *et al.*, 1972).

A change of the extracellular concentrations means a change also of the total intracellular concentration of Ca^{2+} ions (Kürzinger, 1978) and is likely to alter the extremely low cytosolic activity of Ca^{2+} as well. This in turn, could change the activity, e.g., of adenylate cyclase (Lefkowitz *et al.*, 1970). The dependence on Ca^{2+} of the basal activity of cyclic AMP formation and, less compellingly, the effect of Segontin, point to an intracellular action of Ca^{2+}. A simplified picture of adenylate cyclase activity in the hybrid cells has to deal with at least 4 regulatory factors: two act via their receptors from outside: the activating (e.g., PGE_1) and the inhibitory (e.g., opioid) hormones. GTP (Rodbell *et al.*, 1971) and probably also Ca^{2+} ions (Brostrom *et al.*, 1975; Cheung *et al.*, 1975) activate the enzyme from inside the cell. Our results are compatible with the view (Brandt *et al.*, 1978 a) that a hormone cannot activate adenylate cyclase unless the internal factors are present. In this model no change, by the activating hormone-receptor complex, of Ca^{2+} permeability is required for switching on adenylate cyclase (Brandt *et al.*, 1978 a).

The activating hormones (Table 2) raise the level of cyclic AMP markedly even if no Ca^{2+} has been added to the external medium, and opioids are still capable of attenuating this raise (Brandt *et al.*, 1978 a). Thus, extracellular Ca^{2+} ions are not required for the action of opioids on the level of cyclic AMP. Nevertheless, the functional antagonism between

TABLE 3. *Effect of La^{3+} and Co^{2+} on the increase in the level of cyclic AMP evoked by PGE_1*

Experiment 1 (2): 1.7 (4.0)×10^6 viable cells per plate, viability 83% (80%), passage number 19 (22). After removal of the growth medium the cells were washed 3 times with 5 ml of a medium (Kürzinger, 1978) containing 6 mM KCl, 25 mM HEPES, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 20 mM glucose and 145 mM NaCl (pH 7.4 at room temperature). Thereafter, the cells were incubated (10 min, 37° C) with 5 ml of the same medium supplemented with the compounds indicated in the table. After termination of the incubation the intracellular concentration of cyclic AMP was determined (Brandt *et al.*, 1978a).

Expt. no.	Addition (μM) preincubation	main incubation	Cyclic AMP (pmol per mg protein)
1	no preincubation	—	17 ± 10
	no preincubation	$LaCl_3$ (3000)	10 ± 3
	no preincubation	PGE_1 (3)	1010 ± 230
	no preincubation	$LaCl_3$ + PGE_1	530 ± 160
	—	—	36 ± 11 [1]
	$LaCl_3$ (3000)	$LaCl_3$	18 ± 4 [1]
	—	PGE_1	1340 ± 360 [1]
	$LaCl_3$	$LaCl_3$ + PGE_1	180 ± 30 [1]
2	no preincubation	Leu-enkephalin (0.1)	7 ± 1
	no preincubation	$LaCl_3$ (3000)	11 ± 2
	no preincubation	$CoCl_2$ (3000)	7 ± 1
	no preincubation	PGE_1 (3)	740 ± 30
	no preincubation	Leu-enkephalin + PGE_1	290 ± 20
	no preincubation	$LaCl_3$ + PGE_1	220 ± 60
	no preincubation	$LaCl_3$ + Leu-enkephalin + PGE_1	75 ± 7
	no preincubation	$CoCl_2$ + PGE_1	85 ± 40
	no preincubation	$CoCl_2$ + Leu-enkephalin + PGE_1	48 ± 30

[1] After a preincubation in the above medium (30 min, 37° C, ± $LaCl_3$) the main incubation (10 min, 37° C, ± $LaCl_3$) was carried out without prior change of media.

morphine and Ca^{2+} ions (Kaneto, 1971; Weger and Amsler, 1936) can still be understood, if all that matters for the cell is the resulting level of cyclic AMP. Morphine lowers it, Ca^{2+} raises it (Brandt *et al.*, 1978 a).

To our surprise also Na^+ ions are required (Brandt *et al.*, 1978 b) for the effect of the stimulatory hormones of Table 2. Our preliminary evidence points to an extracellular site of action. Presently we are investigating whether it is the absolute concentration or the gradients of Na^+ and Ca^{2+} ions that are important for the effect described.

TABLE 4. *Effect of the "Ca^{2+}-blocker" Segontin and of Leu-enkephalin on the increase in the level of cyclic AMP evoked by PGE_1*

PGE_1 and Segontin were added as solutions in 95% and 30% ethanol, respectively, both giving rise to a final alcohol concentration of 0.3% (v/v). Therefore, the data for the ethanol controls were included in the table. The details of experiments 1 and 2 are identical to those of experiment 1 and 2, respectively, of table 3.

Expt. no.	PGE_1 3 μM[1]	Segontin 100 μM	Ethanol % (v/v)	Leu-enkephalin 0.1 μM	Cyclic AMP (pmol per mg protein)
1	—	—	—	—	19 ± 3
	+	—	0.3	—	1080 ± 290
	+	—	0.6	—	2110 ± 130
	+	+	0.6	—	560 ± 90
2	—	—	—	—	10 ± 3
	—	—	0.3	—	14 ± 2
	—	+	0.3	—	4 ± 4
	—	—	—	+	12 ± 3
	+	—	0.3	—	1680 ± 90
	+	—	0.6	—	2020 ± 270
	+	+	0.6	—	940 ± 280
	+	—	0.6	+	970 ± 260
	+	+	0.6	+	300 ± 90

LONG-TERM EFFECTS OF OPIOIDS

Once short-term effects of opioids had been found in the hybrids, it was not too surprising to encounter long-term effects as well. These might be considered as biochemical correlates of opiate tolerance, dependence and withdrawal. During chronic (range of hours) exposure to morphine (Traber *et al.*, 1975 c; Klee *et al.*, 1975) or enkephalins (Brandt *et al.*, 1976 b; Lampert *et al.*, 1976) tolerance to the inhibitory action of opioids develops in the cells. If one withdraws the opioid, an increased maximal response to PGE_1 is observed. The development of the tolerance effect can be inhibited by cycloheximide (Traber *et al.*, 1975 c; Sharma *et al.*, 1977). The long durations of the experiments and the high inhibitor concentrations used let the conclusion appear premature that protein synthesis is required for the development of tolerance in the hybrid cells.

The analogy in the mode of action of the inhibitory hormones (Ham-

precht, 1977) (Table 2) also holds for the long-term effects, since adrenergic and cholinergic agents cause the same tolerance effects as opioids (Traber *et al.*, 1975 c). This report has been confirmed (Nathanson *et al.*, 1978). From the analogy between the actions of opioids and that of other neurohormones (Table 2) it has been concluded (Hamprecht, 1977) that opioids do probably not principally differ from other neurohormones in their mechanism of action. Thus they assume a special position only due to the striking effects on the physiology of animals and man after oral or systemic application.

In short-term experiments (minutes range) opioids and lack of external Ca^{2+} had the same effects on the hyhrid cells. This is the case also in long-term experiments. Chronic exposure of hybrid cells to low concentrations of Ca^{2+} gradually evokes the same increase in the maximal response to PGE_1 as treatment with opioids (Brandt *et al.*, 1978 b). For the cell it is obviously unimportant which way the level of cyclic AMP is lowered, by opioid or by deficiency of Ca^{2+}. Its reaction is the same in both cases: the specific activity of adenylate cyclase is increased (Traber *et al.*, 1975 c) (has the activity of phosphodiesterase been decreased concomitantly?) to make up for the loss of activity.

Conclusion

The level of cyclic AMP is homeostatically controlled (Traber, 1976). This points to a presumptively highly important function of cyclic AMP in the regulation of nerve cell activity. The only known biological action of cyclic AMP appears to be the activation of protein kinases (Greengard, 1978). Such an enzyme (Uno *et al.*, 1977) and its protein substrate (Ueda and Greengard, 1977) have been isolated from neuronal membranes.

Also Ca^{2+}-dependent protein kinase activity and corresponding substrate proteins are present in membrane preparations from brain (Krueger *et al.*, 1977; Schulman and Greengard, 1978). Adenylate cyclase is also regulated by exogenous (Cassel and Selinger, 1977) or endogenous (Moss and Vaughan, 1978) ADP-ribosyltransferases. It would be important to know if these enzymes are involved in the short-term or long-term regulation of adenylate cyclase activity. Answering these questions would most likely provide a more detailed insight into the mechanisms of opioid action.

It must be stressed that the results obtained in the studies of the model system must be compared with those from proper experiments using animal material. It cannot be taken for granted that, e.g., any

neuron carrying opioid receptors will react to opioids like the hybrid cells do. To emphasize this appears to be especially important since different kinds of opioid receptors have been discovered (Wahlström *et al.*, 1977; Lord *et al.*, 1977; Martin *et al.*, 1976; Kosterlitz and Hughes, 1978). It may well be that they influence different effector systems. Not all of them may exclusively regulate adenylate cyclase or the intracellular level of cyclic AMP. Rather, at least one of them may (also?) be concerned with the regulation of ion permeability of plasma membranes.

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DISCUSSION

NIRENBERG

Did I understand you correctly when you say that the response of the cells to PGE_1 and the increase in cyclic AMP is dependent on calcium?

HAMPRECHT

You know what I said was if you translate it in terms of enzymology, the KM value is not changed, but the Vmax is changed.

NIRENBERG

I understand. Rick McGee in our lab has done a similar kind of experiment and did not find any effect of omission of calcium ions on the response of cells to PGE_1—he got the same Vmax although he did not really test for Vmax, he got the same level of response, the same level of cyclic AMP.

HAMPRECHT

Well, I do not know why he does not find it, but if you do not wash your cells very carefully free of calcium (which is in the culture medium) you of course do not see this effect. You have to wash your cells very carefully free of calcium with the subsequent incubation medium. That may be a point; if you do not wash carefully, you have enough adherent calcium and you blur the thing. We have been fooled by this at the beginning until we resorted to very careful washing.

NIRENBERG

What medium do you incubate the cells with?

HAMPRECHT

This is an incubation medium—we have used actually two kinds—it makes no difference. The first one is based on a medium buffered with bicarbonate but has a reduced bicarbonate concentration. During the incubation period it is sometimes very difficult to adjust the pH value, but if you

lower the bicarbonate concentration, the range in which the pH value would change is very limited. And more recently we have just completely abandoned this medium and we have sort of made an open air incubation on a water bath. In this case we used HEPES buffer without bicarbonate in the medium and we get the same results. So it does not depend on the medium you use. And also it is not important whether you have all the goodies there or not.

NIRENBERG

There are two additional points—we have done similar kinds of experiments with somewhat different results. One is to grow the cells in the presence of PGE_1. There we find a remarkable phenomenon: we lose activity of adenylate cyclase, all the enzyme activity disappears, and this takes time—about eight hours of exposure, 6 or 8 hours, and we lose responsiveness not only to PGE_1 but also to sodium chloride, and it takes about 24 hours for the cells to recover, for the adenylate cyclase activity to come back to normal value and cycloexamide blocks the recovery process.

HAMPRECHT

We have done similar studies but with different results. We have incubated the hybrid cells with PGE_1. This was done by Jörg Traber (in his thesis work) for various lengths of time. After each pre-incubation time, we took the whole cells (we did not measure cyclase) and measured cyclic AMP levels, in response to PGE_1. You find that this is a time of pre-incubation and this is the original response; you find a decrease to very low stimulatability. Now, there could be two possibilities, we thought: either adenylate cyclase is reduced as you mentioned, or phosphodiesterase is induced; if we did this stimulation here, in the presence of the phosphodiesterase inhibitor we came about to this level, so about three-quarters of the original activity, could be found. So we do not think it is affecting much the adenylate cyclase in the attached cells but rather it is inducing phosphodiesterase. The breakdown enzyme is induced so the cyclic AMP level sinks, if you have the same activity of adenylate cyclase. So I disagree on that.

NIRENBERG

Well, in our experiments we do this in the presence of phosphodiesterase inhibitor RO 1720 and one millimolar cyclic AMP, unlabeled, so that reduction due to phosphodiesterase activity is minimized.

HAMPRECHT

I think you should do such an experiment first with the intact cells to see what is going on. Maybe, by dilution during homogenization of the cells you may be losing a factor (or diluting it out too much) which is necessary for maximum activation. I think the discrepancy between these two experiments may be only apparent and may not be real because, maybe, you would find the same thing if you took the intact cells. That is why we always take the intact cells to see how it is there and then next go to the more broken down system.

NIRENBERG

The last thing was the response to the effect of the isoproterenol on the system. We have tried to, but we have done identical or similar experiments and do not find that isoproterenol increases the specific activity of adenylate cyclase. I do not really understand this difference, but we get no effect with ten micromoles.

HAMPRECHT

I was not sure I saw that correctly in your slide during your talk, but you again measured this on adenylate cyclase? (NIRENBERG: Yes.) Well, we have done these experiments over and over again and there is no question about this; we always get this response to isoproterenol. One question we have to ask: if you check the short term effect of isoproterenol on formation of cyclic AMP, do you find that high concentrations inhibit the formation of cyclic AMP via the alpha receptor?

NIRENBERG

No; it has very little effect. We have run concentration curves of isoproterenol and only in very high concentrations—like 10^{-4} and even higher concentrations and we do not see any inhibition of adenylate cyclase.

HAMPRECHT

Well, you know these curves we published several years ago. This is noradrenaline, this is dopamine and this is isoproterenol, minus log concentration and this is cyclic AMP found in response to PGE_1. So each of these three compounds inhibits to the same low levels of cyclic AMP, the only difference being their potency, and they are different by one order of magnitude (shifted to the right) so isoproterenol works in the system, so maybe, again, it may be dangerous to use a broken up cell system to analyze something like this.

ROBERTS

I think something slipped by me: is isoproterenol acting on alpha receptors?

HAMPRECHT

Yes, that is true.

ROBERTS

You say you need a somewhat larger concentration than that which acts on the beta receptors?

HAMPRECHT

No. In comparison to noradrenaline. This is what I have tried to explain with this diagram here. If we measure the inhibition of the formation of cyclic AMP by catecholamines we find such a dose-response curve for noradrenaline. We find one shifted to the right if we use dopamine instead. It is about one order of magnitude difference. If we use isoproterenol, it is shifting to the right by another order of magnitude; so if you use 10^{-5} molar isoproterenol, we can already maximally stimulate the system via this alpha receptor.

BURNSTOCK

Can I introduce a wider issue into the discussion? One always appreciates the value of the model system for studying a particular physiological process. Sometimes it is not possible to work on the systems that would be most desirable. However, when a model is too far removed from a physiological situation, I feel uneasy. I would just like to hear from you, for example, how do you know whether this is really a specific opiate receptor in your model or could you be measuring a generalized membrane effect? What I am really asking is: how good is your model?

HAMPRECHT

That I can, I think, quite easily establish. First of all, the opiate action is stereospecific. If you use an analogue of morphine called levorphanol—it has the same effects as morphine has—it is even more potent. If you use dextrorphane—it is an enantiomer, its optical mirror image—it is completely inactive. So it is stereospecific. Then, naloxone, which is a specific opiate receptor antagonist, as I showed you in one of the slides (we have made more of this kind with all the other compounds), antagonizes the action of any of those opioids tried. So this is the second point. The third point is that

you can of course also try this in other systems. For example, we have one of the parental cell lines. It is a glioma cell line, as I said, and this also responds, although not as much, but it does respond to PGE_1 the same way the hybrid cells do. In this case there is no effect whatsoever of opioids, on the level of cyclic AMP. We have another hybrid line where the C6 glioma cell is fused to a fibroblast line, a mouse fibroblast line. There you have two kinds of hormonal responses, as actually also in the C6 glioma cell. An increase in the level of cyclic AMP via beta receptors and via prostaglandin E-1 receptors. In both cases we cannot inhibit this effect with morphine for example. So I think we can be pretty sure that we are dealing with opiate receptors.

BURNSTOCK

Just one last question on the same topic: what about the cells of origin? Have you any reason to suppose that these substances would have a physiological role on these?

HAMPRECHT

Well, on the C6 glioma cells, they have no receptors, so they do not have any effect, they cannot have any effect. I am not sure what you mean by physiological role. In the neuroblastoma parent—we have not analyzed but I think Werner Klee together with Marshall, they have looked at that parent and, although I do not find the data too convincing because the changes are too small in our view, there are some small changes of level of cyclic AMP or adrenylate cyclase activity if one adds opiates. But they are very marginal; I am not sure they are really significant.

BURNSTOCK

So if the cells of origin do not respond to these substances, how reliable is the system?

HAMPRECHT

Maybe the neuroblastoma does respond—it does a little bit.

BURNSTOCK

Then why would it suddenly begin to respond?

HAMPRECHT

That is a very big question—that applies to all the properties you have seen in that table, and it looks like there is a switching on of genes which

does not occur in the parental cells. But to sort this out is very difficult. We have to know that the glioma cell has about 40 chromosomes; the neuroblastoma cell has about 89, so the sum would be 129. In the smallest hybrid cell line—the one I talked about today—there is a modal chromosome number of 155, so more than one of each of the parental cell lines must have given rise to this cell line. To do genetics on something like this, I think, is impossible. So we just recorded the phenomenon and made use of it, but we cannot explain it. But it is interesting to see that it is these neuronal properties which are all switched on.

TERENIUS

I would also like to make a more general comment which would lead to a specific question, and that is in regard to your use of the word dependence for cell lines. As you know, we are not talking about for instance, dependence on insulin, or acetylcholine or noradrenaline, but of course we are dependent on them. So there is a qualitative difference in the use of the word dependence in relation to opiards. The word dependence is here used in behavioral terms which in some way relates to some qualitative changes occurring after long term treatment. Secondly I have a question. If one performs experiments in animals, one has a phenomenon called "carry-over", which means that if you have made an animal morphine-tolerant and morphine-dependent and then stay around for a couple of months after which the animal is again treated with morphine, the animal becomes tolerant at a much higher rate. I am aiming at a distinction between two things: one is a changed steady state, which you may have under these chronic morphine exposure, and secondly, these long term changes which could be related to memory process or whatever. The specific question I have in regard to this is: Have you ever looked at the phenomenon of carry-over, and have you let your cells be dividing for a long time to see if they will more rapidly become tolerant again?

HAMPRECHT

I think that is a very difficult experiment to do because these cells divide, they keep dividing, and so if you expose cells at a certain time to morphine for a long period, let us say twenty hours or something like this, and then you withdraw the morphine and you just let them go, then you have many more generations of cells occurring in the meantime. That is the reason why we never did such an experiment because we thought that everything that would have happened in the precursor cell during the following generations would just have been diluted out. The experiment has not been done.

MATURATIONAL CHANGES IN THE DEVELOPMENT OF MOTOR INNERVATION IN CHICK EMBRYO

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ABSTRACT

The multipotential mesodermal cell after having undergone an undefined number of divisions comes out of the cell cycle and becomes determined as an immature presumptive myoblast. This event is triggered by neural influences. Thereafter, the process of differentiation from immature myoblasts into myotubes proceeds automatically, although still responding to the modulatory influence of the neural component which gives rise, for instance, to the distinction between slow and fast muscle fibres. Eventually, synaptogenesis occurs. In the fast fibres alone, the sudden fusion of several satellite cells with the myotubes takes place above a restricted area of the sarcolemma, just underneath the terminal branching of a motor axon. The possibility that the nuclei and organelles of fused satellite cells in the plate sole are responsible for the local synthesis of AChE and ACh-R is suggested.

INTRODUCTION

The development of neuromuscular correlations is a story of interdependent maturational events played by three partners, namely nerve, muscle and glial cells. Such a story lasts several days *in vivo* and is marked by a planned sequence of discrete and collective events. Although there is clear evidence for a mutual collaboration, the quantity and quality of the roles played by each partner are as yet not fully understood,

particularly with respect to the role of the glial cells, which is definitely far from clear.

The ultimate purpose of the present paper is essentially to discuss the terminal event of the whole developmental sequence of neuromuscular relationships, i.e. the formation of the synaptic area, which is known to lead either to "en plaque" synapses, typical of fast muscle fibres or to "en grappe" synapses which conversely are typical of slow muscle fibres.

For the sake of clarity, however, we have to start from the very beginning, namely from the earliest transient contacts occurring between mesodermal cells of the somite and limb primordia and early growing nerve fibres: we helieve in fact that mutual early interactions of mesodermal cells with nerve cells provide a very specific basis for the whole subsequent development of neuromuscular correlations.

The determination of the miogenic line

When pioneering fibres first establish contacts with the somites (Sisto Daneo and Filogamo, 1973; Filogamo, 1976; Filogamo and Sisto Daneo, 1977; Filogamo *et al.*, 1978) at the 35th hour of incubation in chick embryos (12 somites, stage 10 of Hamburger and Hamilton), the crucial moment is attained of the determination of the myogenic cell line. This event may be summarized as follows: some cells of the neural tube sprout filopodia toward the perineural space and contact the cells in the somite medial wall which are likewise sprouting filopodia. At this stage filopodia are numerous at the level of the cranial somites and fewer in the caudal ones: and this is in agreement with the cranio-caudal gradient of the neural tube maturation. Upon inspection of serially sectioned somites, we have found that in the somite medial wall, explored by thrusting pioneering nerve fibres (Figs. 1 and 2) presumptive myoblasts first make their appearance and then proliferate to make a sharply defined layer facing the neural tube. The somite has already broken up into three other different cell masses, eacb being characterized by specific cell types, i.e. the dermatome, the sclerotome and the indifferentiated mass (Panattoni and Sisto Daneo, 1977). Upon tbe somite splitting, the tactic behaviour of the presumptive myoblasts is to migrate laterally, eitber individually or in small groups, toward the deep face of the dermatome, where they build up a continuous lamina known as myotome. Their lateral migration is made possible by the simultaneous migration of the sclerotome elements towards the notochord, whereby tbe somitic core is left empty. The presumptive myoblasts undergoing lateral migration

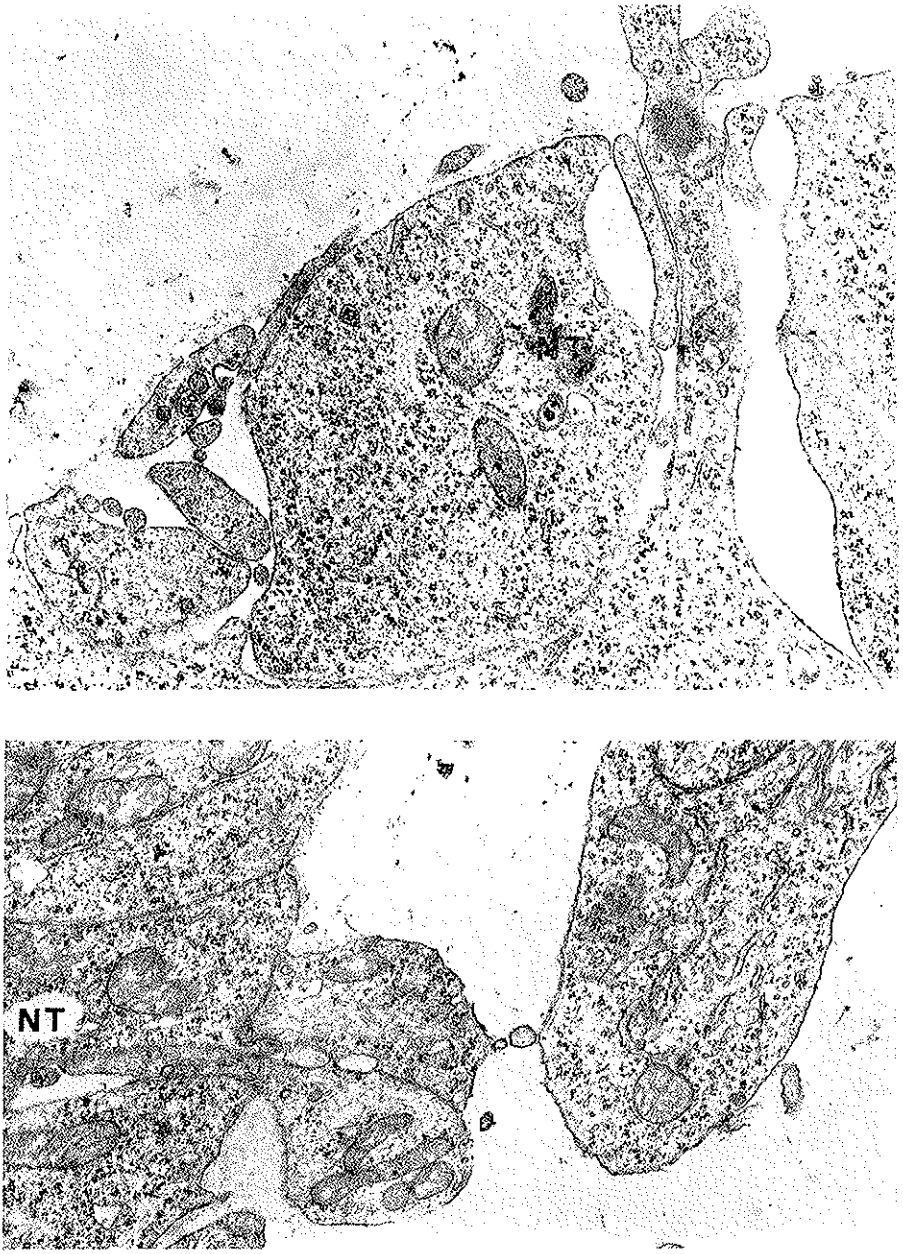


FIG. 1 and FIG. 2. 53 hour chick embryo; 30th somite. Some cell processes are shown sprouting from the neural tube (NT) directed toward the somite medial wall. (Fig. 1 × 20000 - Fig. 2 × 20000).

are partially inermingled with undifferentiated mesodermal cells; they remain in contact with the growth cones of pioneering fibres (Fig. 3) which elongate toward the deep aspect of the myotome which is undergoing differentiation. Exploring fibres coast along this target, but as a rule do not penetrate the myotome lamina. Other interpretations of the genesis of myotomes were forwarded by Langman and Nelson (1968) and by Mestres and Hinrichsen (1976) (see Panattoni and Sisto Daneo (1977) for further details on this subject).

Morphological observations made on chick embryo limb buds on the 4th-5th day of incubation have shown a precise chronologic correspondence between the advancing front of exploring nerve fibres along the limb and the first signs of determination of presumptive myoblasts.

Further studies are required to state precisely whether somitic cells are involved in the formation of limb muscle rudiments; however, such

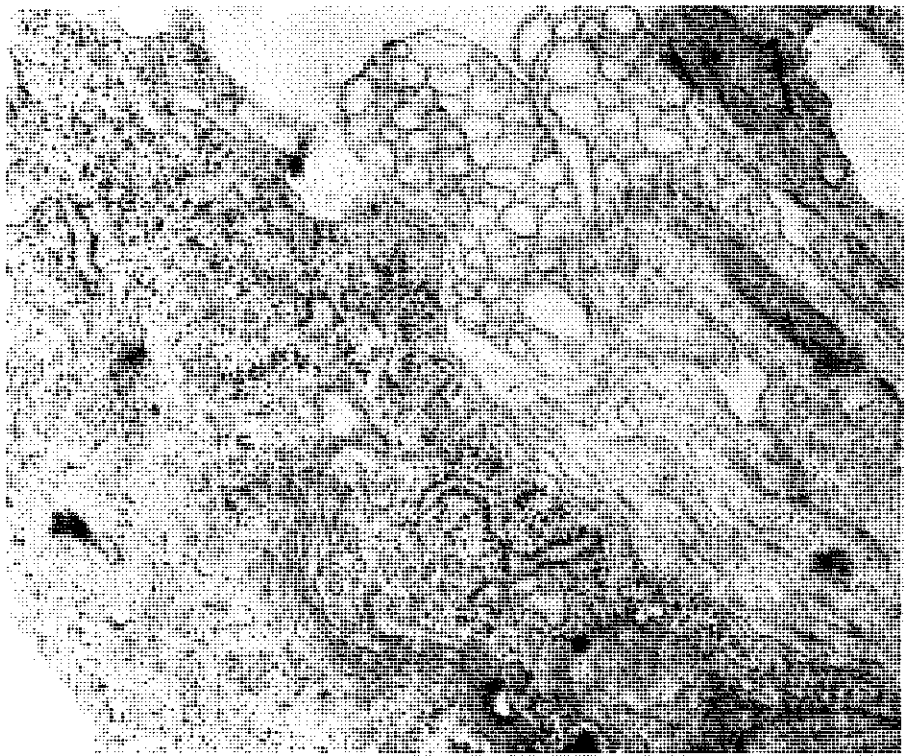


FIG. 3. 3 day chick embryo. The nerve fiber growth cones are frequently seen in contact with undifferentiated mesodermal cells. (× 43000).

a circumstance would not alter the concept that myogenic promotion is induced by nerve fibres.

In an attempt to investigate not only the topo-chronological characteristics of the somite-neural tube relationships, but also the causal mechanism which drives the determination of early myoblasts from somitic mesodermal cells, we have explanted *in vitro*—either alone or in the presence of a neural tube segment—early somites not yet contacted by pioneering nerve fibres (Peirone *et al.*, 1977). This caution is mandatory in order to exclude any possible neural influence already undergone *in vivo*, which would automatically have invalidated our experiment.

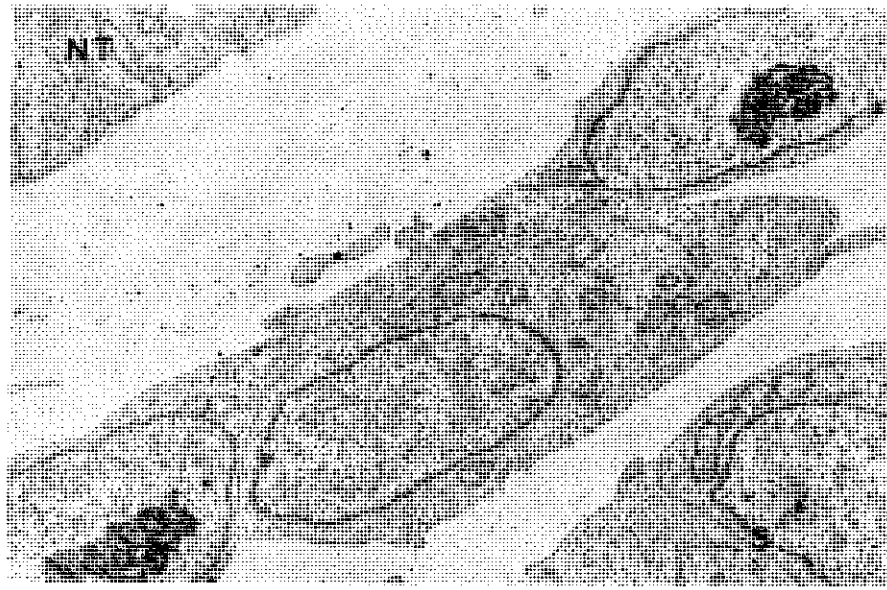
Accordingly, two culture groups were set up. In the first group six pairs of caudalmost somites from stage 10 embryos were co-cultured with neural tube explants of the same stage, while parallel cultures contained somites alone. In the second culture group all the somites of stage 9 embryos were partially associated with neural tube explants of stage embryos.

Myoblasts and myotubes made their appearance, after approximately a week of culture, only in those cultures where explants of neural tube were present. It should be also stressed that substitution of neural tube explants with spinal ganglia failed to support the determination of the myogenic line.

In conclusion, achievement of the myogenic determination of mesodermal cells, which obviously is the expression of an extensive reprogramming of the gene products of these cells may be assumed to require the constructive influence carried by growth cone filopodia of the exploratory axons of young motor neurons (Packard and Jacobson, 1976). At any rate, the potential determining role of neural crest cells which are scattered in the area where nerve cells contact mesodermal cells, should not be overlooked (Figs. 4 and 5).

The importance of the experiments performed by Sandor and Amels (1970) and by Strudel (1955), suggesting that the myotome maintains its usual pathway of differentiation following excision of the axial organs, may be stressed. However, we hold it imperative to take the stage of excision into account. Operations were carried out at 36 to 40 hr of incubation by the former investigators and at 12 to 30 somites by the latter, hence at stages too advanced, in our view, to allow reliable conclusions to be drawn. Moreover, the excised neural tube segment (corresponding to 4-5 somites) was too short.

A striking synchrony has long been evidenced between the pattern of myotome behaviour and the sudden increase in CAT (Giacobini, 1972).



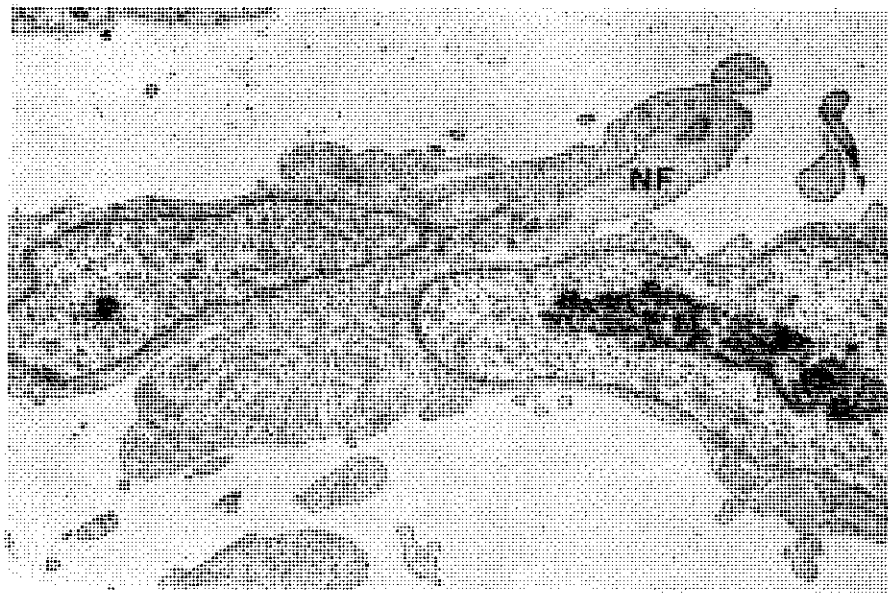


FIG. 4 and FIG. 5. 53 hour chick embryo. Neural crest cells migrating in the perineural space, alone or with nervous fibers.

NT - neural tube NF - nervous fiber. (Fig. 4 × 10000 - Fig. 5 × 11500).

According to Giller (Giller *et al.*, 1977) and coworkers, up to a 20-fold increase in CAT activity takes place in spinal cord cells cultured *in vitro* in the presence of muscle cells explanted from 12-14 day embryonic mice. This observation indicates that neurons and their targets may reciprocally interact. This leads us to postulate that somitic presumptive myoblasts *in vivo* after being determined by exploring nerve fibres, in turn stimulate the growth of the latter by a retrograde effect.

The differentiation of myogenic cells within myotomes

Within the myotome and the muscle primordia of the limb, early myoblasts differentiate into mature elements and later fuse to give rise to myotubes. This crucial event of differentiation is accompanied by structural and functional changes similar to those observed *in vitro*. Tbe changes we have observed include the appearance of AChE and ACh receptors, the development of sarcolemmal invaginations, the T-system and the onset of contractility (Filogamo and Gabella, 1967; Filogamo and Marchisio, 1971).

Experiments performed *in vitro* in our laboratory have shown that the step leading to the shift of immature myoblast to mature ones is accompanied by a dramatic rearrangement of the cell surface structure, resulting in the exposure of twice as many $-NH_2$ groups capable of reacting with the surface probe trinitrobenzene sulfonate (Sartore *et al.*, 1978); the same membrane rearrangement is likely to occur also *in vivo* during the differentiation stage within the myotome.

Although myoblast differentiation into myotubes is a mechanism altogether independent of neural influences, tbis fact does not necessarily mean that the process is not modulated *in vivo* by neural influence. In this connection, a further surprising observation made as early as the 3rd day of incubation, revealed that in the myotomes two types of myogenic cells can already be distinguished (Figs. 6 and 7) (Filogamo *et al.*, 1978). These cells exhibited marked similarities with the "slow" and "fast" myotubes occurring on the 6th day in the slow anterior latissimus dorsi and respectively in the fast posterior latissimus dorsi. Such an early presence of potential slow and fast fibres in myotomes (and in the rudiments of the ahove muscles) is certainly a puzzling problem, since most workers believe that their differentiation occurs much later (Atsumi, 1977).

Since the early differences between the two types of myotubes are a real fact, the problem of their nerve-dependent differentiation is also brought about at such an early stage.

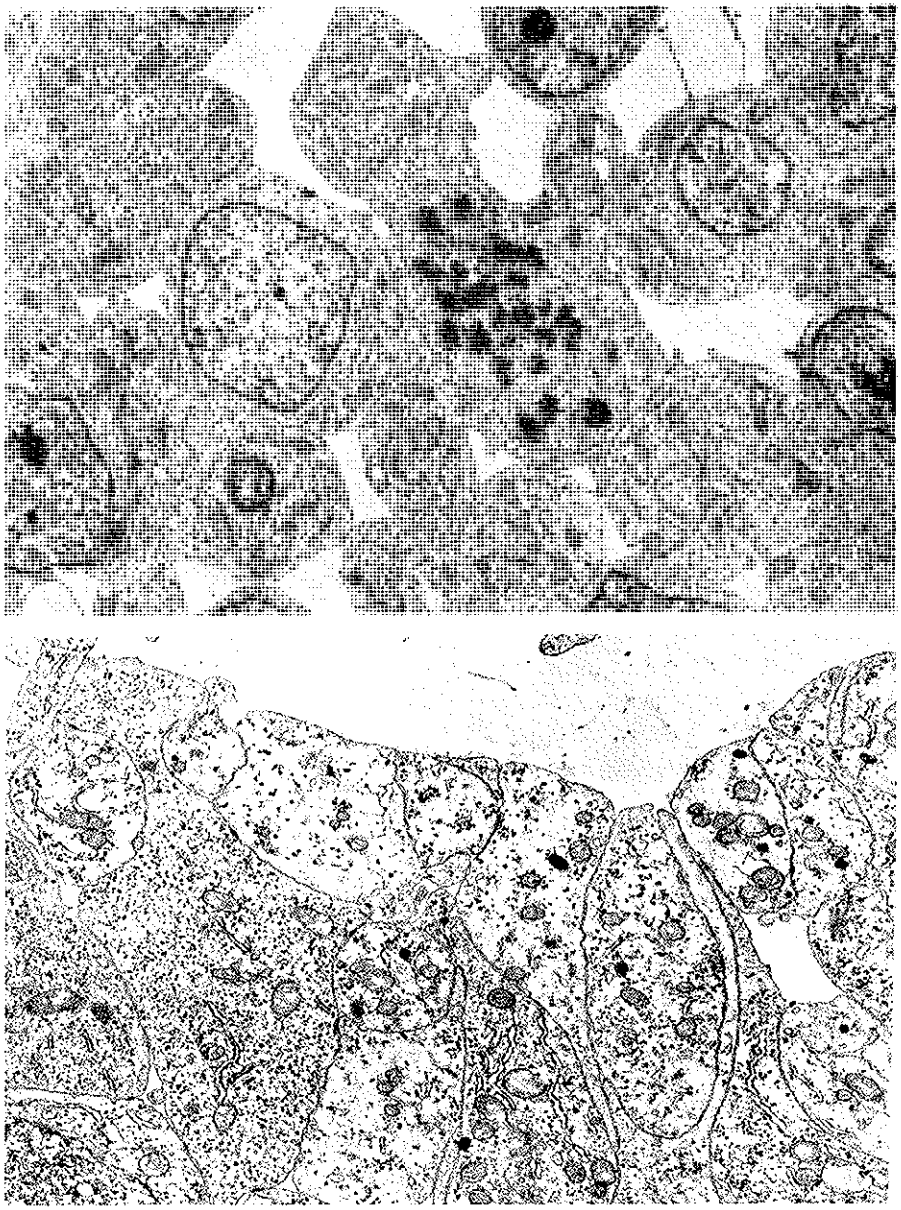


FIG. 6 and FIG. 7. 53 hour chick embryo. In the cranial myotomes, the myoblasts of the anterior and posterior parts are clearly different, the former being similar to slowly twitching contractile elements, the latter to rapidly twitching ones. (Fig. 6: anterior part × 7500 - Fig. 7: posterior part × 7000).

Worthy of note is also the fact that the cells of the myotome anterior portion, which mostly display a slow fibre-like structure, are seen to lie in closer contiguity with nerve fibres: the latter sprout tufts of undulating membranes, tips provided with vesicles and a few actual synaptic endings; conversely, the cells of the posterior portion of the myotome, exhibiting a fast fibre-like structure, are never contacted by nerve fibres on day six. Similarly, nerve fibres tightly contact slow elements in the ALD muscle, while around the fast elements of the PLD nerve fibre contacts are prevented by Schwann cells or by thin laminar fibroblast expansions (Filogamo *et al.*, 1978). Surprisingly, Rubinstein and Kelly's (1978) experiments involving denervation and immobilization in neonatal rats, have clearly shown that "... all the data support the idea that development of slow and not fast fibres is neurally activated".

Also at this stage, as in the preceding one, a retrograde influence may very likely be exerted by developing muscle rudiments upon neuronal maturation. Recently, Pittman and Oppenheim (1978), Laing and Prestige (1978) independently, have observed that neuromuscular blockade, obtained by treating 6-day chick embryos with botulinum toxin, curare or cobratoxin prevents physiological cell death occurring in the ventral horn. According to Oppenheim, the physiological activity of neuro-muscular synapses controls the rate of nerve cell survival. Since the number of synapses between days 6 and 9 is too small, it seems much more likely, in our opinion, that the physiological activity of transient contacts may be responsible for taking up and retrogradely transporting signals to nerve cell bodies.

The formation of endplates

The long-lasting ontogenesis of neuro-muscular contacts attains its final maturational phase with the onset of synapse development. Synapses are very seldom encountered, particularly on fast fibres, before days 10-11 (exceptionally on day 5), but on days 8-10 axons are ubiquitously present in large numbers within the muscle anlagen in chick embryos (free neurotization) (Filogamo and Sisto Daneo, 1977).

The freely growing expansions of nerve fibres spread in veil-like ruffling sheets (undulopodia) which sometimes further extend in to slender finger-like processes (filopodia). These very irregular expansions mostly show an electron-lucent appearance; in some cases, however, a content of smooth endoplasmic reticulum profiles may be revealed together with granules of variable density, distribution and arrangement. Many ter-

minals contain cysternae which seem to open toward the extracellular space: they probably represent the morphological counterpart of the fusion of new membrane patches accounting for axonal elongation according to the model proposed by Bray (1973) even if it cannot be excluded that they represent branching sites of axons as shown previously by Sisto Daneo and Fracchia (1977) in the tectum opticum. Sarcolemmal thickenings and spots rich in AChE activity are commonly found at transient sites of contact between myotubes and terminal expansions of axons: they suggest that a great deal of neuromuscular interactions occur at this stage of free neurotization of the muscle rudiment. Although the significance and the role of these transient contacts is still far from clear, we like to emphasize them because they occur immediately before the definitive impact of freely moving axons on myotubes and their reciprocal capture. Evidence for the release of hitherto unknown regulatory factors beside ACh has been recently provided by Podleski *et al.* (1978), who found that isolated explants of nervous tissue and cell-free nerve extracts may trigger the increase of ACh-R average density and induce the formation of high density patches. According to these workers the substance contained in the extract, and responsihle for the effect on ACh-R distribution, is a macromolecule of probable protein nature.

The mechanism involved in the organization of the synaptic pattern in chick embryonic muscles is triggered mostly between day 9 and day 11 (Sisto Daneo and Filogamo, 1974) even if the earliest synapses may be traced back to day 5. Two types of muscles synapses have been long described. One is tbe so-called "en plaque" synapse whose main feature is the local gathering of many fundamental nuclei and organelles which results in the bulging of the synaptic area in close proximity to branching nerve terminals; these bulging areas have long been known to contain ChE and ACh-R clusters. Tbe second type, called "en grappe" synapse, shows short, multiple contacts over relatively lengthy distances which do not give rise to any bulging area and any particular accumulation of nuclei and organelles; high AChE activity and ACh-R spots are also present.

Over these last few years we have tackled tbe puzzling problem of synaptogenesis by following a research line focused on the operative mechanism of motor endplate formation (Sisto Daneo and Filogamo, 1975).

The simple preliminary consideration we made was that the fundamental sole nuclei, like all the other myotube nuclei, are derived from fused satellite cells. The observation we made (Figs. 8-9-10-11) was that

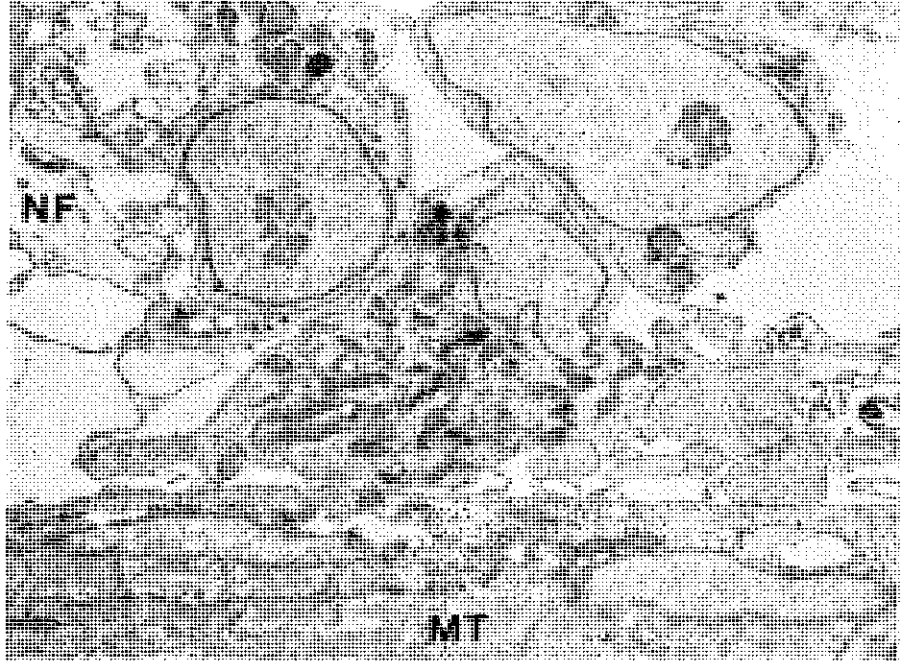


FIG. 8. 6 day chick embryo. In the limb bud some collaterals from a big axon bundle (NF) are reaching the muscle anlage. Some undifferentiated cells (one containing many mitochondria) are lying upon a muscle fiber (MT). (× 7400).

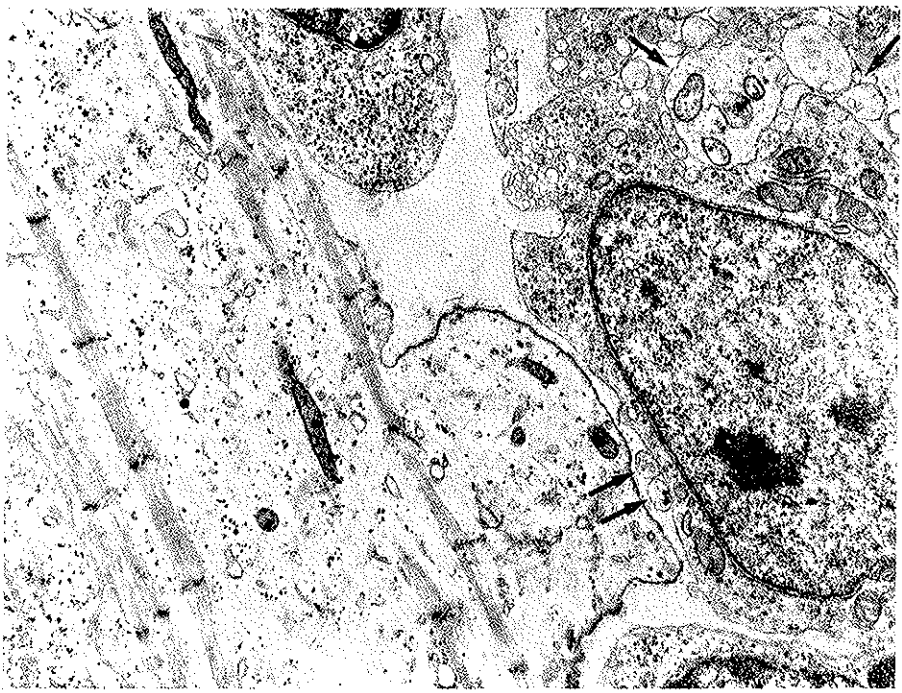


FIG. 9. 12 day chick embryo. In the Pectoralis Major a small axon bundle (arrows) is reaching a myotube's projection; some undifferentiated cells are around. Note the sarcolemmal thickening at the contact point with very small nerve terminals (arrows). (× 12500).

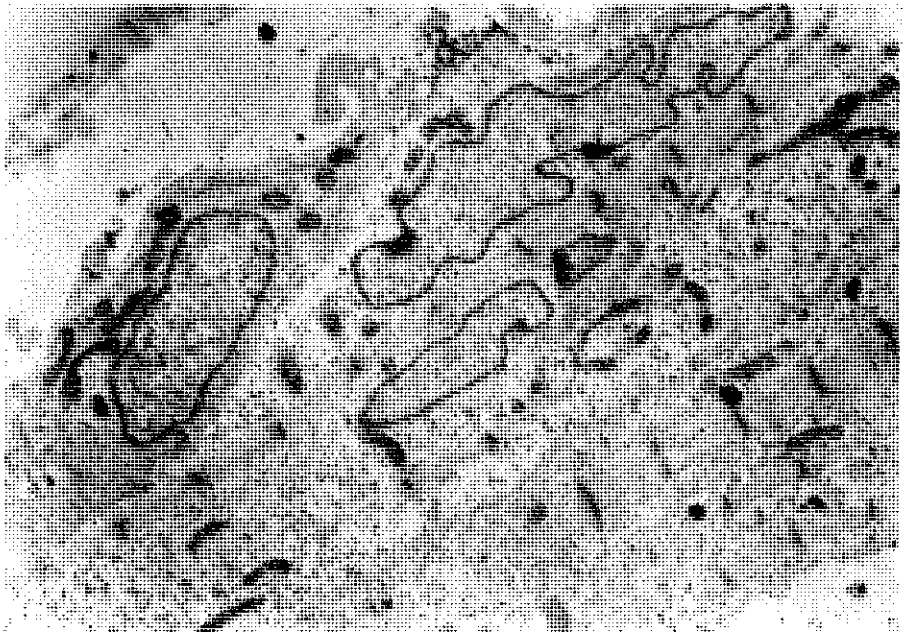


FIG. 10. 12 day chick embryo. Superior oblique muscle of the eye. Two subsynaptic nuclei in the bulging of a focal end-plate; a satellite cell is probably fusing with the myofiber. (× 9500).

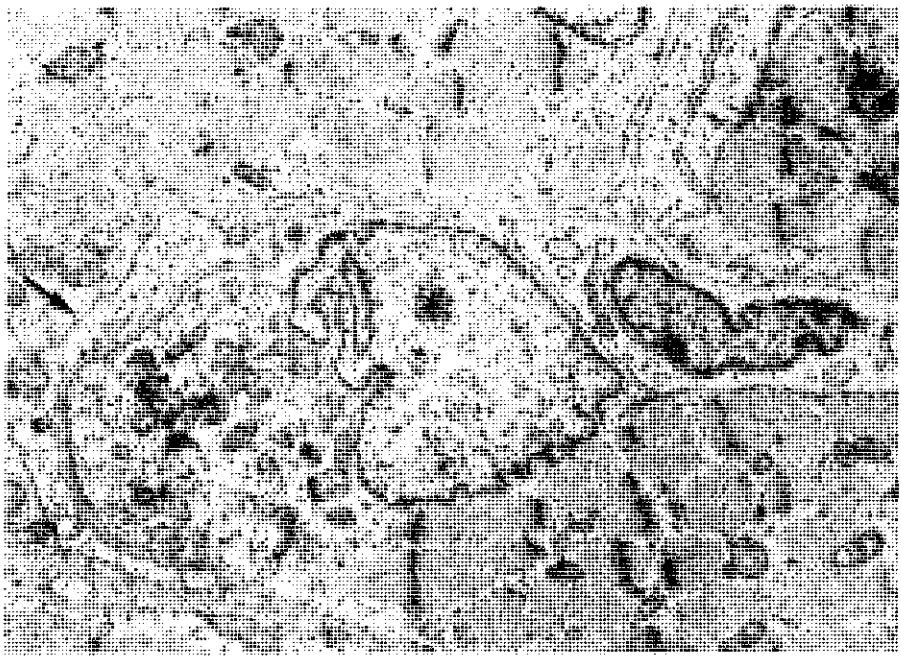


FIG. 11. 18 day chick embryo. Posterior Latissimus Dorsi muscle. A young endplate "en plaque" is showed. Note the hypolemmal nuclei and the great number of mitochondria. At arrow nervous fiber. (× 19000).

in the fibers of fast muscles (where the whole dynamics of development is different from that of slow fibers) the first step of the organization of a focal synapse is marked by the sudden fusion of several mononucleated satellite cells at a myotube area lying immediately underneath the complex terminal branching of a motor axon. The actual fusion step is preceded by the aggregation of a cluster of satellite cells just above a restricted area of the sarcolemma where nerve fibers and glia cells are tightly intermingled; moreover, the fusion event occurs concomitant with a discrete blebbing of the facing sarcolemmal surface and terminates when a well defined hillock appears. The electron microscopical analysis of the hillock demonstrates

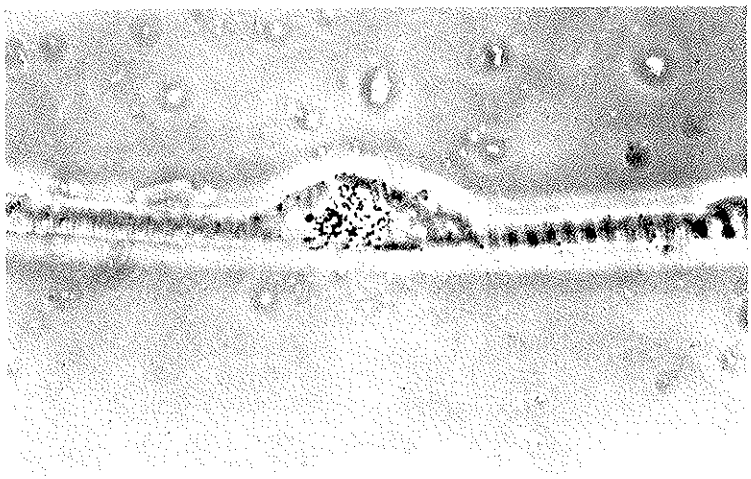


FIG. 12. 16 day chick embryo. Posterior Latissimus Dorsi muscle. Focal endplate. Synaptic ACh-R by optical autoradiography. (× 320) (Courtesy of Dr Bourgeois, 1978).

a local concentration of organelles involved in biosynthetic mechanisms (nuclei, rough ER, Golgi complex, mitochondria, etc.) which unequivocally belong to the fused cells. A frequent constant feature of the newly formed hillock is the appearance of a positive AChE reaction and ACh-R localization (Fig. 12).

Such a mechanism of endplate formation involving the local fusion of satellite cells has been already reported. Very recently, it has been confirmed in rat intercostal muscles (Kelly, 1978). However, some questions are as yet unanswered. A main question concerns the origin of endplate AChE (Vigny *et al.*, 1976) and ACh receptors. Direct evidence is lacking either in favor of or against an autonomous management of a limited pool

of such molecular complexes via lateral diffusion (Changeux and Danchin, 1976; Betz *et al.*, 1977; Varon and Bunge, 1978); anyhow, it is not clear why the synthesis of the above molecules may not be carried out at the level of the plate sole, which contains a full array of nuclei and cytoplasmic organelles (Sisto Daneo and Filogamo, 1976).

Nothing is against the concept that, once the endplate formation has

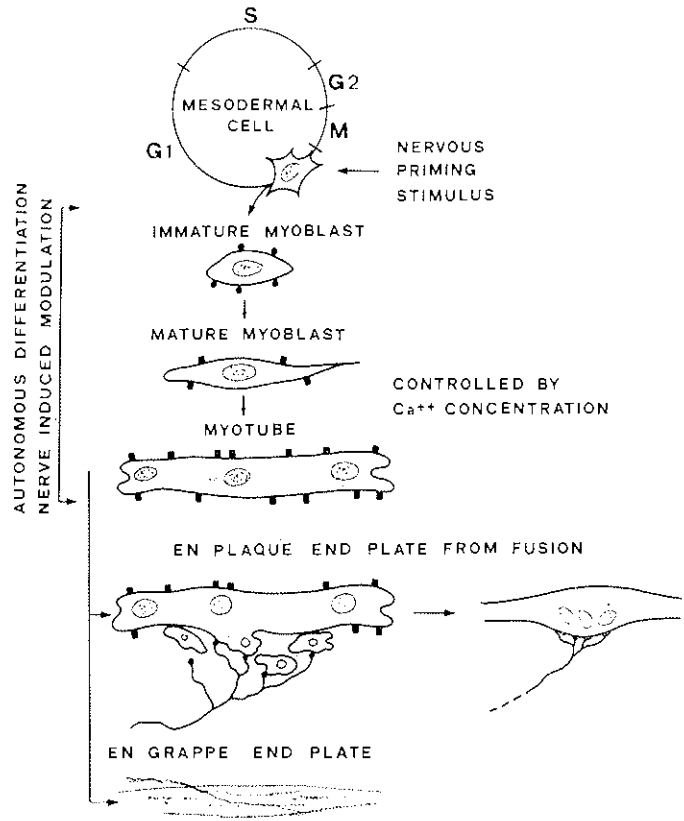


FIG. 13. Neural influences on myogenesis. Scheme.

been achieved, synthesis becomes restricted to this critical area, and is gradually lost by extrasynaptic segments of muscle fibres, which concurrently lose the transient stimulatory contacts provided by the tips of axonal growth cones in the presynaptic period.

On the contrary, the lateral diffusion of molecules is conceivably the essential mechanism in the "en grappe" synaptic contacts.

Conclusions

A widely reported concept claims that the earliest stage of myogenesis takes place independently of any neural contact. Our evidence indeed supports the concept that either somitic or extrasomitic mesodermal cells are subjected to neural influences, which are chronologically and topographically consistent with their pattern of determination into myogenic cells (Fig. 13). The potential determining role of neural crest cells, which are scattered within the area where nerve cells contact mesodermal cells, should not be overlooked. According to us, all the experiments *in vitro* which do not strictly exclude any previous contact of the cultured mesodermal cells with nerve fibres do not support at all the assumption that determination is independent of neural contact. Even if true, this fact does not rule out the possibility that sophisticated media *in vitro* can mimic neural influences.

The second step of myogenesis is the "differentiation" of tbe presumptive myoblast, namely its evolution first into a mature myoblast and then into a myotube. This process may actually occur independently of neural influences, even though the latter doubtlessly modulate their differentiation, such as for instance the nerve-mediated modulating effect on the formation of slow and fast fibres. The differentiation of slow and fast fibres takes place far earlier than is generally believed, within the myotome or at the earliest arising of limb primordia by the 3rd day.

The third step is the process of formation of endplates. We again suggest that the crucial mechanism in fast fibre focal endplate construction is represented by the sudden fusion of a cluster of satellite cells with the myotube. Fusion coincides critically with active localized branching of motoneuron terminals and with the striking increase in CAT within them. Both phenomena are possibly elicited by quantitative and qualitative organizational changes in motor nerve centres.

Since "fusion" of satellite cells with myotubes is the mechanism of focal endplate formation, AChE and ACh-R synthesis in the synaptic area is possibly realized by the organelles of the fused satellite cells; the lateral diffusion of molecules along the sarcolemma toward the endplate may be just a secondary, though important, epiphenomenon.

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DISCUSSION

GREENE

A question on a point on which I was not quite clear. What did you feel was the importance of the early low levels of choline acetyltransferase activity that appeared before synapse formations? Were you implying that during the early contacts choline acetyltransferase or acetylcholine itself was playing a role in interaction between nerve and muscle?

FILOGAMO

Do you inquire about the enzymic levels of the earliest or subsequent stages of myoblast differentiation?

GREENE

No, not the large increase itself, but the earlier period in which there were low levels of choline acetyltransferase activity.

FILOGAMO

The simultaneous appearance of CAT and of the earliest differentiating myoblasts in the same site and day, corresponds to the presence therein of exploratory nerve fibres. The impact of CAT on the determination of the myogenic line or on any differentiation phase is obscure. Botulin toxin, DFP or α-bungarotoxin seem not to affect the earliest development of the myogenic line *in vivo*. Further studies are needed. Only at the moment of the synaptic outburst on day 12 does CAT abruptly increase, simultaneously with an increase both in synapsis number throughout the central nervous system and in embryo motility. Such an increase is essential to synapsis development. During the time interval, a low CAT level accompanies a low level in functional activity; this does not preclude the assumption that the transmission mechanism, even though rudimentary, is operative, hence functional and able to play a modulatory role on the development of neuromuscular relationships.

GIACOBINI

Perhaps I can carry on the discussion on this subject since we have studied similar phenomena, as I will shortly relate, in the peripheral nervous system (autonomic ganglia and iris). I think that the characteristics of this period in the muscle are described in detail in our study. If you remember the slide of Dr. Filogamo, where the enzymes were represented together with the receptor, you will have noticed that the development of the cholinergic receptor and the esterase are simultaneous and followed by the development of the cholinacetyltransferase activity (which is much lower). This will increase at a much later stage. So in my opinion, answering your question with regard to our preparation, you need only a threshold biosynthesis of acetylcholine, as in fact we have found during this first period. What you see later is the "morphological" formation of the synapse. This is a later event. I do not know whether Professor Filogamo is in agreement with me.

GREENE

That is just my point. The question is: does the early presence of choline acetyltransferase activity play a role in the very early development of the myoblast? Not of the synapse, but of the myoblast itself—that was my question.

GIACOBINI

It plays a role in this period of "threshold synthesis", or potential synthesis as it is called. How important this is for the formation of the synapse we do not know.

FILOGAMO

Despite some lack of confidence, I do not believe that CAT plays a determinant inductive role on the appearance of the myogenic line in the chick embryo; as already stated, it is likely to be involved in the modulation of differentiation: how and in what direction have still to be discovered.

NIRENBERG

Do you get synapses formed at this very early stage? Few synapses? Could these be—maybe the later synapses are just following the early ones that form. Do you ever find any evidence for synapse formation at this very early stage?

FILOGAMO

Earliest, few, conventional synapses were consistently detected by Laura Sisto Daneo in our laboratory on day 5 of incubation in a very high number of observations. On days 6 and 7 few synapses are encountered; their outburst takes place on day 11. Nevertheless, the earliest contacts arise at the 35th hour.

HAMBURGER

I think one should clarify some possible misunderstandings. When Dr. Nirenberg just talks about synapses, he actually means probably the first contact. And I think one should make a distinction between the first contact, which is already functional, and the formation of what is commonly termed the synapse, which is a very highly specialized apparatus.

FILOGAMO

I entirely agree.

NIRENBERG

There are two kinds of contact, though. One should distinguish: one would be a functional synaptic contact with transmission, and the other would be a non-functional.

FILOGAMO

All contacts are likely to be functional both as concerns transmission and antero- or retrograde neuromuscular influence. According to developmental stages, however, contact devices change in morphological terms, whereby modulation of the work accomplished by them also changes.

BURNSTOCK

Perhaps I could say what happens during the formation of an autonomic neuromuscular junction. One needs to look at a wide spectrum of criteria when considering whether a junction is functional or non-functional. For example, there may be close contact, with no morphological, biochemical or functional specialisation. During the perinatal development of autonomic neuromuscular junctions, a stage is reached where the geometry of the nerve-muscle relationship and the ultrastructure of vesicles and other organelles in the nerve terminals look like those at a mature functional junction, as does the histochemical localisation of the enzymes involved in transmitter synthesis

and breakdown. However, the junction is not functional since junction potentials cannot be recorded in the muscle for about another week. In other words, morphological and biochemical programming appears to be complete several days before the junction becomes functional. Whether the mechanism for release of the transmitter is not working, whether there is some material in the synaptic cleft which does not allow the transmitter to get through, or whether the receptors are not working, is something which we have not yet solved in our system. So it seems to me that you have raised a question which can only be solved by a very careful multidisciplinary approach in which you take all these different factors into account.

NELSON

At synaptic junctions between hybrid cells and muscle and at early synapses between primary neurons and muscle one can have a very primitive looking apparatus that is still capable of physiologic function. One certainly has to have adequate postsynaptic receptor density and so on, but function can precede an elaborate morphology at least. This function can develop rapidly within a very few hours.

LEVI-MONTALCINI

I wonder whether you studied the effect of the neural tube in the developing chick embryo. It would be very easy to extirpate rather long segments of the neural tube in 12 somite embryos, that is before the onset of the circulation. The operated embryos undergo, as well known, normal development and can even hatch. It would be of considerable interest to see if myoblasts in the trunk level deprived of the spinal cord, would still undergo normal differentiation or if they would fail to differentiate for lack of the "spinal cord factor".

FILOGAMO

So far, we have failed to perform this experiment correctly; further attempts are being planned.

FAMBROUGH

It seems that the question "when does the synapse form?" is not an adequate question any more, because some of us have one idea of what to call a synapse and others have another idea. Thinking about all of the criteria for synapses, they are not met concidentally during development. Now we

have enough technological know-how to ask about individual components in the formation of synapses—when are they organized or expressed during synaptogenesis.

I also wanted to say just a little about the last idea that Dr. Filogamo presented on the genetic regulation of the nuclei in the muscle. In adult muscle fibers there are so many nuclei that if you could line them up end to end, the line would be about as long as the fiber, and yet the region of nerve-muscle contact (which is about one part in 10^4 of the surface of the muscle fiber) is where much of the unique expression of gene products occurs. It makes a lot of sense to have production of the synaptic molecules right at the point of nerve-muscle contact. So how do you go about testing whether that is the case? In the last couple of years some methods have been developed to the point of being sufficiently sensitive for such tests. There is the study by Wake on acetylcholinesterase production in embryonic chick muscle. The interpretation of the histochemical study is that the esterase is first being deposited on the surfaces of the fibers at the myotendenous junctions and later in the central area where the contact with nerve is occurring. There is a remarkable redistribution of internal esterase which probably is an expression of some kind of rearrangement of gene expression through the nuclei of the fiber. On the receptor side, Dr. Atsumi, who has been working with me this past year, has been studying which nuclei are responsible for production of acetylcholine receptors during development. The strategy is to determine which Golgi apparati around individual nuclei are busy producing receptor molecules. Again, it appears that the nuclei in the vicinity of newly forming neuromuscular junctions are the sites of biosynthesis of the greatest numbers of receptor molecules.

CANGIANO

Did I understand you correctly to say that botulinum toxin prevents synapse formation and cell death? What is the interpretation of this?

FILOGAMO

The mechanism of action of α-toxin on the chick embryo neuro-muscular development is not fully clarified at present. Additional studies have to be carried out at the different developmental stages in order to better understand this point.

CANGIANO

What is the interpretation of this?

LEVI-MONTALCINI

As mentioned by Dr. Filogamo, two different groups: Pittman and Oppenheim in the United States and Laing and Prestige in Great Britain, performed independently and at the same time the ingenious experiment of injecting botulin toxin in developing chick embryos, a toxic agent wbich blocks the formation of neuromuscular junctions. Both groups reported that the motor horns in the spinal cord of the treated embryos did not show the severe nerve cell depletion which takes place in normal embryos during early developmental stages. Since however the process of cell death in the ventral half of the spinal cord takes place at very early stages, it seems difficult for me to correlate these effects, as suggested by Oppenheim, with direct interference with the onset of physiological activity of neuro-muscular junctions.

PRESPECIFICATION AND PLASTICITY IN NEUROGENESIS

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INTRODUCTION

The concepts "prespecification" and "plasticity" are often ill-defined: to make them useful, exact criteria as well as the stage of development to which they are applied have to be stated precisely. For instance, plasticity often refers to functional adaptation. But our discussion will deal only with structural modifiability. The term "prespecification" conveys the notion that a developmental process, or phenotypic characteristics of a particular neuron strain, are irreversibly programmed or determined from an early stage on. The neural tube which gives rise to the entire central nervous system represents an early stage. One can inquire whether the undifferentiated neural tube of a 2-day chick embryo has acquired already the *regional specification* for cervical, brachial, thoracic, lumbar sectors which can be characterized by several criteria in later stages. One widely used test for specification is the transplantation experiment borrowed from general experimental embryology. If prospective brachial and cervical or brachial and thoracic segments are exchanged in the 2-day neural tube, they differentiate according to their site of origin, rather than the site of implantation, as shown, for instance, hy the presence or absence of a lateral motor column in the transplant (B. Wenger, 1951). Another criterion would he the circuitry for coordinated limb movements which develops only at limh levels. Transplantation of the brachial neural tube to the lumbar level in 2-day chick embryos results in the performance of synchronus leg movements akin to wing flapping. Alternating movements were never observed; hence segmental specification defined by this criterion is also fixed at that stage (Narayanan and Hamburger, 1971).

If similar experiments are performed on tail bud stages of *urodele* embryos, the outcome is different. Prospective thoracic segments transplanted to the forelimb level differentiate all characteristics of a limb-innervating level, including normal reflexes and support of normal ambulatory movements (Detwiler, 1923). Obviously, irreversible specification of these aspects occurs at a later stage. The data show that taxonomic status and stage of development are two important parameters which have to be identified precisely. Furthermore, the data suggest that in the chick embryo regional plasticity might still prevail in embryos younger than those in which the above-mentioned tests were performed.

A third fundamental parameter in considering the two concepts is level of organization. At a time when the focus is on the cellular and subcellular levels, this aspect deserves special consideration. One of the ground rules established by classical experimental embryology, at least for vertebrates, is the progression of differentiation from larger supercellular units to increasingly smaller subunits. The larger units are called "morphogenetic fields". They are defined operationally as being endowed with a high degree of plasticity or regulative capacity. Relatively large defects are restored; fields cut in half can produce whole organs. For instance, in the rostral part of the neural plate, a regulative forebrain-eye field can be identified; it is set off from an adjacent midbrain field. Transplantation of part of the field to the flank results in the formation of one or two eyes in the transplant and of two eyes in the regulating residual field (Adelmann, 1936). In subsequent stages, the field becomes segregated into two eye fields and a forebrain field, each no longer capable of substituting for the other, but still endowed with regulatory capacity within its boundaries. Following this, pigment epithelium, retina and retinal substructures become specified. Individual cells or small groups of like cells are the last units to acquire specification. The mechanism of this hierarchical sequence of segregation into smaller units, or "self-organization" is obscure. Exchange of information between differentiating areas and cells as well as gradients seem to be involved.

Implicit in this discussion is the insight that specification of definable supercellular aspects occurs before differences can be detected by other presently available methods than transplantation; and, furthermore, that the terms "prespecification" and "plasticity" are not mutually exclusive. The same substructure (field) can be considered as prespecificed with respect to other fields and plastic within its own boundaries. This holds even for individual differentiating neurons (see below).

There are at least three areas in neurogenesis in which the analysis of particulars of prespecification and plasticity is in progress:

1) origin of strain specificity;

2) formation of the stereotyped central axonal pathways and peripheral nerve patterns;

3) selectivity of synaptic connections.

I shall omit the last topic which has been reviewed extensively in recent years (see books edited by Barondes, 1976; Gottlieb, 1976; also Jacobson, 1978). My discussion is limited to the origin of strain specificity and the determination of peripheral nerve patterns.

Origin of Strain Specificity

When and how do the hundreds of different neuronal strains acquire their specific phenotypic characteristics? To what extent are strain-specific characteristics irreversibly fixed and to what extent are they modifiable? How is the population size of neuronal units regulated? These are some of the questions that can be asked concerning strain specificity. We shall address briefly the first question.

With one exception to be mentioned below, different neuron strains are represented by populations of like cells. One can assume that each strain represents a polyclone (Crick and Lawrence, 1975) which is derived from a relatively small number of founder cells. The founder cells would be the smallest units formed in the segregation process discussed in the introduction.

Critical events in the establishment of strain specificity in the central nervous system seem to occur in the neural plate and the early neural tube. Ramón y Cajal (1890) who was the first to apply the silver impregnation method to embryos observed postmitotic differentiating neurons in the spinal cord of 2-day chick embryos: motor neuroblasts and commissural neuroblasts were clearly identifiable by their position and direction of outgrowth of their axons. The extensive and carefully analysed rotation experiments on the medulla level of the neural plate of the *Axolotl,* by C.-O. Jacobson (1964, 1976) have shown that the brain stem nuclei are fairly well stabilized at that stage. However, regulative capacity was observed within areas forming a particular motor nucleus, and the author envisages the neural plate with rising folds as a pattern of small, partly overlapping fields. In other words, we deal apparently with

a critical stage of transition from a pattern of small subunits with restricted plasticity to a pattern of subunits representing specified strains. An early establishment of irreplaceable precursor cells for motor neurons in the spinal cord can be deduced from regeneration experiments in a slightly later stage, the neural tube of early tail bud stages of *Ambystoma* (Holtzer, 1951). Following extirpation of the lateral half of several neural tube segments, regulation occurs predominantly by overgrowth from the dorsal part of the intact side. Regulation is fairly complete histologically, with the exception of motor neurons which are missing. Obviously, plasticity still prevails for other neuron types, but the precursor of motor neurons have been sorted out already. It remains to be seen whether the model suggested by these experiments applies only to large, early differentiating neurons or whether it can be generalized.

There is a unique instance in which it has been possible to define precisely the time of specification of a neuron. I refer to the single pair of giant *Mauthner cells* (M cells) in the medulla of fishes and aquatic amphibian larvae. In a classical study, Stefanelli (1947, 1951) has analyzed the time of origin of these cells in frog embryos. In the late gastrula, the M cells are not yet specified. If the M-cell forming area is extirpated on one side and grown in tissue culture, no M cells differentiate in the explant, but the wound in the donor embryo is closed, and a normal M cell is differentiated. A few hours later, after the appearance of the neural plate, the same experiment results in the differentiation of one M cell in the explant and one in the donor. This, then, is the critical stage of incipient specification. The experiment shows that more than one cell, probably a small cluster, has the capacity to form M cells. In normal development, one cell only emerges as the M cell (perhaps one in central position in a small concentric gradient field). In the experiment, the cluster is divided between the explant and a residual group in the donor, and one cell in each becomes programmed for M cell differentiation. The same situation was found in the above-mentioned experiments of C.-O. Jacobson, using a different experimental design. If the edge of the transplant cuts across the M cell area, one M cell was found occasionally in normal position near the edge and another in the rotated piece. The experiments were done in neural plate stages of the *Axolotl*. Stefanelli found in experiments done at later stages that an M cell was formed only in the explant, indicating irreversible and irreplaceable specification by the time the neural folds close. An autoradiography study by Vargas-Lizardi and Lyser (1974) has shown that the specification follows shortly after the terminal mitosis in the late gastrula. Another

set of large neurons, the transient intracentral sensory *Rohon-Beard* cells in amphibians likewise undergo their terminal mitoses in the gastrula stage (Spitzer and Spitzer, 1975).

At the other end of the spectrum are the neural derivatives of the *neural crest*. The latter originates as a wedge of cells at the point of closure of the neural tube which detach themselves from the epithelia and begin their migration laterally and ventrolaterally. They give rise to a remarkably diverse set of structures. In the head, they form mesenchyme, the trigeminal and root ganglia and their glia, and furthermore part of the cartilaginous visceral skeleton. In the trunk they produce melanophores, dorsal root ganglia, sympathetic para- and prevertebral and enteric ganglia, glia and adrenal medulla.

Experimental tests for specification were preceded by mapping of the migration routes and fates of different regions of the crest. Two methods are being used for mapping: replacement of a small sector by the same sector from a ^{3}H-thymidine labelled donor, or exchange of identical regions between chick and quail embryos, the quail cells being identified by a nuclear marker.

We encounter here again a progression of specification from larger to smaller subunits. In the chick embryo, in an early stage (1½ days), crest cells destined for neural derivatives can be interchanged with cells destined for mesenchyme or cartilage differentiation: they follow the migratory routes characteristic for their new topographic position and subsequently differentiate along the line of the cells for which they have been substituted. This was shown by Noden (1978) using the tritiated thymidine labelling technique. In a slightly later stage, plasticity in the different categories of neural derivatives was demonstrated by Le Douarin and others (1975) using the chick-quail exchange technique. Normally, the neural crest cells at the vagus level migrate into the intestinal tract and form the cholinergic Auerbach and Meissner ganglia and plexuses; crest cells at the level of trunk segments 18-24 form adrenergic paravertebral sympathetic ganglia and adrenal medulla. Transplantation of the prospective adrenal medulla segment of the neural tube and crest of a quail embryo to the vagus site of a 2-day chick embryo results in the migration of the transplanted crest cells into the intestine, where they form typical cholinergic Auerbach-Meissner plexus ganglia. The reciprocal experiment shows the same versatility of the transplants. The plasticity of the neural-crest derived neurons offers the opportunity to analyze the conditions which influence specification. Micro-environmental factors encountered during migration can have an influence (see Patterson, 1978),

but on the other hand, the terminal site of ganglion cell differentiation can also decide the specification of the transmitter under conditions which exclude migration (Smith and others, 1977).

In recent years, extensive *in-vitro* studies in several lahoratories have demonstrated that isolated embryonic or postnatal sympathetic neurons can shift from the production of adrenergic to the production of cholinergic transmitters under a variety of conditions. We shall not discuss these experiments in detail (see reviews by Patterson, 1978 and Varon and Bunge, 1978). Thus the plasticity of this particular category of differentiated neurons is demonstrated for at least one significant phenotypic trait: neurotransmitter synthesis.

Formation of Peripheral Nerve Patterns

Since the pioneer work of Harrison at the beginning of this century, the formation of the stereotyped nerve patterns in limbs has served as a convenient model for the analysis of nerve pattern formation in general. I shall consider primarily the motor component.

Harrison's tissue culture experiment, apart from its major objective to prove the axon outgrowth theory, showed also that the growth cone requires a solid substrate, that is, mechanical guidance. His limb transplantation experiment (in amphibian embryos) gave evidence that pathfinding is an interaction between growth cones and their microenvironment. The finding that foreign nerves can form a fairly typical nerve pattern in transplanted limbs led to the proposition that limb tissues form pathways which guide the nerves to their targets (Harrison, 1907). But it hecame obvious soon that mechanical guidance is only a permissive condition: *directional* outgrowth requires more specific cues. Sperry then directed our thinking along the line of *chemoaffinity* between the growth cone and its suhstrate. The chemoaffinity hypothesis was designed originally to explain selective synapse formation, but it was extended later to the formation of nerve patterns (Sperry, 1963). It postulates matching cytochemical affinities hetween growth cone and specific chemical cues provided by preneural pathways.

Other hypothesis have been proposed. The hypothesis of "myotypic specification" assumes an original random distribution of nerve fibers. The target muscles would then specify the uncommitted axons hy imprinting on them their own biochemical tag. Another hypothesis also assumes random outgrowth, but specification would be imposed by functional validation: only fibers that have established functionally appropriate

connections would survive. It should be mentioned that both hypotheses were based largely on nerve regeneration experiments. They can be ruled out for initial embryonic innervation, since initial outgrowth is not random, as will be shown presently. An argument against functional validation is the simple fact that the basic nerve pattern is formed before function begins.

We ask now the specific question: how do axon bundles from a particular motor pool find their way to their particular target muscle? Before the question can be approached experimentally, a map of motor centers for individual muscles has to be prepared. Landmesser and Morris (1975) have constructed a map based on successive stimulation of the lumbar segmental nerves and recording from individual muscle nerves, in chick embryos, at different stages. The results are unequivocal: the same combination of segmental nerves which innervates a given muscle at a fully functional stage does so at the earliest stage that gives responses, that is, at 6-7 days, when the first spontaneous movements were observed and the individual muscles have just become segregated from a common muscle mass. Stimulation by a "wrong" segmental nerve was not observed. Hence a strong case can be made against initial random distribution and for direct, selective outgrowth from the very beginning. Since the correct innervation pattern is established before onset of natural neuronal death, the latter phenomenon is not implicated in correcting fiber connections with wrong muscles. The elimination of large numbers of motor neurons after day 7 results from unsuccessful competition of fibers belonging to the same or a related motor pool. Miotypic specification is ruled out because the axon bundles find their way along selective routes before they synapse. The chemoaffinity hypothesis seems to give the best explanation of the data.

An experiment done by Dr. Anne Bekoff in the laboratories of Dr. P. Stein and myself strongly reinforces the case for very early specification of the limb motor system. It was mentioned that spontaneous leg motility begins at day 7. The movements are non-reflexogenic since the reflex circuits are not yet closed at that stage. The jerky, convulsive-like character of the movements suggested absence of muscle coordination. To test this point, Bekoff recorded EMGs with floating electrodes in 7-day embryos from a pair of synergists and also from a pair of antagonists, shortly after onset of leg motility. Contrary to expectation, she found that synergists are activated simultaneously and antagonists with a time lag, at that early stage (Bekoff and others, 1975). Coordination is still imperfect; it is refined in subsequent stages. The experiments dealt with

intra-joint coordination; inter-joint coordination exists at least as early as day 9 (Bekoff, 1976). Thus, a different approach from that of Landmesser and Morris leads to the same conclusion: that the stereotyped mature nerve pattern and selective synapse formation are established by initial outgrowth along selective pathways and not by selection from randomly distributed fibers. The experiments go a step further: they demonstrate that the central circuitry for coordination is also prespecified. One has to postulate that at the onset of motility there exist already in the lumbar spinal cord particular interneuron centers which drive individual motor pools and, furthermore, that the centers for different motor pools are linked by excitatory and inhibitory synapses which account for the patterned motor output.

One can hardly expect stronger evidence for early prespecification in the establishunent of nerve pathways and functional neuromuscular connections. At the same time, there exists an equally suhstantial set of data which seems to be incompatible with this notion. I refer to the abovementioned limb transplantation experiments of Harrison which have shown that foreign nerves can grow along the typical pathways and form functional synapses with inappropriate muscles. How can we extricate ourselves from this dilemma?

Recent findings of Dr. M. Hollyday on chick embryos seem to open up a new road to a better understanding of pathway choice, by an analysis of the origin of nerves innervating identified muscles in limh transplants. A prerequisite was the preparation of a map of the motor pools in the lumbar motor column which would be more precise than the one based on motor root stimulation. The method using retrograde axoplasmic transport of horseradish peroxidase (HRP) was employed. HRP was injected into identified muscles and reaction product was localized in the lateral motor column (Hollyday, 1978, 1979). Landmesser (1978) independently obtained almost identical maps for the motor pools of most leg muscles. The maps estahlished for advanced embryonic and post-hatching stages were identical with those found in 10- to 12-day embryos. Both investigators found that there is no correlation between the proximity of muscles in the leg and the topographic position of the motor pools innervating them; nor are the motor pools grouped according to the joints on which they act. However they did find a grouping of motor pools according to embryonic origin of the muscles (see also Ferguson, 1978). Romer (1927) had shown that the leg muscles are derived from two primordia, a dorsal and a ventral muscle mass, respectively. The motor pools for muscles derived from the ventral muscle mass have a medial position in the motor

column, and those for muscles derived from the dorsal muscle mass have a lateral position.

The experimental design of Hollyday was as follows: Supernumerary leg and wing buds were transplanted immediately rostral to the right leg bud of the host, in 2½-day embryos. Normal leg innervation is by segmental nerves 23-30; transplant innervation was by segments 23-25, and right host leg innervation by segments 26-30. Homologous muscles in the host and transplant were injected with HRP at 12 days and the respective motor pools were identified. In the first test, the lateral gastrocnemius, an ankle extensor, was chosen because its normal motor pool is located in the caudal part of the column. Simultaneous injection of the host and transplant gastrocnemius was successful in several cases. The motor pool for the transplant gastrocnemius was found in a medial position in segments 23-25, separated clearly from the normal gastrocnemius pool which is in a medio-dorsal position in segments 27-29 (Fig. 1). The decisive point is the finding that in all 6 cases the same rostral motor pool supplied the innervation for the transplant gastrocnemius. The substitute motor pool normally innervates a thigh adductor; in the experimental situation it supplies prohably both the foreign and its own target muscle. Two conclusions can be drawn: prespecification of motor pools and of pathways, and synapse specificity are not absolute but modifiable by experimental design. Furthermore, nerve ingrowth into a foreign muscle is not from randomly distributed motor neurons; rather, there is consistency in the atypical innervation pattern. We have used the term *selective mismatching* in this context (Hollyday and others, 1977).

In the meantime, the investigation has been extended to 4 other transplant leg muscles, and two transplant wing muscles and the picture was the same as in the first case: a particular atypical motor pool innervates selectively and reliably the same transplant muscle (Hollyday, personal communication). Obviously, we are dealing with a general rule: there exists a selective affinity of second order between transplant muscles and their atypical substitute motor pools. Axon bundles entering a foreign territory make preferential second choices and disregard other possible targets. One can look at the situation in terms of a hierarchy of neuronal specificities. Meyer and Sperry (1976) express a similar idea, in a discussion of retino-tectal specificity. They speak of "preferential graded affinities, not all-or-none specificity" (1976, p. 113).

The next step was to look for rules governing selective mismatching. Several possibilities suggest themselves: functional affinities could play a

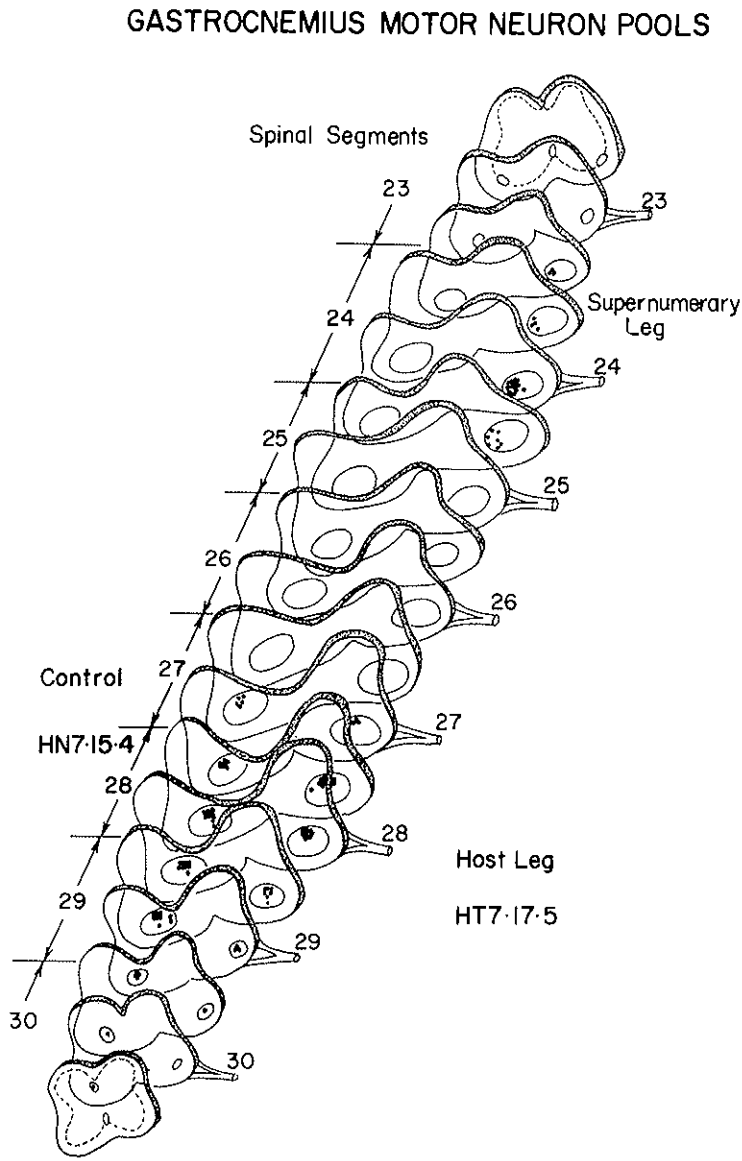

FIG. 1. HRP-labelled motor pools (dark dots) innervating the gastrocnemius of the host leg and of the transplanted (supernumerary) leg, respectively. Note similar medial position of the motor pools and gap between them (from HOLLYDAY, HAMBURGER, FARRIS, in "PNAS", *74*, 3582, 1977).

role in the sense that motor pools tagged for a flexor preferentially seek out another flexor rather than an extensor or abductor. Or topographic position of muscles with reference to a joint could be involved. None of these possibilities are borne out by the data. However, one consistent relation was established: the mismatching is related to the above-mentioned embryonic origin of the target muscles. A derivative of the ventral muscle mass is always connected with an inappropriate motor pool which normally innervates another derivative of the ventral muscle mass; and the same holds for derivatives of the dorsal muscle mass. The validity of this rule is reinforced by the finding that it holds also for wing transplants placed in front of the host leg (Hollyday, 1978, 1979). An autoradiographic study had shown that medially located motor neurons are born earlier than laterally located motor neurons (Hollyday and Hamburger, 1977), hence selective mismatching may also be linked with the time of outgrowth of axons.

Since the axon bundle from a mismatched motor pool is not distributed randomly to all derivatives of the dorsal or ventral muscle mass, respectively, but ends up always in the same transplant muscle, this rule cannot cover the actual situation. An additional, more discriminating selection process must be in operation.

So far, the new results of Hollyday have referred to target specificity: they should now be related to pathway specificity. In her material, the pathways in the transplants were remarkably normal with respect to entrance points into the limbs and intralimb patterns. This is in agreement with the above-mentioned early findings of Harrison and many subsequent studies. However, occasionally nerves grew to their targets along atypical pathways.

To accommodate all data, the original chemoaffinity hypothesis requires an extension, in the same direction as suggested above, that is, in terms of a hierarchical order of specifications. The first choice which motor growth cones emerging from the plexures would be confronted with would be biochemical cues of a rather general nature, provided by the dorsal and ventral muscle mass, respectively. The next branching point would he determined by slight differentials in each of the two original cues, resulting from the segregation of the two muscle masses into four. Eventually each muscle would provide a rather specific cue. By the time a fiber bundle reaches its matching muscle primordium many non-matching fibers have already been split off; hence, the information content near the terminal branching would not have to be great. This consideration and the phenomenon of selective mismatching suggest that the hiochemical

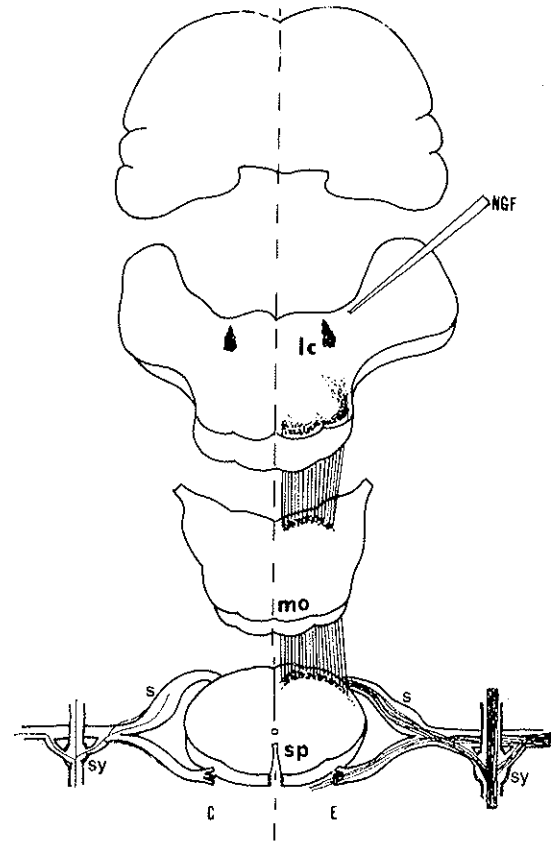

FIG. 2. Injection of NGF near *locus coeruleus* (lc). Note hyperplasia of sympathetic ganglion (sy) and fiber tract in the dorsal funiculus (from CHEN and others, in « Arch. Ital. Biol. », *116*, 53, 1978)

differentials which operate at branching points might be only quantitative, or partially shared molecular configurations. The pathfinding is facilitated 1) by the early invasion of fibers into the limb bud; the growth cones are already in the mesenchyme when it condenses into the 2 primary muscle masses; 2) the short distances between branching points; 3) the creation of mechanical tracks (preneural pathways) during the process of differentiation of limb tissues, as for instance along the interfaces of pre-muscle and pre-cartilage condensations.

Implicit in this model is a modified chemo- or neurotropic hypothesis in which "attraction at a distance" is replaced by guidance along biochemically tagged mechanical pathways. This notion finds strong support in recent experiments of Levi-Montalcini (1976; Chen and others, 1978). NGF was injected into the medulla of neonatal mice and rats near the *locus coeruleus*. NGF reached the paravertebral sympathetic

ganglia, including the stellate and superior cervical ganglia, probably by diffusion along the dorsal and ventral roots. The sympathetic ganglia showed hyperplasia, and massive bundles of sympathetic fibers entered the spinal cord by way of dorsal roots. Within the cord, the fibers travelled to the injection site along the dorso-lateral funiculus, as demonstrated by histo-fluorescence (Fig. 2). Apparently, they followed an NGF gradient. However, they did not establish synapses and got lost near the injection site. Obviously, in this experiment, NGF provides directional or tropic cues to the sympathetic fibers, in addition to its role as a trophic agent. Levi-Montalcini rejects the notion of "action at a distance" and states: "the axonal tip of the fibers moves along gradients of diffusion of trophic and tropic factors released by end organs". C.-O. Jacobson has coined the term "bound gradient" in another context (1976). It can well be applied here.

The authors suggest that the trophic-tropic double role of target structures could serve as a general model of nerve pathfinding in embryos. The mode of outgrowth of embryonic motor fibers, as outlined above, can be harmonized readily with this model. We know that muscle tissue provides a trophic agent which is required for the maintenance of motor neuroblasts (Hamburger, 1958). And in the discussion above we have presented arguments in favor of the assumption that the differentiating muscle masses provide also chemical cues of different degrees of specificity which guide the growth cones to their destination. As a matter of parsimony it would be expedient, here again, to assign both trophic and tropic functions to the same agent. What is called for now is the discovery of the MGF.

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DISCUSSION

PURVES

One way of testing the role of targets would be to remove the target at an early stage and see whether the trajectories of axonal outgrowth were substantially altered. Can you think of a way in which this might be technically possible?

HAMBURGER

We have done limb extirpation experiments. This experiment will show whether or not the motor pools are completely normal—that is, formed completely independently of the targets—and I am pretty sure that that is the way it will turn out. Now, I do not quite understand the experimental design which you have in mind.

PURVES

Landmesser's work suggests that the nerves that eventually innervate the dorsal and ventral muscle masses of the chick hindlimb take relatively independent pathways long before they actually reach the vicinity of the muscle. If it were possible to selectively remove, let us say, the dorsal muscle mass, one might still see such pathfinding in the absence of the target. Such a result would suggest that what you have outlined may not be the case.

HAMBURGER

In the *Neuroscience Abstracts* which have just appeared, Dr. Landmesser reported an experiment in which one half of the limb-innervating segments of the spinal cord was removed. She found that the residual motor neuron pools innervated exactly the same muscles which they normally do—they do not spread into the deprived half of the limb muscles, and this is exactly parallel to the old presentation of Sperry and Attardi, in which they showed that an optic nerve from one-half retina takes exactly the correct path which it normally would take, avoiding the branching at the major branching point and terminating at their normal target areas, leaving half of the tectum devoid of nerves.

NIRENBERG

How many motor pools are there? Is it known?

HAMBURGER

I do not know my comparative anatomy well enough, but I would guess about 40 or 45 muscles in the leg.

NIRENBERG

And there is a separate pool for each muscle?

HAMBURGER

There is a separate pool for each muscle.

NIRENBERG

But neurons from one pool can innervate multiple muscles.

HAMBURGER

Not in the normal development. In normal development they manage to grow straight towards the target and avoid other muscles. In the transplant they will innervate a particular second-choice muscle.

BIOCHEMICAL CONTROL OF SYNAPSE FORMATION *IN VIVO*

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ABSTRACT

Chick embryo autonomic ganglia and their target organs provided a model of synaptogenesis *in vivo*. Tbree molecular constituents of the synapse such as acetylcholine receptors (AChR), expressed by α-bungarotoxin binding, acetylcholine (ACh) and enzymes related to the synthesis and inactivation of ACh or norepinephrine (NE) were correlated at each stage to the development of neurotransmission.

Physiological neurotransmission reaches adult values, both in ciliary ganglia and iris, prior to the establishment of adult levels of cholinergic receptors and ACh, as well as of adult acetylcholinesterase (AChE) and cholineacetyltransferase (ChAc) activity. This implies that the maturation of neurotransmission is independent, within certain limits, of adult levels of neurotransmitter biosynthesis and number of receptors (Giacobini, 1978).

On the other hand, in order to start functional activity it seems necessary for the postsynaptic membrane to develop its specialization (AChE and AChR) even before the presynaptic apparatus (ChAc) for neurotransmitter synthesis has achieved its full capacity. Therefore, the initial rate of increase of AChE activity and the appearance of receptors is greater than that of ChAc activity and biosynthesis of ACh.

Based on these observations the following developmental schedule is proposed for the cholinergic synapse:

I. Phase of Innervation, in the presence of a low number of AChRs and low synthesis and release of neurotransmitter.

II. Phase of Neurotransmission.

III. Phase of Receptogenesis, with a rapid and sustained increase in number of receptors and AChE activity.

IV. Phase of Maturation, characterized by the structural and biochemical maturation of the machinery for neurotransmitter synthesis (presynaptic) and inactivation (postsynaptic).

Experiments using nicotinic blocking agents (Chiappinelli, Fairman and Giacobini, 1978) (α-bungarotoxin and chlorisondamine) demonstrated that while normal synaptic transmission does not appear to be essential for the survival of developing neurons, the developmental course of ganglia and target organs is significantly altered by early receptor blockade. This suggests that synaptic transmission may play a regulatory role on the process of synaptogenesis, and communication between developing neurons and their target organs seems to be essential for normal development of synapses.

Introduction

a) *Differentiation of autonomic ganglia in the chick embryo*

Differentiation of nerve cells presents many different facets. This can be best illustrated by analyzing the maturation of the peripheral autonomic nervous system. Ganglion cells of the autonomic nervous system have their source in the neural crest. Their origin is divided early into "adrenergic" and "cholinergic" areas from which arise sympathetic and parasympathetic ganglioblasts (Le Douarin, 1977), thus a "biochemical differentiation" is taking place during development. On the other hand, primitive sympathetic cells (stem cells) can evolve along two different lines (Fig. 1), one leading to sympathoblasts (sympathetic adrenergic cells in ganglia or ganglioblasts) and the other to adrenoblasts or chromoblasts (adrenergic cells in the adrenomedulla or chromaffine cells). The difference between these two lines of cells is mainly morphological and geographical and not biochemical since both lines of cells synthesize and metabolize catecholamines. However, there is recent evidence that nerve growth factor (NGF) influences and stimulates neuronal differentiation of adrenomedullary cells both *in vitro* (Greene, 1978 and this volume) and *in vivo* (Levi Montalcini, this volume).

The precursor cells do not show adrenergic or cholinergic specialization while in the neural crest (Enemar *et al.*, 1965; Le Douarin, 1977) and

the neurotransmitter (norepinephrine [NE] can be first identified at about 3½ days of incubation [d.i.]).

However, at the 3rd d.i. even before the formation of recognizable ganglia and before the development of NE fluorescence, uptake of (^{3}H)NE can be detected in individual cells located near the dorsal aorta (Rothman *et al.*, 1978). By the 3rd d.i. uptake of (^{3}H)NE can be seen in all primary ganglion cells (Rothman *et al.*, 1978). Therefore the ability to take up NE seems to be an early marker of adrenergic activity in sympathoblasts.

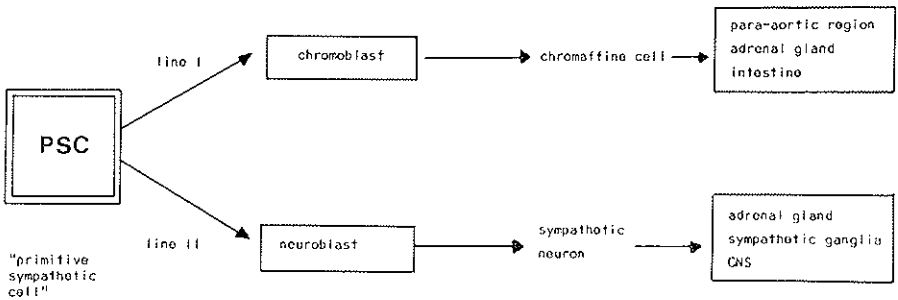

FIG. 1. Diagram of development of the primitive sympathetic stem cell. In *in vitro* experiments the transformation of line I cell into line II cell is possible (See text).

It has also been shown (Rothman *et al.*, 1978) that both uptake of NE and NE fluorescence begin and persist while neuroblasts still replicate. These replicating precursors persist throughout development *in ovo* and some divide repeatedly although an increasing number of them withdraw from the mitotic cycle as developmental age progresses (1977) (Rothman *et al.*, 1978).

As demonstrated by Le Douarin (1977) the orthosympathetic chain (sympathetic adrenergic ganglia) is derived from the level of the neural crest posterior to the 5th somite while the adrenomedullary cells originate from the level of somites 18-24. One should note that levels 1-7 (so called vagal region) provide all cholinergic enteric ganglia. The fate of the ganglioblasts originating from the various regions is not irreversibly fixed. The migration pattern of the crest cells can be changed by transplanting the "vagal region" of the neural crest at the "adrenomedullary" level and vice versa. Under these conditions adrenergic ganglioblasts migrate into the gut and originate cholinergic ganglia, while cephalic neural crest cells develop into adrenomedullary cells (Le Douarin, 1977; Le Douarin *et al.*, 1978). Therefore, the potential for developing into

cholinergic or adrenergic cells appears to be widespread in the whole neural crest.

The expression of either one or the other phenotype is dependent on the tissue (mesenchymal) environment to which the cells are subjected (Cohen, 1972; Le Douarin, 1977). The factors that control the appearance and the choice of neurotransmitter metabolism are still unknown. Neither is it known whether the environment is instructive or merely permits the selective expression of certain prior decisions.

Two recent hypotheses about the developmental mechanisms involved in the chemical differentiation of sympathetic neurons have been suggested on the basis of studies performed in culture of nerve cells *in vitro* (Bunge *et al.*, 1978; Patterson, 1978; Patterson *et al.*, 1978) (Fig. 2 A and B). Both hypotheses emphasize the influence of the environment in the cell's choice of neurotransmitter. According to Patterson (Patterson, 1978; Patterson *et al.*, 1978) (Fig. 2 A) the environment is represented by both non-neuronal cells (releasing presumed "developmental factors") and by other neurons (the electrical activity of which modifies the neurons'

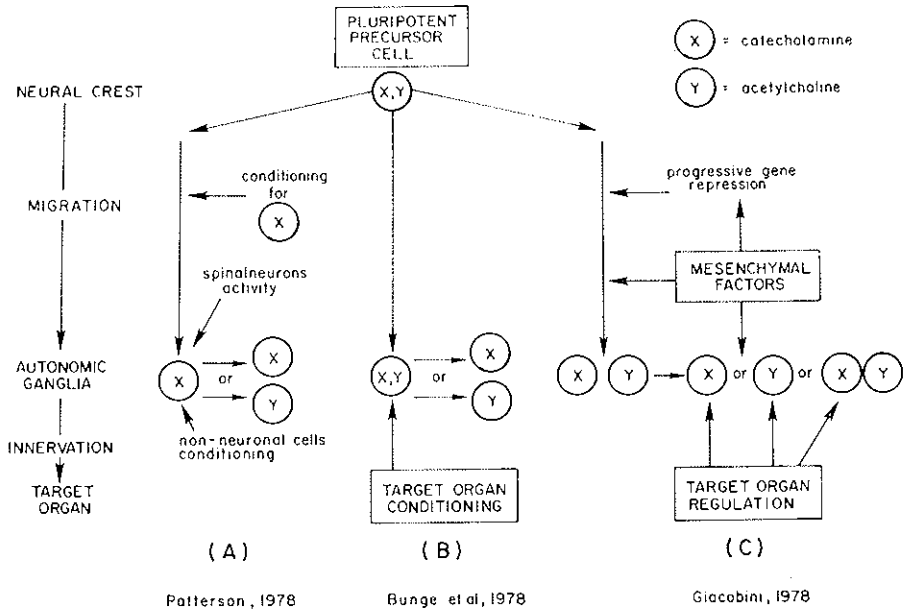

FIG. 2. Three recent hypotheses of developmental mechanisms involved in the chemical differentiation of sympathetic neurons - A) and B) are based on *in vitro* experiments (12-63) and C) on *in vivo* experiments (See text).

response to the factors). This hypothesis implies that the formation of synapses between the central (spinal) neurons and ganglionic neurons determines the choice of transmitter in the latter.

According to Bunge *et al.* (1978) (Fig. 2 B) the differentiation of transmitter mechanism in the neuron is modulated by its contact with peripheral targets and "by some form of nurture from its peripheral field" and in this way the neuron "becomes committed permanently to the expression of this phenotypic characteristic". Both concepts are drawn from the behavior of nerve cells (Bunge *et al.*, 1978; Patterson, 1978; Patterson *et al.*, 1978) under *in vitro* conditions, but contradicted by some experimental data obtained *in vivo*, which will be presented here.

A schematic diagram of the sequence of ganglionic development *in vivo* of chick sympathetic and parasympathetic (ciliary) ganglia is reported in Fig. 3.

The data reported in the figure are derived partially from the literature (Gabella, 1978) and partially from results from our laboratory (Giacobini, 1978). The early stages of ganglionic development can be divided

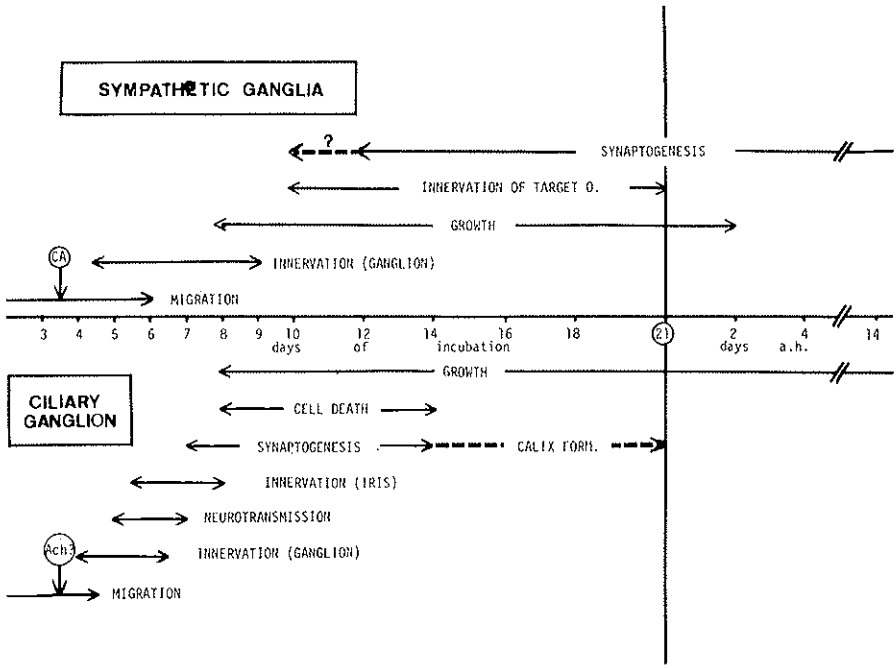

FIG. 3. A diagram of some basic features of ganglionic development of chick sympathetic and ciliary ganglia. For ref. see text. CA = catecholamines ACh = Acetylcholine.

into two periods: (I) period of somal migration and differentiation, including the selection of neurotransmitters; (II) period of ganglionic and target organ innervation, involving axonal growth and penetration of fibers in the ganglionic or target organ.

A later step (III) involves formation of synapses and has been subdivided by us in four subsequent phases (innervation, neurotransmission, receptogenesis and synaptic maturation) of which the first (innervation) can be considered as belonging to stage II. The last phase (maturation) is to be interpreted as a longlasting process which extends itself to a period of several weeks.

This period (III) also includes the phases of cell death and growth in volume of the ganglion cells.

Our study demonstrates that ganglioblasts *in vivo* have achieved their cholinergic or adrenergic differentiation at the earliest detectable stages of ganglionic development (Fig. 2 C). Therefore any specifying influence exerted by the periphery (target organs) (Fig. 2 B) or by other neurons (central spinal) (Fig. 2 A) should be prior to a) their migration to the sympathetic chain or b) their innervation of a peripheral organ.

With regard to sympathetic ganglia in the chick embryo the preganglionic outflow from the dorsolateral cell column of the spinal cord (columns of Terni) can be distinguished as early as 4 d.i. (days of incubation) (Caserta and Ross, 1978), but synaptic contacts are probably established only several days later (Dolezalova *et al.*, 1974; Giacobini, 1975). The first synapses to the neurons of the nucleus of Terni were found to occur (Caserta and Ross, 1978) not earlier than at 10 d.i. Therefore it seems unlikely that this relatively late innervation of both groups of cells (spinal cord and ganglia) could be determining their choice of transmitter, particularly since ganglion cells show an intense fluorescence for catecholamines and the presence of (NE) starting 3½ d.i. and 6 d.i. respectively (Enemar *et al.*, 1965; Jacobowits *et al.*, 1976).

In the chick embryo, adrenergic innervation of target organs occurs at a relatively later period than cholinergic innervation (Chiappinelli *et al.*, 1978; Pappano, 1977). As an example, histochemical methods (Enemar *et al.*, 1965) were unable to detect catecholamine fluorescent structures in the ventricular myocardium of the chick until the sixteenth d.i. The innervation of the pupillary dilator (iris) by the superior cervical ganglion of the chick begins at 13 d.i. but is not completed before 16 d.i. (Kirby *et al.*, 1978). It is therefore difficult to link to the expression of ganglionic, adrenergic and cholinergic function "some form of nurture" from its peripheral field as suggested by Bunge *et al.* (1978). "Specifying" signals

from the adrenergic targets to the adrenergic neuron must exert their influence during the earliest stages of prenatal (3-4 d.i.) development. Any signal (*in vivo*) reaching the neuron at a late prenatal or an early postnatal (or post-hatching) period would be too late. The cell population at this stage has already stabilized, mitotic activity is practically completed (Cohen, 1974; Rothman *et al.*, 1978), cell death has occurred and the cells have already selected and established their complement of neurotransmitter (Cohen, 1974; Enemar *et al.*, 1965; Rothman *et al.*, 1978). When referring to *in vivo* conditions we should therefore speak of "regulatory" influences (Fig. 2 C) rather than "specifying" signals. We should not forget that under *in vivo* conditions often both lines of neurotransmitters (or even several lines, Hökfelt, this volume) can be represented simultaneously in the same adult ganglionic population (Buckley *et al.*, 1967).

We can visualize this phenomenon if we think that as pluripotent neural crest cells of early embryos (1-3 d.i.) differentiate, they progressively lose the potential of expressing several phenotypes, each carrying its complement of neurotransmitter. Normally, under *in vivo* conditions in the ganglia, the neurons are irreversibly restricted to the expression of a single neurotransmitter (acetylcholine [ACh] or [NE]). However, the presence of transmitters other than ACh or NE or modulators, the function of which is still unknown, cannot be excluded (Hökfelt, this volume). If the cells are separated from ganglia at sufficiently early stages of development and grown *in vitro,* they can "regress" to a pluripotent stage and can express one (or more than one) of several types of neurotransmitter phenotypes. The factors involved in this mechanism of selection, in particular the nature of gene transcription changes are unknown. A suggestive scheme of progressive gene repression (rather than selective gene activation) which is consistent with many observations from experimental embryology, has heen recently suggested by Caplan and Ordahl (1978).

b) *Regulation of neurotransmitter metabolism during development*

A substantial body of evidence originated from studies in our laboratory (for ref. see Giacobini, 1978) supports the view that the development of enzymes specifically related to neurotransmitter biosynthesis in chick autonomic (sympathetic and parasympathetic) ganglia is regulated by a) transsynaptic influences, provided by the maturation of pre- and postganglionic interactions and b) retrograde inputs originating in the periphery (target organ) (Fig. 4). Other mechanisms such as NGF, trophic factors

and hormones are discussed by other authors of this volume (see R. Levi Montalcini, L. Greene and A. Cangiano).

In a series of earlier studies (for ref. see Giacobini, 1978) the developmental variations of enzymes associated with the cholinergic synapse and the adrenergic neurons of sympathetic ganglia were investigated in embryonic and posthatching chicken. It was found that the patterns of developmental variations for cholineacetyltransferase (ChAc), dopamine-β-hydroxylase (DBH) and monoamineoxidase (MAO) activity were related to a) the maturation of preganglionic nerve terminals and ganglion neurons and b) a significant change in the functional state of the ganglion along with an intense activity of the synapses following hatching (Fig. 4).

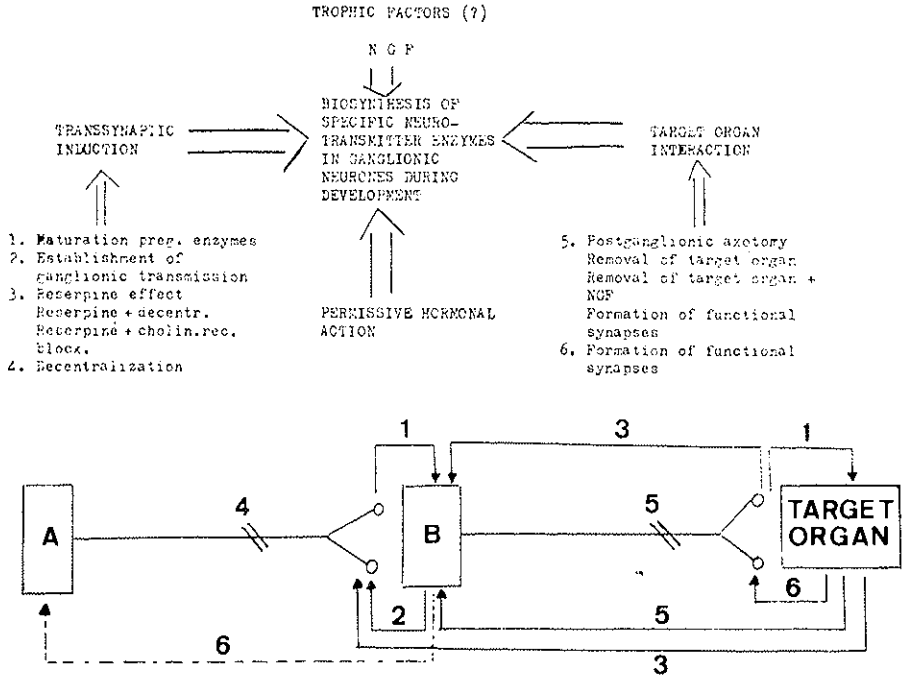

FIG. 4. Mechanisms involved in the regulation of biosynthetic processes during development of the autonomic neuron.

A close temporal relationship in the development of tyrosine hydroxylase (TH) and DBH activity is observed throughout the phases of synaptogenesis and maturation but not during the phase of intense functional activity following hatching (Dolezalova *et al.*, 1974). Our results strongly suggest that before hatching in chick embryo sympathetic ganglia the

cholinergic presynaptic terminals play a role in regulating the development of adrenergic postsynaptic enzymes and of neurotransmitter levels. In the period following hatching, DBH and TH levels in cell bodies are probably regulated by tbe establishment of the functional activity at the target organs. This results in a depletion of DBH, but not TH, through liberation along witb neurotransmitter at the periphery. Reduction of DBH at the terminals might result in increased transport and thereby depletion in the cell body. This mechanism might be responsible for the difference in the pattern of activity of DBH and TH in cell bodies observed in the first week after hatching (a.h.) (Fig. 5).

Additional evidence for a regulatory mechanism of biosynthetic enzymes in ganglia originates from two other observations in our laboratory: a) the effect of a single dose of reserpine administered prior to incuhation on the development of TH activity in chick sympathetic ganglia (Fairman *et al.*, 1977) and b) the effect of ganglionic decentralization on ChAc activity in the chick ciliary ganglia (Giacobini *et al.*, 1978). In both experiments, transsynaptic regulation of TH and ChAc in the cell body is suggested.

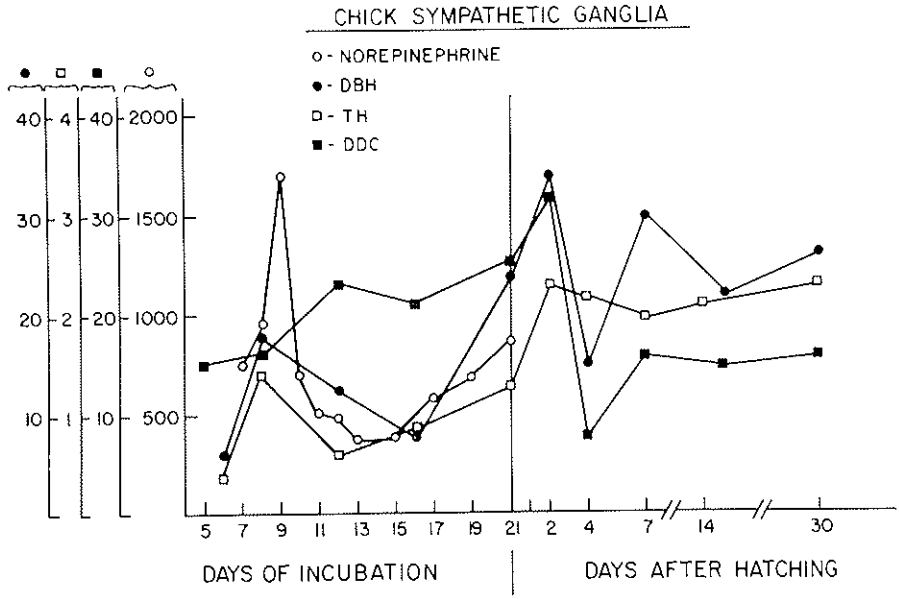

FIG. 5. Developmental pattern of NE, DBH, TH and DDC activity in chicken sympathetic ganglia. O = NE, ng/mg protein; ● = DBH, pmol octopamine/ug. protein/h; □ = TH, pmol CO_2/ug dry weight/h; ■ = DDC, pmol CO_2/ug protein/h. Means and standard errors are not reported (for ref. see text) (The NE data are from Jacobowits *et al.*, 1976).

A diagram of the mechanisms possibly involved in the regulation of biosynthetic processes during development of the autonomic neuron is reported in Fig. 4. Neuron A represents the spinal cord neuron and neuron B the ganglion cell with its target organ. Transsynaptic induction in the ganglia and target organ interaction are the two main mechanisms considered in the diagram. The action of trophic factors or specific factors such as the NGF are also reported together with permissive hormonal action. Several experiments involving preganglionic denervation, axotomy or drug administration in both mammals and birds, substantiate this view (Giacobini, 1978).

Biochemical and pharmacological studies on neuronal control mechanisms of neurotransmitter biosynthesis during the development of the peripheral and central autonomic nervous system, published during the period 1969-1978, have been the object of extensive review by Giacobini (1970, 1971, 1975, 1978), Lanier *et al.* (1976), Hendry (1976), Giacobini and Chiappinelli (1977) and Black (1977, 1978).

Role of ganglionic and peripheral receptors in synaptogenesis

A. *Ciliary ganglia and iris*

A diagram of the ciliary ganglion/iris preparation of the chick is shown in Fig. 6.

The ontogeny of acetylcholine receptors (AChR) in ciliary ganglia and iris has recently been examined in our laboratory by studying the rate of appearance of alpha-bungarotoxin (ABTX) binding sites in both organs (Chiappinelli and Giacobini, 1978). Specific, high affinity binding was found in both tissues (K_D (iris) = 2.5 nM, K_D (ganglion) = 2.7 nM). The binding is saturated above 10 nM toxin concentration and is inhibited by low concentrations (10^{-5}M) of the nicotinic antagonist tubocurarine. The binding appears first in ganglia and then in the iris and is associated with a nicotinic cholinergic receptor in both tissues (Figs. 7-8). As shown in Fig. 8, the amount of binding in the iris begins to increase only after functional innervation of this organ is observed i.e. at 8 d.i. and continues to increase up to four months a.h. In contrast ABTX binding in the ciliary ganglion increases four fold between 7 and 11 d.i. after which the amount of binding remains unchanged up to four months a.h.

In the ganglion, the highest rate of appearance of receptors is posterior to the onset of ganglionic transmission but simultaneous to the rise in both acetylcholinesterase (AChE) and ChAc activity in the cell bodies

(Fig. 7). In the iris, as in the ganglion, the development of the receptor and the increase in both enzyme activities is parallel and almost simultaneous (Figs. 7-8). This fact is interesting since both ABTX binding and AChE activity in the iris are localized in large part in the muscle cells while ChAc activity is exclusively presynaptic (Chiappinelli *et al.*, 1978; Giacobini *et al.*, 1979). If one considers the early period of transmission and synaptogenesis in ganglion and iris (i.e. between 5 and 14 d.i., in

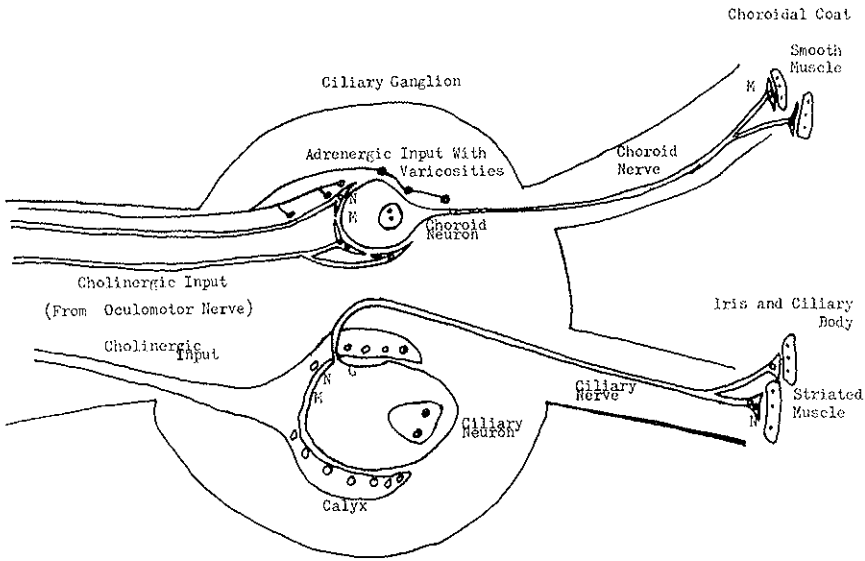

FIG. 6. Diagram of the chick ciliary ganglion and iris. N = nicotinic receptors, M = muscarinic receptors, and G = gap junctions. Illustration is not to scale. (After Landmesser and Pilar, 1972 and Cantino and Mugnaini, 1975).

Figs. 7-8) the following picture is apparent: levels of receptor binding are clearly detectable in the ganglion and iris before initiation of neurotransmission, while only low ChAc and AChE activities, which are probably presynaptically located, are present (Figs. 7-8). Simultaneously with the maturation of neurotransmission, receptor levels and AChE activity also rapidly increase in the postsynaptic elements (ganglion cells and iris muscle) as well as ChAc in the presynaptic elements (terminals in ganglia and iris). When both number of receptors and per cent neurotransmission has reached adult values in the ganglion (12 d.i.), ChAc which has accumulated in ganglion cells starts to be transported in large amounts down their axons to the terminals (Fig. 8). Increased levels of enzyme can then

begin to synthesize the amounts of ACh necessary to maintain neurotransmission at the neuromuscular junction.

If we now consider relative rather than absolute values, a few interesting relationships can be established. While in Figs. 7-8 we have focussed upon the period of development of neurotransmission, a more expanded

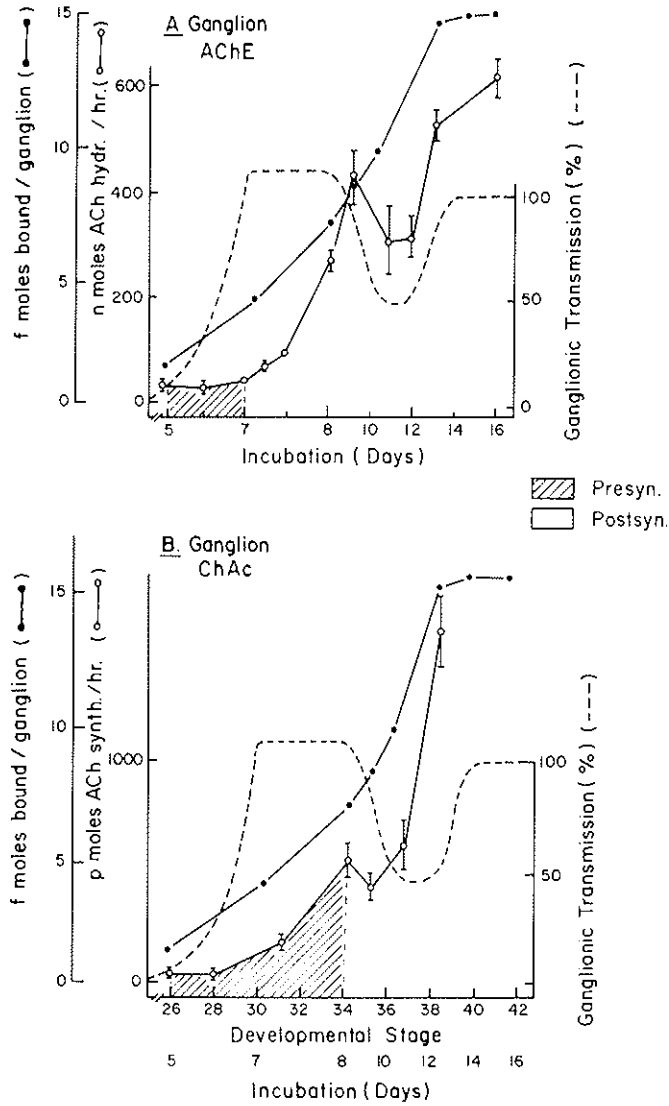

FIG. 7. Relationship between ChAc, AChE activity, ABTX binding and per cent transmission in ciliary ganglion. (From Chiappinelli *et al.*, 1978 and Chiappinelli and Giacobini, 1978).

picture of the events can be seen in Fig. 9 for the ganglion and in Fig. 10 for the iris (Chiappinelli *et al.*, 1978). In order to directly compare the various systems with each other, the relative value of each biochemical parameter at 7 days a.h. was taken as a 100% standard (i.e. all values are expressed as a percentage of the 7 days a.h. value (Chiappinelli *et al.*, 1978). In Fig. 9, it can be seen that in the ganglion both AChE activity

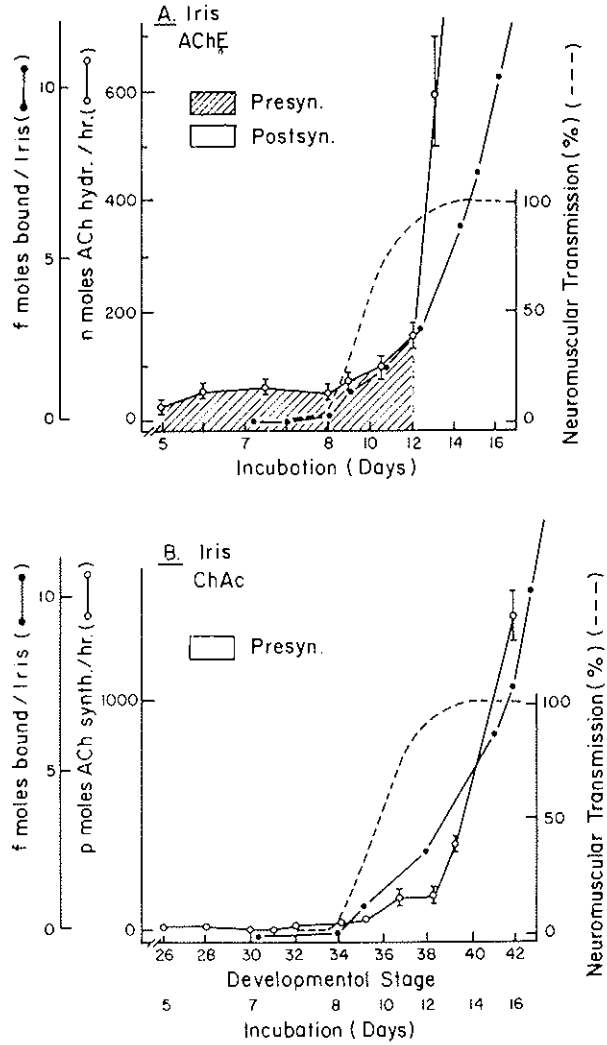

FIG. 8. Relationship between ChAc, AChE activity, ABTX binding and per cent transmission in iris. (From Chiappinelli *et al.*, 1976 and Chiappinelli and Giacobini, 1978).

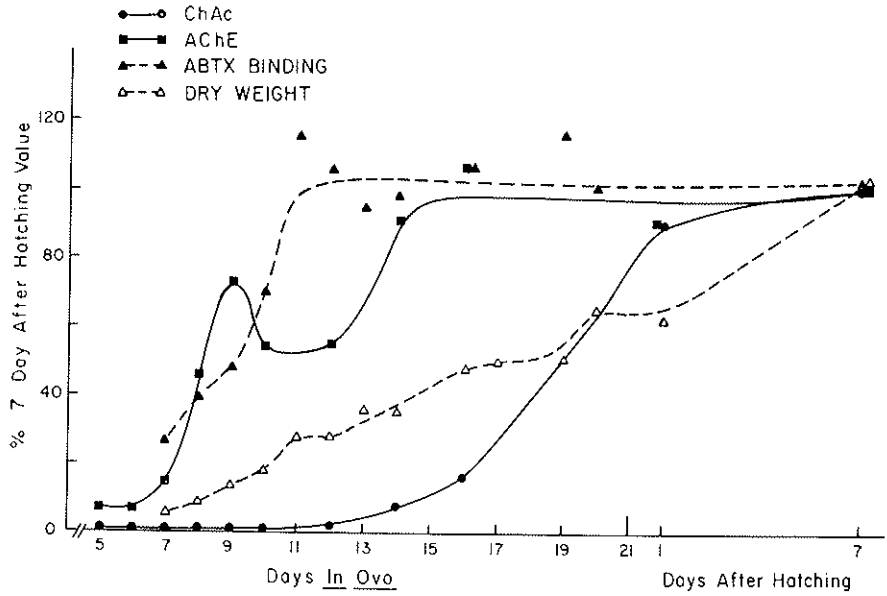

FIG. 9. Biochemical development of the ciliary ganglion. All values are expressed as a percentage of the 7 day a.h. value. (From Chiappinelli and Giacobini, 1978).

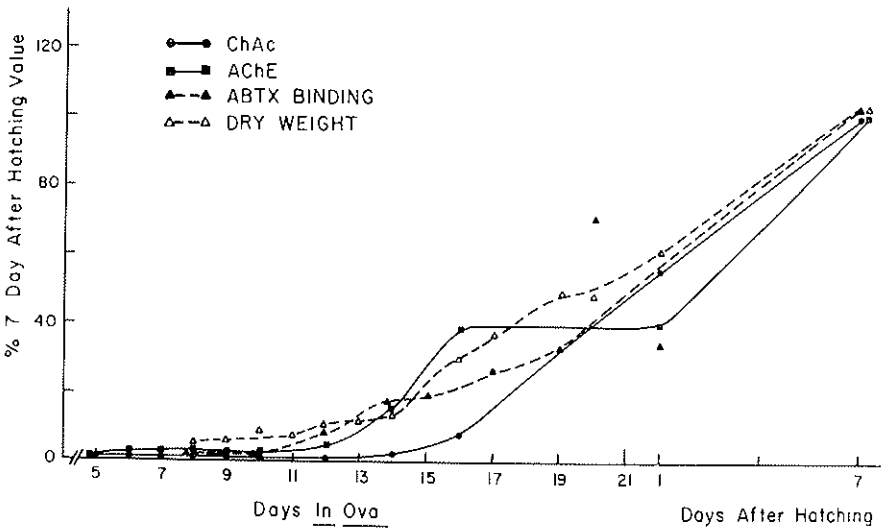

FIG. 10. Biochemical development of the iris. All values are expressed as a percentage of the 7 day a.h. value. (From Chiappinelli and Giacobini, 1978).

and ABTX binding reach approximately 100% of the 7 days a.h. value already at 14 d.i. while ChAc activity is still increasing slowly. This would indicate that the postsynaptic mechanisms involved in transmitter interaction and inactivation develop in the ganglion at a faster rate than the presynaptic specialization, i.e. the ability to synthesize ACh. This sequence seems rational as far as it explains a) why ganglion cells may start their interaction with the central neurons and the target organ (iris) even before adult levels of the neurotransmitter are reached and b) the possibility for a feedback regulation of the presynaptic component.

In the iris (Fig. 10), during the period from 7 d.i. to 7 days a.h. toxin binding increases 70 fold while AChE activity increases 60 fold and ChAc activity 825 fold. Thus both AChE activity and ABTX binding which are primarily localized in the postsynaptic elements increase at approximately the same rate in the same period. In the iris as well as in the ganglion AChE and ABTX increase at a faster rate than ChAc.

B. *Sympathetic ganglia*

Three hasic components of the cholinergic system (AChR, ChAc and AChE) are present in the sympathetic ganglion, beginning at the earliest stages (Fig. 11). Two of them, ABTX binding (Greene, 1976) picks up only after 13 d.i. simultaneously with ChAc activity, correlating to the innervation of the ganglion (Fig. 3). ChAc activity, which is mostly presynaptic, will continue to increase even after hatching, while AChE activity and AChRs will reach practically adult values already at hatching.

If growth of the three components is expressed in relative terms (% of 7 days a.h. values), we can see that as in the ciliary ganglion, AChE activity and ABTX binding increase at a faster rate than both TH and ChAc activity. At the time of hatching, ABTX binding has reached a value of 80%, while ChAc is 65% and TH only 53% of the 7 days a.h. values. This could indicate that a) receptor formation might be related to ChAc synthesis and b) ACh receptor and AChE might be part of the same assembly complex. The latter conclusion is consistent with the finding indicating that at the motor end-plate both macromolecules have the same turnover rate (Inestrosa and Fernandez, 1977).

The curve for NE levels shows (Fig. 5) a distinct peak at 9 d.i. (Brown *et al.*, 1978) which follows TH and DBH developmental peaks by 24 hrs (8 d.i.) and is probably related to the phase of increase in number of small intensely fluorescent cells (SIF-cells) observed by Jacobowits *et al.* (1976). Subsequently, a decrease in NE takes place up

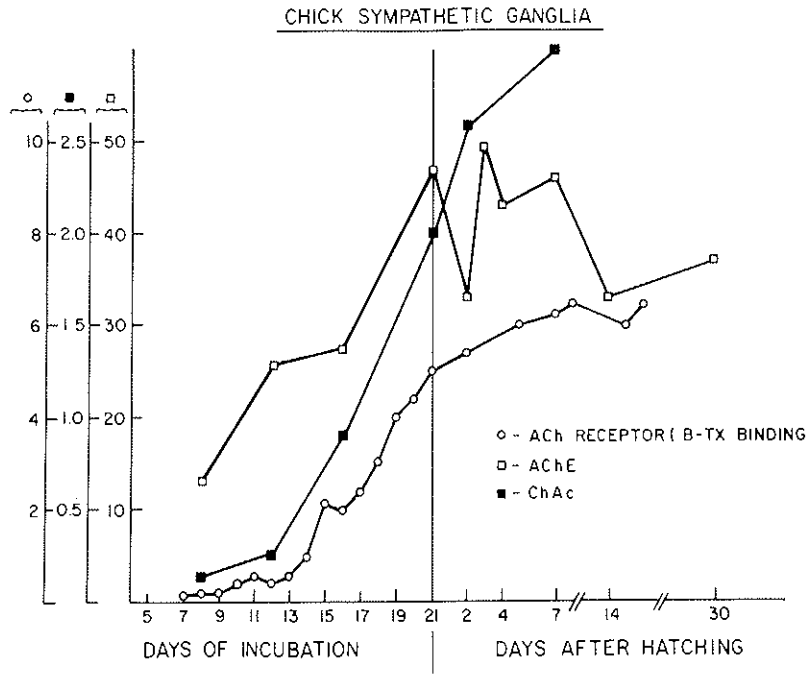

FIG. 11. Developmental pattern of cholinergic receptors, ChAc and AChE activity in chicken sympathetic ganglia. ○ = ACh receptor (ABTX binding), fmol bound/ganglion; □ = AChE, umol ACh/mg dry weight/h; ■ = ChAc, nmol ACh/ganglion/h. Means and standard errors are not reported (for ref. see text). (The ABTX binding data are from Greene, 1976).

to 13 d.i. which coincides with the observed decrease in SIF cells and with the morphological maturation of the ganglion cells. A second rise in NE concentration followed up to 21 d.i. probably reflects an increased synthesis and accumulation of the amine in the cell bodies. TH and DBH activity levels show a parallel trend and will continue to increase up to 2 days a.h. After a short-lasting drop of DBH and dopa-decarboxylase (DDC) activity (but not of TH), steady levels are reached for all three enzyme activities (Dolezalova *et al.*, 1974; Filogamo *et al.*, 1971).

C. *Presence of the neurotransmitter and its developmental pattern in ganglia and target organs*

As stressed in the Introduction, the identification of the neurotransmitter present at the early stages of development becomes a critical issue in determining the degree of differentiation and innervation of the ganglion.

Using a micromethod for ACh assay (McCaman and Stetzler, 1976) levels of this neurotransmitter were measured (with choline) starting respectively at 5 and 7 d.i. in ciliary and sympathetic ganglia (Marchi and Giacobini, 1979). Appreciable levels (1-2 picomoles/ganglion) of ACh were found at these early stages of development indicating the presence of a) cholinergic innervation of sympathetic ganglia and iris and b) ACh biosynthesis (in cell bodies) of ciliary ganglia. The developmental patterns of ACh were followed up to adult stages and showed characteristic features with a pronounced increase after hatching (Marchi and Giacobini, 1979).

D. *Molecular and synapse-related forms of AChE in the ciliary ganglion and iris. Developmental effects.*

The different molecular forms of AChE were examined in the chick ciliary ganglion and iris using both electrophoretic and velocity sedimentation analyses (Scarsella *et al.*, 1978). Four molecular forms were observed (Fig. 12). Forms I, II and III (in order of anodic migration) are present in the "soluble fraction" while forms II, III and IV appear in the "membranous fraction". Ten days after postganglionic axotomy in young chickens the relative proportions of the membranous forms were changed in both the ciliary ganglion and iris, with a marked reduction of form IV. A characteristic evolution of both the soluble and the membranous forms of AChE was evident through different developmental stages (Fig. 12). In the ciliary ganglion the faster components were reduced in favour of form IV. Conversely, the relative proportion of the faster components was increased in the iris.

The marked reduction of component IV following axotomy was interpreted as changes involving synapses and neuronal membranes in the ganglia as well as junctional components related to the muscle. The latter changes related to the muscle occurred between the 4th and 10th day after denervation, at a time when the degeneration of ciliary nerve endings in the iris is already complete.

The developmental study indicated that both form II and IV are nerve-related forms of AChE. The former (form II) is associated with those structures of the iris muscle which continue to grow and differentiate in the posthatching period and the latter (form IV) which decreases both during maturation and after denervation is limited to iris structures that mature early and do not grow in parallel with the mass of the muscle fibers. Such features would be compatible with a junctional form, since in the chick embryo the iris myoneural junctions develop a full functional

activity between the 8th and 13th d.i. (Landmesser and Pilar, 1974), just before a marked increase in AChE activity (Chiappinelli *et al.*, 1978). The developmental relation between AChE activity and the ACh receptor are commented upon in part A of this section.

E. *Effect of cholinergic receptor blockade*

In developing chick sympathetic ganglia, blockade of postsynaptic nicotinic receptors abolishes the reserpine-induced increase of TH (Fairman *et al.*, 1977). In the ciliary ganglion and iris the development of ChAc and AChE seems to be regulated by signals arising soon after the

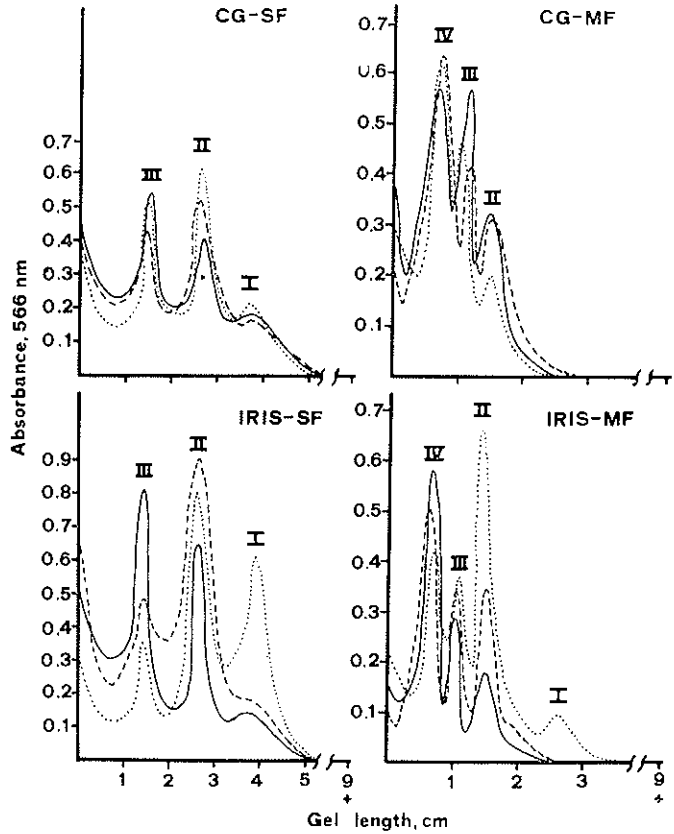

FIG. 12. Variations of the AChE electrophoretic pattern during development. CG = ciliary ganglion; SF = soluble fraction; MF = membranous fraction; (————) chick embryo ten day old; (- - - - -) chick two day old for CG and eleven day old for iris; (. . . . . .) 56 day old chicken. Densitometric recordings of the bands developed by Koelle's modified reaction after gel electrophoresis (Scarsella *et al.*, 1978).

functional innervation of these organs is accomplished (Chiappinelli *et al.*, 1978). In order to understand whether the receptor plays a regulatory role through its interaction with the transmitter during development it was of interest to examine the effect of receptor blockade during specific and crucial pbases of development.

Based on these considerations, the effect of receptor blockade prior to or during the critical period of innervation of the ganglia and target organs (iris) was studied in the two systems using selective receptor blockers or receptor hinders (Chiappinelli *et al.*, 1978).

In order to block nicotinic receptors, at the iris neuromuscular junction (a striated muscle in the chick) ABTX was used; at the ganglionic synapse chlorisondamine and ABTX were used (Chiappinelli *et al.*, 1978). It is known that ABTX does bind to sympathetic ganglia (Landmesser and Pilar, 1976), however, it does not block neurotransmission (Brown and Fumagalli, 1977; Brown *et al.*, 1978; Bursztajn and Gershon, 1976; Carbonetto *et al.*, 1978; Chiappinelli and Zigmond, 1978; Magazanik *et al.*, 1974). ABTX also binds to ciliary ganglia (Chiappinelli and Giacobini, 1978) and blocks nicotinic transmission (Chiappinelli and Zigmond, 1978).

To blockade α- or β-receptors (or both) propranolol and phenoxybenzamine were used (Giacobini and Fairman, 1978).

A total of 300 μg ABTX was injected in the yolk sac at 4 subsequent d.i. (Chiappinelli *et al.*, 1978). The toxin showed a general trophic effect on the iris reducing its weight by approximately 25%; however, the weights of the ganglion and of the embryo were not affected (Fig. 13 A). In the iris, ChAc and AChE activities, both total and specific were also significantly reduced (Fig. 13 A).

Since we have previously demonstrated (Giacobini *et al.*, 1979) that ChAc in the avian iris is selectively localized to the nerve terminals, we conclude that the toxin has had an effect on the development of the nerve terminals, as well as on a) the general development of the organ and b) AChE activity of the iris muscle. Ganglionic ChAc activity was also reduced. By contrast, AChE activity in tbe ciliary ganglion, which is predominantly postsynaptic (i.e. in the cell bodies) (Giacobini *et al.*, 1979), was not affected. Although it is difficult to assign the reduction of ChAc to pre- or postsynaptic structures, the significant reduction of activity at the nerve terminals indicates that, at least partially, this must be postsynaptic. In control experiments on skeletal muscle (biceps femoris) the effect of the toxin was found to be similar to that on the iris, suggesting a similarity in the receptor properties and its developmental role. A similar finding in muscle has been reported by G. Giacobini *et al.* (1973).

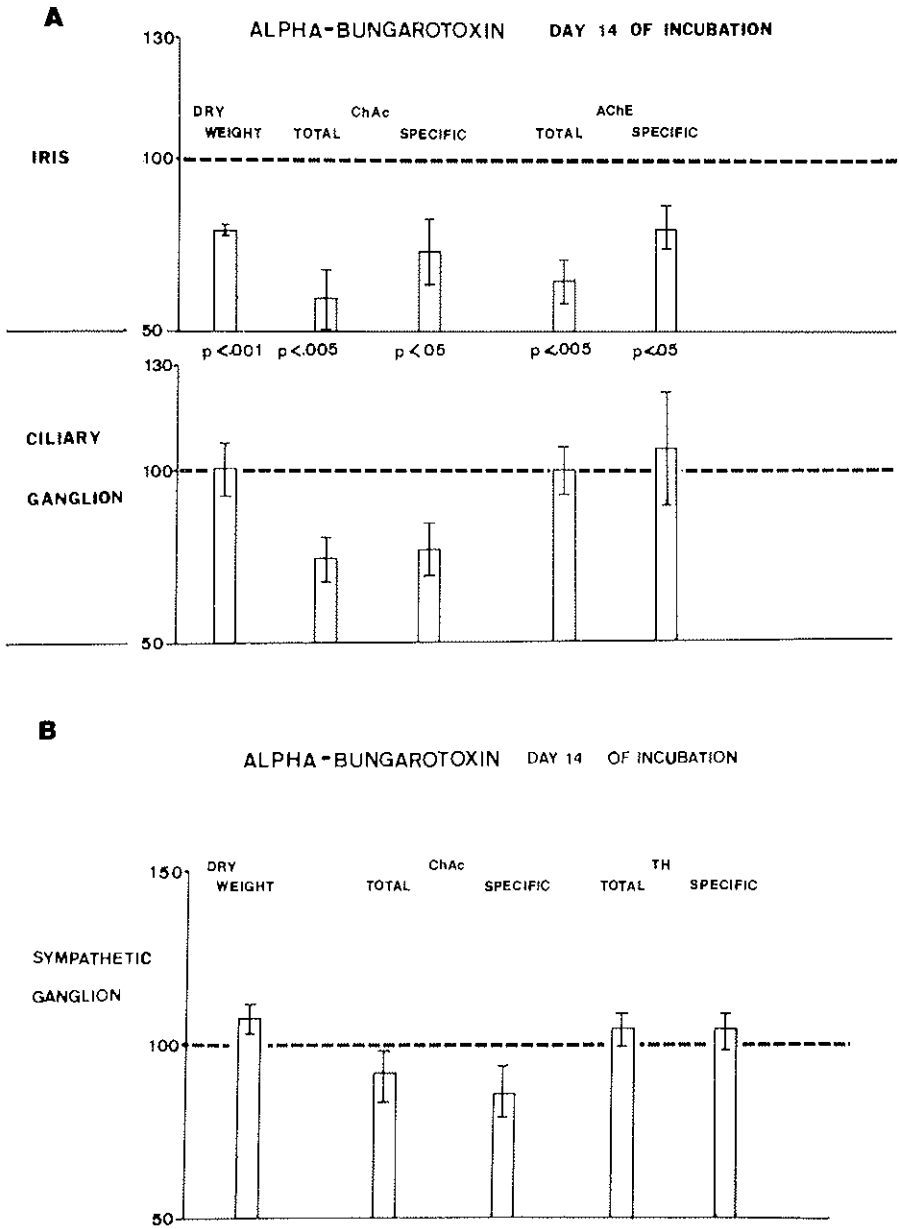

FIG. 13. Effect of alpha-bungarotoxin at 14 days of incubation. A) On iris and ciliary ganglion and B) on lumbar sympathetic ganglia. All values are expressed as a percentage of control values. Bars represent mean ± S.E.M. Number of embroys in each group was 5 (10 determinations per group for iris and ciliary ganglion and 10 to 15 determinations per group for sympathetic ganglia).

Following chlorisondamine administration, weight, ChAc and AChE were all significantly reduced in the ciliary ganglion at both day 14 and 20 of incubation (Chiappinelli *et al.*, 1978) (Fig. 14). This suggests that the drug through its blocking effect on nicotinic cholinergic receptors, affects the development of the ganglion terminals primarily presynaptically (ChAc) although a postsynaptic (AChE) effect cannot be excluded. ChAc activity is also reduced at 14 d.i. in the iris, however, iris weight and AChE activity are normal. This would suggest that, as expected, due to different properties of the receptors (Chiappinelli *et al.*, 1978), the development of the muscular junction is little or not affected by the drug. The reduced ChAc activity in the iris probably reflects the decreased level of the enzyme in the ganglion cells.

At day 20 of incubation the iris has totally recovered its ChAc activity, while the ciliary ganglion still shows reduced weight and AChE and ChAc activity (Fig. 14 B). This would suggest a longlasting effect of the drug or a long-lasting alteration in the ganglion.

In sympathetic ganglia ABTX, administered under the same condition, does not show any apparent effect on either ChAc or TH activity at day 14 of incubation (Fig. 13 B). These results support the view that although ABTX is binding to ganglionic receptors, as it has been demonstrated by Greene (1976), it is not efficiently blocking transmission through the ganglia, as it has been shown by Magazanik *et al.* (1974), Bursztajn and Gershon (1976), Brown *et al.* (1978), and Brown and Fumagalli (1977).

This is in contrast with the effect of chlorisondamine, which causes a significant decrease in weight of the ganglia at day 14 of incubation (Fig. 15). This effect is still present at day 20 and is associated with a significant decrease of ChAc activity. At day 14, TH activity is only slightly affected (Fig. 15), indicating that the drug has interfered with the normal development of the ganglion by acting primarily on the presynaptic terminals.

The effects of chlorisondamine reported here are assumed to be the result of decreased functional activity, or decreased postsynaptic depolarization. The level of activity at the ganglionic synapse is important in the biochemical regulation of the ganglion (Zigmond and Ben-Ari, 1977).

In tbe adult rat and mouse superior cervical ganglion (SCG) and adrenal gland, reserpine causes an increase in TH activity. This increase can be blocked by presynaptic denervation (in both the SCG and adrenal) or by nicotinic blockade by chlorisondamine (in the SCG) (Mueller *et al.*,

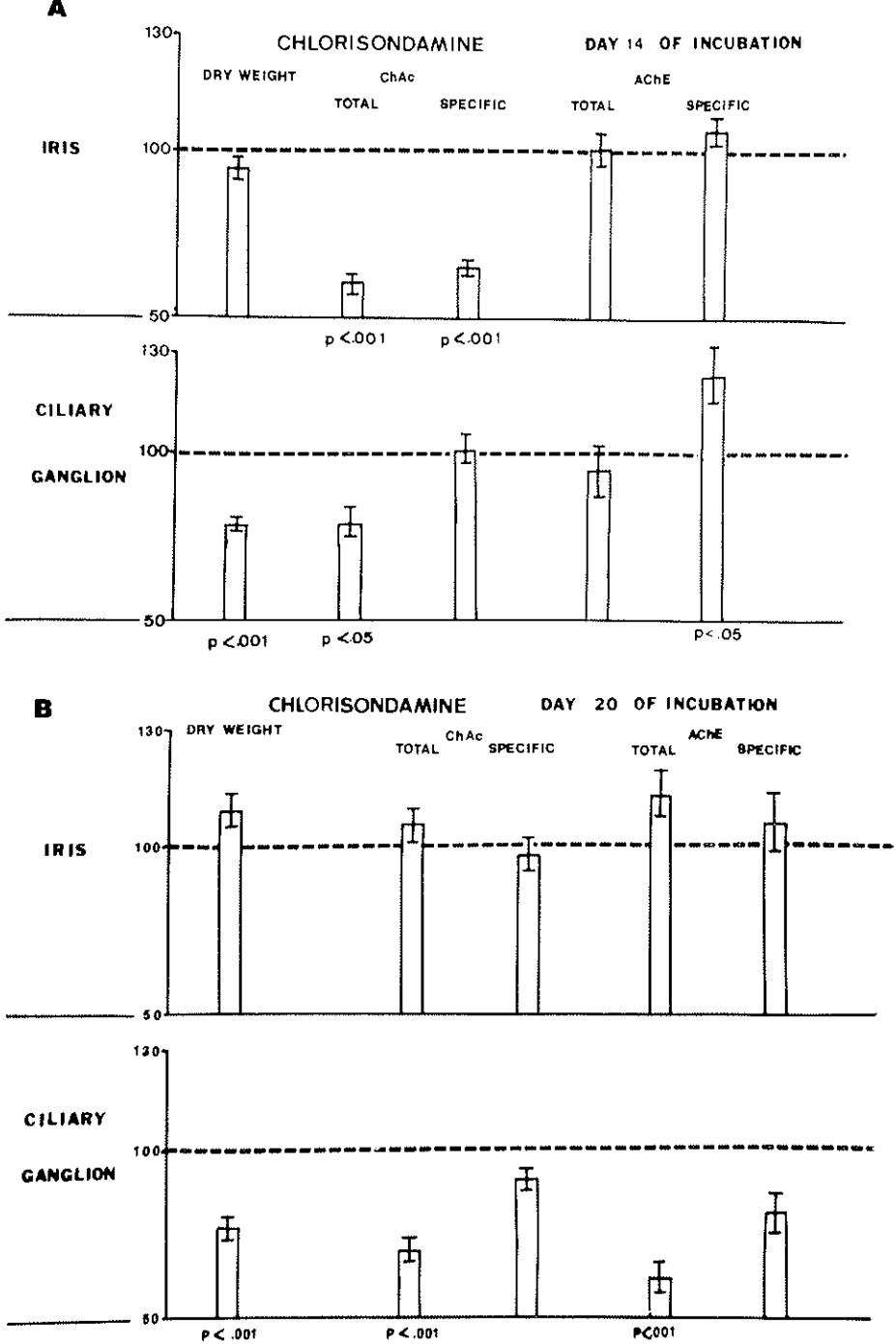

FIG. 14. Effect of chlorisondamine treatment on iris and ciliary ganglion. All values are expressed as a percentage of control values. Bars represent mean ± S.E.M. Enzyme activity is expressed per tissue (TOTAL) and per mg dry weight (SPECIFIC). The significance levels are relative to control values in this and following figure. Number of embryos in each group at 14 d.i. was 4 to 5 (between 8 and 10 determinations).

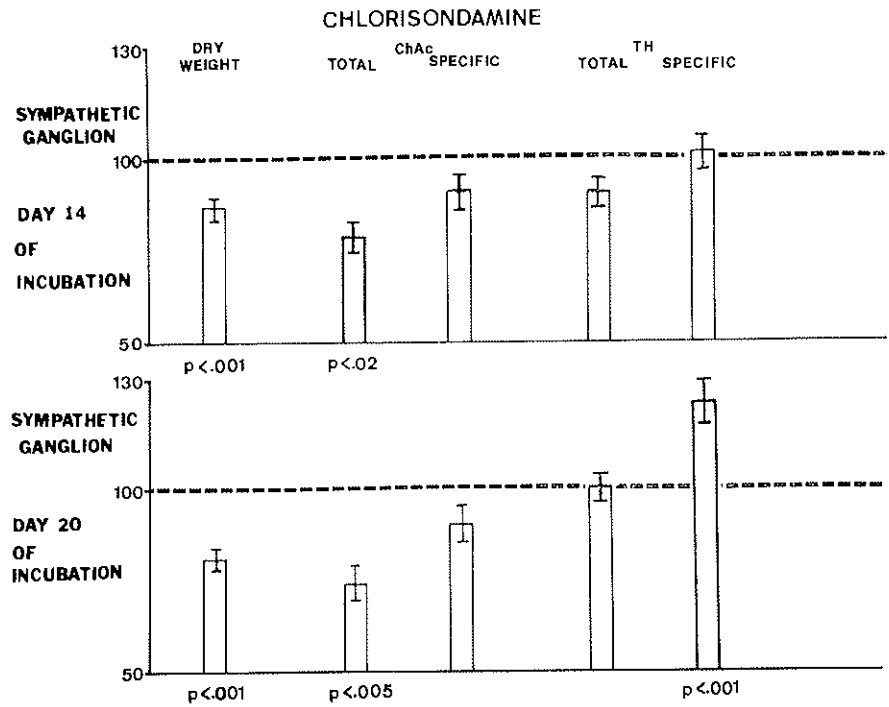

FIG. 15. Effect of chlorisondamine treatment on lumbar sympathetic ganglia. A) At 14 days of incubation and B) at 20 days of incubation. All values are expressed as a percentage of control values. Bars represent mean ± S.E.M. Number of embroys in each group was 8 to 10 (between 15 and 30 determinations per group).

1969; Mueller *et al.*, 1970; Thoenen *et al.*, 1969) demonstrating that the increase is dependent on intact synaptic transmission through the ganglion.

Reserpine treatment during development increases TH activity in the rat SCG and adrenal (Black and Reis, 1975) and in the chick lumbar sympathetic ganglia (Fairman *et al.*, 1977). The increase seen in these studies occurring mainly after functional peripheral innervation is present (at 21 d.i. and 14 and 30 days a.h. for chick sympathetic ganglia), suggesting that reserpine induction during development may depend on an intact peripheral innervation, as does the adult response (Brimijoin and Molinoff, 1971). In addition, the increase seen in the reserpine-treated chick, sympathetic ganglia at 14 days a.h. is completely blocked by treatment with chlorisondamine after hatching (Fairman *et al.*, 1977), demonstrating that the developmental response, like the adult response, is dependent on intact ganglionic transmission.

In the present study chlorisondamine treatment affected the chick sympathetic ganglia at an earlier stage (14 d.i.) than reserpine treatment (21 d.i.). This is probably due to the different sites of action of the two drugs. In sympathetic ganglia, chlorisondamine blocks nicotinic receptors, which begin to appear in increasing numbers around 14 d.i. (Greene, 1976). Reserpine has its greatest effect on the terminals of adrenergic neurons, which do not become functional until around the time of hatching (Pappano, 1977).

F. *Effect of adrenergic receptor blockade*

Single injections of propranolol or phenoxybenzamine separately injected before incubation resulted in a significant increase of TH activity at 8 d.i. (+35%) and 7 days (+37%) a.h., respectively (Giacobini and Fairman, 1978). Neither drug showed an effect similar to that of reserpine reported previously by Fairman *et al.* (1977). However, the injection of a single dose of propranolol (10 mg/kg eggs) associated to phenoxybenzamine (27.0 mg/kg eggs) prior to incubation produces a significant increase (+35%) in TH specific activity of sympathetic ganglia at 20 d.i. The weight of the ganglion is strongly reduced. This effect is similar to the one seen after chlorisondamine at the same age. At age 14 days a.h. the effect is reversed and both total and specific TH activity are significantly decreased. It is possible that a long-lasting blockade of both α- and β-receptors produces a gradual reduction in preganglionic sympathetic activity leading to reduced enzyme activity. A similar mechanism has been suggested by Raine and Chubb (1977) to explain the reduction of TH and DBH in rabbit sympathetic ganglia after long term adrenergic blockade.

G. *Comments on the effect of receptor blockade*

Although the evidence obtained by using two specific receptor binders confirms our previous view that functional innervation is correlated to the development of both ciliary ganglia and iris, the effect of peripheral receptor blockade at the target organ (iris) is not comparable to the removal of the target organ as performed by other authors (Landmesser and Pilar, 1974, 1976).

In the presence of ABTX binding, peripheral junctions can still develop; however, both general effects on weight and specific effects on enzymes are observed. These effects are both pre- and postsynaptic and are similar in the skeletal muscle and in the iris. The effect on ciliary cells is less

pronounced, indicating that normal innervation of the iris is not the only signal which allows the survival of ganglion cells. Moreover, blockade of nicotinic receptors on ganglionic (ciliary and sympathetic) cells exerts a general hypotrophic effect on ganglionic development involving both weight and enzymes. However, in sympathetic ganglia this hypotrophic effect on transmitter enzymes is not apparent indicating that the regulatory signal is not specific. This confirms the findings of other authors (Black, 1973; Black and Geen, 1973, 1974) in developing mammalian sympathetic ganglia. Blockade of ganglionic receptors in ciliary ganglia prior to innervation does not affect target organ synapses (in the iris). In other words, if normal transmission in ciliary ganglia is unpaired but not in the iris, a situation mimicked by the chlorisondamine experiment, the development of the ganglion is retarded, while the target organ (iris) develops normally. In this case, the blockade of ganglionic neurotransmission seems to exert only a "local" effect, involving the structures immediately surrounding the blocked junction, such as nerve terminals and postsynaptic membrane components. On the other hand, if both ganglionic and end organ receptors are blocked, as in the experiments with ABTX, the major effect is seen in the target organ (iris).

In conclusion: normal synaptic transmission expressed by normal receptor function does not seem to be essential for the survival of developing neurons. However, blockade of postsynaptic receptors significantly influences the development of both ganglia and target organs. Thus, we can conclude that synaptic transmission plays a regulatory role in synaptogenesis.

General Considerations: CHEMISTRY, STRUCTURE OR FUNCTION. WHICH COMES FIRST?

A general pattern of development emerges from the analysis of our findings in two systems (peripheral sympathetic and parasympathetic) indicating that initial rates of increase for AChE activity and ABTX binding are greater than that for ChAc. Our findings also clearly show that in the ciliary ganglion and iris, neurotransmission reaches adult values prior to the establishment of adult levels of cholinergic receptors as well as AChE and ChAc activity. In the ciliary ganglion, the highest rate of appearance of receptors is prior to the rise in enzyme activity, while in the iris it is simultaneous. This implies that the postsynaptic system interacting with the neurotransmitter (i.e. AChR and AChE) starts developing simultaneously with or even earlier than full capacity in neurotransmitter synthesis has been achieved in the presynaptic terminals.

Based on our observations, as indicated schematically in Fig. 16 and Table I, the developmental sequence of the basic components of a peripheral cholinergic synapse can be described as follows:

I) Presence of a low number (low density) of cholinergic receptors and low levels of synthesis and release of the neurotransmitter (PHASE OF INNERVATION) (Fig. 16 A). Absence of recognizable synaptic structure and specialization.

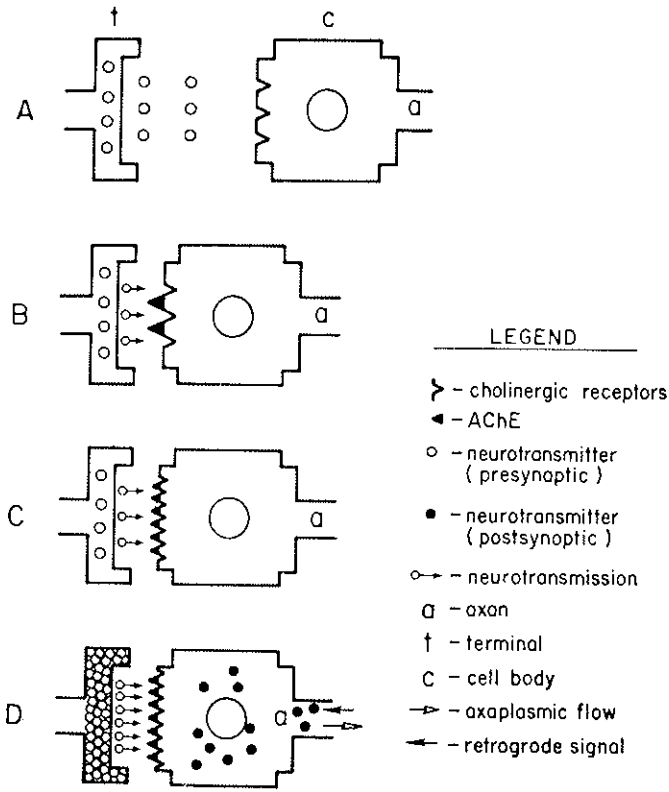

FIG. 16. Developmental sequence of the basic components of a peripheral cholinergic synapse (see text and Table I).

II) Onset and maturation of neurotransmission, first in the ganglion and then at the target organ (PHASE OF NEUROTRANSMISSION) (Fig. 16 B).

III) Rapid and sustained increase in number and density of receptors and AChE activity in specialized areas of the ganglionic and target organ membrane (PHASE OF RECEPTOGENESIS) (Fig. 16 C).

IV) Structural and biochemical maturation of the presynaptic and postsynaptic apparatus for synthesis and inactivation of the neurotransmitter (PHASE OF MATURATION) (Fig. 16 D). This phase can last up to several weeks and extend itself to after hatching (or after birth) periods.

The developmental scheme suggested above is supported by results obtained in structures other than autonomic ganglia such as muscle endplate (Burden, 1977; Giacobini, 1978; Giacobini *et al.*, 1973; Inestrosa and Fernandez, 1977), adrenal gland (Rotundo *et al.*, 1978), retina (Vogel and Nirenberg, 1976), myoblasts cultivated *in vitro* (Betz and Osborne, 1977; Fambrough and Rash, 1971; Fischbach and Cohen, 1973), developing chick heart (Sastre *et al.*, 1977) and brain (Coyle and Yamamura, 1976; Enna *et al.*, 1976; Kouvelas and Greene, 1976). In chick embryo brain, Enna *et al.* (1976) found that both ChAc and AChE activity begin to develop rapidly at about the same time as the muscarinic receptor. AChE activity in particular, develops at a rate quite similar to the postsynaptic muscarinic receptor.

Although the early appearance and maturation of neurotransmission in the absence of a morphologically and biochemically well developed presynaptic apparatus (Phases I-III, Table 1) seem somewhat paradoxical, it should make us acknowledge the fact that early embryonic synapses may function under different and more primitive conditions than adult ones. It seems likely that a relatively low rate of release of the neurotransmitter from a not yet fully developed presynaptic terminal might

TABLE 1. *Course of synaptogenesis in the peripheral nervous system of the chick*

I.	*PHASE OF INNERVATION* low density of receptors, low level of NT synthesis and release. Absence of synaptic structures. Appearance of uptake mechanisms - inactivation of NT is present.
II.	*PHASE OF NEUROTRANSMISSION* onset and maturation of synaptic activity in ganglion and target organ. Only rudimentary synaptic structures are present.
III.	*PHASE OF RECEPTOGENESIS* rapid, sustained increase of receptors density. Primitive synaptic structures. Inactivation of NT increases.
IV.	*PHASE OF BIOCHEMICAL AND STRUCTURAL MATURATION* accelerated synthesis, inactivation and transport of NT. Completion of synaptic structures (vesicles, membrane specialization, etc.).

NT = neurotransmitter

indeed be sufficient to trigger at a distance the process of neurotransmission and the subsequent phases of synaptogenesis (Fig. 16). Biochemical, electrophysiological and morphological evidence for such an hypothesis seems to be well established at least for the ciliary ganglion-iris preparation (Cantino and Mugnaini, 1975; Chiappinelli *et al.*, 1978; Giacobini and Chiappinelli, 1977; Landmesser and Pilar, 1972; Landmesser and Pilar, 1974; Landmesser and Pilar, 1974) but further work is necessary in order to validate and extend it to other structures, including the CNS.

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DISCUSSION

GREENE

Would you expect to see the same kind of mechanisms occurring in the central nervous system as in the periphery, particularly in cases where there is multiple innervation taking place on the same neuron?

GIACOBINI

This is quite a tough question. I do not think we have clear evidence for this mechanism in the CNS; we have, however, some evidence for an intersynaptic (transsynaptic) regulatory system and we can demonstrate it by using some drugs (reserpine, receptor blockers, etc.). We do not have the same type of target organ; however, we have certain systems which can be explored, such as the *locus coeruleus-cerebellum*. We are just working on this model in our laboratory in order to see whether we can demonstrate this mechanism or not. I think it is a little bit premature and therefore I do not wish to generalize on this model. Instead, I would like to hear from some of the experts of the central nervous system, what they have to say about it.

GREENE

For example, one thing in particular that Dr. De Mello's data showed was a clear dissociation between formation of the post-synaptic response mechanism and formation of the synapse itself. Thus, the situation in the retina could be different from that in a ganglion.

FILOGAMO

I think that the increase of cholineacetylase in ganglia is later than in the muscle, because the organization of the central nervous system is important for this increase, and this organization in the spinal cord is very, very early, because it is possible to see synapsis in motoneurons in three or four days.

BURNSTOCK

One parallel result which may be of interest, is the work of Chris Bell, when he was in my lab ten or fifteen years ago. He looked at the innervation of the uterine artery. Normally this vessel is innervated by adrenergic vaso-

constrictor fibres, but in late pregnancy cholinergic vasodilatation can be demonstrated. With electronmicroscopic localization of acetylcholinesterase, these nerves were shown to be present all the time, but in late pregnancy the vessel suddenly becomes a hundred-fold more sensitive to acetylcholine, and at the same time acetylcholinesterase begins to accumulate, on the postjunctional membrane opposite the acetylcholinesterase-positive nerves. After pregnancy this enzyme disperses and the vessel becomes less sensitive to acetylcholine again. So in the development of a cholinergic synapse there is an interesting relationship between acetylcholinesterase localization and acetylcholine sensitivity. I find this sort of thing—the coming and going of functional autonomic junctions in an adult at least as interesting as perinatal development of synapses. However, this is a neglected area and I think more examples will be discovered.

GIACOBINI

Do you want to imply that this is a phenomenon involving switching of the nerve, or switching of the transmitter in the same nerve?

BURNSTOCK

No. Well, it is possible, but it does not look like it in this case.

ON THE REGULATION OF THE NUMBER AND DISTRIBUTION OF ACETYLCHOLINE RECEPTORS IN SKELETAL MUSCLE

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The nicotinic acetylcholine receptor is the most completely characterized neurotransmitter receptor, both in terms of function and of structure. Some of the main features of ACh receptor function are summarized in Table 1, and the main features of ACh receptor structure are summarized in Table 2. Many of these features remain foci of current research, and there is residual doubt on some points, for example, the number and size of polypeptide chains in the isolated receptor units. The features tabulated in Table 1 are based upon electrophysiological studies of neuromuscular preparations while the structural features in Table 2 are derived from studies of solubilized receptor units. It is not entirely clear whether the isolated, detergent solubilized receptor units correspond to functional receptors units *in vivo,* or alternatively correspond to subunits or even incomplete fragments of the functional receptors. This matter has recently been discussed at length (Fambrough, 1979) and is beyond the scope of the present paper. Suffice it to say that evidence for a close correspondence between the detergent-solubilized glycoprotein and the functional ACh receptor unit is very good, consisting of ligand binding studies,

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immunological studies, ultrastructural studies, and reconstitutions of chemosensitive membranes from isolated receptor protein and lipids. There is a very good correspondence between number of ligand binding sites in muscle tissue and the chemosensitivity of the tissue: there are no known discrepancies. Thus it seems justifiable to define the ACh receptor as the functional unit which binds acetylcholine and generates a channel for the passage of small cations, yet to talk about the ligand-binding,

TABLE 1. *ACh receptor function*

Ion Channel
selective for cations
$Na^+ = K^+$
effective diameter $\sim$ 6.4A
10^7 - 10^8 ions/sec
Channels operate independently
2 ACh required to open channel
Open time $\sim$ 1 m sec
depends upon agonist
Each channel contributes about
0.3 μV to depolarization

TABLE 2. *ACh receptor structure*

Glycoprotein $\sim$ MW = 300,000
Carbohydrate $\sim$ 3-5%
mannose
N-acetyl-glucosamine
galactose
N-acetyl-galactosamine
Subunits 5-8 of 1-4 types
MW $\geq$ 40,000
ACh Binding Sites 2-4
Sedimentation Coefficient 9-10S
Isoelectric Point $\sim$ pH 5

detergent solubilized entity as the "ACh receptor", bearing in mind the possibility that the functional unit may contain something more than the isolated unit (for example, bound lipid may be essential for function).

In this chapter we wish to consider what can be called the cell and developmental biology of cholinergic receptors. Beginning with the beautiful electrophysiological descriptions of the distribution of chemosensitivity in embryonic, adult and denervated adult skeletal muscle (Del Castillo and Katz, 1955; Axelsson and Thesleff, 1959; Diamond and Miledi, 1962), it has been recognized that cholinergic sensitivity is a property of skeletal muscle which is influenced by the developmental and physiological state of the muscle. Since the discovery of the exceptionally high affinity ligand, α-bungarotoxin, autoradiographic studies employing radiolabeled α-bungarotoxin have demonstrated that the number and distribution of α-bungarotoxin binding sites in embryonic, adult and denervated adult skeletal muscles corresponds excellently with the level and spatial distribution of chemosensitivity (Fambrough, 1979). Autoradiographs of isolated muscle elements after iodinated α-bungarotoxin was bound to receptor sites are illustrated in Fig. 1. Fig. 1 A shows tissue cultured chick myo-

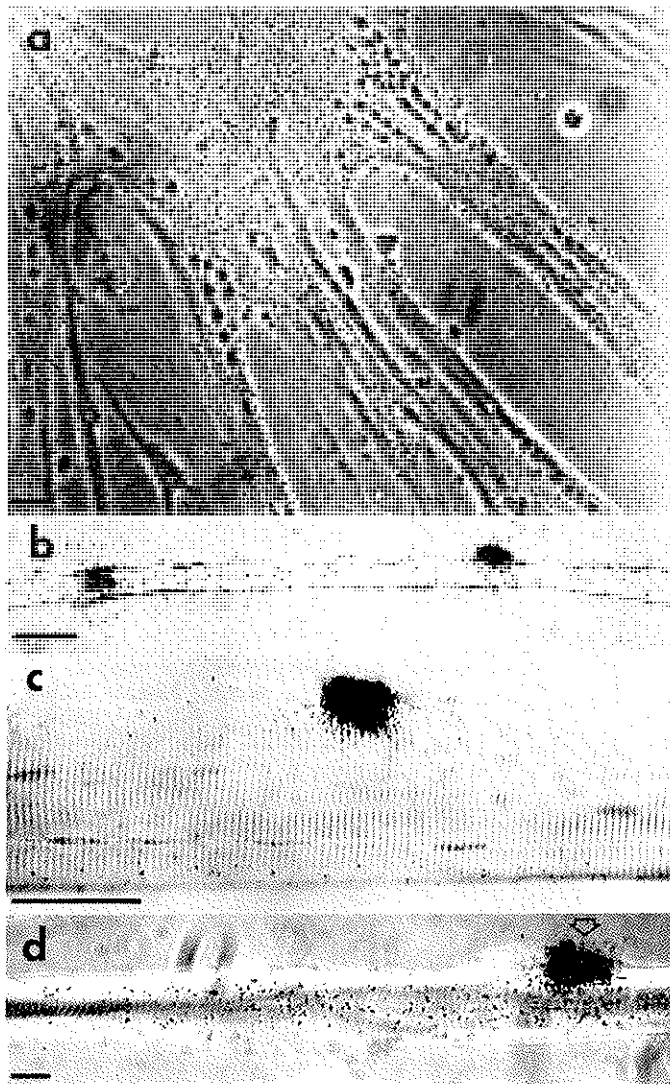


FIG. 1. Light-microscope autoradiograms showing the approximate location of cholinergic receptors in embryonic, adult and denervated adult muscles, as revealed by ^{125}I-labelled α-BuTx binding. (*a*) Embryonic chick skeletal muscle in tissue culture. (*b*) Portion of single muscle fibre from 19-day embryonic chick anterior latissimus dorsi muscle, illustrating extrajunctional receptors and clusters of receptors at the periodic sites of multiple innervation. (*c*) End-plate region of single muscle fibre from normal adult human deltoid muscle. (*d*) Portion of muscle fibre from 10-day denervated rat diaphragm muscle, illustrating the continued presence of dense receptor clustering at former postsynaptic site (arrow) and also nearby extrajunctional receptor sites. Magnification bars represent 20 μm in a, b, and d, 50 μm in c.

tubes with a nearly uniform distribution of binding sites over the multinucleate elements. Fig. 1 B shows a portion of a fiber teased from a 19 day embryo chick anterior latissimus dorsi muscle, a muscle with rather regularly spaced neuromuscular junctions, two of which are illustrated. The intervening area of extrajunctional surface contains a low but easily measured number of ACh receptor sites while the synaptic regions bear dense accumulations of receptors. Fig. 1 C shows the end plate regions of an isolated human deltoid muscle. The dense accumulation of silver grains over the end plate indicates the exquisite localization of receptors to the post-synaptic surface (estimated at 30,000 sites per square micron, Fertuck and Salpeter, 1976) while adjacent surface contains an extremely small number of receptor sites. Finally, in Fig. 1 D a fiber from a 10 day denervated adult rat diaphragm muscle bears large numbers of silver grains in the extrajunctional membrane as well as the cluster of grains over the former neuromuscular junction. This illustrates the reappearance of extrajunctional ACh receptors following denervation together with maintenance of receptor-rich post-synaptic membrane at the former junction. Extrajunctional receptor site density rises in this case to about 650 α-bungarotoxin binding sites per square micrometer of surface and the total number of extrajunctional receptors rises to about 20 times the number of junctional receptors.

The distributions of ACh receptors suggest a number of questions. Among them are the following six:

1. What regulates the turn-on of receptor expression during myogenesis?
2. How is the chemosensitive state maintained in uninnervated cells?
3. What mechanisms bring about clustering of receptors at synaptic sites during the formation of neuromuscular junctions?
4. By what mechanisms are extrajunctional receptors eliminated after innervation?
5. What mechanisms underlie denervation hypersensitivity?
6. What is the reason for the great stability of the post-synaptic surface after denervation?

Clearly there are a number of molecular mechanisms which govern the number and distribution of receptor sites. Among these mechanisms receptor biosynthesis and degradation are central, and regulatory influences operate in large part by modulating receptor metabolism.

Fig. 2 is a cartoon depicting many of the events in ACh receptor biosynthesis and degradation. In this figure a cut-away view of the cell is

shown with nucleus at left and plasma membrane toward the right. ACh receptors are symbolized as triangles associated with lines representing membranes. The arrows indicate directions of transport. This cartoon is based upon biochemical, morphological and kinetic studies, some of which are mentioned below. The data were obtained principally from study of tissue cultured chick myogenic cells but are in excellent quantitative agreement with more fragmentary data for mammalian muscle cultures and for the extrajunctional receptor metabolism in denervated adult muscles *in vivo*.

A feature of the ACh receptor-myogenic cell system which was of particular importance in early studies of receptor metabolism is that the receptor molecules in the plasma membrane are accessible to the ligand α-bungarotoxin, which binds essentially irreversibly to the ACh binding sites of the receptors. Newly synthesized receptor molecules do not interact with α-bungarotoxin because the toxin is unable to penetrate lipid bilayers, and thus labels only receptor sites exposed to the extracellular milieu. Both features of the bungarotoxin-receptor interaction (irrever-

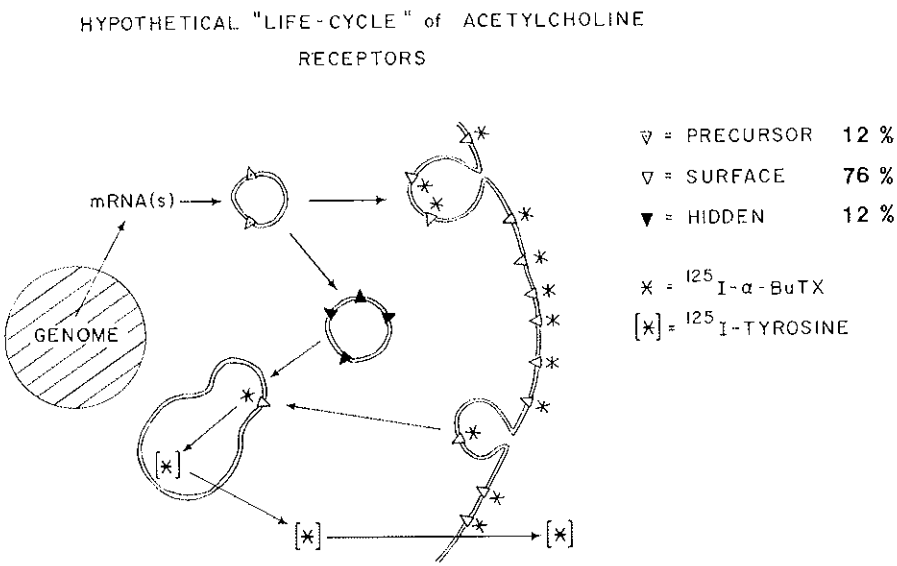


FIG. 2. Hypothetical "life cycle" of ACh receptors in cultured chick skeletal muscle. The figure depicts a cross section through a myotube with receptors symbolized as triangles in membrane profiles. Symbols for precursor, hidden, and surface ACh receptors are indicated on the figure. Also symbols for iodinated α-bungarotoxin and for iodotyrosine are shown positioned as postulated to occur during an experiment in which degradation of toxin bound to ACh receptors is being measured.

sibility and limitation to surface receptors in living tissue) made it possible to develop simple strategies for observing the appearance of new receptor sites in the surface membrane and for following the fate of the receptors during turnover (Hartzell and Fambrough, 1973; Devreotes and Fambrough, 1975). More recently we have used isotopically labeled amino acids to label new receptors through the natural biosynthetic pathway and thus confirm earlier inferences about synthetic and degradative rates (Devreotes and Fambrough, 1976 a; Devreotes *et al.*, 1977; Gardner and Fambrough, 1978). And radioactive α-bungarotoxin has been used in autoradiographic studies to identify the location of receptor sites during biosynthesis and transport to the surface (Fambrough and Devreotes, 1978) and during degradation (Fambrough *et al.*, 1978; Devreotes and Fambrough, 1976 b). Results from all of these sorts of experiments are combined in the following few paragraphs to give a brief description of the life history of extrajunctional ACh receptors.

When myogenic cells are pulse-labeled with labeled amino acids there is a lag of about three hours before labeled receptor molecules begin to appear in the surface membranes (Fig. 3). After this lag period all of the newly appearing receptor molecules are of the labeled variety. In tissue cultured chick muscle during the phase of receptor accumulation the surface population is augmented each hour by the addition of four to five new receptor molecules for every hundred molecules already present. This augmentation is partially offset by receptor degradation, discussed later in this chapter.

As an aside we would like to point out that these labeling experiments employed amino acids with carbon, nitrogen and non-exchangeable hydrogen atoms substituted with the stable, heavy isotopes ^{13}C, ^{15}N and ^{2}H. Labeled receptors were identified by virtue of their altered density. This strategy for labeling was employed because the ACh receptors constitute such a small fraction of total protein in the system (perhaps one part in 20,000) that it would be virtually impossible to reproducibly isolate and completely purify receptors from each labeling time and demonstrate incorporation of radiolabeled amino acids into receptor molecules. The density-shift strategy, as it has been called, can be used to study the metabolism of any large molecule which can be identified after buoyant density or velocity sedimentation. One simply determines the position of the solubilized molecules of interest in the centrifuge tube. If the molecule has been synthesized from heavy isotopically labeled precursors, then the position will be altered. This labeling technique is immediately applicable to the study of various hormone and transmitter receptors in

nervous tissue, the study of peptide hormone and peptide transmitter synthesis, the study of turnover of enzymes related to transmitter metabolism, etc. Some examples of velocity sedimentation profiles for ACh receptors after various periods of labeling with heavy amino acids are shown in Fig. 4.

The lag which occurs before significant labeling of muscle cell surface ACh receptors is due to a three-hour period of receptor packaging, possibly

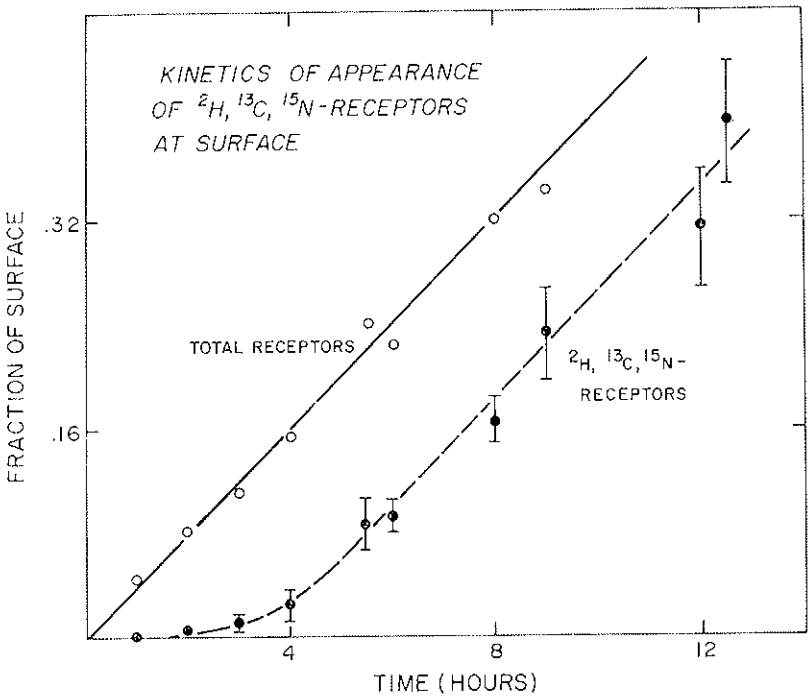


FIG. 3. Kinetics of appearance of total (^{1}H-, ^{12}C-, ^{14}N- plus ^{2}H-, ^{13}C-, ^{15}N-) and density shifted (^{2}H-, ^{13}C-, ^{15}N-) receptors on myotube surfaces during incubation in medium containing ^{2}H-, ^{13}C-, ^{15}N- amino acids. After long-term incubation in medium containing unlabeled α-bungarotoxin, cultures were rinsed to remove unbound α-bungarotoxin and cultured in medium containing ^{2}H-, ^{13}C-, ^{15}N- amino acids. Receptors appearing on the myotube surfaces were labeled by addition of 0.15 μg [^{125}I]monoiodo-α-bungarotoxin/ml to the culture medium during the final 30 min. Unbound [^{125}I]-monoiodo-α-bungarotoxin was removed by rinsing cultures in the cold, and the [^{125}I]monoiodo-α-bungarotoxin-receptors complexes were extracted and analyzed by sucrose gradient velocity sedimentation. The receptors from three to five 100-mm (diameter) cultures were used for each data point. Data from 2 experiments were combined by first subtracting the small background present at zero time and then normalizing the slope for total receptor appearance to 4% addition of new receptors to the total surface receptor population per hour, a typical rate of incorporation. The fraction of ^{2}H-, ^{13}C-, ^{15}N-receptors at each time point was calculated from areas under curves on sucrose gradients (see Fig. 4).

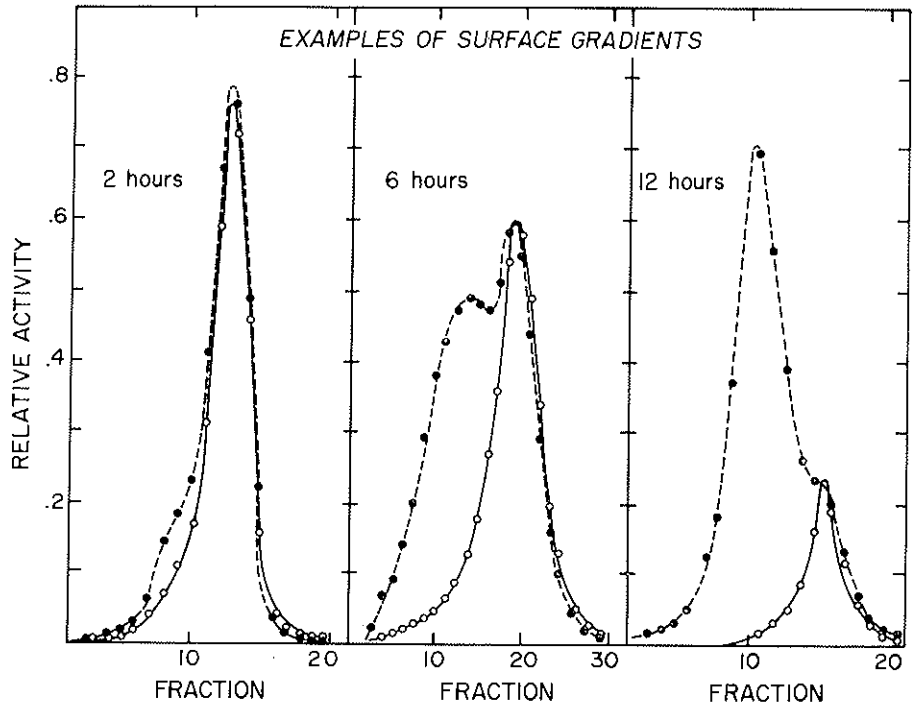
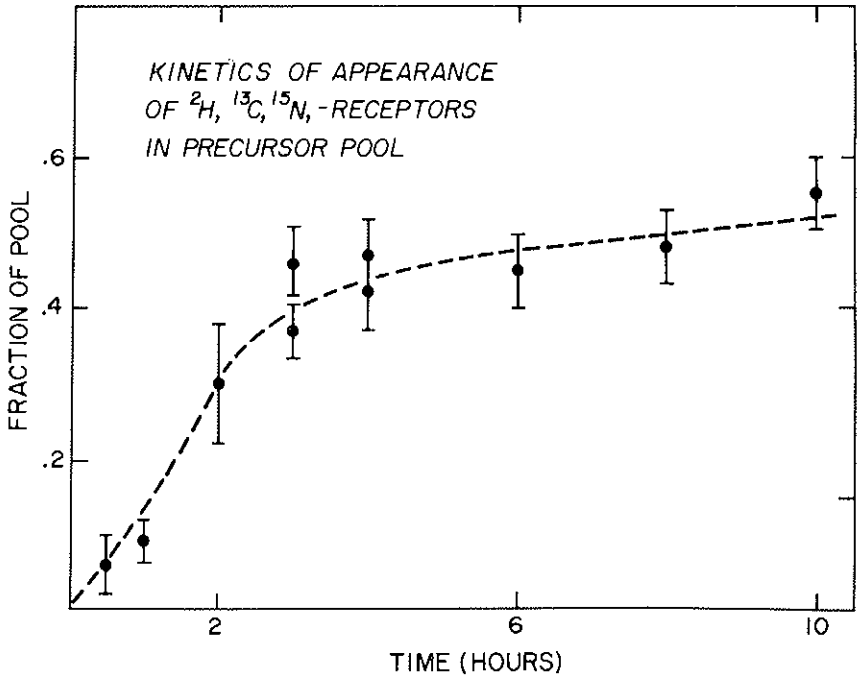


FIG. 4. Sucrose gradient, velocity sedimentation profiles or surface receptors appearing during culture of myogenic cells in medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids. Gradient profiles illustrated here are those from which the data for the 2-, 6-, and 12-hr time points in Fig. 3 were obtained. A marker of [^{131}I]monoiodo-α-bungarotoxin-^{1}H-, ^{12}C-, ^{14}N-receptor complexes was mixed with sample before centrifugation, and its position in the gradient is identical to that of the [^{125}I]monoiodo-α-bungarotoxin-^{1}H, ^{12}C, ^{14}N-receptor complexes. Note the small number of ^{2}H-, ^{13}C-, ^{15}N-receptors present at 2 hr.

processing, and transport within the cell. We have identified a set of ACh receptors which occur inside myotubes and constitute about 20-25% of all the ACh receptors in the myogenic cell cultures. About half of these receptors are newly synthesized molecules in the pathway mentioned above. The kinetics of labeling of this population with heavy amino acids is shown in Fig. 5. The experiments illustrated in this figure show that complete assembly of receptor units from amino acids requires less than fifteen minutes. The remaining lag time before the labeling of surface ACh receptors is due to the intracellular residence of newly synthesized ACh receptors for about three hours. At least the first half of this intracellular residence time is spent chiefly in the Golgi apparatus. Using saponin to permeabilize the membranes of fresh formaldehyde fixed cells,



F):. 5. Kinetics of appearance of ^{2}H-, ^{13}C-, ^{15}N-receptors in the intracellular pool. [^{125}I]monoiodo-α-bungarotoxin-receptor complexes involving the pool receptors from cultures incubated for the designated times in medium containing ^{2}H-, ^{13}C-, ^{15}N-amino acids were prepared. These receptors were analyzed by sucrose gradient velocity sedimentation to determine the fraction of pool receptors which were ^{2}H-, ^{13}C-, ^{15}N-receptors.

it is possible to render the intracellular receptor sites accessible to labeled α-bungarotoxin. Autoradiographs of such preparations show that a considerable portion of labeling occurs over the Golgi apparatus (Fig. 6) and the intensity of labeling of this locus is greatly diminished by inhibition of protein synthesis (Fig. 7).

Inhibitor studies demonstrate some of the properties of the transport and incorporation of ACh receptors into surface membrane. Some inhibitors exert their effect upon the incorporation step directly, this inhibiting appearance of new receptor sites immediately. In this class of inhibitors are low temperature, dinitrophenol, cyanide, azide, the calcium ionophore X 537 A, and colchicine. The colchicine effect is only partial: the rate of receptor appearance is maximally lowered only about 50%, and the dose of colchicine giving maximal inhibition is about 5 μM. Lumicolchicine, a closely related compound which lacks affinity for microtubules,

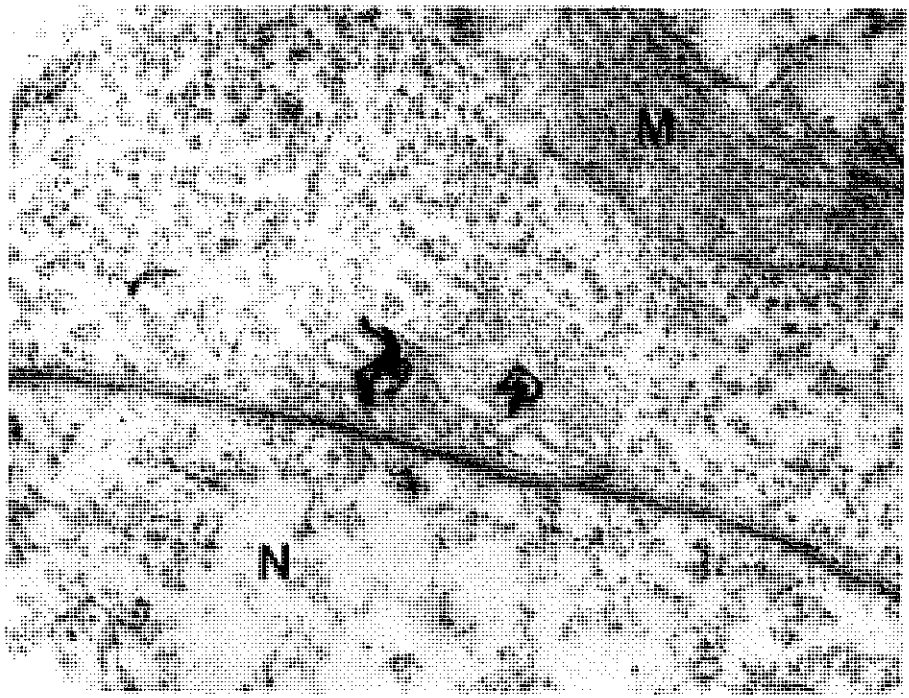


FIG. 6. Electron microscope autoradiograph illustrating the association of ACh receptors with the Golgi apparatus, as revealed by the binding of α-[^{125}I]bungarotoxin to fixed, saponin-treated chick muscle cells. N (nucleus) M (mitochondrion).

has no effect. Cytochalasins B, D and E likewise have no effect upon incorporation. Another set of inhibitors block incorporation of new receptors into plasma membrane indirectly as consequence of their inhibition of biosynthesis of functional receptor units. Among these inhibitors are inhibitors of protein synthesis, puromycin and cycloheximide, and an inhibitor of glycosylation via the dolichol phosphate pathway, tunicamycin (Fig. 8). In these cases incorporation ceases when the pool of internal receptor sites in the pathway of transport to the surface is exhausted. Because of the size of the pool and the rate of incorporation into the surface membrane, this inhibition of incorporation occurs about three hours after administration of the inhibitors. The initial glycosylation of receptors occurs immediately after or possibly during synthesis, for no receptor units resistant to precipitation by the lectin Concanavalin A are detected in detergent extracts of skeletal muscle containing newly synthesized receptors.

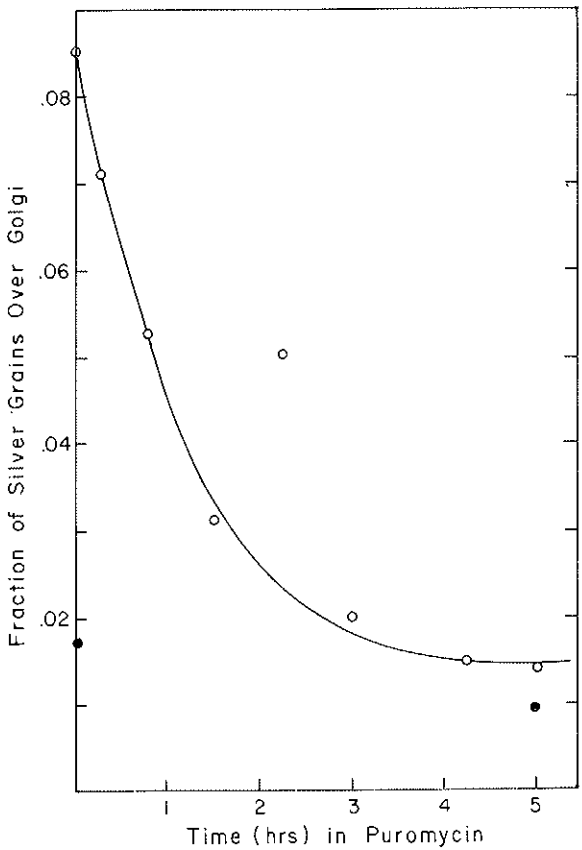


FIG. 7. Association of ACh receptors with Golgi apparatus after inhibition of new receptor synthesis with puromycin (20 μg/ml). Cells were processed for electron microscope autoradiography, and the distribution of silver grains was determined. From left to right, the total grains scored for each point (0) were 1,728, 693, 1,846, 1,004, 701, 247, 780, and 1,157. Closed circles are controls in which specific binding was blocked by 1.5×10^{-4} M d-tubocurarine. Data are expressed as fraction of all silver grains (due to nonspecific as well as specific binding of [^{125}I]BuTx) directly over Golgi membranes and not covering any portion of nuclear membrane.

Incidentally, these inhibitors which slow or block incorporation of receptors into plasma membrane also inhibit the secretion of acetylcholinesterase from the muscle cells. The time course and magnitude of inhibition of AChE secretion is quantitatively comparable to the effects upon receptor incorporation, a matter for further comment later in this chapter. Insertion of new receptor-containing membrane into the plasma membrane is depicted in Fig. 2 to involve membrane fusion. This mechanism seems likely for several reasons, a key one being that the newly synthesized receptors which have not yet reached the surface membrane are located in subcellular particles in such an orientation that the receptor sites are not available for interaction with ligands even after homogenization of the tissue but become available when membranes are disrupted with detergent. We will return to this point later when discussing acetylcholinesterase biosynthesis.

Once displayed on the surface of the muscle fibers the ACh receptors may occur as single receptor units or as aggregates. There is evidence from fluorescence bleaching and recovery measurements that the receptor population in extrasynaptic membrane of cultured skeletal muscle fibers consists of mobile and fixed receptors in approximately equal numbers (Axelrod *et al.*, 1976). In older chick and mammalian muscle cells in culture, clusters of receptors are found in which the packing density of receptors may approach that found in post-synaptic membranes at end plates. In cultured frog somitic muscle cells up to about 25% of the receptors may occur in such clusters (Anderson *et al.*, 1977).

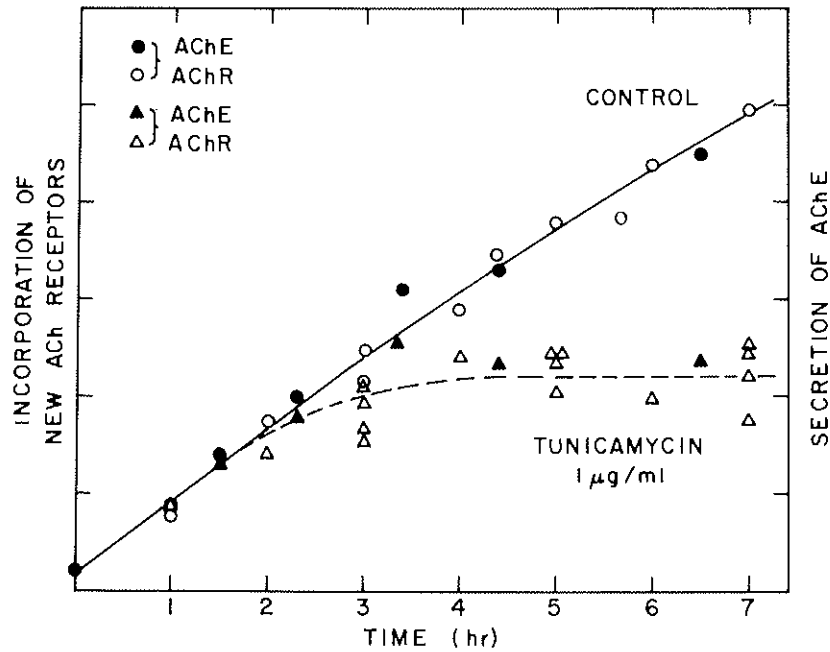


FIG. 8. Effect of tunicamycin (1 μg/ml) on secretion of acetylcholinesterase (●, ▲) and on incorporation of new acetylcholine receptors into plasma membranes (○, △). Control rates of secretion (●) and of incorporation (○) were plotted so as to have the same slope. Data from several experiments with different absolute rates are combined, so the ordinates are in units directly proportional to molecules of acetylcholinesterase secreted and new acetylcholine receptor sites appearing after time zero, but the proportionality constants vary slightly from one set of data points to another. In cultures treated continuously from time zero with tunicamycin in complete medium (DFP-treated in the case of secretion experiment) the rates of acetylcholinesterase secretion (▲) and of incorporation of receptors into plasma membrane (△) were not significantly affected until about 3 hours. Each symbol represents the averaged value for 3 to 5 culture dishes. Data from five separate incorporation experiments are plotted, involving four different samples of tunicamycin.

Returning to the cartoon of ACh receptor life history, we see illustrated a strategy for measuring the degradation rate for ACh receptors. The stars in the figure represent iodine-labeled α-bungarotoxin molecules, which are illustrated having bound to the cell surface ACh receptor sites. The toxin-receptor complexes are shown being interiorized and then degraded, generating iodotyrosine from the proteolytic destruction of the bungarotoxin. The cells are quite permeable to iodotyrosine, and it leaks out of the myotubes into the culture medium or circulation and can be identified by simple chromatographic and electrophoretic techniques. The degradation of α-bungarotoxin and production of iodotyrosine have the characteristics expected of a process which involves active uptake into the cells and destruction in secondary lysosomes. This has been discussed at length (Fambrough, 1979) and only a few bits of evidence are reviewed here. The kinetics of degradation are first-order exponential, as expected of a random-hit process, suggesting equal probability of survival of a given receptor molecule compared to other receptors, regardless of the "age" of the molecule. The median lifetime of receptors in extrajunctional regions of denervated rat diaphragm and from cultured chick skeletal muscle is about 20 hours, as judged from this technique (Fig. 9). When this technique is used in attempts to measure the turnover rate of junctional ACh receptors, a very slow rate of loss of radioactivity from the tissue is found,

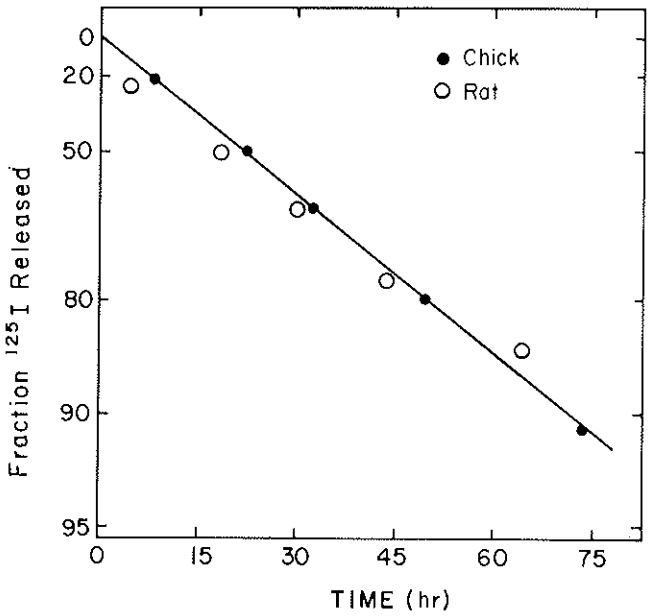


FIG. 9. Release of radioactivity from cultured chick muscle (● ●) and from denervated rat diaphragm *in vivo* (○ ○) after blockage from ACh receptors with [^{125}I]α-bungarotoxin. Chick data are from (7); rat data from (16).

indicating a very slow turnover of junctional ACh receptors compared with extrajunctional ones (Chang and Huang, 1975; Berg and Hall, 1975; Linden and Fambrough, 1979). As expected of an interiorization process, the degradation of bound toxin to yield iodotyrosine is dependent upon cell metabolism to the extent that inhibition of ATP production results in blockade of degradation, and as expected there is a lag of about 45 minutes between binding of the toxin and appearance of iodotyrosine. Associated with this lag, there is an accumulation of radioactivity in secondary lysosomes (Figs. 10 and 11). When lysosomal degradation of proteins is blocked by long term exposure of cells to trypan blue, the uptake into lysosomes continues and after about 6 hours of such uptake nearly 20% of the radioactivity is in the lysosomal compartment (Fambrough *et al.*, 1978).

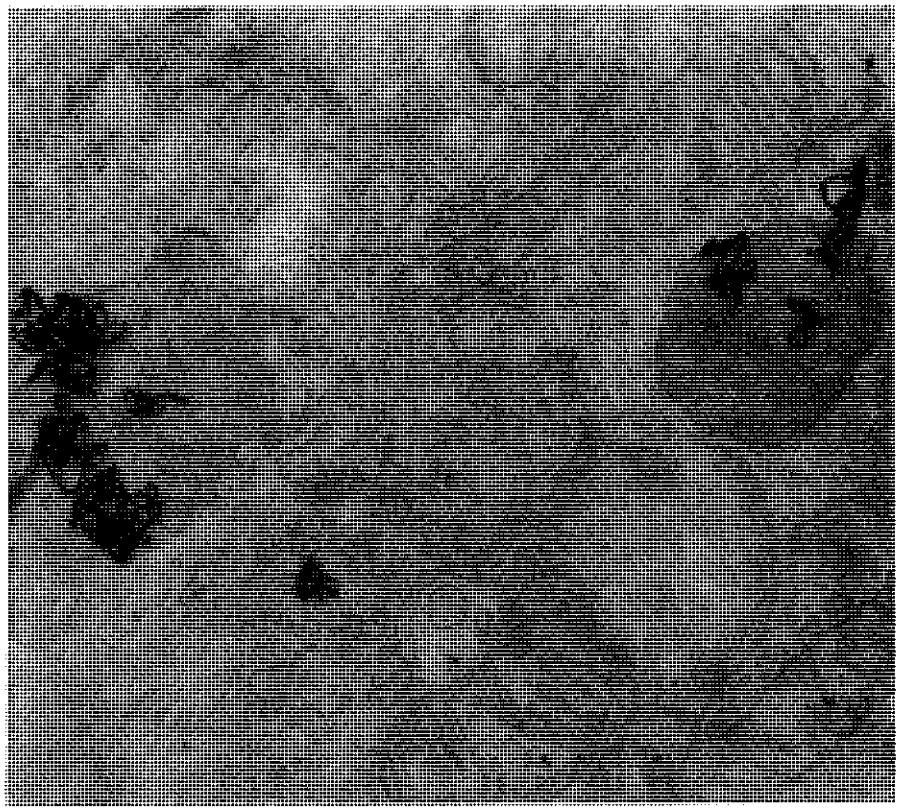


FIG. 10. Electron microscope autoradiograph of chick myotube after saturation of ACh receptors with [^{125}I]α-bungarotoxin, showing grains over electron-dense organelles resembling secondary lysosomes.

Recently we have measured the degradation rate of ACh receptors by pulse chase labeling with heavy amino acids (Gardner and Fambrough, 1978). In these experiments we found that the median lifetime for extrajunctional ACh receptors in cultured chick muscle was 17 hours. Cholinergic ligands carbamylcholine and d-tubocurarine had no effect upon turnover of receptors but α-bungarotoxin slightly prolonged receptor lifetime. Some possible explanations for this small effect are discussed elsewhere. Perhaps the most significant thing about the discovery of this effect of

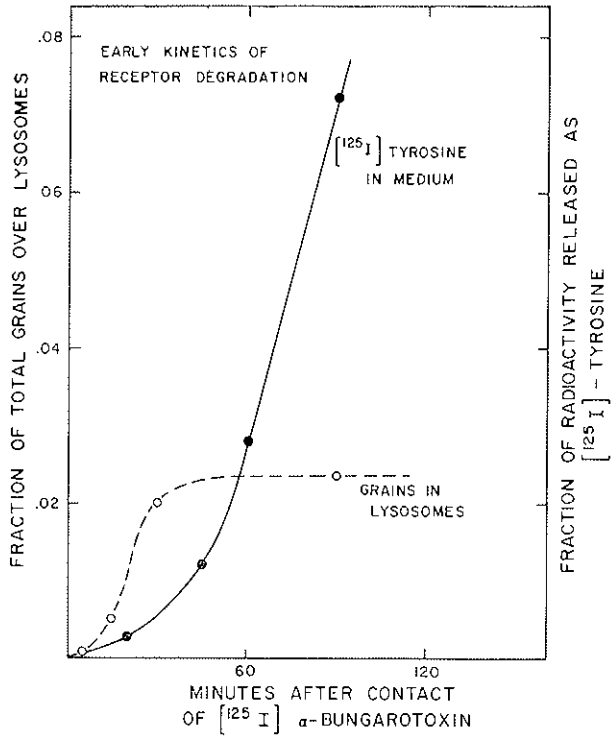


FIG. 11. Kinetics of transport of radioactivity to secondary lysosomes (○ ○) and kinetics of liberation of iodotyrosine from chick muscle cultures (● ●) after brief exposure of muscle to [^{125}I]α-bungarotoxin. Data obtained from electron microscope autoradiograms and from chromatographic analysis of culture medium.

α-bungarotoxin is that it has led to reinterpretation of some very puzzling older observations. At one point we felt that some of the finer aspects of our kinetic data required that we postulate a cycling of cell surface ACh receptors in and out of the plasma membrane (Fambrough *et al.*, 1978; Devreotes and Fambrough, 1976 b). These observations can now be interpreted in terms of a slightly altered receptor median lifetime in the presence of α-bungarotoxin, and we currently have no kinetic evidence for recycling. The only real puzzle remaining in our data on receptor

metabolism is the observation that following labeling with iodinated α-bungarotoxin, there seems to be a rearrangement of receptor sites so that a substantial portion of them are no longer in the plasma membrane (Devreotes, 1978). This could be a preturbation due to the toxin and could be related to the altered turnover rate, or it could reflect tbe occurrence of a very rapid cycling mechanism. The rearrangement is temperature sensitive but seems to occur in the presence of dinitrophenol.

The biosynthetic pathway which leads to appearance of ACh receptors in the plasma membrane resembles the pathway for production and secretion of secretory proteins. Palade (1959) postulated that membrane biogenesis and protein secretion might involve the same cellular apparatus, but before the advent of α-bungarotoxin as a marker for the ACh receptor, there were few identified eukaryotic membrane proteins and none whose biosynthesis could easily be followed (except for some viral coat proteins in virally infected cells). Our studies of the ACh receptor strengthened the case for Palade's hypothesis, but more direct demonstration required that a membrane and a secretory protein be studied in the same cells. This is one of several reasons why we recently began a study of the biosynthesis of acetylcholinesterase. (It had previously been shown that AChE is secreted in fairly large amounts from cultured chick skeletal muscle (Wilson et al., 1973) and from embryonic chick muscle *in vivo*). Another reason for undertaking the study of AChE is that it is the other major functional component of the post-synaptic element of the neuromuscular junction. Actually it is not certain whether AChE should be considered a synaptic cleft component or a true post-synaptic component, although it has long been argued that the AChE at the junction must be contributed predominantly by the post-synaptic cell (Couteaux, 1963). As it turns out, the cell and developmental biology of AChE is at least as interesting as that of ACh receptors. Not only do the number and distribution of AChE molecules vary witb developmental and physiological state of the muscle, as do the number and distribution of ACh receptors, but the AChE is a set of molecular forms which include membrane bound and soluble forms and a form found predominantly at neuromuscular junctions (Hall, 1973; Vigny *et al.*, 1976) which seems to be coupled covalently with a collagen-like component.

An account of our studies of the AChE molecular forms (Rotundo and Fambrough, 1979) and the number and distribution of AChE molecules in cultured chick muscle (Rotundo, in press) would take us well away from the matter of cbolinergic receptors and nerve-muscle interactions. Thus we will only mention a few results which relate to the receptor story.

First, there is a cell surface AChE which consists of two molecular forms. This AChE has the solubility characteristics of integral membrane proteins and has a turnover rate which is similar to that of the extrajunctional ACh receptors in the same cells. The turnover rates may be identical, and that is one point we wish to explore: the possibility that the entire set of integral membrane proteins turnover at the same rate. Second, the bulk of the AChE synthesized in cultured chick muscle is secreted into the culture medium. The predominant secreted form is probably a dimer of active subunits. The kinetics of biosynthesis and secretion of the secreted AChE are identical to those for biosynthesis and incorporation of ACh receptors into plasma membrane. Inhibitor studies have shown that any preturbation of receptor incorporation results in a quantitatively comparable preturbation in secretion of AChE. An example of results of tbis type is illustrated in Fig. 8 where the effects of tunicamycin on AChE secretion into the culture medium are compared with tunicamycin effect of ACh receptor appearance in the plasma membrane. As is the case for the ACh receptor there is an internal pool of AChE which is mostly packaged so that the ACh binding sites are protected even after homogenization of the tissue. Our current postulate is that the AChE molecules and the ACh receptors may even be packaged together in vesicles derived from the Golgi. Fusion of these vesicles with the plasma membrane exposes the ACh receptor sites and the active sites of membrane bound AChE while releasing secretory AChE into the medium. The findings and ideas mentioned in this paragraph are incorporated into the cartoon shown in Fig. 12.

Several other interesting points regarding AChE deserve mention here. Our cultured chick skeletal muscle fails to make the endplate specific AChE molecular form. We hope to use this culture system to determine what influence of the nerve could lead to production of the endplate specific form. Appearance of the end plate form could serve as an endpoint for bioassay of putative trophic substances. Another matter of interest is that, while the number of ACh receptors and the number of AChE active sites are approximately the same at endplates (Barnard *et al.*, 1971), the cultured cells secrete nearly two orders of magnitude more AChE molecules than they synthesize ACh receptors. The regulation of relative molecular numhers at the endplate may involve some fascinating molecular mechanisms. Clusters of ACh receptors in cultured myotubes do not correspond to clusters of AChE molecules. In fact, there has not been an adequate investigation of the distribution of cell surface AChE in cultured muscle. Finally, just as the ACh receptors at neuromuscular

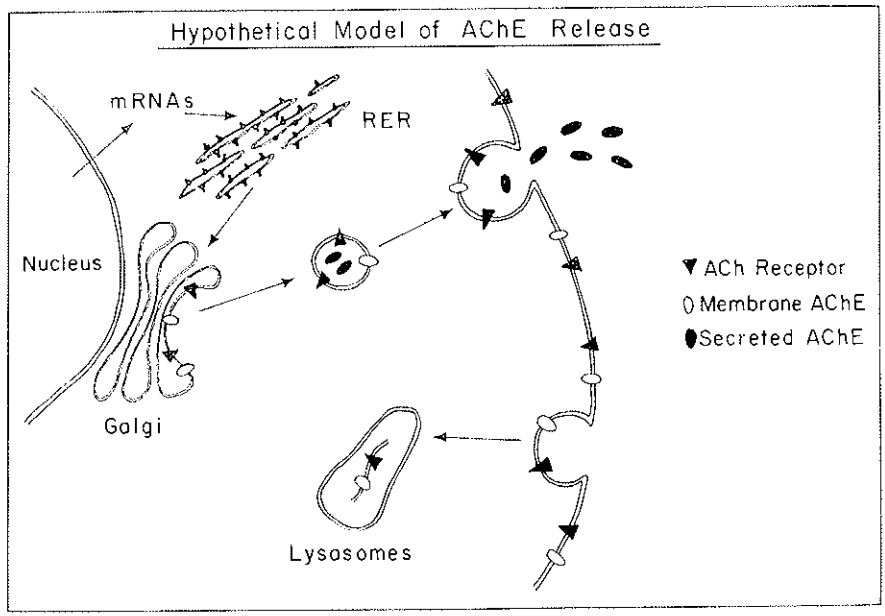


FIG. 12. Hypothetical model of AChE release including biosynthesis and intracellular transport of both the ACh receptor molecule and intracellular AChE.

junctions have a slow turnover rate compared with extrajunctional ACh receptors, the synaptic AChE appears to be stabilized as well. Again, there are no accurate determinations of turnover rate for synaptic AChE and the technical difficulties in such study are imposing. Just as ACh receptors remain at former endplate regions for months after denervations, there remains residual AChE activity at these locations for comparable periods of time (reviewed in Fambrough, 1976).

The control mechanisms regulating ACh receptors may be as varied as the phenomena involving ACh receptors described above. First, let us consider regulation of ACh receptor clustering. This occurs sometimes in extrajunctional regions of denervated muscle and in tissue cultured embryonic muscle as well as at neuromuscular junctions. It is not known whether the extrajunctional receptor clusters are equivalent to synaptic clusters in the mechanisms of spatial arrangement. One obvious difference is that at junctions the receptors are located in membrane underlain by cytoplasmic structures which stain darkly by the usual staining procedures for thin section electron microscopy (Fertuck and Salpeter, 1976), whereas extrajunctional clusters seem to lack submembranous specializations (Vo-

gel and Daniels, 1976). Perhaps correlated with this, junctional receptors seem to show a degree of organization which surpasses clusters of extrajunctional receptors, judging from freeze-cleave studies in which membranous particles at receptor-rich locations are thought to represent some grouping of receptor sites. At junctions the particles occur in rows (Rash

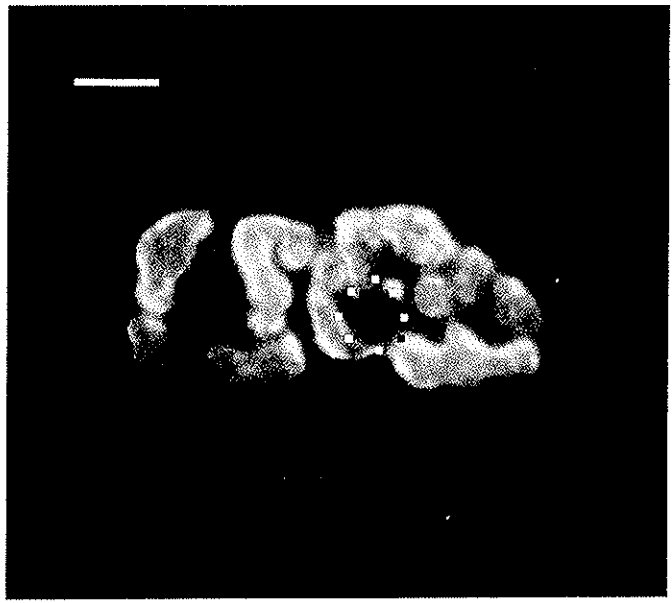


FIG. 13. Fluorescence micrograph showing the postsynaptic membrane of a rat diaphragm neuromuscular junction after incubation of the muscle with tetra-methyl-rhodamine-α-bungarotoxin. Fluorescence is from labeled α-bungarotoxin bound to ACh receptors. Small black and white squares ring a dark, disc-shaped area in which the fluorescence has been bleached by an intense laser beam. The bleaching was done 18 hr before this photograph was taken. In the interim the diaphragm was cultured in Trowell medium at 37°C in a humidified incubator with 95% O_2/5% CO_2. Magnification bar represents 10 μm. From Fambrough and Pagano (1977).

et al., 1978), whereas no such organization is evident in extrajunctional clusters (Peng and Nakajima). In both cases, however, the receptor units are immobilized, as shown by fluorescence bleaching experiments (Axelrod *et al.*, 1976, 1978) such as that illustrated in Fig. 13.

It is now clear that clustering is not directly coupled to a slowing of acetylcholine receptor turnover rate. This was established by autoradiographic studies of loss of iodinated α-bungarotoxin binding sites after the

toxin was bound to receptors in cultured muscle. It seems clear from electrophysiological studies and studies employing fluorescent α-bungarotoxin that ACh receptor clusters in cultured embryonic muscle are sites of preferential insertion of new receptors into plasma membrane (Axelrod *et al.,* 1978; Frank and Fischbach, 1977). It has also been estimated by Burden (Burden, 1977 a; Burden, 1977 b) that the turnover rate of acetylcholine receptors at new neuromuscular junctions in embryonic chick skeletal muscle remains fast (comparable to the turnover rate of extrajunctional receptors) for several weeks before the change to the slow rate characteristic of receptors at adult neuromuscular junctions occurs.

Obviously clusters are not always nerve-induced, in the sense that clusters form spontaneously in culture. There are several recent indications that substances of neural origin may influence clustering of receptors in culture muscle (see the chapter by Nelson in this volume). In the case of the spectacular clusters in *Xenopus laevis* somite muscle *in vitro* the only components in the culture medium are the basic salts, simple organic compounds and vitamins plus some Holmes' α-fetal protein (Anderson *et al.,* 1977). This would appear to be a system of choice for examining the requirement of exogenous substances for receptor clustering.

In some of the most spectacular experiments in the field of nerve-muscle interactions, Anderson and Cohen (1977) found that pre-laheled ACh receptors are spatially reorganized when spinal cord neurons interact with the somite muscle cells. This interaction led to dispersal of previously existing ACh receptor clusters and establishment of clustered receptors along the track of the nerve on the muscle surface. The rearrangement of receptors occurred in the absence of ACh receptor function, for example, in the presence of excess α-hungarotoxin.

There are other changes in ACh receptors which are related to synaptogenesis: changes in channel open time and unit conductance of the functional receptor units and change in isoelectric point of detergent solubilized receptors. The timing of these changes has not yet been established relative to change in turnover rate, but all of these changes seem to occur after clustering and may occur in mammals at about the time that multiple innervation is eliminated.

Some progress has been made toward understanding the mechanisms involved in denervation hypersensitivity of muscle and in the elimination of extrajunctional receptors following innervation. Both of these phenomena involve regulation of ACh receptor biosynthesis. The appearance of ACh receptors in extrajunctional sites following denervation is due to *de novo* synthesis of receptors and insertion directly into plasma membrane

in extrajunctional regions (Devreotes and Fambrough, 1976 a; Brockes and Hall, 1975). This has been shown by direct labeling of ACh receptors with isotopically labeled amino acids (radioactive and stable isotopes) (Fig. 14) and had earlier been inferred from inhibitor studies. It still is undetermined what denervation changes lead to stimulation of

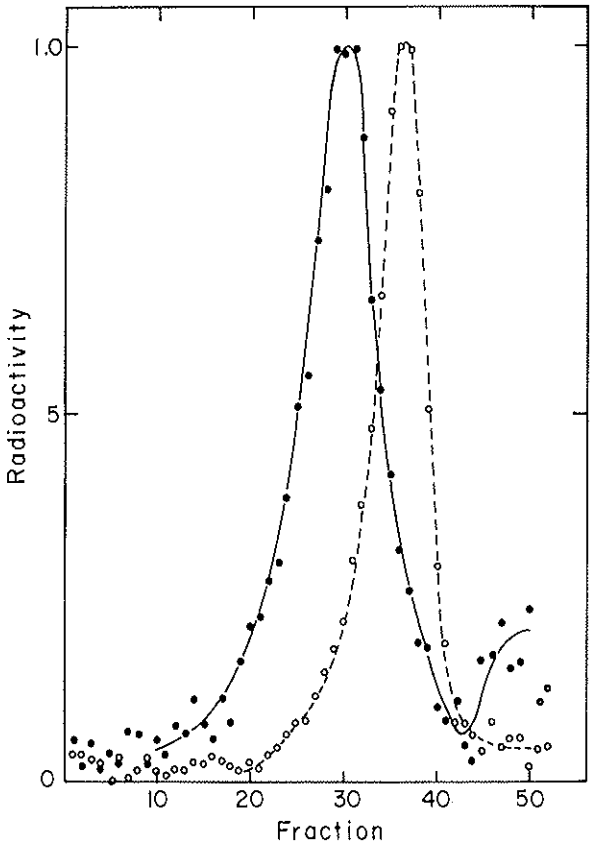


FIG. 14. Velocity sedimentation profiles of ACh receptors synthesized and incorporated into plasma membranes during the last 11 hours of a 42 hour culture period. The newly synthesized ACh receptors, (● ●) complexed with ^{125}I-labeled-α-bungarotoxin, contain ^{2}H, ^{13}C, ^{15}N-aminoacyl residues and therefore sediment about seven fractions ahead of markers of normal ACh receptors labeled with ^{131}I-α-bungarotoxin which included in the gradients (○ ○). Profiles were plotted with the direction of sedimentation right to left.

extrajunctional receptor synthesis. However, a strong cases has been made that muscle inactivity is a key factor (Lomo and Westgaard, 1976). Electrical stimulation of muscle can suppress the onset of denervation hypersensitivity and can reverse preestablished hypersensitivity. There may be other factors. This matter is discussed in the present volume by Cangiano and has been reviewed recently (Fambrough, 1979).

Recent studies by Hall and coworkers (Hogan *et al.*, 1976; Reiness and Hall, 1977) and by us (Linden and Fambrough, 1979) have shown

that the regulation of ACh receptors which is coupled somehow to muscle activity involves regulation of biosynthesis but not regulation of degradation rate. Using degradation of iodinated α-bungarotoxin bound to extrajunctional receptors to estimate the receptor degradation rate, we find no effect of electrical stimulation (Fig. 15), although the same regime of

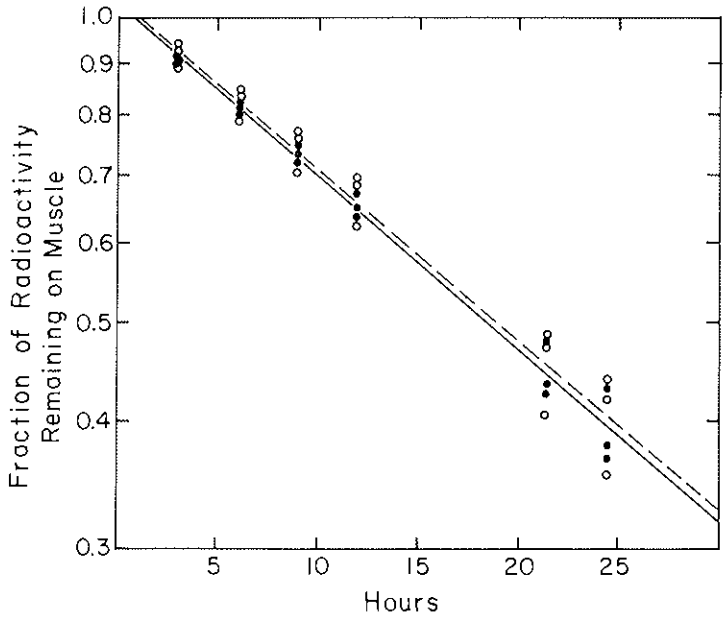


FIG. 15. The effect of electrical stimulation on the loss of radioactivity from 5 day denervated rat EDL muscles maintained in organ culture after labeling ACh receptors with ^{125}I-α-bungarotoxin. On this time scale, loss of radioactivity related to ^{125}I-α-bungarotoxin in bound to junctional ACh receptors is negligible, and the label due to ^{125}I-α-bungarotoxin bound to junctional ACh receptors (measured from contralateral innervated muscles) has been subtracted. Closed circles (● ●) represent muscles stimulated at 100 Hz for 1 sec every 80 sec; open circles (○ ○) represent unstimulated muscles. The inferred half-life of ACh receptors on stimulated muscles was 18.5 hr, on unstimulated muscles 19 hr. Three stimulated and three control muscles were used in the experiment. All data points for the six muscles are plotted.

electrical stimulation suppresses ACh sensitivity and does markedly suppress new receptor appearance in denervated muscle (Fig. 16).

A reasonable conclusion from these studies is that the loss of extrajunctional receptors which follows innervation may occur as a consequence of suppression of extrajunctional receptor synthesis due to muscle activity but no suppression of receptor turnover. Thus, the steady attrition of

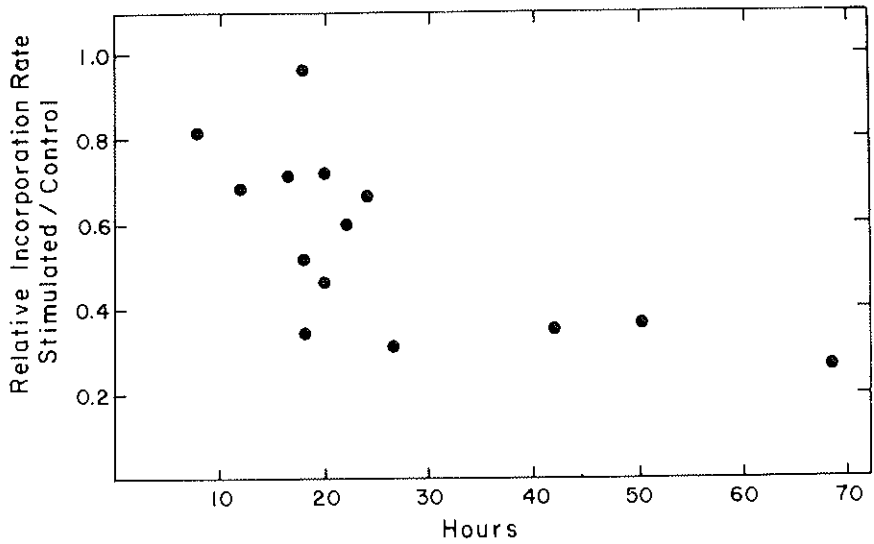


FIG. 16. Effects of electrical stimulation on incorporation of newly synthesized ^{3}H, ^{13}C, ^{15}N-labeled ACh receptors in denervated muscle in organ culture. These muscles contracted vigorously with full tetanic tension, in response to the electrical stimulation. Relative incorporation rate was measured as the amount of ^{3}H, ^{13}C, ^{15}N-containing receptors (identified by velocity sedimentation sucrose gradients) per mg muscle weight in stimulated muscles divided by that measured in non-stimulated muscles cultured for the same time. Incorporation time was 7-9 hr in experiments where stimulation was for 8-18 hr. All longer experiments had incorporation times of 11-12 hr. Each point on the graph represents one experiment, involving two or three stimulated muscles and two or three control, non-stimulated muscles.

extrajunctional receptors without replacement may rid extrajunctional regions of receptors in a matter of days.

This brief discussion of some dynamic aspects of ACh receptors has hit just a few highlights in an exciting field of study. The main point of the discussion, of course, is that ACh receptors reflect many events in the development and innervation of skeletal muscle and in the continuing interactions between muscle and nerve. Thus ACh receptors properly serve as one focal point for study of the cell and developmental biology of membrane proteins, and the number and distribution of ACh receptors serve as interesting parameters in studies of nerve-muscle interactions.

ACKNOWLEDGEMENTS

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DISCUSSION

HAMPRECHT

You mentioned that colchicine blocks the appearance of receptors on the cell surface. Do you also have any evidence whether other disrupting agents such as cytochalasine B would do that?

FAMBROUGH

Yes. First of all, the colchicine effect is interesting because the block is not complete and the dose-response curve is fairly sharp, with the half-maximal inhibition about 3 micromolar and maximal effect at 5. Maximal inhibition is only about 40-50% for the rate of ACh receptor appearance on the cell surface. If you follow the secretion of acetylcholinesterase, you get identical dose-response, identical level of inhibition. In fact, we have followed the ACh receptor appearance and AChesterase secretion simultaneously in the same population of cells and the two are absolutely linked, which I think is what you expect as a cell biologist. The secretory proteins and membrane proteins are very likely to be handled by the same apparatus through the cell. Indeed, there is a three-hour lag before esterase gets from site of synthesis to site of secretion. The colchicine effect says that microtubules are not required but might aid in moving these elements through the cell. The best control for the colchicine effect is a compound called lumicolchicine, which is an isomer of colchicine (made by UV irradiation) which does not bind to tubulin, and even at much higher concentrations does not inhibit these processes. Cytochalasins likewise are not very inhibitory, although they can change the shape of the cells drastically.

We have been very interested in nerve growth and whether new membrane appears at the tips of the processes when they are being extended. Sal Carbonetto did some experiments, using sympathetic ganglion cells in culture. The neurons sent out processes and he asked where do the new alphabungarotoxin binding sites appear, using the heavy-isotope labeling technique.

After the cells had synthesized some heavy-labeled receptor sites, he separated the ganglion from the processes and measured the number of newly appearing receptors in each location. Most of the sites appeared in the ganglion cell surfaces. New sites appeared approximately proportionally to

the number of sites already on the surface in each location. Cytochalasin caused retraction of the processes but did not affect the kinetics of production of these binding sites.

NELSON

Is there an effect on the distribution of the acetylcholine receptor within the surface membrane of treatment with either colchicine or cytochalasin B?

FAMBROUGH

It is my understanding that there is not. Ravdin and Axelrod looked at this very carefully, using more sophisticated techniques than I have used, and they claim that these two do not have effect. What I do not know for sure is whether every combination of disruptive agents has been used.

GREENE

You mentioned that the receptor turns over very slowly in the adult. What about the esterase? Do you have any evidence on that?

FAMBROUGH

There is published work on that and most of the work is of this form: the nerve to some muscle is cut and the cholinesterase activity of the junction is measured as a function of time. Okay, so there are a lot of ifs as you go through this. There are three big ifs, but when you get through with all these hedges, the fall of esterase is a drop of about 50% and takes about a week, and so it has been proposed that that is roughly the life-time of the synaptic-specific esterase. That has really also been difficult to look at. Dr. does probably the neatest experiment of all by a DFP poisoning muscles in real animals and looking at how long it takes the esterase to return—I believe it takes about three days in certain chicken muscles if the muscles are innervated, but if the muscles are not innervated there is no return of esterase at all. The same experiments were done by Sorenson in the rat, and they found that there was a partial return of esterase to the end-plate regions of the innervated muscles.

GREENE

Is there a coordination between regulation of the receptor and the esterase in the adult? From the data you have just described, it sounds as if there is not.

FAMBROUGH

It sounds as though there is not, right. But one thing I should have said that I did not say was that the number of esterase molecules these cells make is vastly greater than the number of receptors they make. So they would not have to utilize the production in the same way—you could get what looks like very different kinetics of the accumulation of the synapse, for instance, with a very tightly coupled original synthesis. Our cells make about 100 molecules of esterase for every one receptor that they make.

ECCLES

The biosynthesis of extrajunctional receptors can be suppressed by neuronally driven muscle activity, but, have you tried muscle activity not neuronally driven?

FAMBROUGH

In our experiments it was electrically driven muscle activity.

ECCLES

By stimulating the muscle directly, or the nerve?

FAMBROUGH

By directly stimulating muscle that had been denervated for 5 to 15 days.

ECCLES

But how can it be neuronally driven as you say?

FAMBROUGH

Well, it is also true the neuronally driven muscle activity will regulate extra-juncture receptors, and Dr. Cangiano will talk about that.

ECCLES

The point I wanted to make was: Is it sufficient to have the neuromuscular junction intact or must it be active?

FAMBROUGH

It is not sufficient just to have it intact, if you will allow that compounds such as botulinum toxin and tetrodotoxin leave the junction intact. Blockade of nerve impulses with these agents leaves the junctions morphologically intact but leads to a rise in extra-junctional receptors.

ECCLES

I think botulinum really kills the terminal and it has to recover by sprouting again.

FAMBROUGH

With tetrodotoxin blockade, would you grant that the junction is probably intact?

FILOGAMO

There is end plate acetylcholine esterase in molecular form. In this case the question is of a rearrangement of acetylcholine esterase from extra-synaptic region or myotube to end plate region. We have two acetylcholine esterase: one extra-synaptic, one synaptic. Rearrangement in these circumstances is very difficult.

FAMBROUGH

Dr. Filogamo has brought up the complication with the esterase which I cryptically alluded to by saying that biosynthesis is much more complicated than receptor. There are at least four molecular forms of the esterase, all of which can be found somewhere or other in some muscle or other. The highest molecular weight form appears preferentially in neuromuscular junctions but it is also found in some other places, like it is found in human intercostal muscle and it is found in genetically abnormal chicken in places where it should not be; but it has been called the synaptic specific form of esterase. Now that form you do not get in abundance in a pure muscle culture, and that form you can induce somewhat by playing with culture conditions. There has been some really nice work done by Vigny and Koenig especially on this matter. So I talked about total esterase and the reason I feel that you can talk somewhat about total esterase is that these forms seem to be interconvertible and all composed of the same basic sub-unit, probably

a part of the production of the synaptic-specific esterase is a late construction of it, using the building blocks that the cell would make whether or not there was a nerve around. There is a lot of nice cell biology to do on this point of forming.

CANGIANO

How can one explain the high number of acetylcholine receptors detectable for a long time after denervation at the neuromuscular junction, even when muscle activity is replaced with chronic direct stimulation? Is this due to persistence of the pre-existing receptors or to continuing incorporation of new receptors in spite of the absence of the nerve?

FAMBROUGH

To give you a short answer, I have to speculate a little bit. I think that receptors are preferentially deposited at end plate in the absence of innervation, to account for the high number continuing for such a long time. Nobody has done an experiment which really clarifies that point. It is a very difficult experiment to do.

BURNSTOCK

Does chronic exposure to either acetylcholine or acetylcholinesterase change your turnover pattern at all?

FAMBROUGH

Chronic exposure to carbachol does not. Chronic exposure to DFP, which blocks the esterase activity, does not. Chronic exposure to curare does not. However, chronic exposure to carbachol does change the production of receptors in a very slow manner. I think John Peacock's lab noticed this, and we have repeated the experiments. It is true that if you give long-term application of carbachol in vitro, the myotubes begin to make fewer and fewer receptor molecules.

HAMPRECHT

Is there any correlation between phosphorylation of the receptor and its appearance in the extra- and intra-junctional area?

FAMBROUGH

I forgot—to get back to iso-electric forms—what Dr. Hamprecht is referring to is the suggestion that the synaptic form and the extra-junctional form might represent a phosphorylated form and an unphosphorylated form. I think that idea should be dismissed on the basis of Diamond's group's work, in which they find phosphorylated and non-phosphorylated form in *Torpedo* where there are not any extra-junctional receptors. There is turnover of phosphate in receptor molecules. I do not think there is any good hypothesis as to the function of the phosphorylation and de-phosphorylation, but that does not seem to be the phenomenon which Zach Hall and Jeremy Brockes described. They found a difference in isoelectric point between the synaptic and extrasynaptic ACh receptors in rat diaphragm. I have heard that they feel phosphorylation is unrelated to the difference they observe.

NELSON

In the morphological work, can the internal, membrane-associated receptors be interpreted as part of the transverse tubular system or sarcoplasmic reticulum membranes or are they clearly independent vesicular structures?

FAMBROUGH

The hidden receptors could be in that system, but that system is very hard to identify in cultured muscle, so we cannot be sure.

SELECTIVE INNERVATION OF MAMMALIAN SYMPATHETIC GANGLION CELLS

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INTRODUCTION

Most neurobiologists would agree that how neurons establish and maintain selective synaptic connections is a major unsolved question in this field. There would be little agreement, however, on the most fruitful preparation by which to pursue this question, or even on the general strategy that should be employed. Most investigations have been carried out on the central nervous systems of invertebrates and lower vertebrates, and on the neuromuscular and peripheral autonomic nervous systems of birds and mammals. These preparations have in common the ability to regenerate synaptic connections, an ability which is attractive because it allows studies to be done in adult animals. Our purpose here is to summarize what is known about the nature of selective innervation in mammalian sympathetic ganglia.

SELECTIVE INNERVATION OF MAMMALIAN SYMPATHETIC GANGLIA

At the end of the last century, the English physiologist J. N. Langley examined the innervation of the superior cervical ganglion in several mammals by stimulating the preganglionic sympathetic outflow of different

spinal segments while observing the responses of sympathetic end-organs of the head and neck (Langley, 1900). He found that stimulation of each of the first seven thoracic spinal nerves produced a different pattern of peripheral effects, even though all the responses are mediated by the superior cervical ganglion (Langley, 1892). The predominant response to stimulation of the first thoracic nerve (T1), for example, was pupillary dilation, and, in the cat, retraction of the nictitating membrane. In contrast, stimulation of T4 caused vasoconstriction of the ear and pilo-erection, but had little effect on the eye. The transition of peripheral effects was a gradual one: T2 affected both eye and ear, but had stronger eye effects, while T3 stimulation affected tbe ear more than the eye. This pattern of *in vivo* responses to ventral root stimulation has subsequently been confirmed by several groups (Murray and Thompson, 1957; Guth and Bernstein, 1961; Njå and Purves, 1977 a and b; Lichtman, Purves and Yip, 1979 a).

In the last few years we have investigated the ganglionic connections responsible for the specific pattern of end-organ responses by recording intracellularly from individual superior cervical ganglion cells (Njå and Purves, 1977 a, b; Lichtman *et al.*, 1979 a). In a small mammal such as the guinea-pig, the superior cervical ganglion can be dissected in continuity with the cervical trunk and the thoracic portion of the peripheral sympathetic system (Fig. 1). The ventral roots of the spinal segments which contribute innervation to the ganglion can then be stimulated and the synaptic responses measured in individual ganglion cells. Each adult neuron receives synaptic contacts from about a dozen different preganglionic axons arising from an average of 4 of the 8 ventral roots that contribute innervation to the ganglion. A striking feature of the innervation is that the preganglionic axons contacting a ganglion cell are nearly always from contiguous spinal segments. For example, innervation to a particular cell might derive from the ventral roots of spinal cord segments T1, T2, T3, and T4, but rarely from T1 and T2, and T6 and T7, with no input from T3 or T4. Typically, one of the ventral roots supplying preganglionic axons to a neuron provides the dominant innervation to that cell (as measured by either the amplitude of the postsynaptic potential or the number of innervating axons), while adjacent ventral roots contribute a synaptic influence that diminishes as a function of distance from the dominant segment.

These rules of contiguity and segmental dominance are also evident in similar recordings made from the stellate and fifth thoracic ganglia (Lichtman *et al.*, 1979 b). As in the superior cervical ganglion, the subset

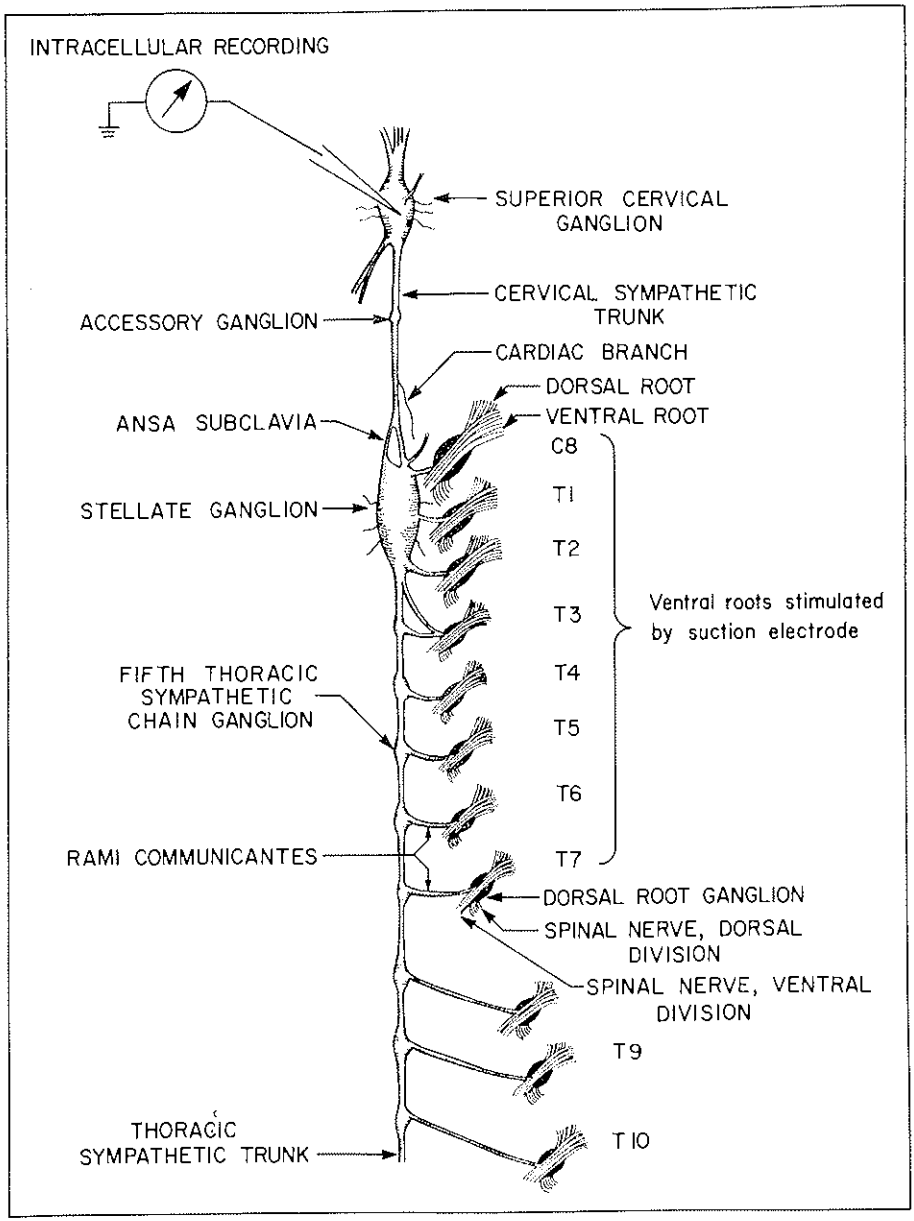

FIG. 1. Diagram of the part of the peripheral sympathetic system studied in our experiments. The cervical and thoracic chain ganglia were dissected in continuity with their communicating rami, spinal nerves and ventral roots. Intracellular recordings from ganglion cells were made while stimulating each contributing ventral root *in vitro* to show how individual neurons are innervated. The particular set of 8-9 ventral roots stimulated depended on which ganglion was under study; as shown, C8-T7 were stimulated when recording from the superior cervical ganglion.

of spinal segments innervating particular neurons is almost always contiguous. Moreover, neurons in these ganglia are generally dominated by a single segment, the synaptic influence of adjacent segments diminishing with distance from the dominant one.

Sympathetic chain ganglia differ, however, in the spinal levels from which their innervation arises and the relative strength of innervation from each level. In general, neurons in the superior cervical ganglion receive innervation from somewhat more rostral levels than neurons in the stellate, which in turn are innervated by more rostral segments than the fifth thoracic ganglion. Most superior cervical ganglion cells receive their strongest innervation from thoracic spinal segments T2 and T3, while the stellate is most strongly innervated by T3-T5, and the fifth thoracic ganglion by T5. These ganglia also differ in the relative homogeneity of their spinal inputs: while no single spinal segment supplies dominant innervation to the majority of the neurons in the superior cervical or stellate, most cells in the fifth thoracic ganglion are dominated by T5.

In summary, the rules of contiguity and segmental dominance are evident in all ganglia examined, and are presumably reflected in the average segmental innervation of each ganglion throughout the sympathetic chain.

The nature of segmentally selective synaptogenesis

Why do preganglionic axons arising from different spinal cord segments preferentially innervate particular ganglion cells? Is segmental selectivity based upon the modality of the pre- and postsynaptic neurons, their position, or some less obvious feature of the system?

1. *Relation of segmental innervation to the modality of postganglionic targets*

A possible basis of segmental selectivity is that the innervation of a ganglion cell reflects the modality of its postganglionic target. For instance, vasoconstriction might be mediated by ganglion cells innervated by certain spinal segments, piloerection by others, and so on; in this case the same set of spinal segments would innervate all those ganglion cells concerned with a particular function.

To investigate this possibility we exposed and stimulated the right ventral roots of T1-T5 and observed end-organ effects in the territory of the guinea-pig superior cervical ganglion (Lichtman *et al.*, 1979 a). In each animal, stimulation of successively more caudal ventral roots caused

piloerection of largely overlapping but progressively more dorsal and caudal areas. Stimulation of roots separated by several segments such as T1 and T5 usually gave responses with little overlap. Similarly, arterioles at different positions in the superior cervical territory were activated by stimulation of different ventral roots: vasoconstriction in the iris and sclera was elicited by stimulation of a more rostral subset of ventral roots than blanching of the pinna (see also Langley and Sherrington, 1891; Langley, 1894). The sympathetic responses of the eye also allowed us to observe the segmental innervation of postganglionic targets of several different modalities at the same locus. In every animal the same spinal roots caused vasoconstriction of the iris and sclera, dilatation of the pupil, and widening of the palpebral fissure (see also Langley, 1892; Langley and Anderson, 1892; Njå and Purves, 1977 a).

Thus end-organs of the same modality located at different positions tend to be innervated by a different subset of spinal segments. On the other hand, end-organs of different modalities located at the same position tend to receive the same segmental innervation.

2. *Direct evidence for the relation of segmental innervation to the position of postganglionic targets*

The accessibility of some postganglionic nerves of the superior cervical ganglion allowed us to confirm directly that sympathetic axons running in postganglionic branches to different destinations arise from neurons receiving systematically different segmental innervation.

The inferior branch of the superior cervical ganglion is comprised of two loosely associated nerves that join the primary ventral divisions of the second and third cervical nerves (C2 and C3; Fig. 2). We found that the compound action potentials in response to thoracic ventral root stimulation were different for those sympathetic fibers travelling ventrolaterally in these spinal nerves compared to those axons travelling dorsomedially (Lichtman *et al.*, 1979a). For both C2 and C3, the largest compound action potentials from ventrolateral-going postganglionic axons were elicited by stimulation of the ventral roots T1-T3, while the compound action potential arising from axons running dorsomedially was comparatively smaller in response to stimulation of T1 and T2, and greater in response to stimulation of more caudal ventral roots.

To determine the segmental innervation of individual ganglion cells projecting to different locations, we recorded intracellularly from 200 neurons antidromically driven by either the ventrolater- or dorsomedial-

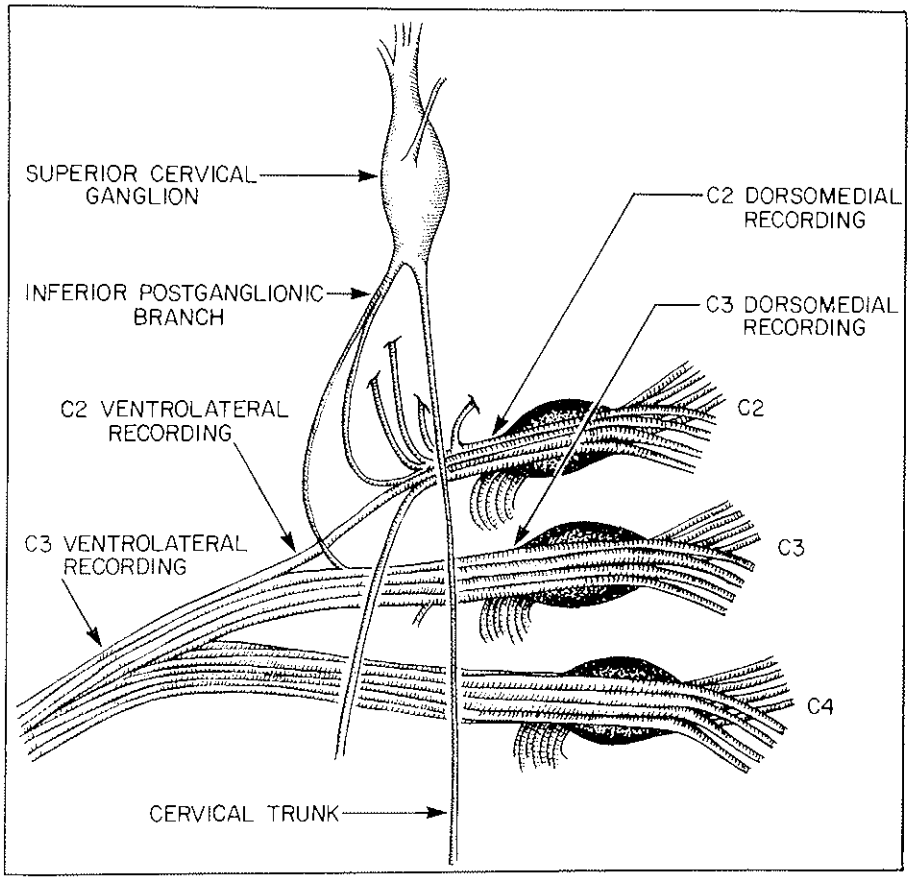

FIG. 2. Diagram of the superior cervical ganglion and the course of its inferior postganglionic branch. Suction electrodes can be applied to the second and third cervical nerves to record sympathetic compound action potentials elicited by stimulation of different thoracic ventral roots. Moreover, ganglion cells travelling to different destinations can be identified by stimulating at these points while recording antidromic action potentials in ganglion cell bodies.

going sympathetic fibers in C2 and C3. In these experiments four suction electrodes were applied to C2 and C3 as indicated in Fig. 2; thus each of the 200 cells impaled was identified as running in one or more of the branches stimulated by the suction electrodes. Only 7 of the 200 neurons travelled in both C2 and C3. However, many axons (60/200) branched to run both ventrolaterally and dorsomedially in a single spinal nerve. Those axons that ran only ventrolaterally in either C2 or C3 (25/200) arose from neurons whose segmental innervation was, on average,

from relatively rostral thoracic segments. In contrast, ganglion cells whose axons ran only dorsomedially in the spinal nerves (115/200) received more caudal segmental innervation. The distribution of the average innervation to neurons whose axons divided to run in both directions was intermediate.

These results show that the segmental innervation of superior cervical ganglion cells is correlated with the destination of their postganglionic axons. Thus a main feature of segmentally selective connections in this system appears to be the matching of positional values of the synaptic partners.

Experiments that further define the nature of selectivity in sympathetic ganglia

Selective synaptic connections imply that appropriate partners amongst the pre- and postsynaptic populations are distinguished in some way. Although segmental selectivity appears to match positional values in the peripheral autonomic system, the experiments described so far say little about the way in which this matching might occur. In this section we describe experiments which shed some light on the nature of selective synaptogenesis in this system.

1. *Position of preganglionic neurons as a factor in the selective innervation of ganglion cells*

It seems likely that the influence of spinal position on preganglionic neurons reflects a continuous rostro-caudal gradient of affinities for ganglion cells projecting to different positions. An alternative, however, would be that all the preganglionic neurons contacting a particular ganglion cell are equally attracted to it. In this case, the increasingly weak connections made by preganglionic axons arising from spinal levels progressively more distant from the dominant level would be due to smaller numbers of preganglionic neurons with identical affinities for that ganglion cell. To distinguish these alternatives we selected all the superior cervical ganglion cells innervated by *both* T1 and T4, and dominated by one or the other of these segments (150 of 912 neurons studied) (Lichtman *et al.*, 1979 a). If the strength of ganglion cell innervation were a function of spinal cord position, then the average synaptic contribution of each axon from the dominant spinal segment should be greater than the average contribution of each non-dominant axon. Conversely, if all the axons contacting a ganglion cell were drawn to it with equal affinity, then the average

synaptic contribution of each axon from dominant and non-dominant segments should be about the same. A rough measure of the average synaptic influence of axons to each cell from dominant and non-dominant levels was obtained by dividing the postsynaptic potential amplitude elicited in response to ventral root stimulation by the estimated number of innervating axons arising from that segment. The result of this comparison was that the average contribution of each axon from dominant segments was about twice as great as the average synaptic contribution made by each non-dominant axon. This result suggests that the location of preganglionic neurons along the rostro-caudal axis of the spinal cord is, in fact, correlated with the affinity of their axons for particular ganglion cells.

2. *Relation of segmental innervation to intraganglionic cell position*

By analogy with other systems in which the selective innervation of neurons is topographically arranged, preganglionic axons might distinguish ganglion cells by their intraganglionic position. To test this idea, neurons that had been impaled in the superior cervical ganglion were divided into groups according to position. We found that the average segmental innervation of neurons located at different points along the major axes of the ganglion was similar (Lichtman *et al.*, 1979 a). The intraganglionic position of the cells sending their axons to particular targets was also determined by injecting horse-radish peroxidase into the eye or ear of a series of guinea-pigs. Following either eye or ear injection, labeled neurons were distributed throughout a large part of the superior cervical ganglion, apparently at random (see also Hendry, 1977). Thus in the superior cervical ganglion there is no obvious relation between intraganglionic position and segmental innervation.

3. *Reinnervation of ganglion cells after interruption of preganglionic axons*

The nature of the signals promoting selective synaptogenesis can also be explored by examining the consequences of one or another experimental manipulation of sympathetic innervation in adult animals. For example, since the superior cervical ganglion is innervated in a stereotyped way, it is possible to ask whether specific connections are re-established when preganglionic fibers regenerate. Such experiments indicate whether mechanisms underlying specific innervation are peculiar to development, and allow one to explore whether the establishment of selective connections involves the correction of initial error. Reinnervation of the superior

cervical ganglion of mammals was first examined by Langley (1895, 1897). He found that end-organ responses normally elicited in the cat by stimulating the preganglionic axons of each segmental nerve were largely restored 2 months or more after sectioning the preganglionic trunk in the neck. Thus, as in normal animals, stimulation of the first thoracic spinal nerve (T1) caused strong eye responses, but had relatively little effect on the blood vessels of the ear; similarly, stimulation of T4 affected the ear but not the eye. The normal pattern of innervation of individual ganglion cells (showing contiguity and segmental dominance) is also readily apparent after reinnervation, and is presumably the basis for the restoration of normal functional effects (Njå and Purves, 1977 h, 1978 b).

A further question is whether synaptic connections made with superior cervical ganglion cells during the earliest stages of reinnervation are less accurate, requiring additional corrective mechanisms to attain the degree of specificity evident after regeneration is complete. To test this possibility, recordings were made from ganglion cells while stimulating the upper thoracic ventral roots within a few days of the establishment of synapses by regenerating axons (Njå and Purves, 1978 b). At this stage many neurons are only weakly reinnervated, or not innervated at all. Those cells contacted by axons arising from more than one ventral root, however, showed as great a tendency to be innervated by contiguous segments and to be dominated by one of them as at later stages of reinnervation. End-organ responses observed *in vivo* during early regeneration were also qualitatively normal: stimulation of the ventral root arising from T1 caused effects on the eye but had little effect on ear, while stimulation of T4 caused vasoconstriction of the ear but gave no appreciable eye effects.

In summary, appropriate reinnervation of ganglion cells shows that selective mechanisms persist in mature animals, and that during reinnervation at least, the normal segmental pattern of innervation is built up by a gradual accumulation of connections which are, from the outset, appropriate.

4. *Reinnervation of ganglion cells by foreign axons*

An advantage for the study of selective synaptogenesis in the peripheral autonomic system is the ability to reinnervate ganglion cells by foreign nerves surgically rerouted to ganglia deprived of their normal innervation. A number of experiments have shown that a wide variety of foreign cholinergic nerve fibers can form synapses with sympathetic ganglion

cells (see Purves and Lichtman, 1978, for references). These results indicate that the selectivity described in the previous sections is not absolute.

This generalization, not surprisingly, also applies to different classes of native axons which sprout after partial denervation of the superior cervical ganglion. Sprouting of ganglionic connections was first suggested by Langley (1894) as a result of observations made after cutting the lumbar sympathetic trunk in the cat. Langley's experiments were extended by Murray and Thompson (1957) who showed that following partial denervation of the cat superior cervical ganglion, stimulation of the remaining axons elicited abnormal end-organ responses. Although axons arising from spinal segments caudal to T3 do not normally mediate pupillary or nictitating membrane effects, these targets were activated by caudal roots several weeks after cutting the sympathetic output from T1-T3. The interpretation given these results was that the remaining intact axons sprouted to form functional connections with neurons that they did not ordinarily innervate.

A more direct comparison of the relative abilities of native and foreign fibers to form synapses during ganglion cell reinnervation is possible if both nerves are allowed to re-establish connections simultaneously. This has been done by sectioning the cervical sympathetic trunk just proximal to the superior cervical ganglion and positioning the cut end of the nearby vagus nerve so that its axons grow into the ganglion with the regenerating native fibers (Purves, 1976 b). Initially about 1/4 of the ganglion cells are reinnervated by the vagus nerve alone, about 1/4 by the native nerve alone, while 1/2 of the ganglion cells receive contacts from both native and foreign axons. About the same fraction of neurons is dually innervated a year or more after the initial operation, showing that native axons do not displace or suppress the foreign endings in these circumstances. Competitive suppression of foreign terminals does, however, occur in the neuromuscular system of some lower vertebrates, and may occur in competition between different classes of native terminals in mammalian sympathetic ganglia following partial denervation (Guth and Bernstein, 1961). The absence of obvious suppression during simultaneous reinnervation by native and foreign axons suggests that the final pattern of ganglionic connections reflects, in addition to selective mechanisms, an indiscriminate desire on the part of innervating axons to make synapses of some sort (see Purves, 1976 b).

5. *Reinnervation of ganglion cells after postganglionic axotomy*

A possible mechanism of selectivity is that ganglion cells receive retrograde instruction from peripheral targets which dictates the innervation they are to receive. This view is attractive in principle since the formation and maintenance of ganglionic synapses depends on a retrograde influence from targets that appears to be mediated in part by the protein nerve growth factor (Black, Hendry and Iversen, 1971 a and b, 1972; Purves, 1975, 1976 a; Njå and Purves, 1978 a). To explore the effect of postganglionic targets on selectivity we asked whether the selective mechanisms are altered when ganglion cells innervate abnormal targets (Purves and Thompson, 1979).

Our experiments were based on observations reported by Langley in 1897. In a single cat Langley (1897) found that the pattern of end-organ responses mediated by the superior cervical ganglion was abnormal after regeneration of the postganglionic nerves. We repeated this experiment in the guinea-pig and confirmed that the effect of stimulating each thoracic ventral root was no longer confined to the region normally activated by that segment. For instance, although T1 normally has much stronger effects on the end-organs of the eye than T4, after postganglionic regeneration these two segments affected the eye about equally. This result could be explained either by a non-specific reinnervation of peripheral end-organs, as suggested by Langley, or by changes in the segmental innervation of the ganglion cells themselves following postganglionic axotomy. While it is not possible to exclude the latter possibility by a direct demonstration that the segmental innervation of individual ganglion cells remains unaltered after recovery from axotomy, intracellular recording from such ganglion cells shows that the relative number of cells dominated by each segment is unaltered, and that the rules of segmental dominance and contiguity are still observed. These observations suggest that ganglion cell axons grow out more or less randomly to reinnervate the territory of the superior cervical ganglion. This view is supported by the further finding that regenerated sympathetic axons traveling to different destinations in the cervical nerves no longer arise from ganglion cells receiving appreciably different segmental innervation (Purves and Thompson, 1979). The apparently disordered outgrowth of postganglionic axons is similar to the regeneration of skeletal muscle motor axons: in this case as well, outgrowing axons appear to contact targets of opportunity (see, for example, Sperry, 1945).

Although other explanations are possible, the loss of end-organ specificity apparent in *in vivo* experiments after regeneration of postganglionic nerves implies that the innervation of abnormal postganglionic targets does not cause a corresponding correction in ganglion cell innervation. If respecification had occurred, the pattern of end-organ responses observed *in vivo* should be normal, even though the responses would not necessarily be mediated by the original ganglion cells.

6. *Reinnervation of transplanted ganglia*

The results described above suggest that ganglion cells are permanently labeled with respect to segmental innervation. If this is so, then ganglion cells transplanted to a novel location should be reinnervated in a way that reflects their original innervation. In order to test this idea, we transplanted T5 ganglia from donor guinea-pigs to the bed of the excised superior cervical ganglion in host animals; transplanted superior cervical ganglia served as controls (Thompson and Purves, unpublished). Normally, these two ganglia receive preganglionic innervation from an overlapping but appreciably different set of spinal segments (Lichtman *et al.*, 1979b): neurons in tbe fifth thoracic ganglion are most strongly innervated by axons arising from T5, while superior cervical neurons receive their strongest input from T2 and T3. Three months after transplantation, superior cervical ganglia were reinnervated much as after reinnervation of ganglia *in situ* (Njå and Purves, 1977 b, 1978 b). Transplanted T5 ganglia, however, were better reinnervated by axons arising from caudal segments and less well reinnervated hy axons from rostral segments, compared to transplanted cervical ganglia. The difference in segmental innervation between these ganglia after transplantation is not as great as normal, perhaps because relatively few axons from caudal segments are available in the regenerating cervical trunk (Njå and Purves, 1977 b, 1978 b). The fact that a difference exists, however, indicates that the same set of regenerating axons can distinguish between the two populations of transplanted neurons. The innervation of the transplanted T5 ganglia appears to be a compromise between connections formed on the basis of information intrinsic to the transplanted ganglia, and the availability of preganglionic axons. This is in keeping with the observation that partial denervation of the superior cervical ganglion leads to the formation of inappropriate connections through sprouting (Murray and Thompson, 1957), and that ganglion cells are readily reinnervated by entirely foreign axons as long as the axons are cholinergic (see Purves and Lichtman, 1978, for discussion and references).

These results support the idea that the normal innervation of ganglion cells reflects, in part, an intrinsic property which appears to be a relatively permanent feature of the neurons involved.

Selective Synaptogenesis During Development

The selective pattern of segmental connections we have described must necessarily arise in the course of normal development. While our experiments say nothing directly about developmental processes, they should also be considered in this context. The initial formation of synapses is likely to involve two general processes: the acquisition of labels by the synaptic partners, and appropriate synaptogenesis. While it is attractive to suppose that synaptogenesis follows, and is indeed dependent on, the acquisition of labels, it is possible that innervation precedes laheling, or is even the cause of it. In this latter view, appropriate connections might initially he established according to availability dictated by the sequential maturation of different groups among the pre- and postsynaptic populations. Whatever the sequence of events that leads to the acquisition of neuronal labels, it is likely that sucb labels play some role in the ontogeny of selective ganglionic synapses. Since different sympathetic ganglia may be comprised of neurons whose labels are different, it may also be possible to define corresponding differences in the chemical composition of these neural groups.

Conclusions

1. Selective segmental innervation is probably a general feature of mammalian sympathetic ganglia.

2. Such selectivity appears to match positional values of the pre- and postsynaptic neurons. For preganglionic cells the relevant value is the position along the rostrocaudal axis of the spinal cord; for ganglion cells the relevant value is the position of the postganglionic target.

3. Tbe identity of ganglion cells with respect to selective synaptogenesis within ganglia appears to be a relatively permanent neuronal feature that can promote appropriate reinnervation in maturity and is little changed by innervation of abnormal postganglionic targets.

4. Whether the identity of the pre- and postsynaptic neurons precedes segmentally selective synaptogenesis and is the basis for it in normal development is not yet known.

ACKNOWLEDGEMENT

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DISCUSSION

HAMBURGER

I just want to make a very general remark. From the functional viewpoint I think you would expect that the programming for selective innervation of muscles has to be very precise; otherwise, coordinated locomotion is in danger, whereas in the target organs of the nerves which Dr. Purves has dealt with, this kind of precision is not necessary. So I would expect less precise rules of innervation and much more mismatching, which would not do much harm.

ECCLES

Many years ago I used to work on the superior cervical ganglion. We thought that there were at least three types of ganglion cells with specific targets. Those we called S-1 were the fast ones with actions on the nictitating membrane and pupillary dilator muscles. They were quite distinctive fibers both presynaptically and postsynaptically. The S-2 group were the more slowly conducting pre-ganglionic fibres as well as of course post-ganglionic fibres; that is, there were two distinct synaptic systems. It makes me feel that there is very much target discrimination in the ganglionic connectivities because it has come out so selectively in the natural situation.

PURVES

I think what you described in 1935 or 1936 is consistent with our observations on the conduction velocity of fibers arising from different spinal segments (Njå and Purves, 1977 a). There is not much difference in the conduction of axons arising from the first three or four thoracic segments, but as one tests axons arising from the fourth through the seventh thoracic segments, the conduction velocity becomes much slower than one would expect on the basis of the increment in distance. The reason for this is not really clear. One possibility is that the more caudal the origin of a preganglionic fibre to the superior cervical ganglion, the more chance it has to innervate thoracic ganglia, and, perhaps as a result, the smaller the axon that is sent on to the cervical ganglion. The result of this would be that the end organs driven by T4, for example, would have a slower preganglionic input, on average, than end

organs driven by T1. That may not be a very definitive answer to your question, but it serves to bring the two sets of results into some sort of harmony.

ECCLES

Well of course I agree those were beautiful results you had and in a way that we never could have dreamt in those days, but we at least had the advantage of the intact reparation, so we could see where the targets were; and that was why I think we came to the conclusion that the T1 segment is practically the only route that supplied the eye with mayhe a little T2. The fast S1 fibres are quite selective in their postganglionic distribution. That is more or less what you would agree on.

PURVES

Yes. Perhaps I should emphasize that selectivity according to the segmental origin of preganglionic fibers is probably only one criterion in the formation of appropriate ganglionic connections; ganglion cells must also be appropriately connected according to their functional modality, for example. Like the visual system, one can imagine that several selective maps are superimposed on one another, expressing different selective criteria through the same set of synapses. It may be that axons with different conduction velocities innervating different targets reflect this aspect of appropriate connectivity. This is a possibility that we have not yet explored.

BERLUCCHI

In connection with Dr. Hamburger's question, I wonder what physiological significance can be attributed to the rather discrete and selective preganglionic innervation which you have described. It is normally thought that in physiological conditions the supraspinal commands to the preganglionic neurons of the sympathetic system are rather diffuse and generalized. I wonder if, on the contrary, it would be possible to activate the pupillodilator sympathetic pathway in a selective fashion.

PURVES

That is a rather difficult question to answer. The conventional view of the organization of the sympathetic system has often been in terms of all or none responsiveness. Over the past 25 years or so a lot of evidence has accumulated to suggest more discrete reflexes. But to answer your question specifically, I would have to say that we really do not know what the advantage is in having the end organs within the territory of the superior

cervical ganglion respond differentially. In principle, such refined responsiveness seems useful, and presumably it is. But if you ask me to give you a physiological example of this usefulness, I would have to confess that I don't know of any. I think, however, that this is only a reflection of my ignorance of how the system works.

BURNSTOCK

The story you have told us is very neat, but unfortunately the recent work of Reiko Yokota in my lab seems to be much more untidy in this area, and I just wonder whether you would care to comment on two particular circumstances that do not seem to fit so well. About 1% of the terminals on nerve cell bodies in the guinea-pig anterior pelvic ganglion are adrenergic. After the ganglionic input is severed, there is about a twenty-fold increase in adrenergic terminals. In other words, adrenergic nerves seem to be capable of occupying the sites previously occupied by cholinergic nerves. Another anomalous situation concerns the gut. Enteric neurons are normally innervated by adrenergic nerves. If a piece of ganglionated intestine is transplanted into the anterior chamber of the eye, adrenergic collaterals from nerves in the eye re-innervate the ganglion cells in exactly the manner seen *in vivo*. However, when a ganglionic intestine is transplanted, a dense, abnormal functional innervation of the muscle occurs, comparable to that seen in a ganglionic colon in Hirschprung's disease. Neither of these examples are tidy.

PURVES

They may not be tidy, but unlike you I do not find them surprising. One experiment which I am sure you are familiar with, is partial denervation of the cervical ganglion. The results of Murray and Thompson's work in 1957 show that if you remove some of the segmental inputs to the ganglion, then the remaining axons sprout and form inappropriate connections, in the sense of mediating abnormal functions. For example, if you cut the ventral roots of T1-T3, then T4, which normally has little effect on the eye, will come to drive the sympathetic end-organs of the eye. It seems to me that this results fit reasonably well with a point I tried to stress during my talk, namely that the selective rules we have studied are really rather weak directives; if you perturb the system and do not give the cells their normal range of choices, they will make secondary choices which may be inappropriate, in some sense. Frankly, what you describe seems to fit rather well with the results of foreign reinnervation and sprouting experiments, although the apparent inappropriateness of transmitter as well as of position and function is certainly a novel finding.

KOSTERLITZ

I would like to support Dr. Eccles' point that fibers from sympathetic ganglia are target oriented. When you record from the nerve innervating the cat nictitating membrane in the orbit, you will obtain a compound action potential due to, amongst others, and C fibers. In order to determine which of the fibers innervate the nictitating membrane, we extirpated the superior cervical ganglion; after about a fortnight, we recorded the compound action potential and also examined the nerve histologically. We found to our surprise that the deflection had disappeared and that the fibers which had degenerated were finely myelinated. This is rather unusual for postganglionic fibers. And with regard to Dr. Eccles' remarks, these are fibers which have to subserve a fairly fast response to the nictitating membrane.

PURVES

Yes. To explore the innervation of ganglia in terms of other selective criteria such as function is certainly one of our ambitions.

NELSON

You found that the mixed innervation you produce after denervation is stable for long periods of time. Didn't Guth and co-workers show that there was a reversion to the normal innervation pattern? Is there a reconciliation of these results?

PURVES

There may be a substantial difference in the two experiments. Innervating axons apparently *want* to make synapses, and will do so in many bizarre situations. In the case I discussed, one has competition between native axons and foreign fibers that have the choice between making highly inappropriate connections or no connections at all. This may be rather different from the situation in experiment of Guth and Bernstein where the competition is between axons which are essentially over-extended, presumably making more than their normal complement of synapses, and axons which are making no synaptic connections whatsoever. It may well be that undercomitted axons can suppress or replace the terminals of overcomitted ones. This might well occur in the absence of any selective force, although the operation of selective rules would presumably aid the correct axons during such competition. Viewed in this way, I don't think there is necessarily any conflict in the two sets of results.

INTERACTION BETWEEN INACTIVITY AND NERVE DEGENERATION IN THE ORIGIN OF DENERVATION-INDUCED CHANGES IN SKELETAL MUSCLE

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It is well known that hypersensitivity to acetylcholine (ACh), resistance of the action potential to tetrodotoxin (TTX), fibrillatory activity and other changes develop in the membrane of skeletal muscle fibres after denervation (for recent reviews see Gordon, Jones and Vrhová, 1976; Purves, 1976; Rosenthal, 1977). The question of what neural influences are lost from the denervated muscles has attracted considerable interest, partly because of the importance of the problem of neural signals in neurobiology. The important role of the loss of nerve impulses and consequent muscle inactivity, has now been firmly established. The two lines of evidence for this are: i) the development of ACh-hypersensitivity, TTX-resistance and fibrillation after chronic blockade of impulse conduction or transmission (Lømo and Rosenthal, 1972; Berg and Hall, 1975), and, ii) most importantly, the prevention or suppression of denervation-induced changes with direct and continuing electrical stimulation of denervated muscles (Lømo and Rosenthal, 1972; Drachman and Witzke, 1972; Purves and Sakmann, 1974; Lømo and Westgaard, 1975; Westgaard, 1975). Several lines of evidence indicate, however, that impulse-independent factors must contribute with inactivity to the origin of the changes induced in muscle by denervation. In experiments of partial denervation of the dually innervated fibres of the frog sartorius, ACh-hypersensitivity develops in the region of the denervated end-plate, in spite of the absence of inactivity in that region (Miledi, 1960). Further-

more it is long known that the shorter the nerve stump the earlier the time of onset of denervation changes (Luco and Eyzaguirre, 1955; Emmelin and Malm, 1965; Harris and Thesleff, 1972). Finally, complete muscle inactivity, although sufficient to produce TTX-resistance and ACh-hypersensitivity, is less effective than denervation (Cangiano, Lutzemberger and Zorub, 1975; Lavoie, Collier and Tenenhouse, 1976; Pestronk, Drachman and Griffin, 1976; Cangiano, Lutzemberger and Nicotra, 1977; Gilliatt, Westgaard and Williams, 1978). Examples of the difference between denervated and paralysed rat soleus and EDL muscles are shown in Fig. 1, tbrough measurements of resistance of action potentials to TTX along bundles of fibres between end-plate and myotendinous regions. The values are significantly higher in the denervated than in the paralysed muscles, at each level between end-plates and tendon. Also to be noted the presence of gradients of TTX-resistance along the fibres, the values being higher at the end-plate region. These gradients tend to level off

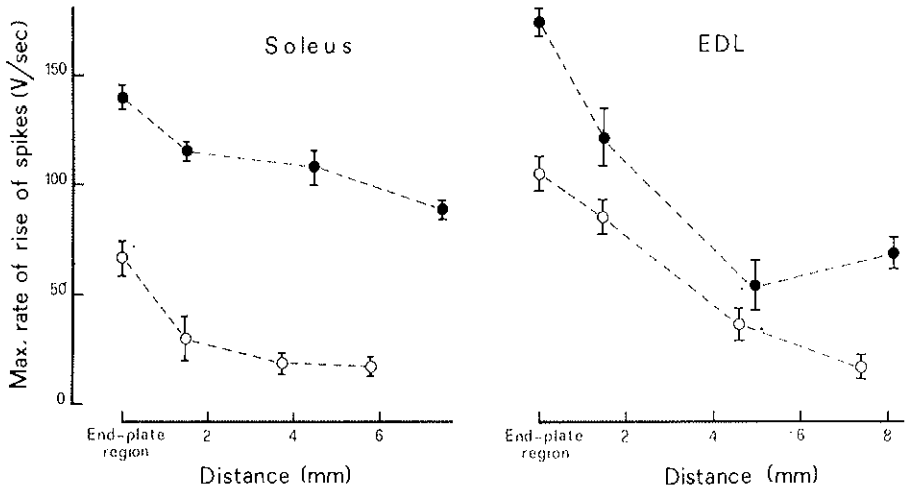

FIG. 1. Resistance of action potentials to tetrodotoxin (TTX) in fibres of denervated (filled circles) and impulse-blocked (open circles) rat hindlimb muscles. The soleus muscles were examined at 72 hours and the EDL muscles at 120 hours. In each muscle, measurements were made from 6-10 adjacent fibres, at different positions along the distance between end-plate region and myotendinous junction. Each point thus represents the mean value for 6-10 fibres and the bar the standard error of the mean. Nerve impulses were chronically blocked in the sciatic nerve with silicone cuffs impregnated with TTX (Lavoie, Collier and Tenenhouse, 1976). Regenerative responses were obtained in single fibres under TTX 10^{-6}M with a double microelectrode technique (Redfern and Thesleff, 1971; Cangiano and Lutzemberger, 1977), while the resting membrane potential was set at — 100 mV. Bath temperature was 27-28°C. The amount of resistance to TTX is expressed as the maximal rate of rise of the regenerative responses.

with time, the values at the myotendinous and intermediate regions growing up to the level of end-plates and surroundings, as was shown for the denervated rat diaphragm (Redfern and Thesleff, 1971). It is interesting to note in Fig. 1 that gradients similar to those seen in denervated muscles exist in impulse-blocked ones, even though in the latter the causal factor, that is lack of action potentials, is uniformly distributed along the fibres (Cangiano, Lømo and Lutzemberger, unpublished observations).

The impulse-independent factor indicated by the lines of evidence presented above, has generally been interpreted as a chemical factor, often termed "trophic", that would normally be released at neuromuscular synapses to keep the muscle fibres in their normal state (Miledi, 1960), in collaboration with muscle activity. According to this interpretation the denervated muscles undergo greater changes than the impulse-blocked muscles because they are lacking two restraining influences on the development of TTX-resistance and extrajunctional ACh receptors, namely nerve impulses and neurotrophic factors. However, experimental blockade of axonal transport (Albuquerque, Warnick, Tasse and Sansone, 1972; Hofmann and Thesleff, 1972; Cangiano, 1973; Cangiano and Fried, 1974) has not provided clearcut evidence in support of "neurotrophic factors" (Cangiano, 1973; Lømo, 1974; Cangiano and Fried, 1977). On the other hand, a radically different proposition can be made about the nature of the impulse-independent factor, if one takes into account the fact that soon after section the nerve starts to degenerate (Miledi and Slater, 1970) and products of axonal degeneration are released in the interstitial spaces between the muscle fibres. These products might then induce denervation-like changes in the membrane of the muscle fibres, through a direct or indirect action via inflammatory reactions (Jones and Vrbová, 1974; Jones and Viskocil, 1975). The situation might be similar to local injury (Katz and Miledi, 1964; Albuquerque and Thesleff, 1968) or the application of a foreign body or piece of nerve on the muscle surface (Jones and Vrbová, 1974; Jones and Viskocil 1975; Lømo and Westgaard, 1976), all procedures known to induce ACh-hypersensitivity. Support for products of nerve degeneration has also come from experiments of chronic electrical stimulation of denervated soleus muscles in which low levels of imposed activity cannot prevent the development of a *transient* ACh-hypersensitivity (Lømo and Westgaard, 1976).

According to the new hypothesis about the nature of the impulse-independent factor, products of nerve degeneration summate with inactivity in producing greater changes in the denervated muscles than in

the impulse-blocked ones. The experiments of comparison of denervation and impulse blockade deserve further comment in this connection. In rats in which the impulse-blocking cuff accidentally damaged some axons, as shown by the presence of some denervated muscle fibres, the paralysed fibres had a significantly higher value of TTX-resistance than that found in paralysed muscles not containing denervated fibres. In these instances the difference between paralysed and denervated muscles was therefore small or absent altogether. This was particularly suggestive of the involvement of products of axonal degeneration, affecting innervated fibres adjacent to denervated ones. The obvious test of this idea was the accomplishment of partial muscle denervation by sectioning only one radicular nerve. In partially denervated rat EDL muscles, innervated fibres developed at the end-plate region resistance to TTX, similarly to adjacent denervated fibres although to a lesser degree (Cangiano and Lutzemberger, 1977). Axonal degeneration appears a necessary step in the production of the signal for inducing these changes in innervated fibres. In fact, in experiments of partial nerve conduction block we found fibres showing no resistance to TTX adjacent to fibres with well developed resistance (attributable to inactivity), indicating that the presence of denervation-like changes in adjacent fibres does not itself constitute the signal (Cangiano and Lutzemberger, 1977).

The difference between denervated and impulse-blocked muscles repeatedly discussed above and shown in Fig. 1, is one effective way of demonstrating the participation, with inactivity, of impulse-independent factors in the origin of muscle denervation-induced changes. Strong support for the idea that such factors must be identified with products of nerve degeneration would come from the demonstration that the signal affecting the innervated fibres in the partially denervated muscles can entirely account for the difference between impulse-blocked and denervated muscles. This hypothesis can be tested by "bringing together" the impulse-blocked and the denervated fibres in the same muscle, so that products of nerve degeneration can act on innervated but *inactive* fibres rather than normally active ones, as was the case in our previous study (Cangiano and Lutzemberger, 1977). We produced this experimental situation by sectioning in rats the radicular nerve L_5 just outside the vertebral canal (obtaining about 50% denervation of the soleus muscles) and establishing at the same time a total conduction block along the sciatic nerve. Other rats served as controls with one side paralysed by conduction block of the sciatic nerve and the other denervated by sciatic section. The different types of soleus muscles thus obtained were

examined in vitro at 56-72 hours for junctional and extrajunctional resistance to TTX as well as extrajunctional sensitivity to ACh. This relatively early time was chosen because the control impulse-blocked muscles, that is not containing denervated fibres, have essentially not yet begun to develop the membrane changes, whereas the denervated muscles already show prominent changes. This is illustrated in Fig. 2 A, which displays the class distribution of junctional resistance to TTX in fibres of impulse-blocked muscles (grey columns) and denervated muscles (open columns), at 56-72 hours. Tbe results from the partially denervated muscles are illustrated in Fig. 2 B and C. Not surprisingly the denervated fibres

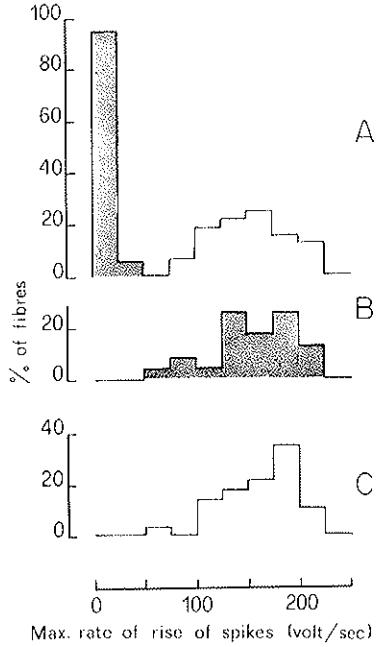

FIG. 2. Distribution of TTX-resistance measured at the end-plate region in populations of impulse-blocked and denervated soleus fibres. Each population is composed of 25-35 fibres from 4-5 muscles at 56-72 hours. (A) represents purely impulse-blocked (grey columns) and purely denervated (open columns) muscles. (B) and (C) represent partially denervated muscles in which impulse conduction was chronically blocked in the sciatic nerve by means of TTX-impregnated cuffs. Innervated (B) and denervated (C) fibres were distinguished on the basis of presence or absence of miniature end-plate potentials (min.e.p.p.s).

(2 C) show high values of TTX-resistance, comparable to those of totally denervated muscles (2 A, open columns). Striking on the contrary appears the behaviour of the innervated impulse-blocked fibres since they also exhibit high values of TTX-resistance (2 B). This is in profound contrast to the substantial absence of TTX-resistance in the impulse-blocked muscles not containing denervated fibres (2 A, grey columns). The fact must be emphasized that the values of TTX-resistance of the innervated, impulse-blocked fibres become as high as those of the denervated fibres

of both partially and totally denervated muscles (Fig. 2). We have obtained entirely similar results for the extrajunctional membrane changes, that is TTX-resistance and ACh-sensitivity. These measurements have been made by shifting the microelectrodes along the same identified fibres (i.e. innervated or denervated) at a distance of 1.5-2.0 mm from the end-plate region. Fig. 3 shows the results obtained for ACb-sensitivity for

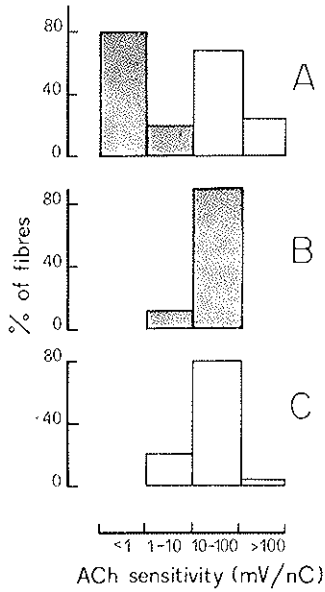

FIG. 3. Extrajunctional ACh-sensitivity (1.5-2 mm from the end-plate region) measured by microiontophoresis in the same fibres shown in Fig. 2. Units of ACh-sensitivity represent millivolts of depolarization per 10^{-9} coulomb of charge passed through the AChCl (3M) containing micropipette. (A), (B) and (C) represent the same types of fibre populations shown in Fig. 2.

the same fibres displayed in Fig. 2. Results similar to those presented above were obtained for the EDL muscle.

It appears therefore clear, from all the measurements performed, that impulse-blocked fibres become after partial denervation indistinguishable from denervated fibres in general, in spite of their intact innervation through which any "trophic factor" should still be supplied. This however might not be the case if partial denervation produced its effects not through a peripheral mechanism, that is axonal degeneration, but instead through a central mechanism. This would involve some retrograde signal affecting the cell bodies of the axotomized motoneurons and then inhibiting the production of "neurotrophic factors" for muscle in the cell bodies of adjacent intact motoneurons. The final change in the intact motoneurons would indeed have to involve a chemical signal instead of action potentials, since they are anyway blocked peripherally by the TTX cuff

placed around the sciatic nerve. Unlikely as it may appear this "central" hypothesis cannot be outright disregarded, and its validity has been tested by us by varying the distance from the muscle of the partially denervating nerve section. One population of animals had the usual section of the radicular nerve L_5 and represented the "distant" partial denervation. The "close" partial denervation was obtained by incompletely sectioning the soleus nerve in another group of animals. In both kinds of preparations a conduction block was established as usual in the surviving axons. In order to achieve the best resolution between the two populations, the soleus muscles were examined *in vitro* when the denervation-induced changes just began to develop. Fifty hours was found to be an appropriate time, quite earlier therefore than the time used previously (56-72 hours). Both innervated and denervated muscle fibres were examined for their junctional TTX-resistance. If the mechanism of action of partial denervation was central in nature, TTX-resistance should begin sooner in the innervated fibres of the "distantly" than in those of the "closely" denervated muscles. At a given time interval, therefore, a higher TTX-resistance should be found in the innervated fibres of the distantly denervated muscles. Such however was not the case since, as illustrated in Fig. 4, we found at 50 hours higher values of TTX-resistance in the innervated fibres of the closely denervated muscles. These results permit us to reject the hypothesis of a central mechanism, indicating instead that the action of partial denervation is peripheral, as required by the hypo-

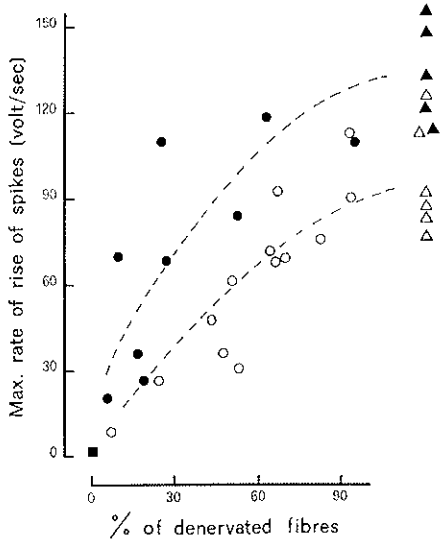

FIG. 4. Resistance to TTX in innervated but impulse-blocked fibres of closely (filled circles) and distantly (open circles) partially denervated soleus muscles at 50 hours. Each point represents the average TTX-resistance measured in 4-15 innervated fibres in one muscle, plotted as a function of the percentage of denervated fibres found in that muscle. Totally denervated muscles at 50 hours are also shown, with close (filled triangles) and distant (open triangles) nerve section. Filled square: impulse-blocked muscles not containing denervated fibres, at 50 hours. Curves fitted by eye.

thesis of products of nerve degeneration. A complete explanation of Fig. 4 requires, however, the introduction of a new and interesting variable, namely the percentage of denervated fibres present in any given partially denervated muscle. In fact, because of the early time period utilized (50 hours) the magnitude of the effect of partial denervation, in terms of TTX-resistance, appeared to be highly dependent on the amount of denervated fibres in each muscle. For this reason the amounts of TTX-resistance found in the innervated fibres of a given muscle after close (filled circles) and distant (open circles) partial denervation are plotted in Fig. 4 against the percentage of denervated fibres found in that muscle. It is clear from Fig. 4 that, i) with increasing percentages of denervated fibres the values of TTX-resistance in innervated fibres become larger, and ii) higher values of TTX-resistance are obtained with close than with distant partial denervation for any given percentage of denervated fibres. Fig. 4 also displays, for comparison, the values of junctional TTX-resistance found at 50 hours in purely paralysed soleus muscles (square symbol) and in totally denervated muscles with either close (filled triangles) or distant (open triangles) section. It must be stressed finally that after partial denervation the values of the denervated fibres, not shown in Fig. 4 for the sake of clarity, essentially matched in each muscle those of the innervated fibres. TTX-resistance in denervated fibres was therefore higher in those muscles where they happened to be more numerous and with close partial denervation. This essentially means that after partial denervation the membrane changes develop simultaneously and to the same extent in denervated and adjacent impulse-blocked innervated fibres. A factor acting from outside with equal intensity on the two kinds of fibres appears involved and products of nerve degeneration released in the interstitial spaces certainly represent a perfect candidate for such factor.

The participation of nerve degeneration products in the origin of muscle denervation changes can be subjected to a further test along the lines presented above, by inducing the release among inactive muscle fibres of products of degeneration of sensory rather than motor axons. For this purpose we surgically removed as much as possible of the dorsal root ganglia L_4 and L_5, without damaging the closely lying ventral roots. This was essential because, as shown in Fig. 4, even a minimal amount of motor denervation can give, already at 50 hours, measurable membrane changes in innervated fibres. A chronic conduction block was established as usual in the sciatic nerve. Three to 5 days later soleus muscles were examined in vitro for their state of innervation and for the presence of

junctional and extrajunctional membrane changes. A large number of fibres (about 50) was first penetrated at the end-plate region to observe the presence or absence of min. e.p.p.s, and only muscles where all the fibres were found innervated are included in this study. Control animals were also prepared with impulse-blocked soleus muscles but completely intact in their sensory and motor innervation. *In vitro* examination at 3-5 days showed that in these control muscles TTX-resistance had not yet appeared or was just beginning to develop. TTX-resistance, when present, was distributed iu gradients with extrajunctionally declining values as reported earlier in this paper. Junctional and extrajunctional values of TTX-resistance in single fibres of purely paralysed muscles for 3-5 days are shown in Fig. 5 B (see also Fig. 1). Removal of the dorsal root ganglia in impulse-blocked preparations, although ineffective in some

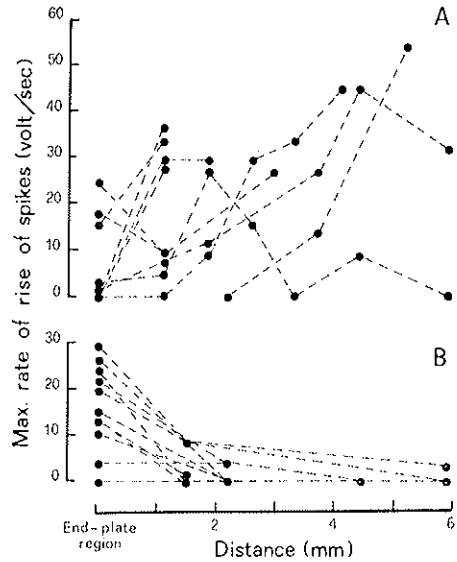

FIG. 5. TTX-resistance measured in single fibres at the neuromuscular junction and at different extrajunctional positions. (A): ten fibres examined in 3 different impulse-blocked muscles, 4 - 5 days after partial removal of dorsal root ganglia L_4 and L_5. None of the 50-60 fibres tested per muscle was found lacking its motor innervation. (B): ten fibres from 3 control impulse-blocked muscles, that is with intact ganglia, at 4 - 5 days.

cases, more often produced clear effects. Although the values of TTX-resistance never reached those seen at comparable times after motor denervation, they were nevertheless higher than in the control impulse-blocked muscles. Another interesting observation was the difference in distribution of TTX-resistance. Indeed in some of the affected fibres the values were higher extrajunctionally, declining to lower values towards the neuromuscular junction where the resistance could be absent altogether (Fig. 5 A). In other cases the difference with respect to paralysed, non

deafferented fibres was less dramatic, essentially consisting in the lack of the gradient normally present at this time, since the extrajunctional values were as high as the junctional ones. The effect of removal of dorsal root ganglia on soleus muscle fibres is certainly due to axonal degeneration because section of dorsal roots L_4 and L_5 central to the ganglia was totally ineffective.

In conclusion, partial denervation of rat muscle has clear effects on membrane properties of the innervated as well as denervated fibres. These effects are strikingly prominent when the innervated fibres are inactive because of impulse blockade in their axons. The results indicate that only release of products of axonal degeneration can adequately explain the effect of partial denervation. In fact this effect develops simultaneously and with equal intensity in the innervated but impulse-blocked and the denervated fibres, it involves a peripheral mechanism, and can be obtained even without sectioning the motor innervation. Thus, muscle membrane changes following denervation appear to be due to the potentiating interaction of inactivity and products of nerve degeneration. Such products, as revealed by the experiments of partial denervation, can entirely explain the difference between denervated and impulse-blocked muscles, making hypothetical neurotrophic factors unnecessary, at least as far as the extrajunctional membrane properties studied here are concerned.

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DISCUSSION

FAMBROUGH

The term "trophic factors" has been thrown around an awful lot, and I believe we must say the following. When we measure different end-points, we may be seeing the effects of different influences. When following acetylcholine sensitivity and TTX resistance, muscle activity seems to be very important. But when you look at muscle weight, you find that the electrical activity which restores the membrane properties does not restore the health of a denervated muscle to give it the size and strength of an innervated muscle. The term "trophic" was originally used in the context of maintaining the quality of the muscle, and it still could be a thing which is mediated by humoral factors from the nerve. Right?

CANGIANO

Yes, I agree. There are many other muscle properties, in addition to the properties of the extrajunctional membrane studied here, and it is certainly a possibility that humoral factors from the nerve contribute to their regulation. As regards specifically muscle weight, activity is a major controlling factor, but other factors could be the load applied to the muscle or else humoral factors from the nerve.

HAMPRECHT

Do you know whether muscle cells or muscle fibres which have become TTX insensitive by denervation still have TTX binding sites? That is, are the TTX receptors or whatever you call them still there, and functional, or they are not?

CANGIANO

I think it has been shown that there is a decrease in binding of tetrodotoxin after denervation.

HAMPRECHT

By binding with radioactive tetrodotoxin?

CANGIANO

Yes, by binding with radioactive tetrodotoxin.

FAMBROUGH

If you are referring to the work of Rogart and Ritchie, my recollection is that they did not see any drastic change in number of TTX sites with denervation.

There are a number of other experiments which you did not have time to go into that appear to support the trophic factor hypothesis. Are there any of these that cannot be explained by the hypothesis you put forward?

CANGIANO

Yes, there is at least one such experimental situation, which regards a different system, that is the development of the action potential mechanism in the slow muscle fibres of the frog. These are peculiar fibres which differ from the majority of frog muscle fibres because they normally lack the action potential mechanism. Interestingly, they acquire that mechanism after denervation. Miledi and Spitzer have shown that muscle inactivity produced by a blockade of neuromuscular transmission with botulinum toxin, is not followed by the development of the action potential mechanism. Denervation appears therefore necessary in this system to have the effect and it seems simplest to assume that this is due to the lack of a trophic regulatory substance. Less likely, although by no means impossible, would be that in this system products of nerve degeneration are much more important than muscle inactivity.

FILOGAMO

Is it known why, when the muscles are immobilized, the acetylcholinesterase activity is very high at the terminal end of the myotendinous junction?

CANGIANO

I am not aware of this effect at the myotendinous junction. On the other hand muscle inactivity decreases acetylcholinesterase activity at the neuromuscular junction and in the muscle as a whole. This has been found, for example, by Drachman and co-workers and recently also by us in collaboration with Dr. Lømo. We found that chronic blockade of nerve impulses for up to fifteen days markedly decreases the sensitivity to acetylcholinesterase inhibitors of synaptic potentials at the soleus neuromuscular junctions.

EVENTS-RELATED POTENTIALS IN THE CEREBRAL CORTEX

J. C. ECCLES

Ca' a la Gra', Contra (Locarno), Switzerland

It has long been known that, with recording from the scalp, afferent inputs to the human brain in the somatosensory, visual and auditory pathways evoke large potentials that initially have a fairly standard time course. The preferred recording arrangements are monopolar with the inactive lead from both earlobes or some other fairly neutral site. Our attention will be restricted to special investigations on the event-related potentials elicited by stimulation of fingers. The neural pathway goes via the contralateral thalamus to the crossed somatosensory cortex that is located in the post-central gyrus as indicated in Fig. 1.

Usually, with recording over the contralateral parietal and adjacent areas of the frontal lobe, a brief finger stimulus evokes a brief initial negativity and a later larger positivity, as illustrated in the N 20 and P 45 waves of Fig. 2 A, the latencies being 20 msec and 45 msec respectively. These waves are interpreted as being generated by the thalamocortical volley and the induced synaptic activity in the primary sensory areas (cf. Fig. 1). Following this initial potential complex there are often complex potential waves (cf. Fig. 2) of diverse polarity and latency that are assumed to he due to transmission from the initially excited focus into adjacent cortical areas and beyond. These later complex wave forms can now he lifted out of background noise by the technique of averaging. In that way potentials of a few microvolts can be recognized with assurance.

Desmedt and Robertson (1977 a, 1977 b) review a large number of investigations on the later complex waves that are generated by a brief somatosensory input. There is general agreement that under favourable conditions (Fig. 2 B and C) a negative wave with a peak latency of about 150 msec (N 150) precedes a positive wave of peak latency 300

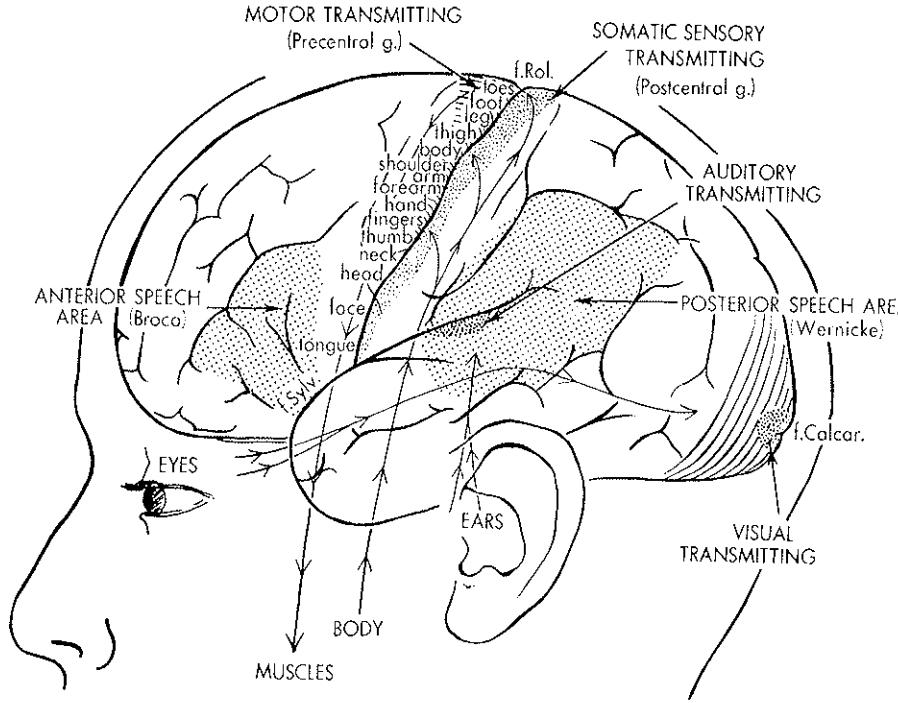

FIG. 1. The motor and sensory transmitting areas of the cerebral cortex. The approximate map of the motor transmitting areas is shown in the precentral gyrus, while the somatosensory receiving areas are in a similar map in the postcentral gyrus. Other primary sensory areas shown are the visual and auditory, but they are largely in areas screened from this lateral view.

to 400 msec (P 300 or P 400). By careful experimental design they have been able to establish the conditions under which these waves are generated, and thus to throw light on the cerebral events that lead to conscious recognition of a touch.

In the standard experimental procedure the subject has bipolar stimulating electrodes fixed to the second and third fingers of each hand. The stimuli to each finger are brief electric pulses of about 3 mA, that were maintained at 30 to 50% above threshold throughout the experiment. The timing of the pulses is controlled by a random generator that delivers the pulses at an average frequency of 150/min, but the actual intervals vary from 250 to 570 msec. Furthermore the pulse applications to any one of the four fingers are also randomized so that any one finger will receive stimuli at intervals ranging from 250 to 2280 msec, the mean

frequency being 37/min. In Figs 2 and 3 the EOG traces show that in averaged records there were no significant eye movement artifacts. Also in Fig. 2 D it is shown that in the absence of finger stimulation, the averaged records have no significant contribution from the electroencephalogram.

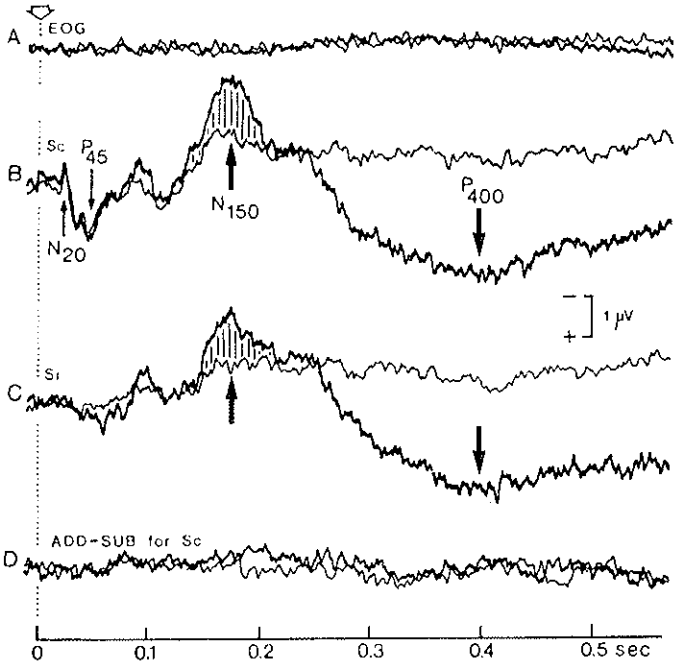

FIG. 2. Cognitive SEP (somatosensory evoked potentials) components N 150 and P 400 at right and left parietal derivations. The EOGs (electro-oculograms) document the absence of any remaining eye movement artifacts. The SEPs were elicited by electrical stimuli of 3.5 mA to the third finger of the right hand in runs when the subject attends that finger (thicker traces) or in other runs when he attends acoustic clicks delivered through earphones (thinner traces). B contralateral SEP recorded from the parietal scalp derivation, 3 cm behind C3. C, Ipsilateral SEP recorded from the symmetrical derivation, 3 cm behind C4. D Add-sub average checks for possible contribution of nonresponse EEG background to the averaged SEP response. The increase of N 150 to target stimuli (cross-hatched area) is somewhat larger contralaterally (B). The P 400 estimated from prestimulus baseline is virtually equal on both sides (Desmedt and Robertson, 1977b).

Fig. 3 illustrates the experimental procedures. The subject is instructed to "attend" to a particular finger. For example in Fig. 3 B it is the second finger of the left hand in the left figurine and of the right hand in the right figurine, the "attended" fingers being shown in black. During a run of many minutes the subject has to count the number of stimuli

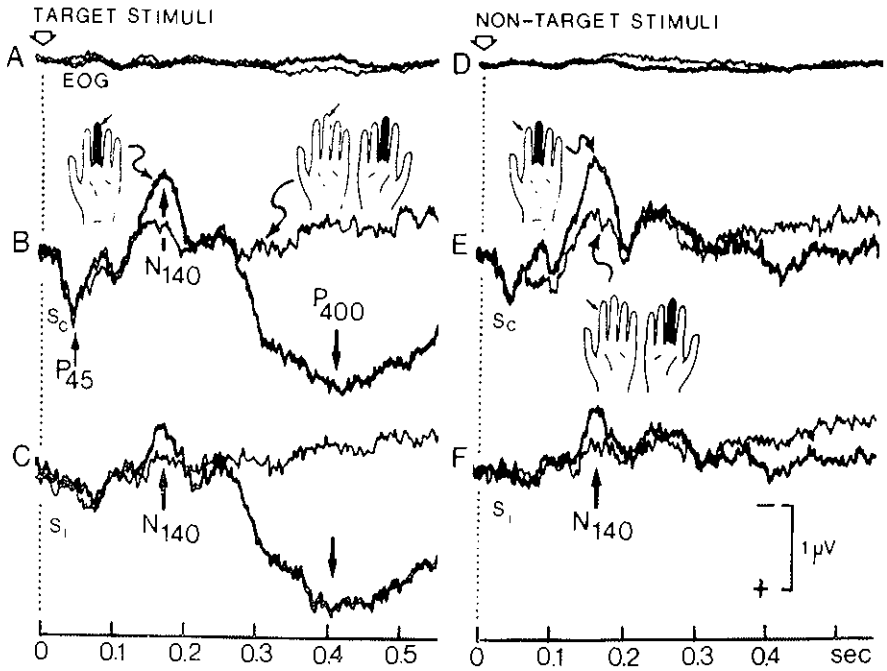

FIG. 3. Experiment with random sequence of stimuli to four fingers. A and D, vertical e.o.g. controls. B and E, s.e.p.s. recorded at the contralateral parietal focus (Sc) by stimuli of 3 mA to the third (B) or second (E) fingers of the left hand. C and F, corresponding potentials recorded simultaneously at the symmetrical ipsilateral (Si) parietal electrode. The subject counts the target stimuli to the third finger of either the left (thicker traces) or the right hand (thinner traces). In the hand figurines, the attended finger is represented in black and the small arrow points to the finger stimulus which evokes the s.e.p. considered. N = 560 (Desmedt and Robertson, 1977a).

felt by the "attended" finger. At the mean frequency of 37/min this task fully engages the concentration of the subject. In a satisfactory run the subject will score about 95% of the correct number, as recorded by the computer. In Fig. 2 B and C the background light trace was recorded when the subject was attending to acoustic clicks delivered by an earphone and not to the stimulated finger. Under these conditions there are in Fig. 2 A large N 150 and P 400 waves, and in Fig. 3 B large N 140 and P 400 waves. These waves stand out from the background records in lighter trace that are registered for the same finger stimulation but when the subject is "attending" to acoustic clicks in Fig. 2 B and C or to the same finger of the other hand, as indicated by the right figurine of

Fig. 3 B. The initial N 20 P 45 waves are superimposed, and the origin of the later N wave varies from 50 to 130 msec in different subjects with a mean value of 77 msec.

The same experimental procedures were applied in Figs 2 C and 3 C, but recording was now over the ipsilateral parietal cortex. In view of the crossed representation of the fingers, the small initial N, P response was to be expected, but it was surprising to find a large late potential complex when the "attended" finger was stimulated. The N 140 or N 150 waves were smaller than for recording over the contralateral hemisphere, but the P 400 waves were approximately equal. Fig. 3 E and F provide an interesting variant in that there was the same "attended" finger, but the stimulation was applied to the first finger either of the same or of the opposite hand. Comparison of the thick traces with the background thin traces shows that with recording from either side there was a small N 140 and later P wave. Thus the effect of attention to stimulation of a particular finger is largely localized to stimulation of that finger. In Fig. 2 B there is accurate superposition of the two traces of the N 20 and P 45 waves, and this also occurs for the P 45 waves in Fig. 3 B and E. This observation is of importance in indicating that attention has not influenced by "gating" the transmission in the pathway from the periphery to the sensory cortex.

Desmedt and Robertson (1977 a, 1977 b) have found that it was important to have the attention of the subject fully occupied in order to secure large late N and P waves. They chose a frequency at the high rate of 150/min for this purpose. The waves were much smaller at a frequency of 40/min for example. Also, if the stimuli were well above threshold, the attention of the subject was not fully occupied. The device of mental counting of the stimuli to the "attended" finger was adopted in order to eliminate complications by the muscle action potentials that would occur if any signalling system were used.

The subject was instructed at the outset to exclude from his mind all the non-target stimuli either to the adjacent finger or to the opposite hand. His attention was fully occupied in the counting of the target stimuli, so we can assume that the other stimuli were not consciously perceived. Thus these experiments lead to two conclusions: that by "attending" to a finger there is a remarkable increase in the spreading neuronal activity beyond the initial activity that its stimulation generates in the primary receiving cortex; and that these later evolving responses are associated with the conscious perception of the stimuli. The effect of attention to a finger is not absolutely restricted to that finger, but to

some extent influences the responses evoked by stimulation of the adjacent finger (Fig. 3 E, F).

It is of very great interest that the spreading neuronal activity induced by attention eventually involves both hemispheres to an equal extent. For example a comparison of the N 140 or 150 waves in Figs 2 B and 3 B with those from the other hemisphere in Figs 2 C and 3 C shows that the contralateral response is considerably larger. On the other hand the P 400 responses are of the same size. In Fig. 4 there is plotting

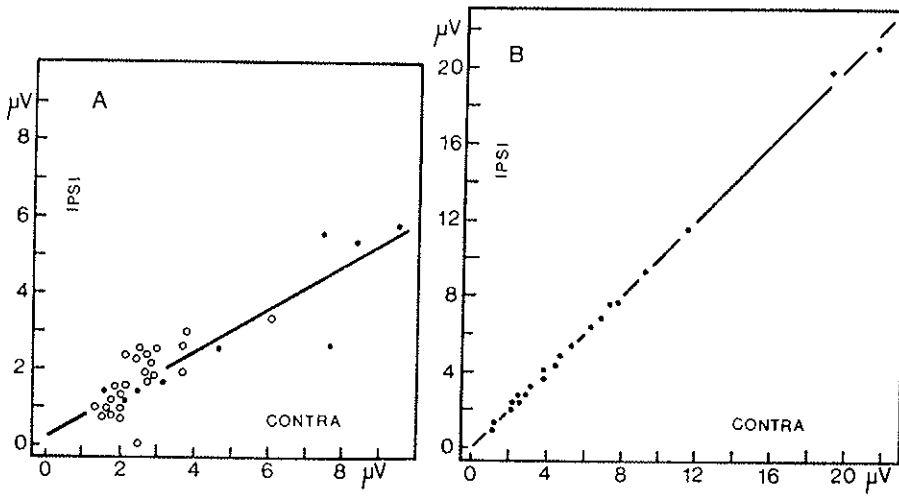

FIG. 4. Lateral distribution of SEP components in fast four-finger paradigms. Comparison of the peak voltages of the cognitive components N 140 (circles in A), P 100 (dots in A) and P 400 (dots in B) at symmetrical parietal derivations referred to the earlobe on the same side. The voltages at the contralateral (abscissa) and ipsilateral (ordinate) parietal locations are compared. The voltages in μV are estimated from the prestimulus baseline. The SEP components P 100 and N 140 are larger contralaterally to the attended hand, no matter whether the target stimuli are delivered to the right or left hand. The P 400 components are equal on both sides (Desmedt and Robertson, 1977b).

of a large number of IPSI and CONTRA responses. Included in the responses plotted in Fig. 4 A are a number of P 150 responses (dots). For some unknown reason the initial "attended" response is a positive wave with a maximum at about 100 msec instead of the N 140 wave. In Fig. 4 A the N 140 responses are larger on the contralateral side, whereas in Fig. 4 B the P 400 responses are very close to the 45° line. Thus the neuronal activity induced by attention is widely spread over both hemispheres. The recorded waves are largest over the parietal lobes

but are also of considerable size over the adjacent frontal lobes. Jones and Powell (1970) have shown by degeneration techniques the sequential relays for neuronal transmission from tbe primary somaesthetic area. The spread is initially largely to Brodmann areas 5 and 7 in the superior parietal lobe but later Brodmann areas 6 and 46 in the frontal lobe are involved. Moreover all these parietal and frontal areas are very affectively connected to the contralateral side through the corpus callosum.

The effect of attention in developing a late positive wave is illustrated in Fig. 5 for a quite different task (Desmedt and Robertson, 1977 b).

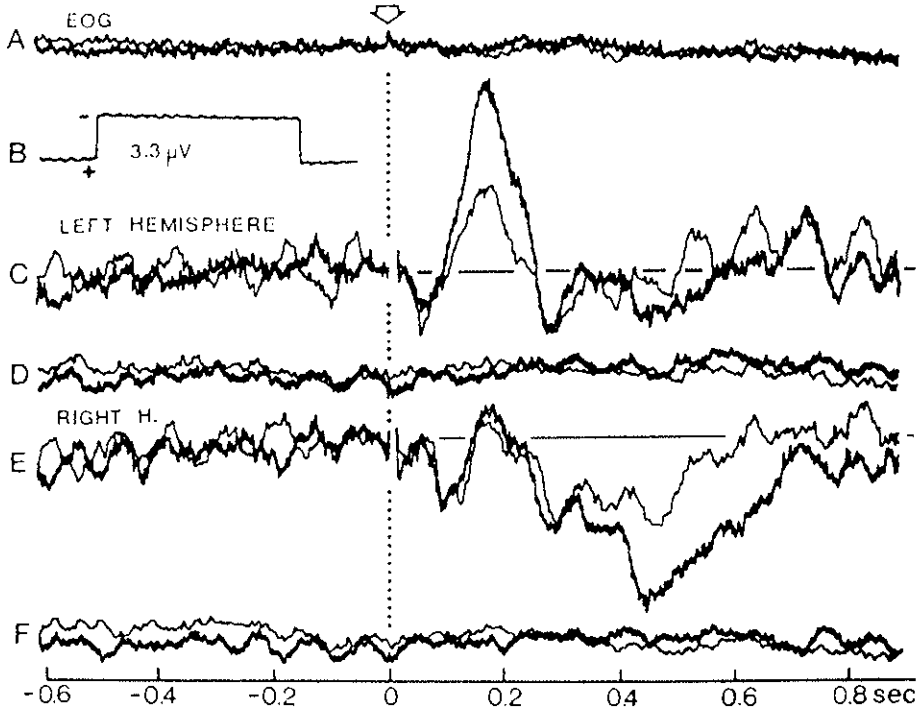

FIG. 5. Right parietal increase in ERPs during active touch in a right-handed subject. At the time indicated by the hollow arrow and the vertical dots, the right index finger is dropped by a mechanical device onto a circular ridge on a perspex rod. Palpation allows the subjects to identify the orientation with respect to his own body of a narrow groove in the ridge (thicker traces). The thinner traces correspond to other runs carried out in the same way but with the ridge replaced by a smooth perspex surface. A Vertical EOG documents the absence of any eye movement artifact in the samples. C-F Cerebral potentials simultaneously recorded from left (C, D) and right (E, F) parietal regions, 3 cm behind the central locations C3 and C4; the active electrodes are referred to the earlobe on the same side. C, E averages displaying the ERPs. D, F Add-sub averages of the same EEG samples which check that the nonresponse EEG background does not contribute to the waveforms of the ERPs (Desmedt and Robertson, 1977b).

The right index finger was dropped by an electro-mechanical device on to a circular ridge on a plexiglass rod. The test stimuli were provided by a gap in the ridge that could be oriented for successive tests in 4 directions (90° apart) relative to the body of the subject. In the control stimuli there was no gap in the ring. In Fig. 5 the thicker traces correspond to the tests with the gap in the ring. In Fig. 5 C the initial large N wave with a peak at about 180 msec is probably equivalent to the N 140 wave of Figs 2 and 3, and is larger on the contralateral side as would be expected, as also the larger size for the gap contacts. In the subsequent course the two traces in Fig. 5 C do not appreciably differ. On the contrary there is in Fig. 5 E after 300 msec a much larger positive wave for the gap stimuli despite the fact that the initial input of the stimulus was into the left hemisphere. A similar late long positivity was also observed over the right hemisphere when the active touch was applied to the left hand. This special electrical response of the right hemisphere is in accord with the attribution of spatial discrimination to the right hemisphere of right handed subjects. Furthermore this late response arises because the attention of the subject is directed to the task of identifying the gap and its orientation.

The influence of attention and intention on the neural machinery of the brain

Let us consider the sequence of events when attention is effective in recognition of a weak finger stimulus that otherwise goes unnoticed, as in the experiments of Figs 2 and 3. In both cases there is the same afferent input to the somatosensory cortex, as is shown by the identical N 20 and P 45 waves. Under the influence of attention which fully engages the subject there is an extensive development of neuronal activity which spreads widely on the ipsilateral side and then progressively invades the contralateral side so that by 400 msec there is symmetrical involvement of both sides (cf. Fig. 4). I would propose that only in these latter stages of wide-spread neuronal activity is there the signal to the mind that gives the conscious experience of the finger stimulus which is mentally counted. This long latency of a conscious recognition matches the experimental measurements of Libet (1973) using weak repetitive stimulation of the somaesthetic cortex of the conscious human subject.

It is now possible to give a more detailed explanation of the mechanism of spreading activation of the neocortex. It has been shown by radio-

tracer injections that the association cortex is composed of modules that are about 250 μm across and that function as units both in receiving input and in projecting ipsilaterally and contralaterally to other modules (Goldman and Nauta, 1977; Szentágothai, 1978). So we have to envisage that under the influence of attention there has been a development of a spatio-temporal pattern of modular activities. It is this pattern which eventually is detected by the conscious mind in the process of attention. This is shown diagrammatically in Fig. 6 for communication from the activated modules of the liaison brain to the conscious mind. But, furthermore, in Fig. 6, there is shown communication from the mind to the modules of the brain. Presumably this is the channel of activity for the influence of the conscious process of attention whereby it affects the development of neuronal activity from the initial input to the somatosensory cortex. The process of attention illustrates both of the channels of communication illustrated in Fig. 6. Thus it provides important em-

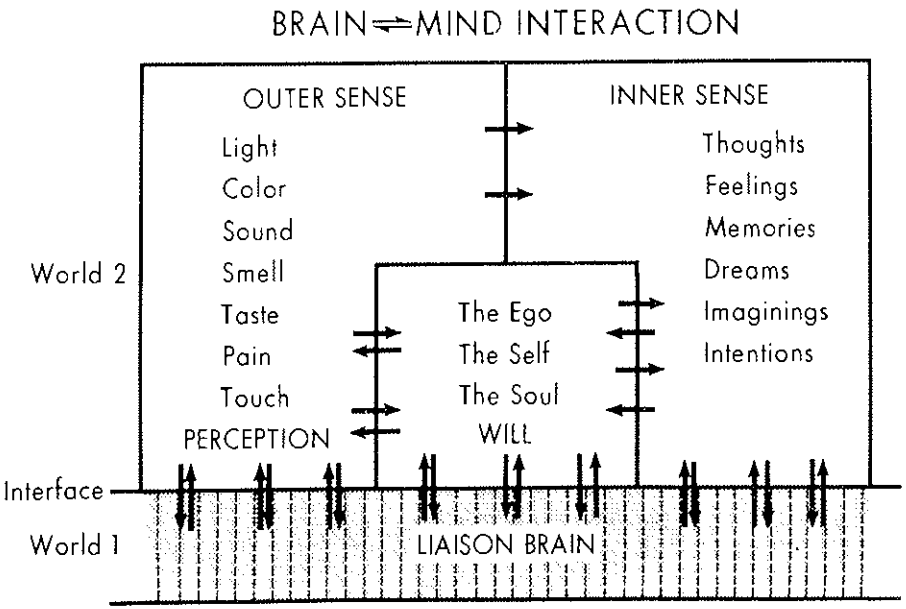

FIG. 6. Information flow diagram for brain-mind interaction. The three components of World 2: outer sense, inner sense and the ego or self are diagrammed with their communications shown by arrows. Also shown are the lines of communication across the interface between World 1 and World 2, that is from the liaison brain to and from these World 2 components. The liaison brain has the columnar arrangement indicated by the vertical broken lines. It must be imagined that the area of the liaison brain is enormous, with open modules numbering many hundreds of thousands, not just the two score here depicted.

pirical evidence for the dualist-interactionist hypothesis of the relationship of the mind to the brain (Popper and Eccles, 1977).

The potentials related to the recognition of the ring-gap and its orientation similarly involve the spread of neuronal activity from the initial input to the somatosensory cortex. But in this case the mental effort to recognize and judge the orientation of the gap results in the modular spread being diverted to the right bemisphere where there is a special aptitude for spatial judgment in right handed subjects. So we are introduced to the concept that the development of the modular pattern of neuronal activity in the neocortex is dependent not only on the attention of the subject, but also on the interest in extracting meaning from the sensory input. Desmedt and Robertson (1977 b) have opened up a rich field of investigation on the mind-brain problem.

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DISCUSSION

GIACOBINI

I have some difficulty in reconciling some concepts and therefore I would like to have some explanation about this effect of the mind upon the body, which are two separate entities. In this experiment, actually you are dealing with stimuli acting upon sensory pathways and then you are superimposing "something" which I do not quite understand and that you call attention to. Now this "attention" depends as a matter of fact on the state of the pathway itself. Well, if you anesthetize the finger or if you manipulate the cortex, you can also manipulate the "attention". If the individual is, for instance, under the influence of certain drugs which are known to either eliminate or to focus attention, such as amphetamines, LSD, cannabinols or local anesthetics, you can manipulate the stimuli as well as you can manipulate the cortex. I do not see there any separation.

ECCLES

The point is if you disturb the experiment in these ways, you would not get these late potentials and the subject does not count mentally the signals. It is all described by Desmedt. Attention has to be the strongly focused, undivided attention of the subject. It is very fatiguing and cannot be done for long periods. This is a mental act. He is not making any movements. He is just there attending concentratedly to that finger and counting the signals that come in there. That is the experiment. But if you put any distractions in, the experiment is ruined. He does not count and you do not get these late waves. Thus Desmedt has taken attention into what you could call a very carefully controlled situation.

GIACOBINI

Yes, but what you call attention is just immobilization of certain pathways which can be manipulated at libidum physiologically or pharmacologically.

ECCLES

No, I do not call that attention. The recorded waves are what happens as a result of attention, but attention is something other. It is a *mental*

effort to concentrate on some particular sensory input regardless of anything else. We are always doing that. We are always attending to one thing or another. You know that you can attend to writing something and there can be music or noise or conversation and it is all unrecognized. It is what happens with pain of a severe injury in a battlefield. The soldier is attending to the struggle to survive and does not notice the pain, which is being conveyed into the brain by the other pathways. I think attention is a very important mental phenomenon.

BERLUCCHI

I wonder if the same right hemisphere potentials would be observed when the subject has to count, rather than localize, the stimuli. Possibly an opposite asymmetry in favor of the left hemisphere would become manifest when the subject is concentrating on time, rather than on space.

ECCLES

The point is that he has not to think of the other fingers at all. He does not ignore them in an active sense. His task is simply to attend to the stimulations of the target finger.

BERLUCCHI

Has this investigator repeated the experiment by stimulating one finger at a time and by asking the subject to tell the number of times he has been stimulated - in this situation you can tell the discrimination between fingers. When you count in time, not in space, when you count successive stimuli, I think you probably depend on the left hemisphere, so you should find an asymmetry, opposite to the asymmetry you showed for the recognition of the position.

ECCLES

Probably it is in the latest stage of cerebral processing that you get this gap recognition.

BERLUCCHI

But in the latest stage of the other experiment, with the counting the number of shocks given to the given finger, as far as I understand there was no difference between the hemispheres.

ECCLES

It depends on the crossed inputs to the cortex. If the recording is from the ipsilateral hemisphere, then the first waves are small and the N 140 wave is less, but the P 400 wave is just as large as on the contralateral side.

BERLUCCHI

Of course this can be solved by a very simple experiment, by giving shock to a simple stimulus and by asking the subject to count and seeing whether the P-400 wave is as pronounced as in those situations. But is it so pronounced?

ECCLES

There is lessening of the late waves under all conditions that diminish the attention. For example, with halving the rate of stimulation. The waves are reduced because the attention of the subject is now wandering; he is still counting, but the counting is less accurate.

BERLUCCHI

But suppose you have efficient counting, the subject is 100% correct, but you give the stimuli to only one finger, what is the amplitude of the P-400 wave with respect to the condition where you ask the subject to discriminate between fingers?

ECCLES

It would be just the same I expect. He won't know that you are playing a trick on him, that you are stimulating only one finger and not the four, as long as he is attending to this stimulus.

BERLUCCHI

But you tell him before ...

ECCLES

You tell him you have to attend concentratedly.

BERLUCCHI

Oh no, if it is a counting task, you have to concentrate. ...

ECCLES

The attention would be right down for such a simple task. Similarly, if you put the stimulus strength high, attention is diminished and the slow waves are much reduced.

BERLUCCHI

But this is in a sense agreeing with what I am saying. You need a discrimination. When you need a discrimination between fingers, you may need greater attention; and for the other fingers maybe you do not need to ask the subject to discriminate at all, but you can simply see that you find differences between those waves if you give different probabilities to the stimulus.

ECCLES

The answers to your questions can be found in the original papers. I have not given the full story. There has not been time. You will find that all of these questions about levels of attention are fully considered. They had to get this 37-a-minute average for stimuli to one finger by empirical discovery. Under these conditions there was undivided attention. If the stimuli are too fast, the subject gets confused and cannot count and the late waves go off. Desmedt requires a 95% counting accuracy from his subject; otherwise he rejects the pictures.

WEISKRANTZ

I had a number of questions, one of which is similar to Dr. Berlucchi's. I wonder if in addition to having an eye movement control, he ever had electromyographic recordings to monitor "sub-vocal" speech? But that is a detail. There is one other detail that I think arises. The main potentials you say were recorded over the parietal cortex. The next relay from this region of cortex in Jones and Powell's study of cortical pathways is to the depths of the superior temporal sulcus.

ECCLES

Well, that is just one site. It also relays in areas 6 and 46, in the frontal lobe.

WEISKRANTZ

Yes, but from area 7 the next relay is to the fundus of the superior temporal sulcus which, because of the geometry, would be less likely to yield good potentials recorded at the scalp.

If one could record in it, one might find the asymmetrical responses of the kind that Dr. Berlucchi is talking about, especially as it is rather close to the speech area.

ECCLES

Yes. The superior temporal sulcus is at the third relay. The observed scalp potentials are the sum, the total performance of what Jones and Powell mapped by global degenerative techniques. We may hope that the radiotracer techniques will lift this into a new level of discrimination.

WEISKRANTZ

Can I turn to some questions that do not involve details so much but matters of principle? I think the work you reviewed is of fundamental importance and there is no question that information getting to the cortex is being distributed or processed differently under different conditions. Now, you said that there is no gate, but I do not see why in principle there cannot be a gate between S-1 and subsequent regions having the same status in the real nervous system as any gate would have lower down in the nervous system.

ECCLES

You have got to explain how these late waves are generated, and that involves, if you like, gating, or it involves amplification, under the influence of attention.

WEISKRANTZ

Except that it could still be at a neural level. All one would be doing is delaying the stage or the point at which the gate occurs, but it could in principle be the same kind of gate.

ECCLES

It could be independent of attention.

WEISKRANTZ

Not independent of attention but involving neural events associated with attention.

ECCLES

Well, that is my story.

WEISKRANTZ

I am just trying to use a different terminology.

ECCLES

Yes, I know. I know that I am frightening you, but I am only saying that there is the brain action. All of this late activity is in neural circuits, and attention comes in and modifies this activity. If you do not have attention, you do not get those late N and P waves. I think you would agree that attention is a mental event, a mental happening.

WEISKRANTZ

There are all sorts of mental events, and I accept that there are changes in the brain associated with them. The question is: what is the neural basis or the neural structures within which these events are said to be operating. I would prefer to put as much as possible in the real nervous system without appealing to extracerebral influences.

ECCLES

Of course. And that is what I am trying to do too.

WEISKRANTZ

May I ask one more question about the details of the modular arrangement? There are a number of different views as to what is involved in conscious discrimination. Yours may seem an attractive one from some points of view, but of course there are alternatives. I wonder how, with your kind of modular arrangement or modular pattern, you would deal with the sorts of phenomena that emerge from Sperry's work, where there are very high level discriminations which must involve complex modular arrangements of which however the subject is unaware. Similar findings emerge from the work that we have been involved in.

ECCLES

But I think even 99% of these patterns we may be unaware of. The brain has got an immense amount of operation and you only concentrate on one or another selectively.

WEISKRANTZ

But even with concentration, you cannot get to some places, and there seem to be very specific routes.

ECCLES

I think that we all have this problem. That is why we may eventually go to a psychiatrist, when there are so many of these areas in the brain that we cannot control or get at and that are getting at us instead!

WEISKRANTZ

I was really talking about the very specific interruptions of pathways that we know, as it were, disconnect awareness while the discrimination nevertheless can be carried out efficiently, and moreover of which a *normal* person *is* aware. The question is at what stage precisely in that modular pattern would you put such a cut that gets rid of the awareness?

ECCLES

I will leave you with this interesting problem, but I do suggest that these ideas of our discussion might be helpful.

TERENIUS

I think I will be coming back to a question similar to the one that Dr. Giacobini raised. I do not mind using the word mental event I think that it can be used in some circumstances, but I think if we do, we should look a little more into what the nature of a mental event could be. I would like to bring in some observations, some extra-mental observations, mainly done in animals but also in humans, with peptides and attention—perhaps acquisition or consolidation—maybe we should say conservation of certain modular patterns which has been carried out in the Rudolph Magnus in Holland (E. G. D. de Wied, in "Characteristics and Fractions of Opioids", eds. J. van Ree and L. Terenius, Elsevier, Amsterdam, pp. 113-122, 1978) where it is shown that ACTH and certain of its fragments which have no

hormonal activity, seem to act as conservating certain behavioral events. Similar studies have also been done in humans and also with the above-mentioned evoked potential techniques. And one may see changes due to peptide treatment. So the only thing I would like to add is that if we are talking about mental events—I think we may be allowed to do so—one should be very careful in bringing in what has been said to be the ghost in the machine in a scientific discussion.

ECCLES

I thank you for your comments. I understand the great inhibitions. If you have been conditioned for a whole lifetime against the views that I express. I hope that I can also talk to people who have not been so heavily conditioned. They may see that there is a future in this way of thinking which otherwise would be denied them.

HAMPRECHT

Is it possible to improve a person's ability to pay attention by rewarding successful attention?

ECCLES

You know it is a strange thing. You reward chimpanzees and monkeys, but you do not reward humans. The performance is sufficient for them. These volunteers knew the game. They were all carrying out these observational tasks. There is no use giving them a banana afterwards or something like that! It would be only a distraction. That is the great advantage of human experiments. The reward is not in some present. The reward for them is in knowing that they have carried out the task in an exemplary fashion.

HAMPRECHT

Do all subjects have exactly the same duration and kind of patterns?

ECCLES

You saw that the plotted curves were very similar. The late P wave reaches a maximum between 300 and 400 msec. There is surprisingly little difference. There is more variance in the late N wave. Some subjects give an early P wave instead of an N wave, P-100 instead of N-140, for reasons unknown. Further progress in identifying the generation of the late waves

depends on leading from the exposed cerebral cortex. That has not been done, but it will be done soon. You can get conditions in operations where this might be possible. About the variations, Desmedt finds out strangely enough that older subjects tend to do better. They make bigger potentials. He now has some very elderly in Brussels who can give quite nice pictures with a single trace, with no averaging. Thus subjects show large variations for unknown reasons.

ROBERTS

I have never heard a lecture by Sir John Eccles that did not arouse a tremendous amount of interest and discussion. I think that coffee breaks, lunches, and other opportunities will have to be taken advantage of to clarify the various issues brought up by him.

THE PROBLEM OF HEMISPHERIC SPECIALISATION IN ANIMALS

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There is a story of a visitor to Dublin who noticed that there were two clocks in the forecourt of the railway station, each of which showed a different time. "What's the point of that?" he asked a porter. "What's the point", replied the porter, "of having *two* clocks if they both tell the *same* time?". This paper is concerned with the same dilemma. Two brains in the same head are redundant if they are duplicates, but if they are not duplicates why have two?

There would seem to be no justification for the quantitatively impressive communication system between the two hemispheres (in the cat it has been estimated that there are 700,000 fibres per square millimetre cross-section of the corpus callosum; Myers, 1959) if there were nothing to communicate, i.e. if each of the two hemispheres did not have information or functions quite unique to it. Therefore, even though it is commonly assumed that hemispheric specialisation does not occur in animals other than man, *a priori* one is led to assume that the two hemispheres *are* specialised. Indeed, that the two hemispheres are specialised in animals cannot be doubted, if only because each has a stronger relationship to the contralateral side of the body (and to the contralateral visual field) than it does to the ipsilateral body. Were each hemisphere to receive equally strong projections from both sides of the body then no doubt there would be less need for a corpus callosum. Putting the matter in this way makes it clear that we must distinguish between specialisation and dominance, although the two terms are often used synonymously, together with another term, lateralization.

The problem of *dominance* arises from evidence of an asymmetry of specialisation, i.e. that the two hemispheres are differently engaged irrespective of their symmetrical specialisations with respect to contralateral sides of the body. The strongest evidence for this derives from injured brains, either through accident, disease or surgery, in that lesions to one hemisphere may be more destructive of a particular capacity than lesions to the other hemisphere, or if the commissures between the two hemispheres are cut, information directed to one hemisphere may be dealt with differently than if it is directed to the other hemisphere. Where there is asymmetrical specialisation, then the need for a good interhemispheric communication pathway is presumably much greater than that required merely for symmetrical specialisation, because in the latter case adjustments of peripheral parts of the body are normally taking place more or less continuously so that both hemispheres are engaged in the same task, e.g. both visual hemi-fields are stimulated by tbe same display as the eyes scan it.

In man the evidence for asymmetrical specialisation is overwhelming. Evidence for asymmetrical specialisation in infra-buman animals is said to be weak or non-existent. I accept the paucity of positive evidence, but I wish to re-examine the assumptions on which the assertion and the relevant studies are based. I want to ask first of all what advantages asymmetrical specialisation might impart to any creature, human or non-human. Let us accept as given tbat the evolution of the mammalian nervous system has already imposed a high degree of symmetrical specialisation, i.e. that each hemisphere is more directly in contact with the contralateral side of the body and the retinal half-field of each eye than with the ipsilateral side, without examining the evolutionary background to this arrangement. Tbe arrangement is, of course, neither necessary nor even efficient: even though the body may be roughly symmetrical in shape, not all internal organs are symmetrical or duplicated. But let us accept the approximate anatomical symmetry of both the peripheral body and the brain as an organ as our starting point.

If we do, then we encounter what might historically be one of the first *a priori* arguments for the evolutionary advantage to an animal of a degree of asymmetry superimposed on the fundamentally symmetrical plan. It was put by Ernst Mach in his *Analysis of Sensations* in 1886 (but he claims to have first expressed the idea in a lecture in 1861). The notion has recently been considerably elaborated by Corballis and Beale (1970) and also by Webster (1977 a). It is that a bilaterally symmetrical nervous system could not tell left from right, as defined relative to the axis around

which the body itself is symmetrical. "The idea that the distinction between right and left depends upon an asymmetry, and possibly in the last resort upon a chemical difference, is one which has been present to me from my earliest years" (p. 112). The diagram from Webster illustrates the point (Fig. 1). The fact that man *can* tell the difference between left and right can be taken as evidence, it follows, that asymmetry does exist somewhere in the nervous system. But animals can also tell

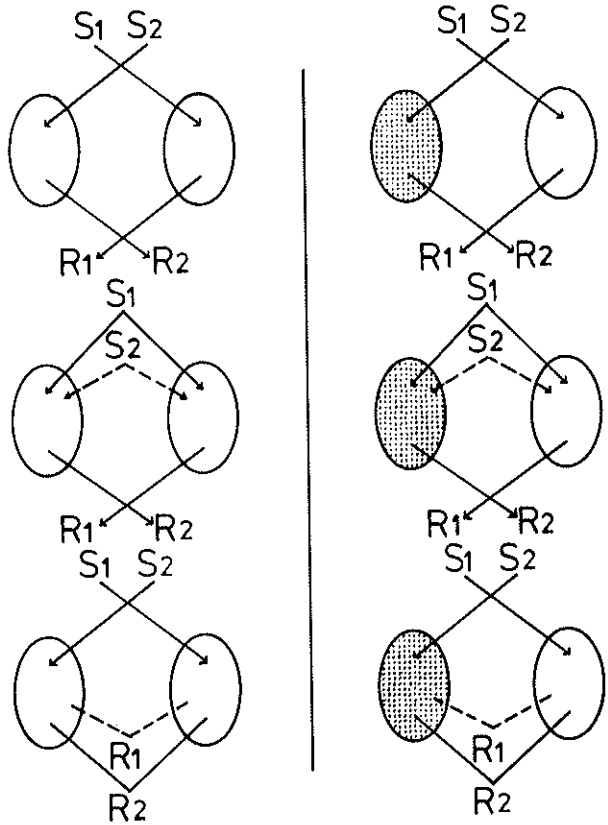

FIG. 1. Diagrams illustrating argument that bilateral asymmetry is required to discriminate left from right. *Left:* bilaterally symmetrical hemispheres. *Right:* asymmetrical hemispheres. *Upper diagrams:* left-right responses required to mirror-image stimuli. *Middle diagrams:* left-right responses required to non-spatial stimuli. *Lower diagrams:* responses other than left-right required to mirror-image stimuli. Argument is that middle and lower left combinations are impossible (from Webster, 1977a).

the difference between left and right—it is very common for animal workers to train rats or monkeys to make a left-going response given one centrally placed stimulus and a right-going response for another stimulus. It may be that the animal studies have not attempted deliberately to ensure that the external environment is entirely homogeneous or symmetrical with respect to right vs. left—this has not been the aim—but certainly many situations approach this, as in delayed alternation between

left and right positions carried out successfully by monkeys in darkness between responses (Ettlinger *et al.*, 1966).

Taken at face value, this argument would seem to provide strong presumptive evidence for asymmetry of the nervous system in the rat and monkey. But of course we cannot take it at face value. In fact, I regard it as a relatively weak argument. First, as Mach himself pointed out, the asymmetry can be muscular or neuro-muscular at the peripheral level. "The whole human body, especially the brain, is affected with a slight asymmetry ... which leads, for example, to the preference of one (generally the right) hand, in motor functions" (p. 111). This slight motor asymmetry can reinforce itself by association, for example, with writing and so "a confusion of those vertically symmetrical figures with which the art and habit of writing are concerned no longer ensues" (p. 111). "I learned by chance", says Mach, "from a retired Army-officer that on dark nights or in snow-storms, when external landmarks are absent, troops will move approximately in a circle of large radius so that they almost return to their point of departure, though all the time they are under the impression that they are marching straight forward. An analogous phenomenon is narrated in Tolstoi's story, *Master and Servant*. Probably the only way to understand these phenomena is to assume a slight motor asymmetry" (p. 112).

Even without an appeal to motor or other peripheral asymmetries, to the extent that right-left discriminations are important, man at least can easily create his own asymmetrical markers. The most extreme example is of those recruits in Czarist Russia who were "so stupid that they could not distinguish right from left" and were required to wear a bundle of hay tied to the left leg and a bundle of straw to the right, so that they could correctly obey the commands "turn left" and "turn right" (Fritsch, 1968, who cites Elze). In fact, even admitting that some people have right-left difficulties, I do not see that logically we are compelled to assume bodily asymmetry at any level in those persons who are free of this difficulty, *provided these persons are capable of simple learning*. After all, even the simplistic S-R theories of Hull were created very largely to account for the learning of rats who are rewarded for turning either right or left. There was much else wrong with these theories, hut not with their logical assumption that it should be possible differentially to reward left or right responses irrespective of how and where the separate mediating pathways happen to be situated in the brain. It is actually advantageous for the two hemispheres to be organised symmetrically with respect to body space, because the probability of occurrence of important

events requiring action is equal both to the right and to the left of us, and I see no need for a superimposed asymmetry to distinguish left from right given that simple learning is possible.

But if the right-left discrimination need not detain us, there is another aspect of spatial perception that is much more relevant to the present discussion. First, let us consider another *a priori* assumption about one of the advantages of asymmetrical specialisation. The evidence for asymmetrical specialisation in man derives mainly from the fact that in the large majority of persons damage to the left hemisphere is much more likely to interfere with linguistic function than is damage to the right hemisphere, at least beyond a certain young age. Language is, by its very nature, a complex abstract system that is not related to the left or the right half of the body. It seems reasonable to assume that it would be much less efficient for the nervous system to duplicate all the machinery in both hemispheres than it would be to have just one piece of machinery, but with good access to it through the corpus callosum. At any rate, that broadly speaking is how it appears to be organised in man.

Rats and monkeys have not, as yet, been credited with language (although the chimpanzee is a possible candidate). But certainly rats and monkeys and many other creatures, including birds, do have a capacity to learn about spatial relationships in their environment, independently of the parts of their own bodies. That is, they can appreciate extra-personal space. A simple example of this was provided years ago by Tolman and his colleagues (1947). A rat is trained to reach a goal by turning right in a simple T-maze. If now another entrance is provided to the maze directly opposite the original entrance, the rat at least under some conditions will still make a correct turning to the goal, even though this now requires a left turn. On the basis of this and many related experiments, Tolman postulated that rats can form "cognitive maps" of their environment. Mach, in fact, was well aware of the distinction between personal and extra-personal space. "For the animal organism, the relations between the parts of its own body are, first of all, of the highest importance. An alien object only acquires value by standing in relation to parts of the animal's own body. In the lowest organisms the sensations, including the space sensations, are sufficient to secure adaptation to primitive conditions of life. But as these conditions become more complicated they force on the development of the intellect. Then the mutual relations of those functional complexes of elements (sensations) which we call bodies, acquire an indirect interest. Geometry arises from the spatial comparison of bodies with one another" (p. 190).

It is clear that the appreciation of extra-personal space is also independent, as is language, of the right or left side of the body, as such, and hence of symmetrical hemispheric specialisation. It is also clear that many members of the vertebrate family enjoy such an appreciation, sometimes to an exquisite degree, as in many migrating and in some territorial creatures. It is also a capacity which has obvious and important evolutionary advantages. It seems reasonable to assume, therefore, that it would be inefficient for all the machinery necessary for extra-personal space to be duplicated in the nervous system in more than one locus or system. In man damage to the posterior right hemisphere is well known to produce visual-spatial difficulties, but more central to the present argument is the question of whether personal and extra-personal spatial difficulties can be dissociated hemispherically. McFie and Zangwill (1960) report that 5 out of 8 cases with left hemisphere damage had a right-left discrimination impairment, while none out of the 21 right hemisphere cases did. Conversely, 9 out of 18 right hemisphere cases had a difficulty with visual topography, whereas only one out of 8 left hemisphere cases did. Even on relatively simple tests of visual position discrimination (cf. Warrington and Rabin, 1970), right posterior damage produces an impairment relative to other loci in either hemisphere (Taylor and Warrington, 1973). In a much earlier study, Semmes *et al.* (1963) implicated both hemispheres in impairments on their tests of personal and extra-personal space, but the pattern of foci of lesions was quite different for deficits in the two kinds of spatial tasks in the two hemispheres. (But I would question whether their tests of extra-personal space were really free of personal-space judgments. When I tried the test myself I tended to do it directly in terms of left-right turns). Within the right hemisphere, the focus for loss of topographical perception and memory seems to lie in the posterior parietal lobe (De Renzi *et al.*, 1977). In a single case report, De Renzi *et al.* have also found a clear visual maze learning impairment in a patient with right posterior damage who showed a topographical memory loss in everyday life. Such visual maze learning impairments are well documented as being associated with right hemisphere lesions (Milner, 1965), and these have been found by De Renzi *et al.* (1977) in a group study to be associated with right posterior damage.

The topic of visual-spatial and topographical disorders in man is complex and there are clearly a number of dissociable deficits within the complex. Fortunately it is not necessary for me to argue that extra-personal space is a unitary capacity, or that there are not many neuropsychological tasks in which personal and extra-personal space require-

ments are inter-twined (e.g. Butters and Barton, 1970). All that is necessary for present purposes is to argue that extra-personal space and personal space, no matter how or in what way reducible, are dissociable, that they depend upon different neural substrates, and that critical links in these substrates tend to be located in different hemispheres, as the evidence of McFie and Zangwill indicates.

It would seem reasonable, therefore, to look for evidence of asymmetrical hemispheric specialisation of extra-personal space in animals, especially given the ethological importance of this capacity in many species. Here, as far as I can judge, we draw a complete blank. There are no studies explicitly designed to examine the possibility. Admittedly, Pohl (1973) reported an elegant dissociation in the monkey between tasks designed to depend on either personal or extra-personal space—he found that the former were impaired by frontal lesions and the latter by posterior parietal lesions. The posterior parietal effect was confirmed by Mendoza and Thomas (1975) but only under certain rather critical conditions that suggest an interpretation in terms of visual spatial attention rather than discrimination of relative spatial relations as such. Brody and Pribram (1978) also designed tasks explicitly to distinguish between extra-personal and personal spatial discriminations, and they too found that monkeys with parietal lesions were significantly impaired on the extra-personal task but not on the more difficult personal spatial task. But in all three of these studies the lesions were bilateral, and so while they help to confirm the dissociation between the two kinds of space, they are irrelevant to the question of asymmetrical specialisation.

One recent study on the rat, however, bears rather more directly on the point, although it started from a somewhat different premise. Denenberg and colleagues (1978) have accepted the claim that in man there is a greater involvement of the right hemisphere in emotional responses. They argued, further, that emotional dispositions in the rat are critically shaped and modified by stimulation in infancy. It is already known (Zimmerberg *et al.*, 1974) that the dopaminergic nigro-striatal pathways of the two sides of the brain of rats may contain different concentrations of dopamine, the animals having a tendency to turn in a direction contralateral to the striatum containing more dopamine. These effects are consistent within individual animals, but over-all there are no more right than left dopamine dominant rats in the population as a whole. Denenberg *et al.* suggested that critical experience in early infancy might induce a consistent hemispheric asymmetry and studied their animals in an open-field situation, which measures both emotional and exploratory

behaviour. Litters were either handled or not handled between birth and weaning, and then reared either in laboratory cages or in enriched environments for a further 4 weeks. Later they were subjected to right or left neocortical ablation (or sham operation or no surgery). When tested in the open-field situation, it was found that ablating either the right or left neocortex increased the activity of non-handled controls, but there was no evidence of lateralization. In the groups handled in infancy, however, the results were different: there *was* lateralization. Ablating the left brain did not significantly increase activity, but ablating the right brain produced extreme scores. The most dramatic finding was that the handled plus enriched environment animals had near-zero scores in the test: Denenberg *et al.* suggest that "the right brain is the repository for the interactive effects of handling and environmental enrichment".

In interpreting their results Denenberg *et al.* draw attention to a recent provocative speculation by Webster (1977 a) that asymmetry of hemispheric specialisation may have evolved in connexion with territoriality in animals. Locomotor activity in the open-field situation reflects emotional responsiveness and exploration, both of which are important for a territorial animal. Denenberg *et al.* consider that their findings "are in essential agreement with Webster's hypothesis concerning laterality and territoriality". My own position would be somewhat broader. Exploring the environment is of enormous value to an animal in building up a schema of its extra-personal space, whether or not it happens to be a territorial animal, although for a territorial animal it is admittedly virtually a *sine qua non*. I arrived at the position in terms of economy of brain systems in not duplicating important and stable functions that are independent of each half of the body. But the final position and predictions are similar, although discriminable in principle.

Webster found the origins of his speculation in the evidence for asymmetric neural control of bird song, because one of the functions of song in birds is to define and defend territory. Here the evidence is compelling and much more solid than anything else we know about asymmetry in non-human animals. Nottebohm (1977) has found in the adult chaffinch and canary that song is more severely degraded by section of the left than the right hypoglossal nerve, which innervates the organ of song production (the syrinx). The asymmetry is also found more centrally in the hemispheres themselves, both in terms of effects of lesions and in morphology. Canaries that have their left nerve cut during the first two post-hatching weeks develop normal song repertoires, which are then under the sole control of the right nerve. "It seems fair to conclude that either tracheo-

syringealis nerve and its corresponding syringeal half has the potential to assume a dominant role in song control, yet this role is normally bestowed on the left side" (1977, p. 31). It is, of course, tempting to draw parallels with control of speech in man, but regardless of the aptness of that parallel, there is strong evidence of clear hemispheric asymmetry at this phylogenetic level, together with considerable evidence as to the actual critical neural circuitry.

Language and extra-personal space are, as we said, abstract systems that are independent of body space. Discrimination and production of temporal sequences of stimuli, similarly, can be independent of leftness or rightness of the body, and of extra-personal space. Aside from the evidence of the Gardners (1971) and Premack (1976) on the chimpanzee, we know little about the possible symbolic productive capacities of non-human mammals, and we know nothing at all about the relevant neuro-psychology of chimpanzees like Washoe and Sarah. But some years ago, Dewson, Cowey and I speculated that the perception of auditory sequences might be a candidate for hemispheric asymmetry in the monkey. We do not know if the monkey can comprehend speech—perhaps it can—but perception of auditory sequences must be a prerequisite of the comprehension of speech, and so this capacity might be an evolutionary forerunner of asymmetric specialisation for linguistic processing. We taught monkeys, in effect, to play a two-key piano, except that one key (on the left) always produced a tone and the other (on the right) always produced white noise. The animal had to listen to a pair of sounds presented randomly (noise and tone in any of the 4 possible combinations) when it pushed the "observing key", and then to reproduce the sequence by pressing the keys in the correct order, for which it obtained a food reward.

After the animals learned the task we systematically varied the duration of each member of the sound-pair and also the interval between the members of the pair so that we could establish the temporal limits within which each monkey could perform. Then the animals were given *unilateral* lesions of auditory association cortex in the superior temporal gyrus, sparing primary auditory cortex (and, of course, some animals were given control lesions).

The result was both encouraging and discouraging. We found that even a unilateral lesion is sufficient to produce an enduring deficit in the task (Dewson *et al.*, 1970), reflected in the narrowing of the range of temporal parameters within which the animal could perform successfully. That in itself is rather remarkable, because no one else had demonstrated a durable impairment after a unilateral lesion of association cortex in the

monkey. There was no suggestion, however, that damage to the left hemisphere in the group as a whole (nor to the hemisphere contralateral rather than ipsilateral to the preferred hand) caused a greater difficulty than damage to the other hemisphere. Since that time, Cowey and I have been pursuing the question of the nature of the deficit itself, e.g. whether it might be an auditory short-term memory impairment (Cowey and Weiskrantz, 1976) akin to that found in man after posterior temporal lesions in the left hemisphere (Warrington and Shallice, 1969; Shallice and Warrington, 1970, 1977).

But Dewson followed a different tack upon his return to Stanford. He altered and simplified the task in a very significant way. In our original situation the animal had to press one key on the left and another on the right in the correct order. Therefore it is possible that the animal remembered each auditory sequence to be reproduced as a left-right pair of responses. Dewson has removed the spatial factor. The animal has to press one of two keys, but the spatial location of the key is irrelevant. Instead, for a particular sound it has to press (after a brief but controlled delay) a red key and for another sound a green key, but the actual location of red and green on the keys varies randomly from trial to trial. There is no simple way in which an animal can remember the sequence according to a left-right spatial code (although of course an animal may sometimes invent mediating responses). The remarkable result is that Dewson and colleagues now find that only lesions of the *left* hemisphere, and *not* of the right, interfere with performance on this task (Dewson, 1977). Dewson acknowledges that his findings are preliminary, although since his original report he has added further animals to the original group (of six) and the finding of asymmetry has held up (1978, and personal communication). The importance of the result is such that it obviously deserves further replication and elaboration. The effect seems dramatic, but one must remind oneself how exceedingly rare are studies deliberately designed to examine the possibility of auditory asymmetry or auditory memory in the monkey. Or, for that matter, of any other type of hemispheric asymmetry. The common generalisation that asymmetry is absent in the monkey can hardly be said to rest on a solid or extensive bed of evidence.

The "split-brain" approach has yielded such a rich harvest of evidence for asymmetry in man, as will no doubt be reviewed by others, that it would also seem to be a promising approach to the question in animals, although it is worth bearing in mind that the patients had a history of severe epilepsy prior to surgery and also that they were of an age when asymmetry was already consolidated. (On *a priori* grounds one might

well expect a different outcome in infantile commissurotomy or in cases of agenesis of the commissures: if the two hemispheres cannot communicate one might think there is little to be gained and something to lose by specialising). There have been some tantalising but inconclusive pieces of positive evidence with split-brain cats and monkeys (Gazzaniga, 1963; Hamilton *et al.,* 1974; Trevarthen, 1974; Hamilton and Lund, 1970; Robinson and Voneida, 1973; Webster, 1972). But undoubtedly the most exhaustive and searching study is a recent one by Hamilton (1977) which yielded entirely negative results. This was a study of retention after commissurotomy of visual habits taught pre-operatively, and also of new learning of visual habits by one or the other hemisphere after cutting of the commissures in the monkey. No evidence was found of asymmetry either in the way memories were laid down prior to commissurotomy or, more critically, in the speed with which each isolated hemisphere acquired the discriminations. The learning problems included discrimination of line orientation, direction of rotation of a spiral, monkeys' faces, perspective drawings, and two-dimensional patterns. Hamilton acknowledges his disappointment, which one can appreciate given that he himself had found preliminary positive evidence using the same preparation.

Hamilton comments that "since the null hypothesis can never be proven, the key question about these experiments centres on the adequacy of the tests used for hemispheric specialisation. Although by no means exhaustive or perfect, these tests, particularly those with faces, should have detected asymmetries of the magnitude found with human patients. For example, Levy, Trevarthen and Sperry (1972), using roughly comparable stimuli, have found clear hemispheric differences in perception of faces by human split-brain patients" (1977, p. 58). The evidence from studies of unilateral cerebral damage had already led to the conclusion that learning to recognise new faces is a right hemisphere function in man (Warrington and James, 1967; De Renzi and Spinnler, 1966; De Renzi *et al.,* 1968; Milner and Teuber, 1968). [In fact, the split-brain evidence of Levy *et al.* (1972) might be thought to be equivocal in the present connexion, as it appeared that both hemispheres of patients were capable of constructing faces out of the chimeric figures, sometimes even simultaneously, depending on the response mode.] Given the importance that Hamilton rightly gives to perception of faces as a test, one wonders whether the monkeys might not have perceived the pictures of faces as *faces,* i.e. whether the pictures were not treated as just some more examples of meaningless patterns to be discriminated. Some years

ago Pratt and I (unpublished) taught monkeys to discriminate a long series of pairs of real objects. After they had mastered each pair, they were shown good coloured photographs of the same pairs of objects. The animals performed at chance—they did not treat a photograph as representing the real object. In contrast, they showed immediate transfer to real objects of the same shape but transformed in size, in spatial orientation, and in texture. (But cf. Zimmerman and Hochberg, 1970). In fact, I am not certain that commissurotomized patients would have more difficulty in discriminating with one hemisphere than the other the patterns typically used in monkey experiments, with conventional monkey testing methodology. One must share Hamilton's disappointment and admit that "hemispheric specialisation in monkeys, if present, will not be easily detected" (p. 60). Or, at least with split-brain monkeys or with conventional discrimination training procedures. But the training procedure has turned out to be crucial in perpetuating other long-standing supposed discontinuities between monkey and man. For example, it was long thought that the monkey could not carry out cross-modal perceptual tasks, but with special training procedures this in fact appears to be a relatively easy task for the monkey (Cowey and Weiskrantz, 1975; Weiskrantz and Cowey, 1975; Elliott, 1977; Jarvis and Ettlinger, 1977; Bolster, 1978). Some other supposed neuropsychological discontinuities between monkey and man would seem also to depend upon differences in methodology (Weiskrantz, 1977). Hamilton himself, in fact, makes useful suggestions for alternative methods of testing for asymmetry in the monkey and so, especially given other positive leads, the matter cannot yet be considered to be closed.

Aside from unilateral lesion and split-brain studies, there are three other major sources of evidence regarding asymmetry in man, all of them based on normal people. The first is of electrophysiological or eye-movement correlates of different cognitive or behavioural tasks or states. There is hardly any animal evidence in this area, but it is worth noting the interesting finding of Stamm *et al.* (1977) who recorded the steady potential shift in the prefrontal lobes of monkeys performing the delayed response task. For monkeys trained to respond with one hand the magnitude of the steady potential shift was greater in the contralateral than ipsilateral prefrontal and precentral areas, but more interestingly when the animals were over-trained the prefrontal (but not the precentral) electrical changes continued to be found in the original hemisphere even when the other hand was used. As the electrical response is correlated mainly with the delay period in the task, Stamm *et al.* conclude that

"overtrained monkeys seem to utilise only one prefrontal area for mediation of the transient memory required by the task" (p. 400). (Another example of "acquired" or "induced" asymmetry is provided by Trevarthen, 1974, p. 193.) Webster (1977 b) has also reported changes in hemispheric electrical asymmetries in the cat during different phases of sleep.

The next source of evidence from man is from reaction time studies of responses to stimuli directed initially to one or the other hemisphere via the left or right field. There is a large and far from clear literature on this topic, although the evidence is often interpreted as supporting the view that linguistically symbolic stimuli are reacted to more quickly when they are in the right half-field (which projects to the left hemisphere) and non-verbal stimuli more quickly in the left visual field. Parallels also occur for the dichotic listening procedure. As Cohen has commented, the results are "not absolute, not constant, and not simple". "The main task now confronting researchers investigating lateral asymmetries", she adds in a more recent article (Cohen, in press) "is not to demonstrate their existence, but to account for their variability. The performance difference between left and right hemispheres is a highly unstable phenomenon. The magnitude and direction of the observed differences can shift from task to task, from individual subject to individual subject, and from trial to trial. Changes in experimental design, stimulus materials, instructions, level of practice, task difficulty and subject population are all liable to produce these shifts. Quite a number of published findings are difficult to reconcile with each other, and these probably represent only an iceberg tip. Many more failed replications, unpromising pilot studies, and uninterpretable results remain submerged in the researchers' file". The fragility of the visual effect is well illustrated, for example, in an interesting recent paper by Hardyck *et al.* (1978) who found that visual lateralization effects disappeared when new stimuli were presented on every trial. But, as I can find no experiments using this approach in normal animals, we are relieved of the burden of reviewing the field of complex results with human subjects here.

Finally, there is gross anatomy. The findings of Geschwind and Levitsky (1968) on human brains are well known: they demonstrated in a large series of normal adult brains that the planum temporale is significantly larger on the left in 65% of the specimen brains (left was equal to right in 24% of the brains). More recently Wada *et al.* (1975) have confirmed this in a larger series of human brains, and report that the planum temporale is larger on the left than the right in about 90% of both adult and infant brains. It is interesting that the left Sylvian

fissure was already reported to be longer than the right in adult brains as long ago as the 1890s by Cunningham and Eberstaller (see Rubens, 1977, for confirmation and review) but this received little attention or was dismissed. As the planum temporale lies within Wernicke's speech area, it is natural to link the asymmetry in anatomy to the functional asymmetry for speech, although the functional validation with evidence of known speech lateralisation remains to be carried out. It is of interest that Cunningham also found a more acute Sylvian angle (greater upward slope, as in man) on the right in the chimpanzee, orang-utan and baboon (cf. Rubens, 1977) and LeMay and Geschwind (1975) also showed that the termination of the right Sylvian fissure tended to be higher than the left in the gorilla, chimpanzee, and orang-utan. Yeni-Komshian and Benson (1976) reported a significantly longer left Sylvian fissure in the chimpanzee, and a similar but insignificant trend in the rhesus monkey. Given the recent indications of at least a measure of linguistic capacity in the chimpanzee, it is not surprising that the morphological asymmetries of the Sylvian fissure are not unique to man, but can also be seen in the higher apes.

Given the supposition that the appreciation of extra-personal space would confer a selection advantage for asymmetrical hemispheric organization, it would be of interest to seek hemispheric asymmetries outside the region of the Sylvian fissure or its homologue in non-human mammals, and particularly in the posterior parietal cortex (and perhaps also in the prefrontal zone to which the posterior parietal cortex projects). In 1975 Webster published a brief note about anatomical hemispheric asymmetries in the cat. Otsuka and Hassler (1962) had already classified the sulcal patterns in the cat into 4 types, and Webster added a fifth type that he saw rather frequently. Almost half of the cats' brains (out of a total population of 39) were asymmetrical, i.e. there was a different category of sulcal patterns in one hemisphere than the other. When I went through his table in detail, I was struck by the fact that there was no example of one particular class (Otsuka's Type 2) in any of the right hemispheres, and no example of another particular class (Type 5) in any of the left hemispheres. Webster now tells me (personal communication) that this dissociation holds up in a much expanded series of brains (total of 94). Forty-three of the brains were asymmetrical. Among the asymmetrical brains, there are 20 Type 2 hemispheres, and 17 of these are left hemispheres. Of the 13 Type 5 hemispheres, 10 are right hemispheres. In the diagram of these two types of brain (Fig. 2), it will be seen that in the "left" hemisphere type the lateral sulcus is discontinuous with the

posterolateral sulcus, whereas the "right" hemisphere type shows a continuous sulcus posteriorly and anteriorly. Needless to say, such a difference would be highly significant by X^2 test. It illustrates that there may be rich opportunities for studying asymmetries in non-primate animals, and that the probability that they exist can by no means be excluded.

In the monkey there have been a few studies that have focussed on the region of the temporal lobe found to be asymmetrical in man, with

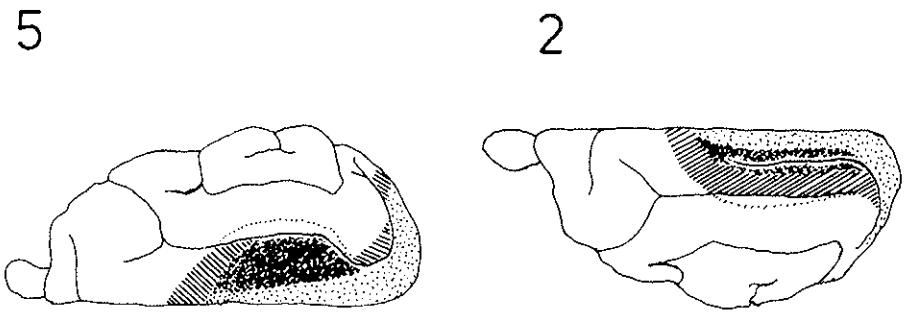

FIG. 2. Of those cat brains that are asymmetrical, the left hemispheres are predominantly Type 2 and the right hemispheres are predominantly Type 5 (Webster, 1975, and personal communication). Diagrams based on Otsuka and Hassler (1962) and Webster (1975). Light area corresponds to area 17, the dark grey area to area 18, and the hatched area to area 19.

negative results (LeMay and Geschwind, 1975; Wada *et al.*, 1975; Yeni-Komshian and Benson, 1976). Recently, an extensive study has been carried out by Falk (1978) of 107 endocasts from a variety of Old World monkeys, using a computer analysis of 150 points from which linear and areal dimensions could be derived for the main metrical features of the whole of the lateral cortical surface. "In summary", states Falk, "t-tests between measurements in right and left hemispheres of cercopithecoids reveal gross asymmetry in cortices of some genera of Old World monkeys. Most of the variables showing asymmetry are located in 1) prefrontal cortex and 2) parietal cortex along the caudal portions of *s* (Sylvian sulcus) and *ts* (superior temporal sulcus)" (p. 80). These two sulci are longer in the right hemisphere, and there is also a greater gap between the principal and the arcuate sulci on the right.

Other features, especially topological as contrasted with metrical ones, may come to light only if they are searched for specifically. In discussing the question of anatomical asymmetry with my colleagues Alan Cowey

and Dick Passingham, both commented spontaneously that they had sometimes noted differences between right and left hemispheres of monkeys during bilateral surgery, the former in the posterior cortex and the latter in prefrontal cortex. Cowey has noticed that the inferior occipital sulcus sometimes bifurcates as it courses rostrally. In a preliminary survey this appears to happen about twice as often in the right hemisphere as in the left, but it would be rash to say more than this at the moment. But it again reinforces the view that the null hypothesis can by no means be accepted without further exploration, especially in the cortical areas we know to be important for spatial analysis. It is interesting that both Webster and Falk find the discriminating asymmetrical features lie in the parietal and prefrontal areas (in Falk's case, in the frontal eye fields, which receive a projection from parietal lobe). Anatomical asymmetries, of course, need not necessarily entail functionally asymmetrical areas, but it would be reasonable to assume that they do, especially if the gross anatomical differences are also reflected in cytoarchitectonic differences. Fortunately the question can be put to direct experimental test.

Let me recapitulate. A distinction was drawn between personal and extra-personal space, which appear to be dissociable both in animal and human neuropsychological studies. While Mach and more recent authors have stressed the need for asymmetry to help with left-right (i.e. personal-space) discriminations, in fact it can be argued that hemispheric asymmetry would be of particular benefit where the animal possesses any abstract cognitive system or construction that is independent of body space as such, of which extra-personal space perception is a good example. Evidence from Denenberg and his colleagues on asymmetrical effects of unilateral lesions in rats handled in infancy would be consistent with this position. It is also consistent with Webster's hypothesis that asymmetry evolved in connexion with territoriality in animals, which in turn was derived from the clear evidence of asymmetrical neural organisation of bird song in canaries and chaffinches, as discovered by Nottebohm. Next we considered a capacity that must be necessary, if not sufficient, for appreciation of spoken language, namely the discrimination of auditory sequences. Dewson's evidence for a differential effect of left but not right auditory association cortex lesions on an auditory sequence task in monkeys, while still preliminary, is an exciting development which provides yet further evidence that asymmetry may not be unique to man. Next we considered some of the evidence with commissurotomised animals, and while there are some gleanings of positive evidence of asymmetry, the most searching

study that has been carried out with monkeys, by Hamilton, yielded negative results. Some consideration was given to the question of whether the tests, the methods, or the preparation might have been optimal. Finally, some anatomical asymmetries have emerged in studies of animal brains, such as those by Webster and by Falk whicb, interestingly, mainly appear to lie in, or at least overlap with, regions of visual association cortex and frontal eye field. Given the impossibility of proving the universal negative, given the positive if somewhat preliminary leads, and given the advantages to an animal in not duplicating neural processing but in evolving specialised systems, especially for abilities that are independent of body space and that would confer a considerable evolutionary advantage, there seems good reason for pursuing the hypothesis that asymmetrical organisation may be found in both birds and mammals. I must confess my bias, finally, in being reluctant to assume that there is a sharp discontinuity in hemispheric organisation between man and all other animals, and that the richness and profusion of commissural pathways and connexions have evolved merely to allow the two halves of the body to be joined, or to allow each balf of the body to enjoy the luxury of having its own store of memories.

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DISCUSSION

LEVI-MONTALCINI

The experiments that you mentioned by V. H. Denenberg and co-workers were published in the 22 September "Science" issue. These results seem to me of very great interest since they provide strong evidence in favor of the role of the right hemisphere in the recognition of spatial clues, a property which is of outstanding value mainly in territorial animals.

WEISKRANTZ

Well, as far as I know, there is no evidence that allows us to distinguish these two possibilities. His assumption, as you say quite correctly, is that it is from the emotional base that asymmetrical organization emerges. But the open field test is one in which the animal explores, and exploration reflects both emotional and spatial factors. I think until a lot of experiments are done with unilateral lesions using different spatial tasks we will not reach a final understanding. I think one great value of the paper by Denenberg and his colleagues is that it provides a technique for further analysis. But just as a suggestion, for example, consider the hippocampus which is, according to some people, part of the limbic lobe controlling emotional responses (but according to some people there is hardly any part of the brain that is not part of the limbic lobe!). Recently it has been discovered by Olton at Johns Hopkins University that bilateral lesions of hippocampus produce a devastating impairment in the rat's ability to learn what he calls a radial maze—this is a maze with lots of different arms arranged in a radial pattern in which all of the arms contain food when the animal is put into the maze. The animal is helped by remembering which arms he has visited because there is no point in going back to those places where he has already eaten the food. Normal rats have a really quite remarkable ability to perform this maze very efficiently. But animals with hippocampal lesions are very severely impaired. Now there is a big argument as to whether that is the only kind of capacity the hippocampus is controlling—a big argument that I do not want to get into—but it is natural to look at this situation straight away in terms of left versus right hippocampal lesions. You can measure the spatial memory component quite specifically—largely independently of emotional factors. So there are ways of dealing with the problem, but I do not know of any evidence that has yet dealt with it.

PURVES

Are other animals handed?

WEISKRANTZ

This is an area that there has been some work on. The general answer to this is, at least among the other primates, that the clear pattern of handedness found in man is not found in other primates; but for specific tasks animals may prefer one hand, for another task it may be the opposite hand. In any case—and I am sure Dr. Levy will go into this matter—I am not sure there is a tight connection between handedness and other aspects of hemispheric asymmetry. It is still the case that among left-handed people it is predominantly the left hemisphere, not the right hemispbere, which is responsihle for control of speech, for example; so that there is some degree of independence. Handedness may be independent of other kinds of asymmetry. Hamilton used the split-brain monkey preparation and compared left versus right hemisphere processing by directing the stimulus input to one or other eye and hence, in this preparation, to one or other hemisphere. He could not find any difference between left and right, or any difference between the hemisphere that was contralateral to the arm that the animal was allowed to used in that task as opposed to the ipsilateral hemisphere. Webster is the only person who has specifically analyzed his results in terms of the paw preference of split-brain animals. Now Webster did find positive evidence; he found that the hemisphere contralateral to the preferred paw of the split-brain cat had an advantage in learning a visual discrimination task, but as he would admit himself, the evidence is not conclusive because he always trained the animal first with that hemisphere rather than the ipsilateral one, and so there is a possibility of an artifact due to an order effect. It would be necessary to randomize the order to be certain of the conclusion.

BERLUCCHI

I am certainly willing to accept the possibility of a continuity between man and other animals in terms of hemispheric differences. However, it must be conceded that the evidence for right-left functional asymmetries in the brain of animals is scanty and controversial. Usually it does not come from a single case, as it is the rule in human neurology, but must be brought out by statistical analysis of population results. Studies reporting hemispheric differences of this kind in groups of animals are often difficult to replicate.

I also like to emphasize the lack of satisfactory relations between anatomical and functional asymmetries of the hemispheres of the human brain. The planum temporale appears to be larger on the left in about 65% of the human subjects studied by Geschwind; if one wants to relate this morphological asymmetry to the functional asymmetry concerning the lateralization of language, one is immediately faced by the fact that the language centers are on the left in more than 90% of the total population. As concerns animals, it is my knowledge that the functional asymmetry in the brain of songbirds does not have a morphological cerebral counterpart.

WEISKRANTZ

Yes, both in lesions and in anatomy. It is really excellent work. They are dealing with the question of anatomical asymmetry in relation to functional asymmetry. Let us leave aside the question of Broca's area and Wernicke's area: I think the traditional evidence from neuropsychology in fact may provide much better evidence than the split-brain evidence for that particular question. But of course one cannot argue directly from the size of a cortical area as such, particularly from anything as crude as just trying to measure the area. Even making the section in the right plane is difficult. All one has are correlations between the angle, the length of tbe Sylvian fissure, and the area of the supratemporal plane. The question remains to be studied intensively in two further ways—one of these has already started, and for all I know the other has as well. In the first place, one wants to study the anatomy in terms of its detailed cellular architecture, not merely the gross area, to examine the actual configuration of the cortex itself and its laminar configuration, i.e. the cytoarchitecture. But even considering gross area, the finding that the supratemporal plane is larger in the left than the right hemisphere in only 65% of the brains in Geschwind and Levitsky's sample must be set beside the fact that the right was larger than the left in only 11% of the brains, i.e. 24% were judged to be equal. That judgment may reflect caution and the limits of measurement. The figure that emerged from the study by Wada *et al.*, it should be noted, was 90% greater on left than right. But, in the second place, what one wants to know is the relationship between size or some other anatomical feature and the actual functional hemispheric location of speech in the same subject. Such a correlation is in principle possible to discover using modern radiological techniques such as the CT scan together with reversible unilateral treatments of the brain, such as intra-carotid injections of sodium amytal or application of unilateral electro-convulsive shock; these treatments are both in clinical usage in any event and reveal which hemisphere

is dominant for speech. The size of the Sylvian fissure and the supra-temporal plane just happens to have been an anatomical difference that many workers seized upon because it was an obvious one ever since Eberstaller in 1890 and Cunningham in 1892.

BERLUCCHI

I think that Geschwind and his colleagues have now indicated that the relative width of the planum temporale on each side is correlated with the ability which is specific of each hemisphere. Thus, if a subject has a larger planum temporale on the left, it simply means that he is better at verbal tasks than at visuospatial tasks. If the right planum temporale is larger than the left, this means better visuospatial abilities.

WEISKRANTZ

I do not think there is going to be much future in trying to settle this by a vote. The only way to settle it is to determine the correlation within subjects between anatomical configuration and the functional capacity measured in some independent way. Then we will know the answer. Let me just turn to the animal evidence that you cited. You are quite right: there have been a number of two-stage studies. Typically the effects of a one-stage lesion are more severe than those of a two-stage lesion—I mean the two-stage operation produces a smaller deficit even after the second operation than a bilateral lesion made in one-stage, and of course usually a much smaller effect after just the first lesion. So there may be a basement effect that restricts the analysis. But the one thing that has come out of the analysis that I tried to present is the following: the kinds of task that are sensitive or I think are likely to be sensitive, on the very slim evidence that we have so far, are not the traditional discrimination tasks. I am not sure that they would be very sensitive in man either, with the exception perhaps of faces. They are, for the left hemisphere, sequential tasks—and all we know about animals so far is an auditory sequential task—we do not know about a visual sequential task. And for the right hemisphere, I am advocating, again from the human evidence, the study of extra-personal space. For the two kinds of tasks the animal literature is practically virginal! I am not arguing merely that until they are studied more fully we cannot be sure of the issue of asymmetry in animals; it is that I think we have a good reason for assuming that these tasks should be sensitive. And in the few cases where they have been looked at, they have been surprisingly promising.

MILNER

First I would like to say that I share your hope that we shall find asymmetrical representation of spatial functions in the monkey brain. I would also like to follow up your comment that you were not sure that the monkeys in Hamilton's experiment treated the faces as faces. I think that in people with right temporal-lobe lesions one finds just as striking impairments in the recognition of nonsense patterns (Kimura, 1963) as in the recognition of human faces, although there may indeed be something special in the biological significance of faces. Similarly, in the tactile mode, it was astonishing to me to see the ineptness of the left cerebral hemisphere at tactile form perception, as indicated by the inability of the commissurotomized patients to match simple, wire shapes, when using the right hand, in contrast to their relatively efficient performance with the left hand (Milner and Taylor, 1972). I would therefore expect Hamilton's experiments to have uncovered any analogous hemispheric functional asymmetry in the monkey, if such exists.

WEISKRANTZ

Yes, I would have hoped so too, and concerning a negative result I would have to reply that either this is a case where there is a genuine difference between man and other animals, or there may be differences in the ways in which human and animal subjects are tested. And I do not mean simply in the superficial aspects of the situation, or with the stimulus material out of context. (In passing I do not agree with Professor Eccles that monkeys work only for a reward—monkeys like to solve problems and do it for the aesthetic pleasure as well as for the food reward. And as Lukas Teuber once remarked, the monkeys quite often store food in their pouch, and whenever they make a mistake they just reach into their pouch and give themselves their own reward. So in fact they are rewarding themselves for making an error. Anyway that is an aside, not really very important.) Obviously it may be relevant that we do not normally give human subjects a piece of banana for a correct answer. But I think there are two aspects that are important. First, the usual mode of exchange with a human subject is verbal, even though the task itself may not be verbal. And as you know, I think, and this could be another whole lecture, in certain areas of neuropsychology, simply putting a question in a verbal form gives an entirely different result with human subjects than if they are trained in the way in which you would train an animal, i.e. to make forced-choice discriminations. And I think that could be crucial; we know people who, with ordinary clinical testing, are said to be blind in half of their visual fields. But if they are required to discriminate—*not* to tell us what

they "see" or to tell us whether they "see" this or that but to make a forced-choice even in the "blind" field—their discriminatory capacity can in fact be quite remarkable in some cases. I would argue the same for some types of amnesia—there can be genuine and undisputed learning and memory without acknowledged awareness of this by the amnesic subject. So that is one aspect, the verbal-nonverbal difference in procedure, and the type of question we put. In fact, we typically ask a human subject for an implicit commentary and we ask the animal subject another type of question. There is another difference in procedure which I think one may not be able to generalize to all testing situations. Doreen Kimura's situation, as I remember, was a recurring figure situation in which the task was one of recognition over a relatively short interval. The tasks that were used by Hamilton, and I would suspect in all the unilateral animal cases, were not recognition tasks but discrimination learning tasks, i.e. associative tasks. I had a real shock when I tested some of Professor Sperry's split-brain patients because I found to my surprise—this was of course many years after the operation—that when they were told the *class* of stimulus that they had to discriminate, and I presented one of them in the left half-field, which projects to the "non-verbal" hemisphere, they had no trouble whatever in discriminating the stimuli; they became very indignant if I suggested they could not see the stimulus normally. They could describe the shape, the size, the position accurately. If I said it was going to be a letter of the alphabet, for example, and flashed up a particular letter, then they were correct. And I think that may be rather comparable to the animal discrimination learning situation, where in effect we say to the animal, for example, "Today we are going to have a triangle versus a circle"; one or the other is going to be "correct". And we also have good reason to believe that the neural mechanisms involved in a recognition task are quite different from those involved in an associative task.

MILNER

But I was commenting on the ineptness of the left hemisphere, not the cleverness of the right, on the tactile form-matching task.

WEISKRANTZ

I was commenting on Kimura's nonsense shapes. Yes, the ineptness of the left hemisphere, that is fine, but we do not know how inept it would be in man if tested with the typical monkey testing paradigm. And I am not of course that much of an optimist to assume that the monkey has a fully

developed speech system in the left hemisphere. I think he is entitled to certain differences from us, but I am trying to see the extent to which these are continuities, i.e. quantitative rather than qualitative differences.

J. LEVY

I just want to make a brief comment regarding handedness in animals. In almost all species that have been studied, including rats, mice, monkeys, and apes, 50% have been found to be right-pawed and 50%, left-pawed. Further, even in genetically homozygous mice, a 50-50 distribution in left-right paw preference is observed, ruling out the possibility that paw preference in mice could be due to genetic factors. In rats, 7 generations of selection for paw preference do not change the 50-50 proportion, again demonstrating an absence of genetic control over paw preference. Though no selection experiments have been performed in monkeys and apes, the lack of a species-specific asymmetry in handedness suggests that handedness in these animals, also, results from random, nongenetic factors, and is probably due to accidental contingencies of reinforcement in the environment. Particularly in the primates where manual usage is rather highly skilled, we would expect to see a bias toward right-handedness if the left cerebral hemisphere, as is the case in the majority of people, were specialized for the programming of sequential manual movements. While the absence of any dextral bias in monkeys may be due to the inadequacy of the tests that have been applied, this would seem to be an unlikely explanation in view of the fact that dextrality in people is apparent even with very crude measurements in quite young children.

WEISKRANTZ

Yes. Well, again handedness has been looked for in the monkey by a number of people—Ettlinger was probably one of the first—but I am not sure whether it has been looked for in the way you have described, which seems important. Some people argue that the two hands, even in the human infant, tend to play different sorts of roles—one is generally supportive, and the other is manipulative.

J. LEVY

In Trevarthen's studies of baboons in which the animals were required to open complicated locks, I do not recall his ever having reported a simple left-right asymmetry in hand usage. My recollection is that different animals utilized the two hands in different ways.

WEISKRANTZ

Yes, you are right. But Trevarthen did report with a split-brain primate something rather close to what Stamm has also reported, that is an acquired handedness, based on the animal's having experience mainly with one hemisphere rather than with the other.

TERENIUS

There is some evidence from brain damage in early ages in humans that there is certain plasticity in the lateral specialization. And I wonder if one should look upon this whole problem of man and animal as a quantitative phenomenon. Maybe even redundance may be useful in a creature like a rat or a cat—damage to one side of the brain will be of no problem. But when we come to such a young evolutionary species as the human being there was a need for lateralization. Maybe that is the answer, actually, to the experiment you mentioned, tbat if one would give a lot of information to an animal one would see an increasing extent of lateralization.

WEISKRANTZ

Yes. Well, that is the way I would like to interpret Denenherg's results rather than in terms of emotionality. I think to get into the question of plasticity in the young human brain would be opening up a very large topic; there is certainly not plasticity for all aspects of behaviour. But the degree of plasticity may in fact relate to the environmental demands placed on the animal. What do we have to cope with most of all—what is most important from an evolutionary point of view? We have to remember our physical environment, its configuration; we have to remember certain contingencies and we have to remember the objects and people, other organisms—faces, objects, external space, sequences, and the relationships among them. And I do not see that these pressures are any greater for man than they are for the monkey. If there is an evolutionary advantage in not duplicating the cerebral mechanisms unnecessarily, then the monkey may also have already benefited from such an evolutionary pressure.

COMPLEMENTARY FUNCTIONAL SPECIALIZATIONS OF THE HUMAN CEREBRAL HEMISPHERES

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It is now well-established that the human cerebral hemispheres, so similar in their morphology, differ markedly in their functional specialization. Already by the early 1860's, observations such as those of Dax and of Broca, in patients with unilateral brain disease, had pointed to a major role for the left hemisphere in the speech processes of most right-handed subjects, but it has taken much longer for the complementary functions of the right side to be recognized. In spite of the suggestion of Hughlings Jackson, in 1874, that posterior regions of the right hemisphere may play a special role in "visual ideation", the real breakthrough came much later, when a series of careful studies in patients with well-lateralized brain injuries revealed the full importance of the right parietal cortex for spatial functions (Brain, 1941; Paterson and Zangwill, 1944; McFie, Piercy and Zangwill, 1950; Hécaen, Ajuriaguerra and Massonnet, 1951; Hécaen, Penfield, Bertrand and Malmo, 1956). This evidence was later strengthened by work in Montreal on the temporal lobes, which disclosed specific perceptual and memory disorders after temporal lobectomy that varied with the side of removal (Milner, 1958, 1962, 1967; Kimura, 1963). Since then, convergent evidence from many sources has led to the overthrow of traditional concepts of cerebral dominance in favour of the notion of a complementary specialization of the two halves of the brain.

The evidence is of three kinds. First, there have been studies of large groups of patients with focal brain lesions of one or other hemisphere, permitting one to delineate specific cognitive deficits that vary with the side and site of injury and that appear against a background of otherwise

normal intellectual functioning. Much of the work that I shall review below falls into this category. Second, it has been possible by appropriate techniques to elicit evidence of complementary functional asymmetries in normal subjects. This has usually been achieved by simultaneously channelling competing inputs to the two ears (Broadbent, 1954; Kimura, 1961, 1964) or to the two visual half-fields (e.g., Klein, Moscovitch and Vigna, 1976), or else by comparing accuracy of recognition (Kimura, 1966) or speed of response (Rizzolatti, Umiltà and Berlucchi, 1971), to visual stimuli presented briefly to left or right of fixation. The slight but orderly laterality differences that emerge from such experiments have provided independent confirmation of clinical findings. Last, and most compelling, have been the observations made by Sperry and his colleagues, on patients in whom the two cerebral hemispheres had been surgically disconnected as a treatment for intractable epilepsy. In this unusual clinical situation, it is possible to confine sensory input and motor output to one side of the brain and thus bring out the contrasting specializations of the two sides (Sperry, Gazzaniga and Bogen, 1969; Sperry, 1974; Franco and Sperry, 1977).

The aspect of the commissurotomy studies that has so captured the public imagination has been Sperry's demonstration of a divided consciousness within the same person, together with the suggestion that the separated hemispheres exhibit radically different cognitive styles (Levy, 1969; Sperry, 1974). Yet there are also other findings of major interest, including the discovery that the surgically-isolated right hemisphere, though essentially mute, does have some limited understanding of speech (Sperry and Gazzaniga, 1967; Gazzaniga and Hillyard, 1971; Zaidel, 1978). This had not been predicted and serves as a reminder that the hemispheric specializations we demonstrate are apt to be relative rather than absolute (Teuber, 1978).

Hemispheric differences as revealed by focal brain lesions

We have seen that among the three ways of exploring the functional asymmetry of the human cerebral hemispheres, the oldest is that of analysing the effects of circumscribed brain lesions. This method still has certain advantages, because it allows one to examine the question of hemispheric differences in greater detail by looking at the form these asymmetries take in the case of particular cortical areas. In what follows, I shall try to delineate the complementary roles of the left and right anterior temporal

regions in memory processes, while at the same time situating the temporal-lobe findings in the broader context of hemispheric specialization. The work is based throughout on the study of patients undergoing unilateral cortical excisions for the relief of focal epilepsy.

Differential Effects of Focal Left-Hemisphere Lesions

Figure 1 shows the critical areas in the inferior frontal and posterior temporo-parietal regions of the dominant hemisphere where electrical stimulation of the exposed cortex at operation may cause a speaking patient to become momentarily dysphasic, and where injury in the adult may result in lasting language impairment. In the surgical treatment of epilepsy, these primary speech areas are always spared but extensive removals can be made from other parts of the left cerebral cortex without

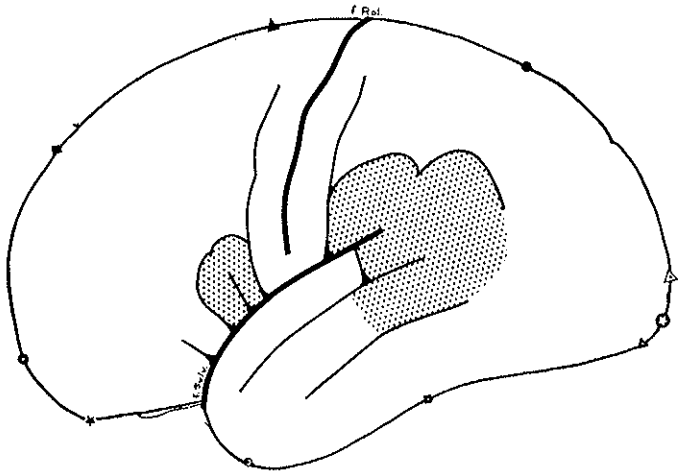

FIG. 1. Diagram of the left cerebral hemisphere showing the primary speech areas, as indicated by stimulation and ablation studies (from Rasmussen and Milner, 1975).

risk to speech (Penfield and Rasmussen, 1950; Penfield and Roberts, 1959; Rasmussen and Milner, 1975). Nevertheless, in such cases one does observe specific verbal deficits that vary with the locus of excision (frontal, central, or temporal) and that are not seen after a corresponding removal from the non-dominant hemisphere for speech.

Thus, in the case of left frontal-lobe lesions that spare Broca's area, patients show a conspicuous reduction in spontaneous narrative speech and writing (Kleist, 1934; Milner, 1964; Luria, 1966, 1969; Zangwill,

1966), although they are not otherwise dysphasic and have no articulatory disorder. This reduction in verbal fluency can be easily demonstrated on formal tests (Thurstone and Thurstone, 1943) that require the subject to produce as many words as possible beginning with a particular letter within a given time limit, either in writing (Milner, 1964) or in speech (Benton, 1968; Ramier and Hécaen, 1970; Perret, 1974).

A still more marked reduction in word fluency is seen in patients whose left-hemisphere lesions involve the cortical face area (in the lower central region, between the frontal and parietal speech zones). In addition, such patients have unusual difficulty with any task that requires them to analyse the phonetic structure of the speech sounds they hear (Taylor, Milner and Darwin, cited by Milner, 1975b). Thus, they cannot identify phonemes embedded in nonsense words, nor spell correctly to dictation; yet they are not clinically dysphasic and they achieve normal scores on most other verbal tasks.

Although I shall not be considering in detail the effects of frontal and central lesions, it is worth pointing out that on a nonverbal fluency task, in which subjects were asked to produce as many novel abstract drawings as they could within a specified time, the greatest impairment was seen not after left, but after right frontal and frontocentral lesions (Jones-Gotman and Milner, 1977). These results thus extend to the domain of fluency our earlier finding of complementary deficits in the temporal ordering of recent events after left and right frontal lobectomy (Milner, 1974).

Verbal memory functions of the left temporal lobe. Left temporal lobectomy (in the dominant hemisphere for speech but anterior to the critical language zone) does not result in any of the verbal disabilities described above. Instead, it selectively impairs the learning and retention of verbal information (Meyer and Yates, 1955; Milner, 1958), irrespective of whether the information is presented aurally or visually (Blakemore and Falconer, 1967; Milner, 1967), and irrespective of how retention is tested (Milner, 1958, 1967; Milner and Teuber, 1968). Such lesions do not, however, affect memory for spatial locations (Corsi, 1972), faces (Milner, 1968), melodies (Milner, 1962; Shankweiler, 1966) or abstract visual patterns (Kimura, 1963; Taylor, 1969).

The verbal memory defect is usually demonstrable before operation, in patients with longstanding epileptogenic lesions of the left temporal lobe (Milner, 1958, 1967, 1975a; Fedio and Mirsky, 1969), but the deficit becomes more marked after left temporal lobectomy (Meyer and Yates,

1955; Milner, 1958), with detectable residual loss many years later (Milner, 1967, 1975b), even though there is no dysphasia and no decline in verbal intelligence as measured by standard tests.

This kind of verbal-memory impairment is relatively rare in other cortical lesions (except for those causing dysphasia, in which case the verbal memory loss forms part of a more complex language disturbance). In an effort to elucidate the source of the memory difficulty, Wilkins and Moscovitch (1978) have suggested that left anterior temporal-lobe lesions may disrupt semantic systems that involve verbal or lexical representation, hut leave intact those systems that involve visual or analogue representation. Although there is some support for this view (Wilkins and Moscovitch, 1978; Jaccarino-Hiatt, 1978), the puzzling fact remains that the verbal memory disorder is still disproportionate to the defects on other cognitive tasks. It will be seen below that a complementary material-specific memory disorder follows removal of the right anterior temporal cortex.

Right-Hemisphere Dominance for Face Perception and Face Recognition

I have chosen to illustrate right-hemisphere specialization by means of a few tasks involving face perception or face recognition, rather than attempt a comprehensive survey of right-hemisphere capacities as revealed by focal lesions.

Left visual-field bias in face perception

It is a common observation that human faces are not perfectly symmetrical about the midline, despite the general similarity of the two sides. This has led to speculation as to whether, in forming an impression of what another person looks like, we tend to be more influenced by one side of the face than the other. In an experiment that has been extensively replicated (McCurdy, 1949; Lindzey, Prince and Wright, 1952; Gilbert and Bakan, 1973; Lawson, 1978), Wolff (1933) was able to show that the right side of the face tends to be more salient than the left for most ohservers.

The experimental method is illustrated in Figure 2. It entails splitting full-face photographs (and their mirror-images) down the middle, then rejoining the corresponding halves to make two symmetrical composite photographs, one created from the left side of the face, the other from the right. Thus, in Figure 2, we see the normal face on top and the two composite faces below. The subject's task is to indicate as quickly

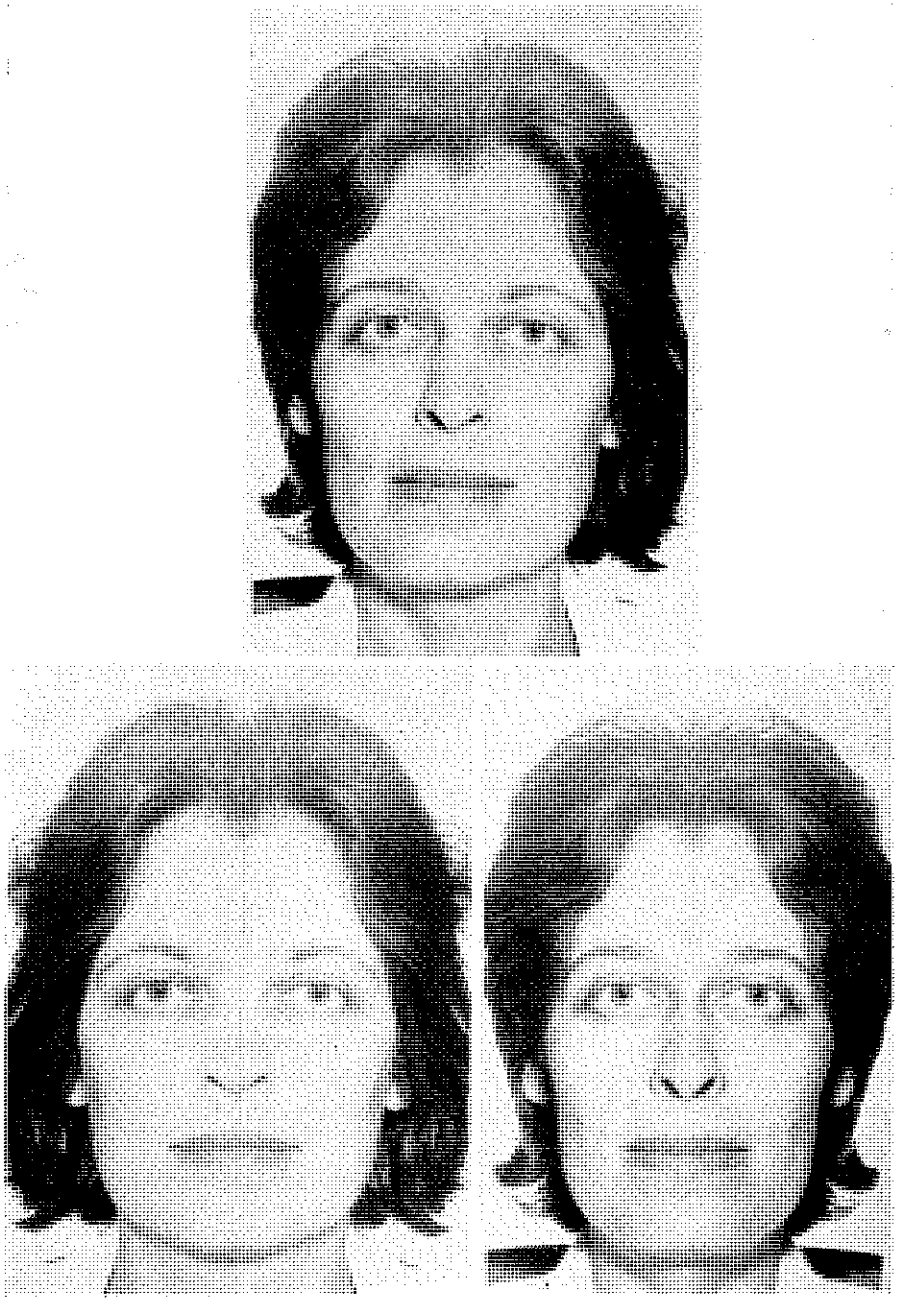

FIG. 2. Sample item from Kolb's version of the Composite-Faces task. *Above:* normal photograph showing facial asymmetry. *Below:* two symmetrical faces made by pairing, respectively, the left or right half of the normal face with the corresponding mirror image. The subject must indicate which of the composite photographs more closely resembles the real face.

as possible which of the composite pictures resembles the real face more closely. To control for any tendency on the observer's part to point to one side of the page rather than the other, the relative position of the composite faces is balanced across test items.

The experimental finding was that there is a significant bias in favour of the right half of the face (i.e., the part of the photograph in the viewer's left visual field). Contrary to earlier belief, this result is not due to any greater expressiveness of the right half-face, since reversing the orientation of the whole face does not reverse the left-field bias (Gilbert and Bakan, 1973; Lawson, 1978). Nor can the finding be attributed to a left-right scanning habit acquired through reading, because habitual readers of Hebrew also show a left-field bias in face perception (Gilbert and Bakan, 1973). It seems, rather, that the greater salience of the left visual-field, as demonstrated on this task, reflects the greater contribution of the right hemisphere to test performance. This conclusion holds for right-handed subjects only, left-handers showing no consistent bias in either direction (Gilbert and Bakan, 1973; Lawson, 1978).

Effects of focal brain lesions. My colleague, Dr. Bryan Kolb, has since had the opportunity to administer a composite-faces task to 52 patients with well-documented focal cortical excisions, as well as to 20 right-handed normal control subjects. Figure 3 shows the mean percentage of left-field choices made by the control group and by patients grouped according to side and site of lesion.

In line with previous studies, Kolb's control group showed a substantial bias in favour of the left visual-field, and the same was true for patients with left temporal, left frontal or left parietal-lobe lesions. It is important to note that these left-hemisphere groups showed no exaggeration of the normal bias, although the patients with left parietal lesions had right homonymous visual-field defects, which might have led them to favour the left side.

Quite different results were obtained for the right hemisphere: here, patients tested after a right frontal lobectomy showed a normal left-field preference, but those with right temporal- or right parietal-lobe excisions had no bias to either side (Fig. 3). Such patients gave no evidence of contralateral neglect and their atypical results cannot be attributed to visual-field defects, since, as we have seen, there were patients in the left-hemisphere groups, with comparable losses in the opposite visual field, who yet performed normally. Instead, the results for the right temporal- or right parietal-lobe groups are congruent with neurological

findings implicating the right posterior cortex in face perception and face recognition (De Renzi and Spinnler, 1966; Warrington and James, 1967a; Benton and Van Allen, 1968; Milner, 1968; Tzavaras, Hécaen and LeBras, 1970; Yin, 1970; Newcombe, 1973; Meadows, 1974), as well

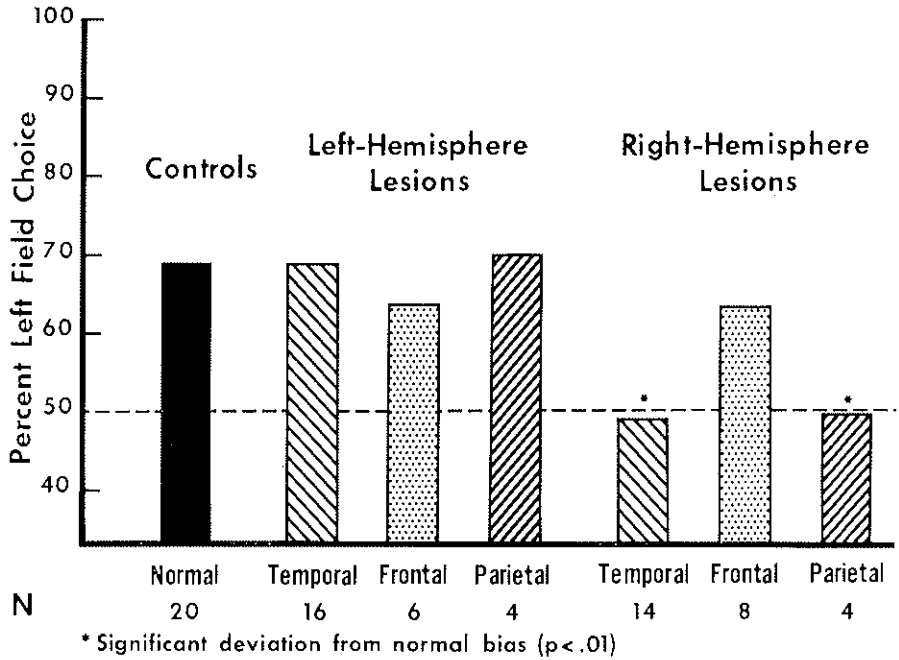

FIG. 3. *Composite-Faces task:* Histograms indicating mean percentage choice in favour of the left visual field. Results for Kolb's normal control subjects and for groups of patients classed according to side and site of cortical excision.

as with the more general claim that the right hemisphere is dominant for visually-directed attention (Dimond, 1976; Geschwind, 1977). They are also consistent with the findings of Levy, Trevarthen and Sperry (1972) for the perception of chimeric faces after cerebral commissurotomy.

Right posterior cortical contribution to visual closure

Further evidence for the importance of the right posterior cortex in face perception comes from another task, the so-called Closure Faces Test of Mooney (1956). This consists of a series of 44 incomplete representations of faces, in which the highlights and shadows are exaggerated but the contour is poorly defined (cf. Fig. 4, for a typical item). The subject

is allowed up to 30 sec in which to discover the face and state the sex and approximate age of the person depicted.

Figure 5 shows the mean error scores for 125 patients grouped according to side and site of cortical excision; all patients were left-hemisphere dominant for speech. Since the mean error-score for an appropriately-matched normal control group was 6.7, it is apparent that neither left frontal, left temporal, nor left occipital lobectomy affected

FIG. 4. Representative item from Mooney's Closure Faces Test.

performance on this task. The latter finding is particularly impressive, because each of the patients with left occipital-lobe lesions had a complete right homonymous hemianopsia.

It can be seen from Figure 5 that the right-hemisphere groups show a clear anterior-posterior gradient in test performance. Whilst the patients with right frontal-lobe lesions (including those with some invasion of the anterior temporal region) performed insignificantly *better* than the control subjects, the patients tested after right temporal lobectomy had a mild impairment (confirming an earlier finding by Landsell, 1968) and those with right posterior lesions (most of which were temporo-occipital) had the most severe deficits of all.

There is an evident parallel between these results and Kolb's findings for the composite-faces task, although there was little or no overlap in

the patients tested. Several points deserve comment. First, the fact that greater deficits follow the more posterior lesions of the right hemisphere accords well with earlier findings for this kind of perceptual task (Ettlinger, 1960). One should note however that the critical posterior cortical area implicated in complex perceptual tasks of this kind appears to be different from the right-parietal area maximally involved in topographical

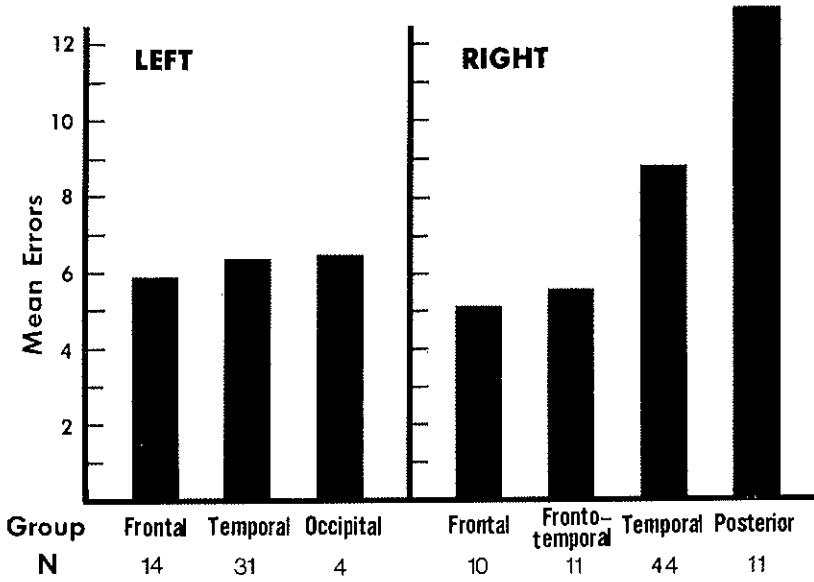

FIG. 5. *Closure Faces Test:* Mean error scores for different patient groups classed according to side and site of cortical excision. The mean for the control group was 6.7 errors out of a total of 44 items.

orientation and maze learning (Newcombe and Russell, 1969). A second point of interest is that the right frontal-lobe group, despite their rapid and efficient performance on this test (as well as on tests of face recognition) were found by Kolb to have a severe impairment on a task that required the matching of emotional expressions across changing faces. This deficit (seen also in the right temporal group but not in any of the left-hemisphere groups) confirms recent results with normal subjects that point to a right-hemisphere dominance for the perception of emotional expression, whether in the face (Buchtel, Campari, De Risio and Rota, 1978; Ley and Bryden, 1979) or voice (Haggard and Parkinson, 1971; Safer and Leventhal, 1977).

Visual memory functions of the right temporal lobe

The visual disorder associated with right anterior temporal-lobe lesions affects both perception and memory (Milner, 1958, 1967, 1968; Kimura, 1963; Milner and Teuber, 1968), but the perceptual changes tend to be slight and variable (Milner, 1958; Ettlinger, 1960; Lansdell, 1968; Kimura, 1963; Meier and French, 1965; Warrington and James, 1967b), as was the case for the closure results reported above. In contrast, severe and lasting deficits are found on tasks that require the recall or recognition of visual patterns after a delay. Thus, when patients with right anterior temporal lesions are asked to study, for 45 sec, the faces shown in Figure 6, and then, after a 90 sec delay, are required to select these faces again from a larger array, they show a marked failure of visual recognition,

FIG. 6. Material from Munn's (1951) face-recognition task. This set of photographs is shown to the subject for 45 sec. After a short delay, he is asked to pick these faces out again from a larger group (from Milner, 1968).

often trying unsuccessfully to do the task by verbal coding of the stimuli (Milner, 1968). A similar deficit, but in the recognition of snapshots of famous people, has been described by Warrington and James (1967a) for patients with right temporal-lobe lesions.

At this point it must be emphasized that these patients with unilateral neocortical lesions do not have an agnosia for the faces of people around them, and indeed the bulk of the evidence suggests that such major disturbances of face recognition usually indicate a bilateral lesion, though the right temporo-occipital component is the most constant feature (Meadows, 1974).

It is not necessary to use photographs of faces in order to bring out the visual memory impairment after right temporal lobectomy. It can be seen equally well when the task requires the subject to learn to recognise abstract patterns of the kind shown in Figure 7 (Kimura, 1963),

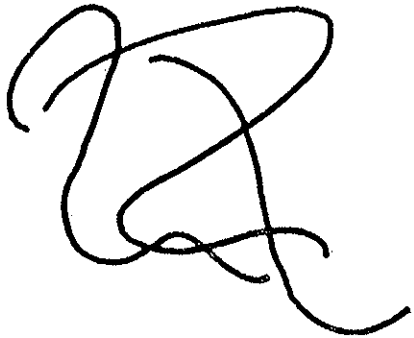

FIG. 7. Difficult item from Kimura's Recurring Nonsense Figures test (from Milner, 1967).

or to recall a complex geometric figure that the subject has already copied with reasonable accuracy (Taylor, 1969). It is tempting to see in this visual memory impairment an analogue of the deficit in visual discrimination-learning that has been observed after bilateral lesions of inferotemporal cortex in the monkey (Chow, 1951; Mishkin and Pribram, 1954; Iversen and Weiskrantz, 1964). More recent experiments by Iwai and Mishkin (1968) and Cowey and Gross (1970) point to a further functional differentiation within the inferotemporal and prestriate region, with the critical area for pattern discrimination far posterior and the anterior temporal cortex more directly implicated in visual memory. Such a distinction would be consistent with our finding of greater perceptual deficits with the more posterior lesions, hut we have not so far been able to study the effects of discrete lesions in the posterior zone, in patients where the anterior temporal region was functioning normally.

The findings reviewed in this section have served to delineate a material-specific memory disorder seen after right anterior temporal lobectomy, and one that complements the verbal memory impairment found after comparable lesions of the left hemisphere.

Complementary Effects of Left and Right Hippocampal Lesions

In the operation of unilateral temporal lobectomy, it is often necessary to remove not only the anterior temporal neocortex but also the amygdala and parts of the hippocampus and parahippocampal gyrus. Yet it is known that combined bilateral damage to these medial structures, sparing the neocortex, results in a profound and lasting amnesic syndrome (Scoville and Milner, 1957; Penfield and Milner, 1958), although lesions restricted to the amygdala have no such effect (Sawa, Ueki, Arita and Harada, 1954; Scoville and Milner, 1957). The question therefore arises as to whether the severity of the specific memory deficits that accompany unilateral temporal-lobe lesions may not also be a function of the medial extent of removal.

The answer clearly depends on the kind of material used to test memory. With abstract visual patterns (Kimura, 1963; Taylor, 1969), and possibly with faces, the neocortical lesion appears to be the critical factor in the deficit seen after right temporal lobectomy, and we find no relationship to the extent of hippocampal excision (Jones-Gotman and Milner, 1978). In contrast, when all that has to be recalled is a simple spatial location (Corsi, 1972; Milner, 1974) or the correct sequence of turns in a maze (Corkin, 1965; Milner, 1965), the magnitude of the deficit after right temporal lobectomy is directly related to the amount of hippocampal destruction.

This distinction is less clearcut in the case of the verbal memory impairment after left temporal lobectomy, since on most measures we have found the deficit to be related both to the lateral and to the medial extent of removal (Milner, 1967). Nevertheless, here also the hippocampal lesion appears to be less critical in the recall of material that has its own intrinsic structure (as with words that have been already grouped into particular semantic categories) than in the case of random lists of words (Jaccarino-Hiatt, 1978) or strings of unrelated consonants (Corsi, 1972; Milner, 1974; Samuels, Butters and Fedio, 1972).

In humans, as in lower primates (Kimble and Pribram, 1963), sequence-learning appears to be particularly vulnerable to hippocampal lesions (Drachman and Arbit, 1966). This fact led Corsi (1972) to use

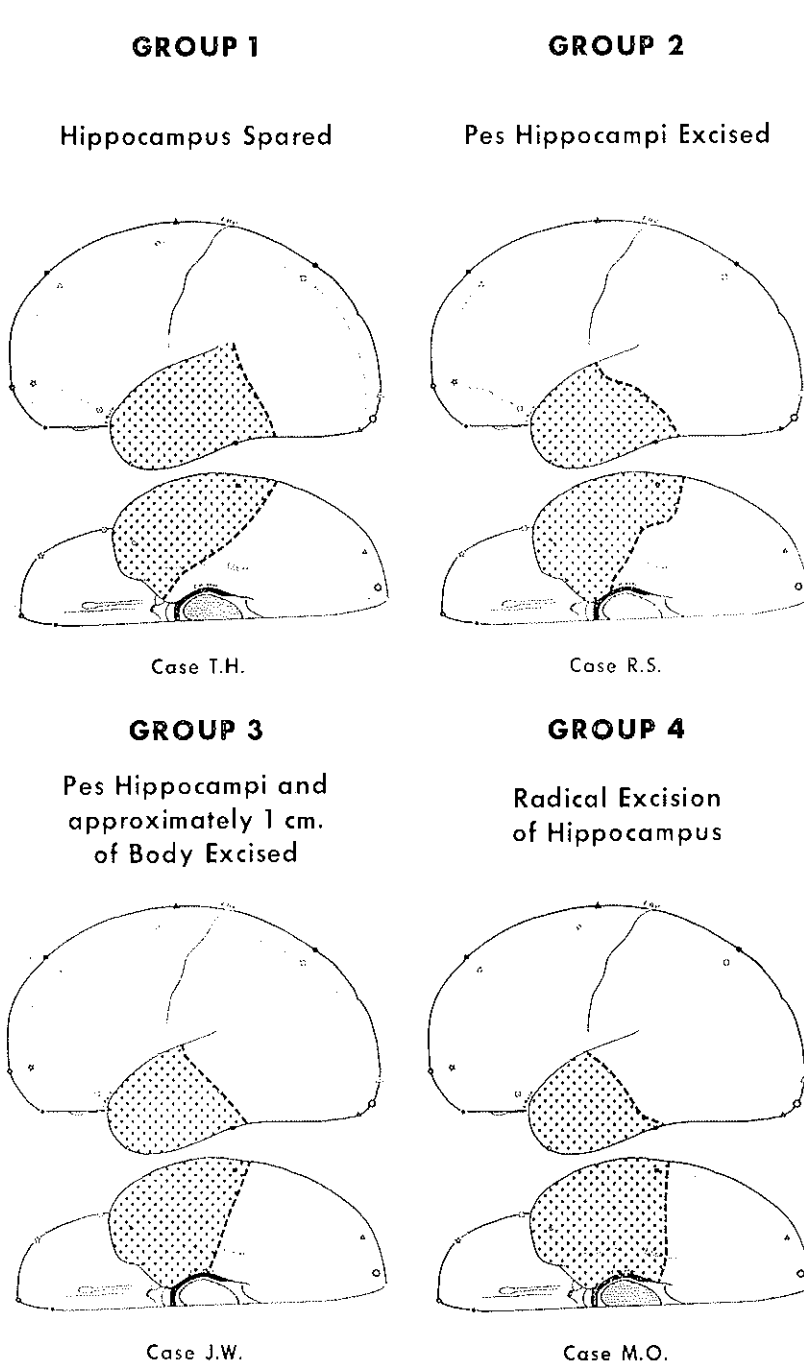

FIG. 8. Brain maps, based on the surgeon's drawings at the time of operation, showing representative left temporal lobectomies in four groups of patients classed according to the extent of hippocampal destruction. The mean extent of lateral neocortical removal was the same for all groups. Stippling indicates extent of cortical excision. *Above:* lateral surface. *Below:* inferior surface (from Milner, 1970).

two formally similar sequence-learning tasks (one verbal, the other spatial) to bring out complementary material-specific deficits after unilateral temporal lobectomy and to show that the occurrence of these deficits was contingent upon damage to the hippocampus and neighbouring tissue. This work is described briefly below.

In the verbal task (invented by Hebb, 1961), 24 random strings of digits, each exceeding by one digit the subject's immediate memory span, are presented successively for immediate recall. Unknown to the subject, the same sequence recurs every third trial, the intervening sequences occurring only once. Under these conditions, normal subjects learn the recurrent sequence, while continuing to make errors on the non-recurrent ones (Hebb, 1961; Melton, 1963). The fact that the recurring string becomes progressively easier to recall, despite the intervening items, is taken by Hebb as proof of the early formation of a structural trace.

As would be predicted, Corsi found an impairment on this verbal-learning task after left temporal lobectomy and not after right. More interestingly, when the left temporal-lobe patients were subdivided into four groups according to the extent of hippocampal removal (Fig. 8), it was found that the larger the hippocampal lesion the more salient the deficit. Figure 9 shows, for each presentation of the recurrent sequence, the proportion of subjects recalling that sequence correctly in each of the above groups and in the normal control group. Groups 1 and 2, with complete or near-complete sparing of the hippocampus, showed normal or near-normal learning; Groups 3 and 4, with more extensive encroachment upon the hippocampal zone, failed to progress within the limits of testing. It is noteworthy that this marked impairment in supra-span learning is compatible with a normal immediate memory span for the same material, a discontinuity that supports the distinction between a primary memory process (undisturbed by medial temporal-lobe lesions) and a secondary process by which the long-term acquisition of new information is achieved (Waugh and Norman, 1965; Shallice and Warrington, 1970).

Corsi's nonverbal analogue of the digits task is illustrated in Figure 10. The test material consists of nine black wooden blocks irregularly distributed over a black board and permanently fixed there. On any given trial, the examiner taps a subset of these blocks in a random sequence, and immediately afterwards the subject tries to touch the same blocks in the same order. The first step is to establish the immediate-memory span for block-tapping (i.e., the longest sequence that the subject can reproduce reliably after one exposure). Then, 24 test trials are carried out in which

different spatial sequences one block longer than the span are presented. As with the digits, the same sequence is repeated every third trial but the intervening sequences are never repeated. Again, normal subjects learn the recurrent sequence, whereas performance on the non-recurrent ones remains at a low level.

On this spatially-cued task, Corsi's results were the reverse of those that he had obtained for the digit sequences. This time there was no deficit after left temporal lobectomy, even in cases of radical hippocampal resection, hut patients with right temporal-lobe lesions now had an impairment that was proportional to the amount of hippocampus excised. There is thus a double dissociation (Teuber, 1955) between the effects of left and right hippocampal lesions on the performance of these two tasks.

These experiments by no means exhaust the situations in which the severity of the material specific-memory loss after unilateral temporal lobectomy has been found to depend upon the extent of hippocampal removal (Jaccarino-Hiatt, 1978; Jones-Gotman and Milner, 1978; Jones-Gotman, 1979). Taken together, the results point to a constant inter-

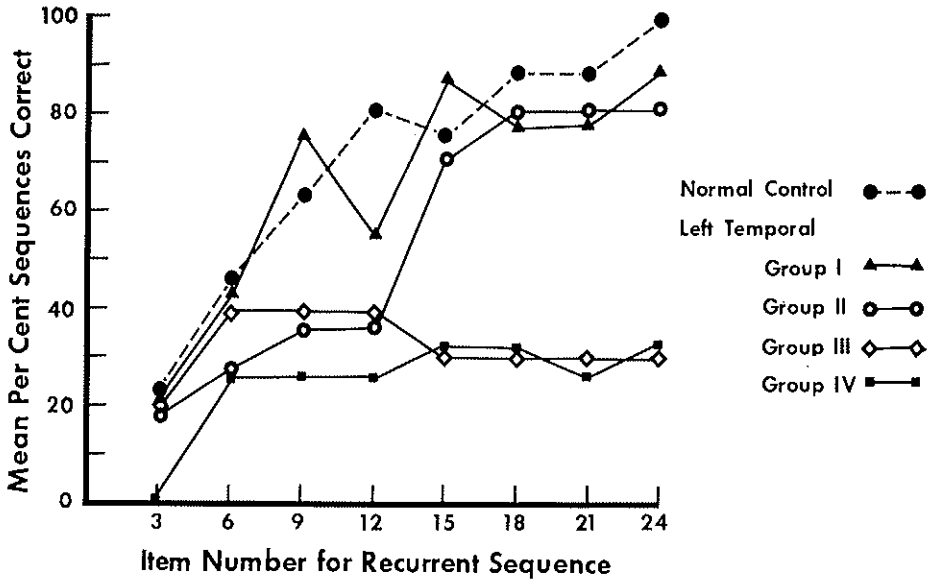

FIG. 9. *Digit-sequences task:* learning curves for the four left temporal-lobe groups (cf. Fig. 8) and the normal control group. The ordinate represents the proportion of subjects correctly recalling the recurrent sequence, the abscissa the ordinal position of the sequence (from Corsi, 1972; Milner, 1978).

play between the hippocampal region (by which I mean both hippocampus and parahippocampal gyrus) and those neocortical areas of the same hemisphere that are involved in the encoding, storage, and reevocation of information in different sensory modes. This view has been strengthened by recent anatomical studies that demonstrate multiple afferent and efferent connections linking the hippocampal region to widespread areas of the ipsilateral neocortex, and thus bringing it, directly or indirectly, under the influence of all the major sensory systems (Van Hoesen, Pandya and Butters, 1972, 1975; Van Hoesen and Pandya, 1975a, 1975b; Rosene and Van Hoesen, 1977).

Duality of Memory Encoding

Thus far I have been arguing for the existence of partially separable memory systems for words and for visual patterns, such that one system can be disturbed by a focal unilateral lesion while the other one continues to function normally. To prove this dissociability, we have deliberately used memoranda that could not be handled equally well by both systems, at least under the time constraints and information loads imposed by our tasks. In everyday life, however, one is most often confronted by events

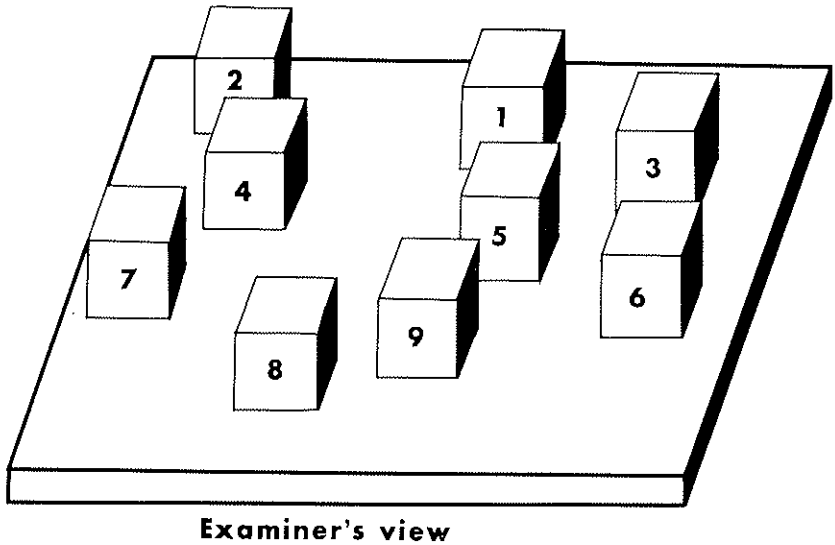

FIG. 10. *Block-tapping task:* sketch showing the approximate position of the nine black blocks (1¼ inch cubes) on a black board (8×10 inches). On the examiner's side the blocks are numbered for ease in recording performance, but the numbers are not visible to the subjects (from Corsi, 1972; Milner, 1971).

that can be coded in more than one way, and there is by now a large body of research with normal subjects that points to a duality of memory processing, verbal and imaginal, whenever the conditions permit it (as, for example, when recalling concrete words or representational drawings) (Bower, 1970; Paivio, 1969, 1971; Paivio and Csapo, 1973; Shepard, 1966).

That the two codes acting together may be more effective than either alone was demonstrated by Jaccarino (1975) in another study of patients tested after unilateral temporal lobectomy. In this experiment 25 line-drawings of common objects (Fig. 11) were presented one at a time, in

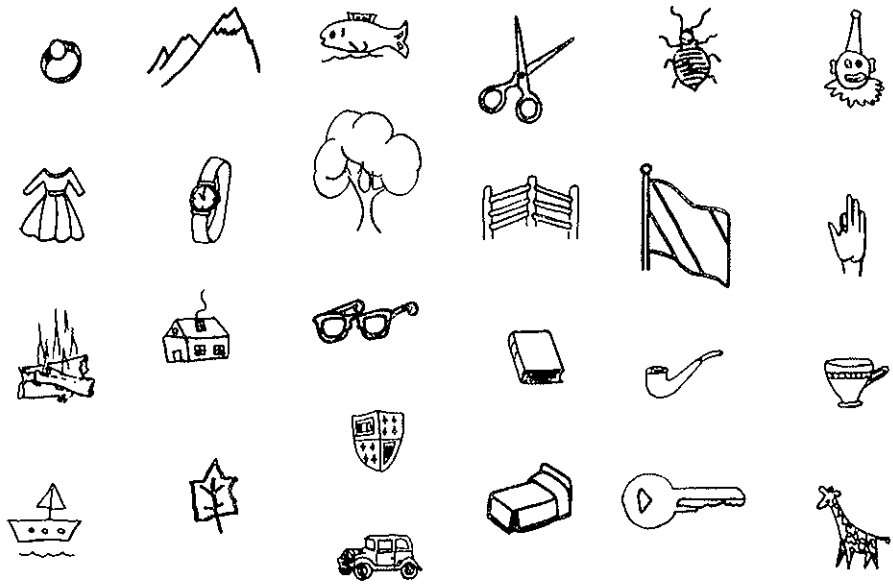

FIG. 11. Drawings used by Jaccarino (1975) to test recall of picture names (material supplied by H. P. Bahrick and derived from Bahrick and Boucher, 1968).

varying order, over six trials, after which the subject was immediately required to name from memory as many of the pictures as he could. A second recall was elicited, 24 hours later, without forewarning.

Figure 12 summarizes the main findings for Jaccarino's normal control subjects and for her right and left temporal-lobe groups. The well-known stability of memory for pictures (Shepard, 1966) is evident in the results of the normal subjects. Although the initial memory load was high, resulting in only 58% recall immediately after presentation of the pictures,

there was little further loss the next day, when the normal subjects still achieved 56% recall. This stability contrasts with the poor delayed recall of both patient groups. In immediate recall, the right temporal group performed normally and the left temporal group showed only the slight impairment consonant with their known difficulty in evoking names. When tested the next day, however, both these groups showed a pro-

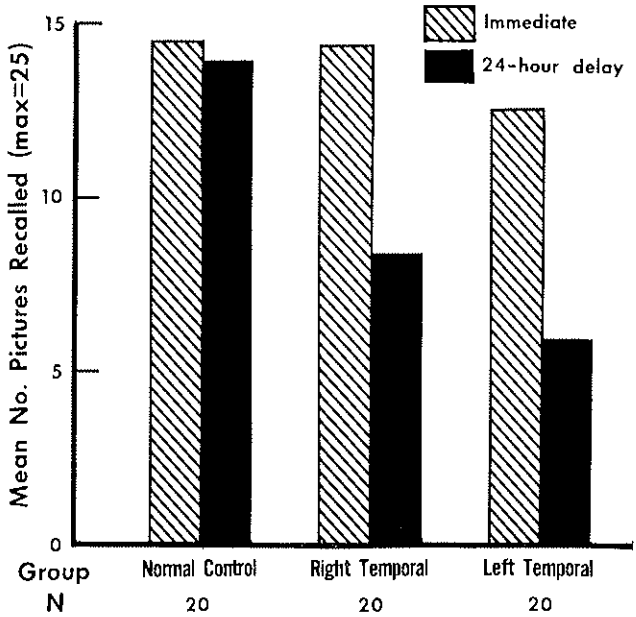

FIG. 12. *Free recall of picture names:* Mean scores for normal subjects and for patients tested after right or left temporal lobectomy (from Milner, 1978; adapted from Jaccarino, 1975).

nounced impairment of recall. From these results Jaccarino concluded that the initial recall of pictured objects can he mediated verbally (where no disadvantage would be expected to accrue from a right temporal-lobe lesion), but that recall after a longer interval is partially dependent upon the evocation of a visual image.

The deleterious effect of unilateral temporal lobectomy on the delayed recall of pictures was unexpected, because we had thought that in such a case recall could have been mediated adequately by either a verbal label or a visual image. Instead, we found that, far from heing redundant, these two modes of processing, partially separable in their cerebral organization, each play a critical role in the recall of representational material by normal

subjects. Such findings also serve to remind us that the rich and varied cognitive achievements of the intact brain depend upon the continuing integrative activity of the two sides, though the contributions of each differ so widely.

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DISCUSSION

BERLUCCHI

On the object recall test, were there errors of commission, and was it possible to differentiate between lesions on the basis of these errors?

MILNER

Intrusion errors (or errors of commission) were extremely rare and did not contribute to the group differences. The analyses were therefore based entirely on the number of objects correctly recalled by the various groups, in immediate and in delayed recall. We found that normal subjects forgot some of the items after 24 hours that they had recalled initially but, interestingly, they also showed some reminiscence, recalling a few items the next day that had not been recalled initially. We believe that this is consistent with the notion that on this task the processes mediating recall of the picture names after a delay were somewhat different from those mediating immediate recall (as the results for the right temporal-lobe group would also suggest).

BERLUCCHI

And so it is impossible to tell a difference between the right and the left lesions?

MILNER

The mean recall scores of the right temporal lobe group are slightly higher than those of the left but Jaccarino did not observe any qualitative difference in the types of errors made by the two groups. By extrapolation from other tasks, I would expect patients with frontal-lobe lesions to show more intrusion errors than either temporal-lobe group, but we have no direct evidence bearing on this point.

WEISKRANTZ

Did she ever try just recognition of the common object rather than recall?

MILNER

Yes. Jaccarino's original reason for doing the study was to investigate the effect of picture-naming on subsequent recognition of the pictures. As in the experiment of Bahrick and Boucher (1968), the subject had to pick out the specific picture from five similar ones (e.g., a particular watch from an array of six watches). No clearcut group differences emerged from this aspect of the study.

CEREBRAL ASYMMETRY AND ITS VARIATIONS IN THE HUMAN BRAIN

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Until the first half of the 19th century, there was no evidence to suggest that the human brain differed in any fundamental way from that of lower primates except for a certain size advantage for the former. However, in 1836, Marc Dax, a French neurologist, presented a paper at Montpellier in which he reported that unilateral damage to the left side of the brain produced disorders of thought and language. In spite of the remarkable implications of that observation, it was little noted in the neurological community, quite probably because Dax failed to publish his findings. A quarter century elapsed before the idea that man's brain is asymmetric gained widespread acceptance. In 1861 Broca published his classical paper on speech loss following lesions to the third frontal convolution of the left cerebral hemisphere (Broca, 1861), and over the next 100 years a vast literature accumulated supporting and expanding the initial investigations. Not only did the left hemisphere appear to be dominant for speech, but also for the comprehension of language, for sensory integration, for the planning of behavior, and, indeed, for all cognitive activities. The right side of the brain came to be considered as a mere input-output unit for its dominant partner, sending information to and receiving information from the thinking left half-brain. The human animal had been reduced, in effect, to a half-brained species whose organ of cognition was hardly larger than the brain of an ape. The wastefulness of an evolution that would functionally discard half the brain mass in a creature that was the epitome of its creation was never considered to be a problem since it was never considered at all.

Hughlings Jackson was one of the few early researchers who even entertained the possibility that the right side of the brain was more than a transfer station. In 1864, describing a patient with a right hemisphere tumor, he said, "She did not know objects, persons and places ... there was what I would call 'imperception', a defect as special as aphasia" (Taylor, 1958). He went on to suggest that, perhaps, the right side of the hrain had its own special mode of thinking. Nevertheless, such an idea had little impact on the views of most neurologists, and even Weisenberg and McBride's report (1935) that patients with injury to the right side of the brain displayed a specific defect in the manipulation of forms and in the appreciation of spatial relationships did not significantly affect the generally held belief that thought and behavioral control were centered in the left hemisphere.

A number of studies of patients suffering from unilateral brain damage emerged during subsequent years, all suggesting that the right hemisphere was as superior to the left in certain capacities as was the left to the right in language (Paterson and Zangwill, 1944; McFie, Piercy, and Zangwill, 1950; Hécaen, Penfield, Bertrand, and Malmo, 1956; Ettlinger, Warrington, and Zangwill, 1957; Reitan and Tarshes, 1959; Hécaen and Angelergues, 1962), and though many researchers were coming to doubt the validity of the concept of a dominant left hemisphere, Alajouanine and Lhermitte (1963) maintained that the various findings confirmed the classical view. In particular, they attributed the symptoms of right hemisphere injury to a pathological release of activity in the left side of the brain due to loss of inhihition from the right. Thus, neurological theory was at an impasse. Since any behavioral consequences of a neurological lesion could be explained as reflecting disinhibition of intact neural tissue, no amount of data from patients with unilateral cerebral injury could establish superiority or dominance of one hemisphere over the other for any domain of cognition. The conclusion that deficiency syndromes resulting from damage to the left hemisphere were indicative of left hemisphere specialization for the disordered functions, while deficiency syndromes following damage to the right hemisphere were indicative of the release of pathological activity on the left was, of course, without scientific justification. As Kuhn (1962) has emphasized, mere facts are forced into conventional paradigms of thought until such time as assimilation is not possible, and it is only when the old paradigms utterly fail in the face of accumulating evidence that a paradigmatic revolution occurs in which the theoretical framework itself accommodates to the observations. As it happened, Kuhn's analysis of the nature of scientific revolu-

tions was published in the same year that a revolution in neuropsychology was beginning.

In 1962 Bogen and Vogel (1962) published a case report of a patient in whom all the neocortical commissures had been severed, and in the same year Gazzaniga, Bogen, and Sperry (1962) reported various aspects of this patient's behavior. Fourteen years ago at a Study Week sponsored by this Academy on "Brain and Conscious Experience", Roger Sperry described the nature of the human split-brain syndrome: "Everything we have seen so far indicates that the surgery has left these people with two separate minds, that is, two separate spheres of consciousness. ... When we deliberately induce different activities in the right and left hemispheres ..., it becomes evident that each hemisphere is oblivious to the cognitive experience of the other". Examinations of many subsequent commissurotomy patients have strongly confirmed Sperry's initial descriptions and have shown, beyond doubt, that the right side of the human brain is a perceiving, motivated, reasoning organ of thought that, further, is as superior to the left side of the brain in its own domain of specializations as is the left to the right in language.

In some of the earlier studies (Gazzaniga, Bogen, and Sperry, 1965; Bogen and Gazzaniga, 1965), the right hemisphere was found to surpass the left in visuo-constructive tasks such as drawing and copying of designs with colored blocks, skills that may have reflected merely a superiority at the motor-executive aspects of constructional tasks or, alternatively, a more basic superiority in the understanding of form and space. In a later study, Levy-Agresti and Sperry (1968) compared the abilities of the two separated hemispheres to match a tactually perceived three-dimensional form with its "unfolded" visual representation, a task which, though requiring no constructional skills, depended on a high-level capacity for visualizing transformations in a three-dimensional mental space. The right hemisphere was found to he vastly superior to the left and in all except one patient, the left hemisphere performed at chance. It was apparent from this investigation that the right side of the brain was not merely skilled at manual construction, but was greatly superior to the left in the *understanding* of spatial relationships. It should he noted, however, that there are still a minority who deny the right hemisphere any special cognitive abilities. Le Doux, Wilson, and Gazzaniga (1977) have claimed, as Gazzaniga *et al.* (1965) originally suggested, that the right hemisphere exceeds the left only in what Gazzaniga is now calling "manipulo-praxic" abilities. The observations of Levy-Agresti and Sperry (1968) are attributed to the left hand's superiority at manipulating

objects in order to determine their sensory properties. Even the "manipulo-praxic" capacity, Gazzaniga suggests, is not a true specialization, but rather a behavioral dominance designed to relieve the cognitive burden on the left. His conclusion derives from the fact that the two hemispheres are equally good at matching two visual patterns, in contrast to the right hemisphere's advantage in visuo-tactile matching. However, so simple is the task of making visuo-visual matches that both hemispheres perform almost at ceiling for pictures of ordinary objects, and a cognitive superiority of the right hemisphere can only be expected when a task is sufficiently difficult that it challenges the specialized hemisphere. With visuo-tactile matches, form invariants must be extracted, significantly increasing the difficulty of the task.

In spite of the yearning of some researchers to return man to an earlier and simpler world in which his half-brained status was unchallenged, a great deal of evidence is now available, not only from studies of split-brain patients and patients with unilateral brain damage, but also from investigations of normal populations, to demonstrate beyond any reasonable doubt that the right side of the brain is superior to the left in its imagistic memory, in its understanding of form, in its ability to derive mental maps, and in its ability to encode sensory experience that is resistant to verbal description (see, e.g., Dimond and Beaumont, 1974; Dimond and Blizard, 1977; Bogen, 1969). During the past several years, it has also been found that under certain experimental conditions, the right side of the brain overwhelmingly dominates behavioral pathways, even under competitive conditions when both sides of the brain receive equal sensory input and both sides are equally competent at guiding the simple motor response required. Thus, the right side of the human brain not only exceeds the left in a wide range of cognitive processes, but dominates behavioral control when it imagines itself to be more competent for the task at hand. In the following section, some of the more recent split-brain studies will be reviewed that illustrate both the nature of the right hemisphere's special capacities, as well as its striking behavioral dominance in certain competitive situations.

Competitive perception in split-brain patients

Figure 1 shows one of several tests given to split-brain patients in a series of studies designed to investigate both relative hemispheric abilities, as well as relative dominance of the hemispheres over behavioral control pathways (Levy, Trevarthen, and Sperry, 1972; Levy and Trevarthen,

1976; Levy and Trevarthen, 1977). As patients maintained fixation, a split face was rapidly flashed in a tachistoscope so that the right hemisphere saw the left half-face and the left hemisphere saw the right half-face. Under such conditions, each isolated hemisphere effects a perceptual completion so that a whole face is actually perceived, different for the two sides of the brain. Neither hemisphere has any awareness whatsoever that it has only received information from half a stimulus, and the split-stimulus technique has the great advantage that information can be projected to the foveal fields of both hemispheres, serving not only to increase the amount of information that can be processed in a brief tachistoscopic presentation, but also enhancing perceptual awareness.

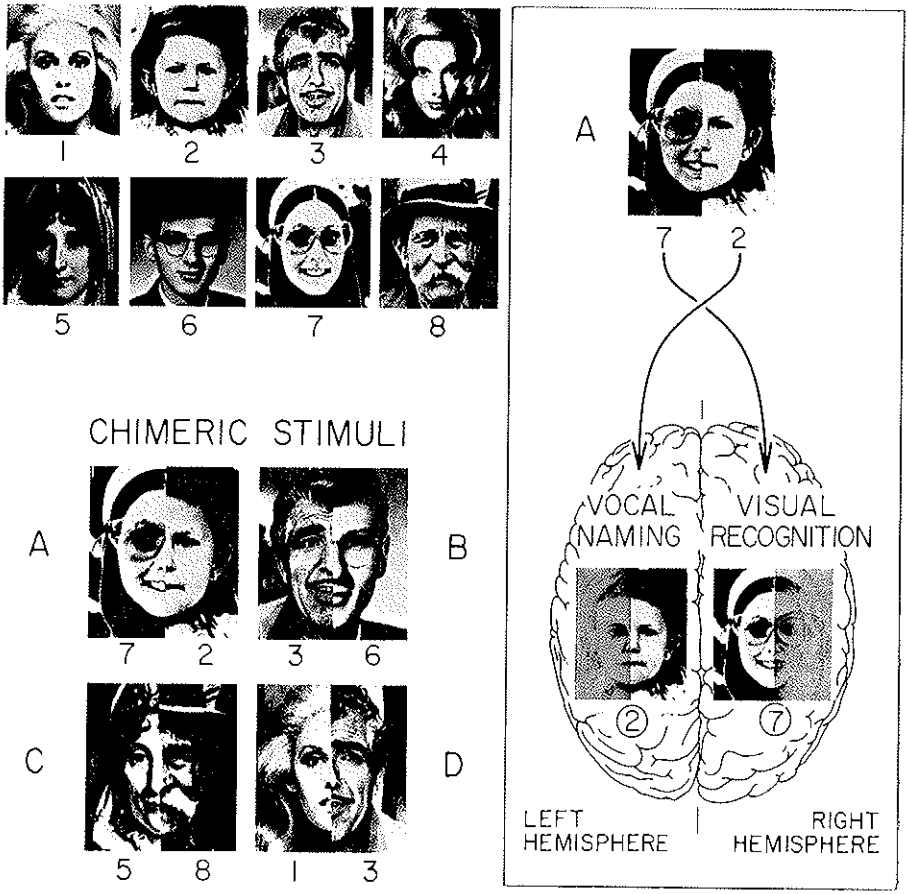

FIG. 1. A test of face recognition given to split-brain patients (reprinted from "Brain", *95*, 61-78, 1972).

Since only the left hemisphere can speak, if a verbal description of the face perceived is requested, the face shown to the left hemisphere is described and the right hemisphere manifests no behavioral responses indicating that it disagrees with the description it hears. If names of faces are taught to the patient (a task split-brain subjects find extremely difficult and can accomplish only by the use of verbal mnemonics such as "Ralph has glasses"), then the left hemisphere typically names the face it sees, though names of right hemisphere faces are given considerably more frequently than chance, suggesting that the right hemisphere is probably capable of occasionally producing spoken words. In the naming task, only three faces were used to construct the split stimuli and the patients only had to learn three names. In spite of the relative simplicity of the task, errors constituted 15% of all responses, indicating the difficulty suffered by the left hemisphere in retaining the facial image sufficiently well to identify it correctly, the difficulty of the right hemisphere in providing spoken names, or both. To learn and remember face-name associations at a highly accurate level may require the integrative functions of both hemispheres. Interestingly, one of the patients, N. G., asked one day whether Dr. Sperry was well, mentioning that she had not seen him in some time. Shortly after being told that he was in good health and was, at that time, somewhere in the laboratory building, N. G. walked out of the testing room and passed Dr. Sperry in the hallway on her way to the water fountain. After returning to the testing room, she asked, "Who was that man in the hallway? He looked kind of familiar, but I don't know who he is". N. G.'s speaking left hemisphere clearly had some form of memory code for the man she called "Dr. Sperry", but that code, evidently, did not include any accurate representation of his face.

In a further test of face recognition, three whole faces were displayed in free vision and patients were told to select from the set of choices a face matching the one they saw in the "machine" (tachistoscope). On half the trials, patients were directed to point to their choice with the right hand and on half with the left. Pointing hand had no effect on responses: on 82% of trials, the right hemisphere matched the face it saw, on 15% the left hemisphere matched the face it saw, and erroneous matches were made on 3% of trials. The very strong dominance of the right hemisphere in a task for which either hemisphere was competent with respect to the simple pointing response required, suggested that the right side of the brain displayed the dominance it did, not because of

any motoric specialization, but rather because of the cognitive demands of the task. It should be noted, also, that the left hemisphere showed no behavioral evidence, verbal or otherwise, of any dissatisfaction with the choice it saw the right hemisphere make. The nonresponding hemisphere behaved as if it were totally unaware of having seen a face.

We could not be certain, in fact, that two different percepts were being generated in the two hemispheres under conditions of bilateral presentation. The response patterns of the patients suggested that an actual perceptual extinction was occurring for stimuli presented in the visual field contralateral to nonresponding half-brain. In an attempt to gain some understanding of the reasons for the apparent extinction, a new series of trials was given, with occasional interruptions of the patients' intended responses. Before a patient could complete a matching response, he was interrupted and told to describe the face seen. The face described was that seen by the left hemisphere, but the face subsequently selected from the set of choices was that perceived by the right hemisphere. On other trials, a patient's intended verbal response would be blocked and he would be asked to point to the face he had seen. The right hemisphere face was selected, but the subsequent description was of the left hemisphere face. These observations demonstrated that sensory information was reaching both hemispheres and was accessible for perceptual processing. However, they did *not* demonstrate that in the *absence* of an interruption, two percepts were actually generated. The interruption itself may have served to activate an otherwise inattentive hemisphere which was then able to retrieve sensory information from a short-term memory buffer and to process it up to the level of perceptual awareness. It would appear that the nature of task instructions selectively aroused one or the other hemisphere, preparing it for information processing and the behavioral response concomitant with that processing, while leaving the other hemisphere inattentive and ill-prepared to process sensory input up to the level of conscious awareness. As Roger Sperry inferred more than a quarter century ago, "Perception is the preparation to respond" (Sperry, 1952).

The only obvious interpretation of our observations is that both hemispheres process task instructions and reach some conclusion regarding their abilities to follow them, that, depending on the conclusion reached, descending signals are sent to the brain stem activating system that elicit ascending arousal to the cortex, and that arousal is asymmetrically directed to the hemisphere whose elicitation commands are dominant. This model

would suggest that a hemisphere's dominance over behavioral pathways is more a function of a hemisphere's imagined than actual abilities. Indeed, subsequent studies lend weight to this conclusion.

For both the face test and a test of nonsense-shape recognition (see Fig. 2), the matching ability of the right hemisphere surpassed the left hemisphere's ability at verbal identification, and for both tasks, the right hemisphere dominated the matching response. However, the right hemi-

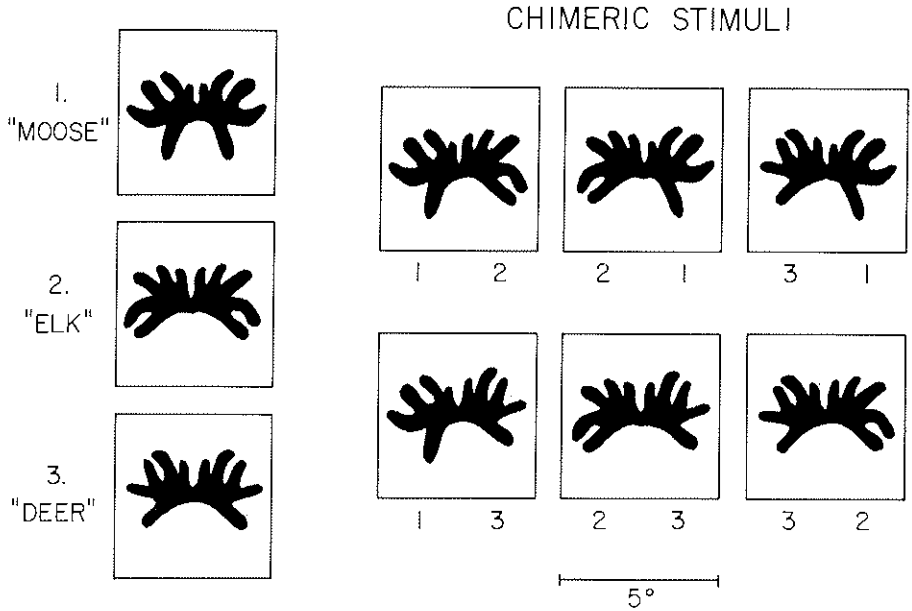

FIG. 2. Stimuli and choices in a test of nonsense-shape recognition administered to split-brain patients (reprinted from "Brain", *95*, 61-78, 1972).

sphere was also strongly dominant for matching pictures of common objects (Fig. 3) and for three-element arrays of X's and squares (Fig. 4), in spite of the fact that on the common objects test, the left hemisphere's verbal identification was as accurate as the right hemisphere's matches, and on the chain pattern test of X's and squares, the left hemisphere's verbal accuracy was significantly *better* than the right hemisphere's matches. It appears that the instruction to make a match between two physically identical visual stimuli is a task for which the right hemisphere believes itself to be particularly competent and that, in consequence, it assumes dominance over behavior. The fact tbat, in actuality, it was

relatively poor at matching patterns whose ordering of elements was arbitrary had no effect on reducing its dominance in taking control of behavior.

In terms of comparative ability, the right hemisphere surpassed the left in discrimination and memory for pictures that were complex and had no verbal labels stored in long-term memory, and the two hemispheres were equal for pictures of common objects with well-known names, con-

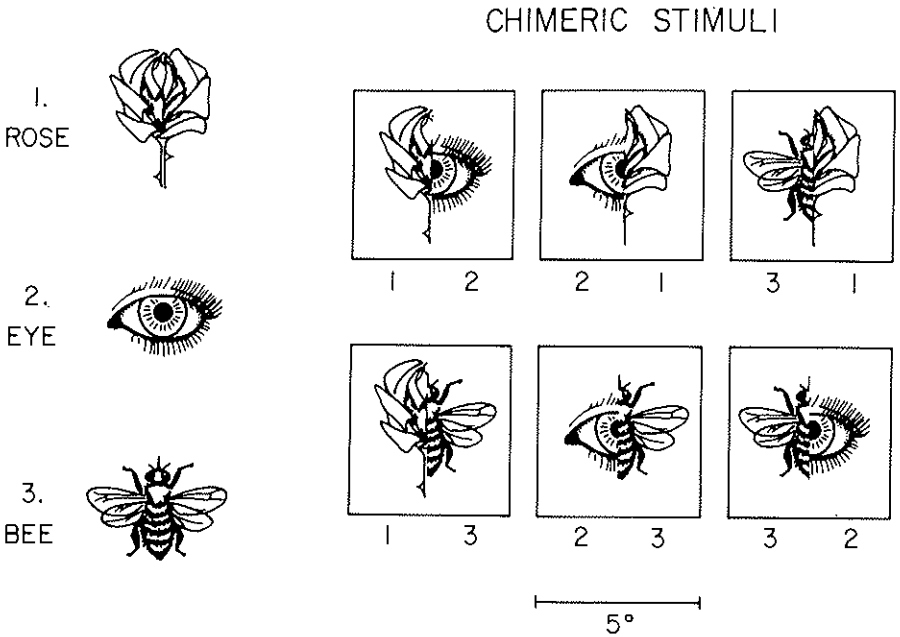

FIG. 3. Items used to test recognition and identification of pictures of common objects in split-brain patients (reprinted from "Brain", *95,* 61-78, 1972).

firming Milner's earlier findings with patients having unilateral cerebral damage (Milner, 1967). The left hemisphere's superiority on the chain pattern test resulted from its better ability at maintaining the order of arbitrarily related stimulus features. The right hemisphere, though excelling in the appreciation of form, appears to extract only those aspects of a visual stimulus that define its meaning and spatial invariants. When stimulus elements are constrained neither hy meaning, by good Gestalt, nor configurational invariants, their ordering in the right hemisphere's representation is often randomly permuted. Such permutations are also seen in the performance of normal, intact individuals.

Perceptual asymmetries to lateralized sensory information are observed in normal people in consequence of cognitive differences in the two sides of the brain. Though the cerebral commissures can convey information to both hemispheres from either side of sensory space, there is a certain degree of information loss entailed in transcommissural transmission which is reflected in asymmetries of perceptual accuracy or reaction time. Thus, if, for example, nonsense syllables are flashed to the left or right visual half-field and subjects are required to pronounce the syllable, the typical right-handed individual is more accurate in identifying syllables projected to the right and received by the verbal left hemisphere. In contrast, if a dot is flashed somewhere in the left or right visual field and subjects must determine its relative location with respect to some frame of reference, a task for whicb the right hemisphere is specialized in right handers, performance will be more accurate for dots projected to the left and received by the right half of the brain. Levy and Reid (1978) found that the proportion of errors in syllable identification that were due to

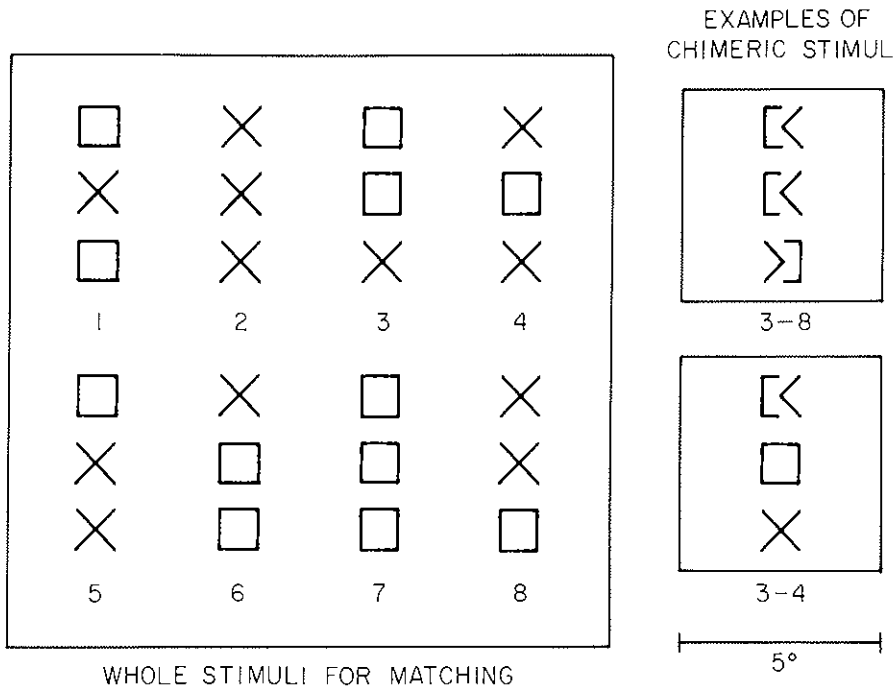

FIG. 4. A test of recognition and identification of chain patterns of X's and squares given to split-brain patients (reprinted from "Brain", *95*, 61-78, 1972).

permutation of the letters was much higher for syllables projected to the nonverbal hemisphere than for those projected to the verbal hemisphere both in normal right handers with left hemisphere language and in left handers with right hemisphere language, findings perfectly in accordance with the split-brain studies. The ability of the verbal hemisphere to retain the order of arbitrarily related stimulus features may be fundamental for its linguistic skills.

In later studies with split-hrain patients, we found the left hemisphere to dominate a matching response when the relation between stimulus and choice depended on semantic (see Fig. 5) or phonetic (see Fig. 6)

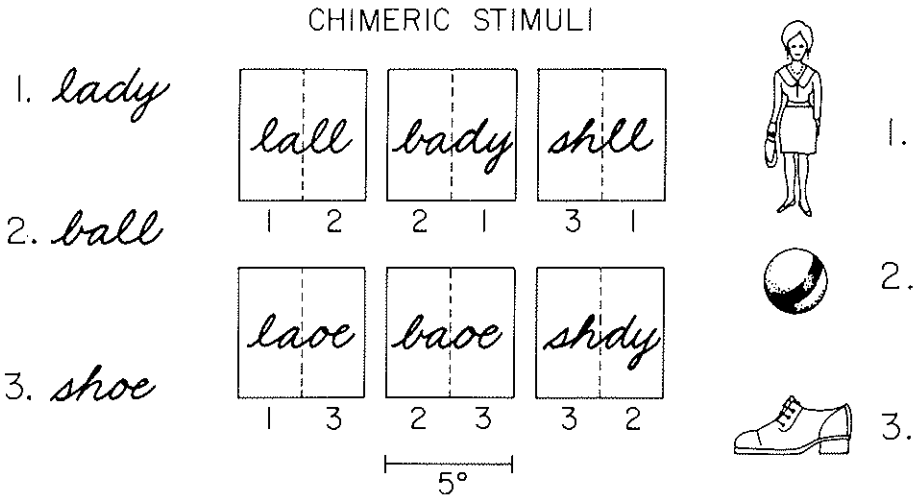

FIG. 5. A test of reading. Split-brain patients were required to point to a picture in free vision named by the word they perceived (reprinted from "Brain", *100*, 105-118, 1977).

associations. When two half-words were projected to the two hemispheres, and patients were required to point to a picture named hy the word, a strong left hemisphere dominance emerged, in spite of the fact that in a non-competitive situation the right side of the brain is perfectly capable of reading at this simple level. The left hemisphere's dominance, therefore, could not be attributed to any incapacity of the right hemisphere, but, instead, must be attributed to its stronger disposition to process words for meaning. In the phonetic test two half-pictures were projected to the two hemispheres, and patients were required to point to a picture in free vision whose name rhymed with the name of the stimulus perceived. Thus, in English, the word pairs "rose-toes", "eye-pie", and

"bee-key" have identical sounds except for the initial consonant, and matching of rhyming pictures depended on the capacity to derive the sound image of each picture and to analyze those sound images for phonetic identities. Not only was the left hemisphere strongly dominant for this task, but in non-competitive conditions, the right hemisphere was found to be totally incompetent. When whole pictures were projected solely

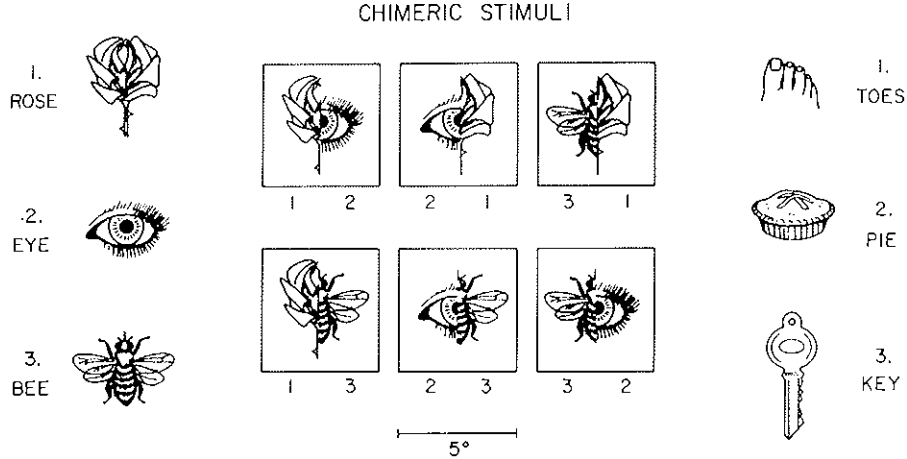

FIG. 6. A test of phonetic analysis. Split-brain patients had to match pictures with rhyming names (reprinted from "Brain", *100*, 105-118, 1977).

to the right hemisphere and patients were asked, "Is it the ..... (rose, eye, or bee)?", they could indicate by a head nod or shake their agreement or disagreement with almost 100% accuracy. However, if they were asked, "Does it rhyme with ..... (toes, pie, or key)?", responses were at chance level. We see, then, that though the right hemisphere can decode spoken language for meaning, it has no capacity for phonetic analysis. It appears that words are processed as acoustic Gestalts which have direct access to the lexicon, with no intermediate analysis of phonetic elements.

This conclusion has been confirmed and expanded by Zaidel who found that though the comprehension vocabulary of the isolated right hemisphere is approximately that of a 12-year-old child (Zaidel, 1976 a), it is almost totally incompetent at pointing to a printed letter representing the initial consonant sound of a spoken nonsense syllable (Zaidel, 1976 b). In addition to the radical differences between the two sides of the brain

in phonetic analysis, the right hemisphere is also quite defective at syntactical processing. Gazzaniga and Hillyard (1971) found that it was unable to distinguish singular from plural, present from future tense, and subject from object in semantically unconstrained sentences. Even in infantile hemiplegics with total left hemispherectomy, though the remaining right hemisphere develops a high-level ability at language production and comprehension, it is likely to interpret passive voice sentences such as, "The cat was chased by the dog", as meaning that the cat chased the dog (Dennis and Kohn, 1975). Zaidel (1977) found that the ability of the right hemisphere to interpret grammatically complex sentences having no semantic constraints was no hetter than that of 4-year-old children. The right hemisphere's language is not the language of the left, and though it is generally mute and deficient at certain other aspects of linguistic processing, it is obvious that the right half of the human brain thinks, and with respect to some cognitive operations, better than does the left.

Lateralization of hemispheric function in the human brain allows for the development of two separate neurological organizations, one in each hemisphere, each designed for the processing and integration of a restricted set of functions different from that on the other side. Perfect symmetry of function, while permitting symmetry of sensory analysis and motoric reactions to the two halves of space, implies complete redundancy of cognitive operations and an effective halving of the neurological space available for higher integrative processes. A cat with only one cerebral hemisphere is hardly less intelligent than one with two (Bogen and Campbell, 1960), but hemispherectomy in man produces disorders of language and its cognitive correlates if the left hemisphere is removed, or of the appreciation of form, nonverbalizable aspects of sensory experience, and spatial relationships if the right hemisphere is removed. The deduplication of function inherent in lateral specialization almost douhles the cognitive power of a brain with no increase in mass. In an animal for whom intelligence is the primary mode of adaptation to its world, cerebral lateralization must have been as strongly selected in evolution as was brain size itself. Quite probably it was a trait that was heginning to emerge some 25 million years ago, before the human line had diverged from that of apes, since anatomical asymmetries are observed in the ape brain homologous with those in man (Yeni-Komshian and Benson, 1976; LeMay and Geschwind, 1975). Such asymmetries are not seen in the monkey brain (Yeni-Komshian and Benson, 1976) and split-brain monkeys reveal no evidence of functional lateralization (Hamilton, 1977 a, h). Thus, in the line of evolution from the insectivores to man, it appears

that only the hominoids were the beneficiaries of cerebral asymmetry and the cognitive advantages it confers.

There is, however, a rather serious conceptual difficulty with the notion that hemispheric lateralization has been subject to selective pressures for so many millions of years, for, if so, the human population would be expected to be as invariant in the pattern of brain asymmetry as it is in its bipedalism or in the capacity to acquire speech. Yet a great deal of evidence has now accumulated to suggest that there is a substantial genetic variation among people in both the degree and direction of cerebral asymmetry, and, quite probably, in the nature of hemispheric specialization itself (see Levy, 1976, for review). In the following section, recent studies are described on individual differences in the patterns of functional brain asymmetry, and speculations are offered regarding the developmental, neurological, and evolutionary factors that might be responsible for these variations.

Individual differences in functional brain organization

It has been known for many years that left and right handers differ in the lateral organizations of their brains. In particular, well over 90% of dextrals have language functions specialized to the left hemisphere and visuo-spatial functions specialized to the right, with only a small fraction displaying either partial bilateralization of cognitive functions or a reversal in direction of cognitive asymmetries. In contrast, left handers are highly heterogeneous, substantial proportions being lateralized in the same direction as most right handers, in the opposite direction, having strong degrees of cerebral asymmetry, and having weak degrees of cerebral asymmetry. Though some of the variation in laterality patterns, both between and within handedness groups, may be due to undiagnosed brain damage suffered during early development that produced a shift in handedness, language lateralization, or both, this cannot be the complete explanation. First, Hécaen and Sauguet (1971) found that though left handers from dextral families almost always had linguistic functions lateralized to the left hemisphere, those from sinistral families were mixed with regard to both the direction and degree of hemispheric asymmetry, suggesting that in the former group, sinistrality resulted from undiagnosed damage to the hand control region of the left hemisphere with language remaining centered on that side of the brain, while in the latter group, the direction and/or degree of asymmetry derived from genetic variation. In an investigation of normal subjects, Carter-Saltzman (1978) confirmed these

observations, finding that familial sinistrals were variable in their brain organization patterns, while nonfamilial sinistrals typically had left hemisphere language. She also found that the familial group was nonsignificantly superior to right handers in their reading skills, but that the nonfamilial group was significantly inferior, data congruent with the interference that familial sinistrals represent normal genetic variants, while nonfamilial sinistrality may often reflect early cerebral pathology. Right hemisphere language or partial bilateralization of linguistic function is more prevalent in both left and right handers having left handed relatives (Luria, 1970; Zurif and Bryden, 1969; Hines and Satz, 1971; Hécaen and Sauguet, 1971; Hicks, 1975) than in those without, additional evidence in support of the genetic origin of much of the observed variation.

Considering only right handers with left hemisphere language and left handers with right hemisphere language, the neurological basis of the hand-brain relationship is fairly obvious. The great proportion of the pyramidal motor fibers are crossed, writing depends on access to the linguistic processing regions, and, in addition, the planning and execution of sequential manual movements are specialized to the verbal side of the brain (Geschwind, 1975; Kimura, 1973). Thus, it is to be expected that the dominant hand is contralateral to the verbally dominant hemisphere. Why people exist in these two varieties is an evolutionary question, and the answer may depend on gaining an understanding of whether and how cognitive structure varies with the direction of brain asymmetry.

A difficult neurological problem arises from the fact that in a small fraction of right handers and in more than half of left handers, the dominant hand is *ipsilateral* to the language dominant hemisphere. Agraphia in conjunction with aphasia is quite common in left handers following unilateral damage to the left hemisphere (Hécaen, Angelergues, and Douzens, 1963), ruling out the possibility that in "ipsilateral" individuals, language is sufficiently bilateralized so that the linguistically subdominant hemisphere is in sole control of the writing hand. If it were, no agraphia would be expected in sinistrals following left-sided damage. The two possibilities that remain are that the final common path to the hand arises from the contralateral hemisphere which receives essential linguistic information via the cerebral commissures from the other side of the brain or that the visuo-motor control entailed in writing and most other skilled unimanual activities is mediated through the direct, uncrossed pyramidal tract. Regardless of whether the first or second proposed mechanism proves to be a valid description of reality, we would still be left with a number of unanswered questions. Under the bihemispheric-

control hypothesis, it would be difficult to understand how such a complex neural program developed, either ontogenetically or phylogenetically. Under the ipsilateral-control hypothesis, we would be confronted with having to explain why manual control is mediated through the smaller, uncrossed tracts rather than through the larger, crossed tracts. Even if we could provide an evolutionary account of the many varieties of functional neural organization seen in the human population, the anatomical pathways mediating manual control in individuals having the dominant hand ipsilateral to the language hemisphere would still be unknown. From our current understanding of neuroanatomy, such people should not exist.

In an attempt to gain some understanding of the extent and nature of variations in manual and cerebral asymmetry in normal populations, Levy and Reid (1976, 1978) examined perceptual asymmetries to lateralized verbal and spatial stimuli in a group of university students classified by handedness, sex, and hand posture during writing. As mentioned, accuracy of perception is higher for stimuli presented in the sensory field contralateral to the hemisphere specialized for their processing. In consequence, the direction and degree of hemispheric lateralization may be inferred from the direction and degree of perceptual asymmetries on tasks requiring the specialized functions of one or the other half-brain.

The verbal test consisted of the presentation of vertically oriented three-letter nonsense syllables to the left or right of a fixation point, with subjects required to pronounce the syllable, a task dependent on phonetic analysis and specialized to the verbal hemisphere. In the spatial task, a dot was flashed in one of twenty locations within a rectangular frame to the left or right of fixation, and subjects had to identify the relative position of the dot from a 4×5 array displayed in free vision. In a pilot group of right-handed males from dextral families, consistent with expectations from the neurological literature and establishing the validity of the two tasks as measures of cerebral asymmetry, all subjects showed a right visual field (left hemisphere) superiority on the syllable test and a left visual field (right hemisphere) superiority on the dot location test.

A total of 25 right handers and 48 left handers was tested in the final experimental sample. Handedness was classified by writing hand except that subjects catagorized as right handed had to manifest a dextral preference also on all unimanual skills assessed. Though all left handers used the left hand for writing, they varied in manual preference for other skills. Figure 7 depicts extreme examples of two different hand postures adopted by individuals during writing. The typical right hander positions his hand below the line of writing and the tip of the pen tends to point

toward the top of the page, a posture also adopted by a substantial fraction of left handers. However, an inverted hand posture is occasionally seen in right handers and quite frequently in left handers, in which the hand is positioned above the line of writing and the tip of the pen tends to point toward the bottom of the page. The critical relationships in classifying hand posture have to do with the position of the writer's hand relative to the line of writing and the direction in which the pen is pointing relative to the top and bottom edges of the page. Whether the hand is bent with respect to the forearm or whether the pen points toward or away from the writer's body is irrelevant and is predominantly a function of how the paper is oriented. The presumed motor programs that guide writing would vary with changes in the relationship between the writing

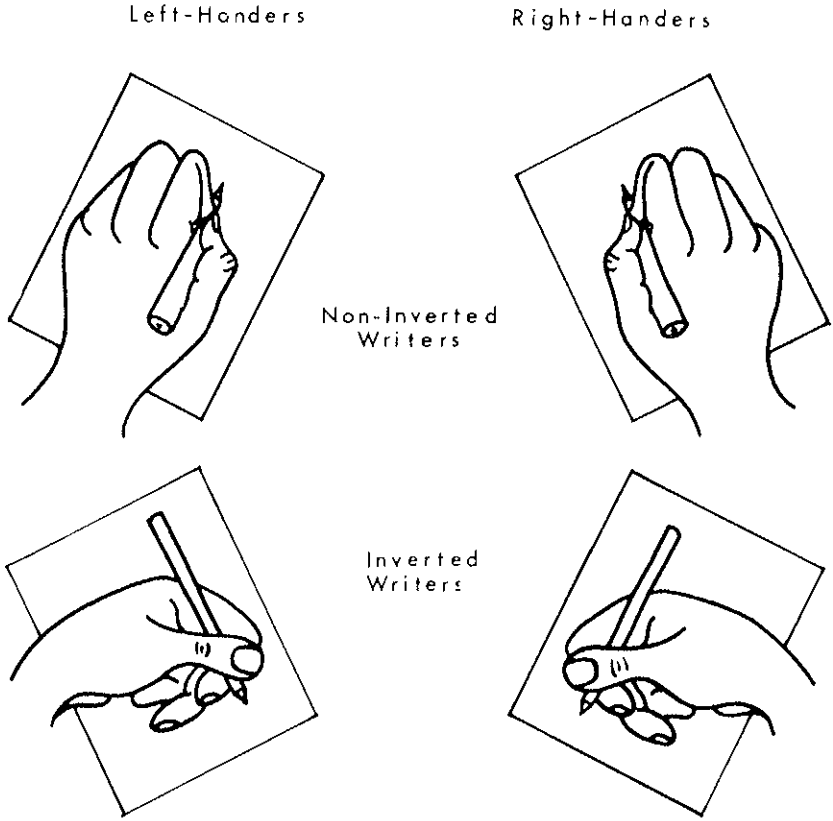

FIG. 7. Extreme examples of the inverted and noninverted hand postures adopted by different individuals. The inverted posture is common in left handers and very uncommon in right handers (reprinted from "Science", *194*, 337-339, 1976).

hand and the line of writing, but not necessarily with changes in the hand's position relative to the body and arm.

We decided to investigate the relation between hand posture and brain organization for several reasons. First, the inverted posture in right handers suggested that the hand position utilized by a writer could not be simply explained as a consequence of the direction of writing in interaction with the hand used for writing. Secondly, this conclusion was strengthened by observations of hand inversion in left-handed Israeli writers while writing Hebrew which proceeds from right to left and in Chinese writers in which writing proceeds from top to bottom. Thirdly, the frequency of hand inversion in left and right handers is close to that of ipsilateral language inferred from the neurological literature, and we thought it not unreasonable to entertain the possibility that when the linguistically specialized hemisphere was ipsilateral to the writing hand, an inverted posture might be adopted. Of the 25 dextrals tested, 24 used the noninverted posture and 1, the inverted posture. Left handers were chosen so that one group of 24 used the noninverted posture and 24, the inverted posture. The inverted right hander (subject RI) was a female. Of the other groups (RN, LN, and LI), half were male and half female.

For the verbal test, the proportion of correct responses for left visual field stimuli were subtracted from the proportion of correct responses for right visual field stimuli, and for the dot location test, the reverse subtraction was made. All difference scores were then normalized (transformed to z-scores) over the entire group of 73 subjects. Figure 8 displays the results. Among male subjects, 100% of right handers manifested a right visual field (left hemisphere) superiority on the syllable test and a left visual field (right hemisphere) superiority on the dot location test, while 100% of left handers with the noninverted posture (group LN) showed the reverse. Left handers with the inverted posture (group LI) were lateralized in the same direction as right handers, but to a significantly smaller degree as seen by the fact that their scores are closer to the axes of symmetry. Females in all groups (RN, LN, and LI) displayed smaller asymmetries than males, but the same group-dependent directions and degrees of asymmetry. Subject RI was the only right hander to have a left visual field (right hemisphere) superiority on the syllable test, as well as a right visual field (left hemisphere) superiority on the dot location test. When lateralization was indexed by the average of the two z-transformed difference scores, 70 out of 73 subjects yielded indices consistent with group means. One LI male and two LI females failed to show

the left hemisphere language/right hemisphere spatial lateralization typical of their group. Group LI, however, was so weakly lateralized that any unreliability or lack of perfect validity in the measures could easily produce erroneous results, i.e., the error variance for this group, given the small asymmetry observed, was necessarily larger than for other groups.

Reid (1978) subsequently applied two tactile measures of cerebral laterality to groups of 5- and 8-year-old children classified by handedness, hand posture, and sex, replicating the results of Levy and Reid (1976, 1978). Smith and Moscovitch (1978) also confirmed the relation between hemispheric lateralization and hand posture on visual tests similar to those of Levy and Reid, but found no association using a verbal test of dichotic listening. Whether the failure to find the hand posture effect with dichotic tests reflects invalidity or unreliability of the dichotic measure, or whether there is a modality-specificity in the relationships that obtain, is unknown. Of possible relevance is the fact that Milner (personal communication) found no relationship between hand posture and cerebral

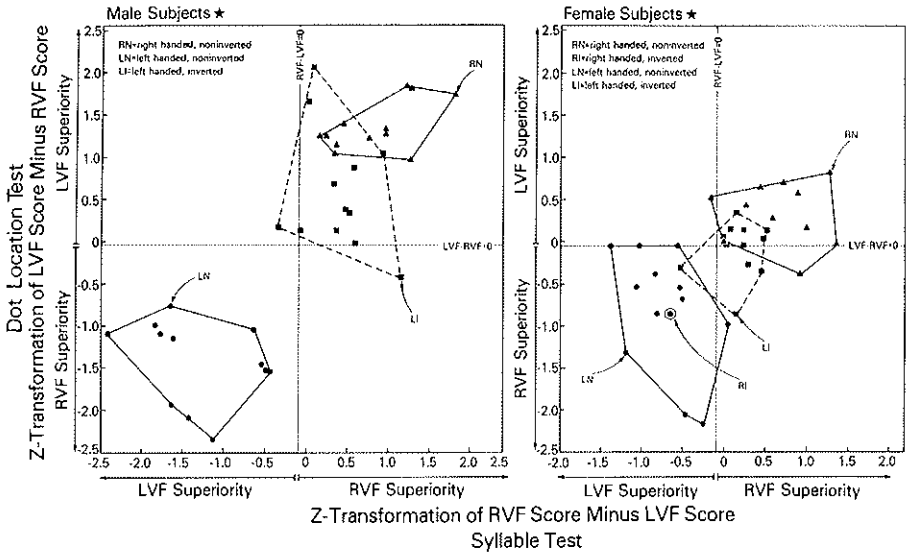

FIG. 8. Normalized perceptual asymmetry scores of individual subjects classified by handedness, hand posture during writing, and sex. Scores to the right of the vertical axes indicate a right visual field (left hemisphere) superiority on the verbal test, and scores above the horizonal axes indicate a left visual field (right hemisphere) superiority on the spatial test (reprinted from J. LEVY, *Lateral differences in the human brain in cognition and behavioral control*, in "Cerebral Correlates of Conscious Experience", P. A. Buser and A. Rougeul-Buser, eds., Amsterdam, Elsevier/North-Holland, 1978).

lateralization for speech, as assessed by the Wada test, in a group of epileptic patients. These results must be interpreted with caution, however, since pathological sinistrality is considerably higher in epileptics than in normal populations and since the magnitude and effect of sociocultural pressures on both handedness and hand posture are unknown in this group. Nonetheless, the possibility must be borne in mind that the relationships among handedness, hand posture, and cerebral organization differ as a function of the modality of specialized hemispheric processes. Conceivably, the acoustic-articulatory aspects of language differ from the visual-kinesthetic aspects of language in some subgroups of the population in terms of their hemispheric organization. It is obvious that individuals having linguistic functions specialized to the hemisphere ipsilateral to the dominant writing hand have an unusual brain organization, and functions that may be almost invariably associated in the majority of people may be dissociated in this group.

Using chronometric analyses in which manual reaction times to lateralized stimuli were compared for the two hands and sensory fields, Moscovitch and Smith (1978) attempted to determine the neural pathways for hand control in people having the noninverted or inverted hand postures. Earlier work by other investigators had already shown that under proper experimental conditions, chronometric analyses could be used to infer the anatomical pathways used in manual reaction times. In particular, reaction times were found to be faster when the responding hand was ipsilateral to a lateralized stimulus by about 3 to 10 msec, even if the response key itself was on the opposite side of space from the stimulus and, thus, required the subject to depress a key on the left with the right hand or a key on the right with the right hand (Berlucchi, Crea, Di Stefano, and Tassinari, 1977). The reaction time superiority for ipsilateral hand-stimulus conditions is to be expected since the hemisphere receiving the stimulus is the same hemisphere that controls the hand when the motor pathways to the hands are crossed. When a stimulus and responding hand are contralateral, no response can he initiated until stimulus information is transcommissurally conveyed to the other hemisphere, entailing a delay of a few milliseconds. For visual stimuli, the anatomical effect can be detected both in simple reaction time tasks in which a fixed response is always given on every trial within a block to a fixed stimulus, as well as in the Donders' *c*-reaction (Donders, 1868) in which a fixed response is given to one class of stimuli and no response to another class of stimuli. In discriminated reaction time tasks, in which one response must be given to one stimulus and another response to another stimulus, anatomical

effects cannot be observed since they are overwhelmed by the much larger effect of spatial compatibility between the location of a stimulus and the location of a response key. For tactile and acoustic stimuli, the anatomical effect can only be detected in simple reaction time tasks, spatial compatibility effects dominating in both the *c*-reaction and discriminated reaction time tasks.

In the Moscovitch and Smith (1978) study, the *c*-reaction paradigm was used for visual, tactile, and acoustic stimulation. In response to tactile and acoustic stimuli, all subjects, regardless of hand posture, had faster reaction times to ipsilateral hand-signal conditions, but in response to visual stimulation, subjects with the inverted hand posture had faster reactions to the *contralateral* hand-signal conditions. The only simple interpretation of these latter results is that these subjects were relying on the uncrossed motor pathways for control of the manual response. The failure to observe the contralateral superiority to tactile and acoustic stimuli can be attributed to spatial compatibility effects which overrode the anatomical pathway effect, though Moscovitch and Smith suggest that their results imply a peculiar visuo-motor organization. The resolution of this question will require the use of a simple reaction time paradigm where spatial compatibility effects are directly assessed by having subjects respond under two conditions: with the response key ipsilateral to the responding hand and with the response key contralateral to the responding hand. Relative reaction times as a function of anatomical pathways should remain invariant under the two conditions, but should be opposite for the two conditions to the extent that spatial compatibility effects are operative. It might be noted that the Moscovitch and Smith results on visual reaction times have been replicated by Day (personal communication, 1978) who also used the *c*-reaction paradigm, but subjects in the Day experiment were run in both a hands-crossed and hands-uncrossed condition to control for possible effects of spatial compatibility. There was no effect of hand position relative to location of the response key, confirming that the observed reaction times actually derived from the arrangement of anatomical pathways. The only difference in Day's results versus those of Moscovitch and Smith was that the superiority of the contralateral hand-signal conditions in subjects with the inverted hand posture failed to reach significance, though the interaction between hand-signal relationship and hand posture was highly significant.

If the hands are controlled by uncrossed motor pathways in individuals using an inverted hand posture, one possible explanation is that in these

subjects the direct pyramidal tracts are larger than the crossed tracts. However, under this assumption, homolateral hemiplegia should be observed in about half of all left handers following unilateral lesions. Not only is this not so, but Milner has never observed an instance of ipsilateral paralysis following brief anesthetic inactivation of one cerebral hemisphere in any of the epileptic patients submitted to the Wada test (personal communication). One must conclude, therefore, that even in people whose language-dominant hemispbere is ipsilateral to the writing hand and who, according to the Levy and Reid (1976, 1978), Reid (1978), and Smith and Moscovitch (1978) studies, use an inverted hand posture, the majority of motor fihers, as is the case in other individuals, are crossed. Given this conclusion and the findings of Moscovitch and Smith (1978) and of Day (1978), we are led to infer that, for unknown neurological reasons, visuo-motor control of the hands in that subgroup of the population using an inverted hand posture, is mediated via the smaller, uncrossed pyramidal tracts, and not, as some have suggested, via a transcallosal pathway. The available data are not incompatible with the view that spontaneous manual activity that is not under guidance of visual input may depend on ordinary crossed control, but they strongly suggest that visually guided manual performance, including writing, drawing, or any other manual behaviors that rely on visual feedback, is controlled in this subgroup by the ipsilateral hemisphere by means of the ipsilateral motor tract. It is as if the visual processing regions of the hemispheres have access only to those motor pathways whose projections are uncrossed, and it is likely, given the Reid (1978) observations, that the same may be true of the somesthetic processing regions of the hands. If kinesthetic feedback from hand movement projects to the contralateral hemisphere, and if, further, that information must be transcallosally conveyed to the ipsilateral hemisphere before it can be utilized to modulate manual activity, an unusually complex neural pathway must underlie kinesthetic-motor integration. Perhaps the inverted hand posture is an adaptation to this integrative program and functions to amplify, in some way, kinesthetic signals. Considerably more research will be required before any real understanding of the relationship between ipsilateral visuo-motor control and the inverted hand posture will be obtained.

With respect to the question regarding the cognitive implications of variations in the degree and direction of cerebral asymmetry, a few suggestions are beginning to emerge. Levy (1969) had found that in a group of graduate science students, selected for very high verbal-analytic abilities, left handers had an unusually large discrepancy between verbal and spatial

skills, being relatively better than right handers in the former and worse than right handers in the latter. Among architects, Peterson and Lansky (1974) found that left handers, rather than being inferior to right handers in spatial ability, were greatly superior. In the Levy and Reid study (1976, 1978), the weakly lateralized left handers in the inverted hand posture group (group LI) displayed large discrepancies in performance on the syllable and dot location tasks, some being superior in one and some being superior in the other. The various observations, though meager, suggest that weakly asymmetric brains may either have bilateralized verbal functions, enhancing verbal ability to the detriment of spatial ability, or bilateralized spatial functions, resulting in the opposite cognitive effects. Small degrees of lateralization may, in other words, be correlated with cognitive specialization for either verbal or spatial processes.

Until very recently there was no evidence to suggest that variations in the *direction* of hemispheric specialization had any cognitive consequences, and, indeed, from a conceptual standpoint, a mirror reflection of a typical right hander should be cognitively identical to the right hander himself. Though parity may be overthrown in the world of elementary particles, it is not overthrown in the world of brains. The real question, then, is whether left handers with right hemisphere language are actually mirror reflections of right handers with left hemisphere language.

A number of studies have been consistent in showing that among right-handed children, the left hemisphere of girls and the right hemisphere of boys mature more rapidly than the other side of the brain (Rudel, Denckla, and Spalten, 1974; Witelson, 1976; Kimura, 1967; Bryden, 1970; Pizzamiglio and Cecchini, 1971; Van Duyne and D'Alonzo, 1976), findings consistent with the generally found female superiority in verbal fluency and the male superiority in maze tasks and spatial visualization. If left-handed children with right hemisphere language are perfect mirror images of right-handed children with left hemisphere language, then in the former group, it should be the right, verbal hemisphere of girls and the left, spatial hemisphere of boys that show the more rapid maturation, preserving the sex-dependent invariance of cognitive maturation and structure. If, on the other hand, the earlier maturing hemisphere is always the left in girls and the right in boys, independently of the direction of cerebral asymmetry of function, cognitive development and structure within a given sex would vary as a function of the direction in which the brain is lateralized. In this case, a verbal right hemisphere would not be equivalent to a verbal left hemisphere, nor would a spatial left hemisphere be equivalent to a spatial right hemisphere.

Reid (1978), as discussed, confirmed the handedness and hand posture effect on brain organization in children. However, she also found that in her younger group (children 5 years of age), hemispheric asymmetries emerged on only one of the two tasks administered. The lack of asymmetry was correlated with relatively poor performance and was indicative of immaturity of the hemisphere specialized for the task. In children with left hemisphere language, girls manifested a left hemisphere superiority on a task designed to test memory for temporal patterns, a specialized function of the verbal hemisphere, and no asymmetry on a spatial task, while boys showed a right hemisphere superiority on the spatial task and no asymmetry on the temporal pattern task. On standardized cognitive tests, the girls excelled on verbal measures and boys on spatial measures. These results are in complete congruence with those of others on sex differences in the rate of left and right hemisphere development. In children with right hemisphere language (group LN), Reid found a reversal of the usual cognitive sex differences, girls excelling on spatial function and boys on verbal function. Further, the left, spatial hemisphere of girls was superior to the right hemisphere on the spatial laterality task, and no hemispheric asymmetry was seen on the temporal pattern task. In boys, the right, verbal hemisphere was superior to the left hemisphere on the temporal pattern task, and there was no hemispheric asymmetry on the spatial task.

The Reid data show that, regardless of the direction of hemispheric lateralization, it is always the female left and the male right hemisphere that have the developmental advantage. In consequence, within neither sex, is there an equivalence of organization for a verbal right versus a verbal left hemisphere, nor for a spatial left versus a spatial right hemisphere. "Mirror image" left handers are not, in fact, laterally reversed reflections of right handers. The direction in which the brain is lateralized evidently matters due to the sex-dependent asymmetry of hemispheric development. Verbal functions are enhanced in girls with left hemisphere language and boys with right hemisphere language and spatial functions are enhanced in the opposite groups.

Some speculations on human variation

The patterns of cerebral asymmetry in the human population are various, differing with respect to the direction and degree of hemispheric lateralization and differing with respect to their relations with handedness and sex. These variations appear to have important cognitive conse-

quences, affecting the generality or specificity of cognitive abilities, as well as relative capacity for processes specialized to the two half-brains. In a social species, differences among members in propensities, skills, and capacities would serve to increase group cohesion and stability, reinforcing the social bond through mutual interdependence. While the human animal is highly plastic in terms of what he can learn to do and the social roles he can come to fill, his plasticity is not infinite, and more importantly, even if he assumes a particular role and does so with competence, this does not assure that he is self-fulfilled. Tbe freedom to exercise those abilities for which one has a special propensity and special skill brings benefits beyond those conferred on the group. It brings satisfaction to each member of the culture and a powerful strengthening of social structure. If biology could assure that a social species was composed of members innately disposed and competent to fill the various social niches necessary for the adaptive fitness of the species, it would surely do so.

In the case of human beings it is not difficult to imagine how a non-teleological evolution could have accomplished precisely this end. Selective pressures emerging from the social group itself would serve to protect rare and valuable skills. Such rare individuals would, in consequence, have a higher probability of survival and procreation than the more common members of the group. The result, of course, would be a progressive increase in the frequency with eacb generation of people having the once-rare skills. Yet, as rarity decreased, so too would the special benefits conferred by the group. By means of such frequency-dependent selection, a balanced polymorphism could be reached in whicb the cognitive variations among people would serve group fitness and social stability. It may be that our humanity is intimately tied, not only to the functional asymmetry of our cerebral hemispheres, but to the many variations of its manifestations.

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DISCUSSION

PURVES

There is one thing you said that I am confused about. You talked about left-handers who have bilateralization of verbal abilities and score well on verbal IQ tests.

LEVY

These left-handers were a highly selected group, namely, graduate science students at Caltech.

PURVES

Were they deficient in spatial ability? The implication is that there is really not room in a hemisphere for both functions.

LEVY

There is no conflict between the notion that verbal organization of a hemisphere interferes with spatial organization and the findings with infantile hemisplegics. Infantile hemisplegics with hemispherectomy are not, in fact, cognitively normal. Their average IQ's are far below normal, and while some of the IQ decrement may be due to pathology, I do not believe this is the whole story. When one cerebral hemisphere is compelled to bear the burden of two, I believe there is a profoundly retarding effect on the level of cognitive development that can be reached.

PURVES

Since the brain is so remarkably adaptable, it still seems odd that if a "normal" person has language on both sides his spatial abilities should be diminished.

LEVY

I see nothing odd in the notion that bilateralization of verbal functions should interfere with spatial abilities. A cerebral hemisphere is a finite computer, and if we have a set of neural networks that is optimally organized to

serve one set of functions, there is no reason to suppose that it is also equally competent to serve another set of functions. I am suggesting that the neural organizations for verbal and spatial functions are different and that if a particular organization is designed for verbal operations, then, by definition, it is not designed for spatial operations. One cannot do an infinite number of things with a finite number of neurons.

LEVY

I do not think it is correct to think that higher cognitive functions can be localized in one particular area *within* a cerebral hemisphere. Studies of split-brain patients strongly suggest that each hemisphere functions as a whole and complete brain; there is no other obvious explanation for the fact that functional integrity remains so high following total neocommissurotomy. In contrast, no region *within* a cerebral hemisphere functions as a complete brain. The normality of function of the isolated hemisphere is presumed to be due to the fact that interactions among all regions of that hemisphere are still possible. Though particular areas within a hemisphere may be especially critical for a given function, I believe it is incorrect to say that the function itself is actually located in that region. Integrity of function, in my view, depends on the integrated activities of all regions within a hemisphere, and, in consequence, one could not confine verbal abilities to one region and spatial abilities to another. As Dr. Milner's work has shown, even the hippocampus is laterally specialized, left hippocampectomies interfering with verbal memory and right hippocampectomies interfering with memory for nonsense shapes. My central point is that if a hemisphere is partially organized for verbal functions, to that extent it is incompletely specialized for spatial functions.

HAMBURGER

I would expect among monozygotic twins a very high percentage of both brain and handedness asymmetry. Is that true?

LEVY

In monozygotic twins, there are two factors that affect the laterality pattern that are not operative in most singletons. One is the increased pre- and perinatal pathology associated with twinning and that also occurs in dizygotic twins. The second is ectodermal *situs inversus*, an effect that is confined to MZ twins. In some MZ twins, one sees mirror-imaging of fingerprint patterns, birthmarks, handedness, and probably cerebral asymmetry. Perinatal

stress can produce brain damage that leaves no obvious neurological signs, but that induces a developmental reorganization of hemispheric laterality patterns. With respect to mirror-imaging, Charles Boklage has examined the concordance rate for schizophrenia in MZ twins who were concordant for right-handedness or not since studies in recent years have shown that schizophrenia is associated with a specific left hemisphere disorder. In twins concordant for right-handedness, 92% of the pairs were also concordant for schizophrenia, but of those who were not concordant for right-handedness, only 25% were concordant for schizophrenia. These observations suggest that cerebral mirror-imaging, superimposed on genetically controlled developmental lateralization of the brain, may have resulted in bilateralization of functional organization so that even if the left hemisphere were disordered, the right hemisphere could subsume its functions, protecting from manifest schizophrenia. The Boklage analysis is the only direct evidence we have to date that MZ twinning can induce mirror-imaging of cerebral laterality, but this conclusion is totally congruent with the fact that left-handedness is greatly increased in MZ twins compared to both singletons and to DZ twins. We clearly need more data, but Dr. Hamburger's suggestion that laterality patterns should differ in MZ twins compared to others is certainly confirmed by observation.

WEISKRANTZ

Going back to chimeric figures; we started a conversation about one question informally but never finished it. Has there been any information of this sort on cases of agenesis of the corpus callosum?

LEVY

I do not have any personal observations on adult patients with callosal agenesis except for one case I observed at Caltech. However, Roger Sperry and Ron Saul have examined some of these patients.

CORTICAL AND SUBCORTICAL ROUTES FOR VISUAL INTERHEMISPHERIC TRANSFER IN THE CAT

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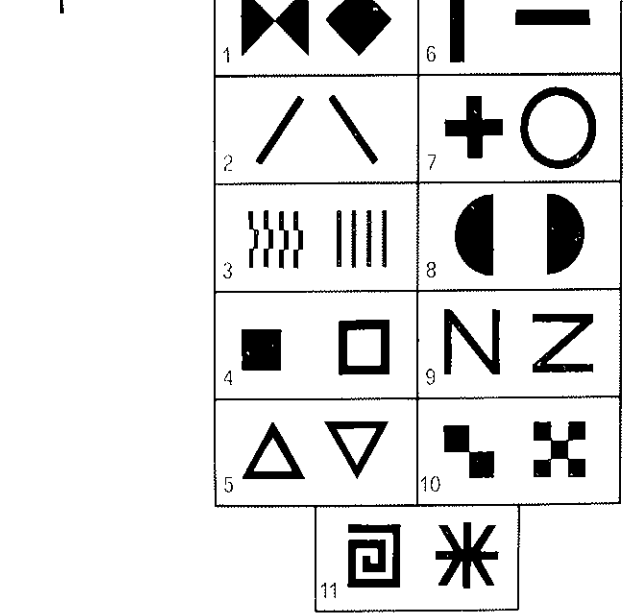
ACKNOWLEDGEMENT

A cat with a midsagittal section of the optic chiasm (split-chiasm preparation) can learn visual pattern discriminations using one eye only, and can subsequently transfer the learned response to the untrained eye. However, if the corpus callosum is sectioned in addition to the optic chiasm (split-brain preparation), monocular learning is still possible, but interocular transfer fails, in that the rate of relearning with the second eye is similar or identical to the rate of learning with the first eye (see Fig. 1). This outstanding experiment, first performed by Myers and Sperry (1953; see also Myers, 1955, 1956; Sperry *et al.*, 1956), shows the crucial importance of the corpus callosum for the interhemispheric communication of a visual input restricted to one hemisphere. Subsequent work in split-brain primates, including man, has confirmed the primary role of the corpus callosum in the interhemispheric processing of visual information, thus lending generality to the original findings in the cat (see Sperry, 1968, 1974; Gazzaniga, 1970).

Several anatomical and electrophysiological studies have provided evidence potentially useful for the analysis of the neural bases of visual interhemispheric transfer in the cat, and I have reviewed this evidence elsewhere (Berlucchi *et al.*, 1972). This analysis has recently been deepened by attempting to identify the cortical areas specifically involved in the transfer mechanism, and by investigating the possibility of a participation of subcortical centers to such a mechanism. This work is briefly reviewed in what follows.



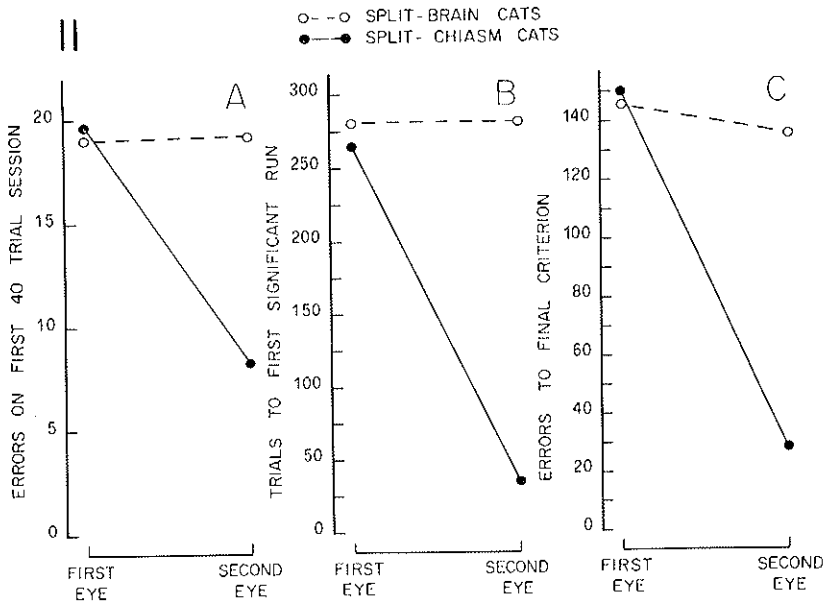


FIG. 1. Interocular transfer of pattern discriminations in split-chiasm and split-brain cats. I (above) shows the pairs of pattern used for discriminations. II (below) shows the mean performance of four split-brain cats and four split-chiasm cats on tests of interocular transfer of the discriminations shown in I. A shows the performance with the first eye and the second eye on the initial session of training (data are errors in a 40-trial session); B shows the number of trials required for performing the first significant sequence of correct responses (chance probability equal to or lower than .01) allowing for one error; C shows the number of errors required for attaining the final criterion of learning (two consecutive 40-trial sessions with at least 36 correct responses in each sessions). Note successful interocular transfer on all measures for the split-chiasm cats (continuous line) and absence of transfer in the split-brain cats (interrupted line). In the latter cats the scores for the two eyes on each measure are almost identical (from Berlucchi *et al.*, 1978a).

VISUAL INTERHEMISPHERIC TRANSFER AFTER SELECTIVE CORTICAL LESIONS

Among the cortical areas with visual functions (see Sprague *et al.*, 1977), areas 17, 18 and 19, as well as the lateral suprasylvian area, are known to project contralaterally via the corpus callosum. Visual information transmitted via the corpus callosum from areas 17, 18 and 19 appears to be limited to a relatively narrow region of the visual field hordering on the vertical meridian (see Berlucchi *et al.*, 1972; Shatz, 1977 a, b). By contrast, visual callosal information issuing from the lateral suprasylvian area probably originates from cortical areas representing a larger extent of the visual field (Heath and Jones, 1970, 1971). Similarly, callosal connections of the areas on the convexity of the suprasylvian gyri (areas 7, 20 and 21) are likely to be instrumental in transposing a large portion of their representation of the contralateral visual field onto the homotopical areas of the opposite hemisphere (see e.g. Dow and Dubner, 1971; Sanides, 1978). Since these various cortical areas participate in the integration of visually guided behavior (see Sprague *et al.*, 1977), one wonders whether any of them is selectively involved in the emission and the reception of the callosal information necessary for the interhemispheric transfer of visual discriminations.

In an attempt to test this possibility, split-chiasm cats with unilateral or bilateral lesions of areas 17, 18 and 19 have been submitted to tests of interocular transfer of monocularly learned pattern discriminations (see Berlucchi *et al.*, 1978 c). It will be recalled that areas 17, 18 and 19 are the recipients of most projections from the lateral geniculate nucleus, one of the main targets of the retinal input (see Sprague *et al.*, 1977), and that these areas contain neurons with highly differentiated capacities for processing visual information (Hubel and Wiesel, 1962, 1965). Learning of visual pattern discriminations was retarded in split-chiasm cats with tbese cortical lesions, as compared with split-chiasm cats witb an intact cortex, only if area 19 was heavily encroached upon by the lesion. Learning in split-chiasm cats with lesions limited to areas 17 and 18 was within the limits typical of control split-chiasm cats, in agreement with data obtained in cats with intact optic pathways (see Sprague *et al.*, 1977). However, regardless of the presence or absence of a learning deficit, interocular transfer of pattern discrimination was as successful in split-chiasm cats with these cortical lesions as in control split-chiasm cats, even though the sites of origin and termination of the callosal connections of

areas 17 and 18, and in some cases of area 19 as well, had been removed by the cortical ablation (Fig. 2).

A different result was obtained when the cortical lesion spared areas 17 and 18 and invaded areas in the middle and posterior suprasylvian gyri (Berlucchi *et al.*, 1979, see Fig. 3). Split chiasm animals with unilateral suprasylvian lesions showed a marked learning deficit when using the eye on the side of the lesion and, more interesting, were largely unable to transfer pattern discrimination learned with the eye on the intact side to the eye on the injured side. A high degree of transfer was instead observed when the eye on the injured side was trained first and the intact side received the transfer.

Preliminary results (Antonini, Berlucchi and Sprague, in preparation) suggest that bilateral suprasylvian lesions in split-chiasm cats have an effect on interocular transfer similar to that of a callosal section, i.e. they suppress transfer.

These results argue for the dependence of visual interhemispheric transfer on the callosal input to visual areas in the suprasylvian gyri, rather than on the callosal connections of areas 17, 18 and 19. This conclusion is in agreement with studies of interocular transfer in cats lacking the normal convergence of binocular information onto neurons in areas 17 and 18. In Siamese cats, for example, the visual input to areas 17 and 18 on each side is largely limited to the contralateral eye; yet these cats show a normal interocular transfer (Marzi *et al.*, 1976), presumably because convergence of binocular information takes place in other brain areas, such as the superior colliculus and at least some suprasylvian cortical area (Antonini, Di Stefano, Marzi and Legg, in preparation). The hypothesis that visual interhemispheric transfer in the cat relies on callosal connections of cortical areas different from 17 and 18 also receives indirect support from parallel findings in monkeys, showing that transfer is sustained by inferotemporal cortical areas (Gross and Mishkin, 1977). These cortical areas in the monkey resemble the suprasylvian areas of the cat in that they receive a visual input both from visual cortical areas innervated by the lateral geniculate nucleus, and from posterior thalamic nuclei receiving projections from superior colliculus and pretectum. In addition, inferotemporal cortex in the monkey and suprasylvian areas in the cat return fibers to the superior colliculus (Kawamura *et al.*, 1974; Kuypers and Lawrence, 1967). This brings us to the consideration of a possible role of the superior colliculus in visual interhemispheric transfer in the cat.

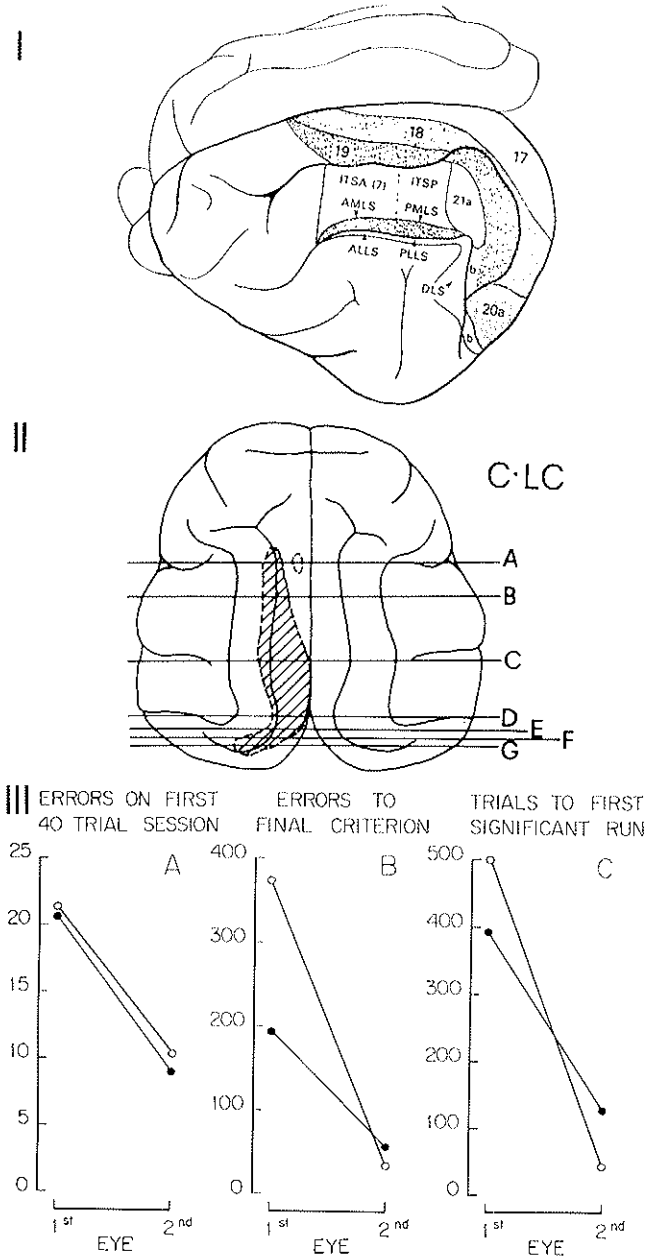


FIG. 2. Effects of cortical lesions on interocular transfer in split-chiasm cats. I shows cortical areas important for visually guided behavior in the cat (from Sprague *et al.*, 1977). II shows a case of unilateral lesion of areas 17, 18 and 19. III shows mean scores for interocular transfer in cats with a unilateral 17, 18 and 19 lesion. For measures of transfer see previous Figure. Note that learning is worse on the injured side, but transfer is successful regardless of whether the eye trained first is on the injured side or on the intact side (from Berlucchi *et al.*, 1978c and 1979).

SUPERIOR COLLICULUS AND CROSSED TRANSFER OF VISUAL INFORMATION

The absence of interocular transfer shown by split-brain cats on pattern discriminations may not occur with other visual tasks. Meikle and Sechzer (1960) and Meikle (1964) described positive interocular transfer of a simple light-dark discrimination in cats after a chiasmatic and callosal section. Interocular transfer of this discrimination was absent in split-chiasm cats with a much more radical midline transection affecting telencephalic as well as most diencephalic commissures (Meikle, 1964). It may be suggested that the function of a cortical area or a subcortical center in visual discrimination can be inferred from the effect of the section of their respective commissures on interhemispheric transfer of different visual tasks. According to this suggestion, pattern discrimination would be mediated by the cortex, since it cannot be transferred between the hemispheres after a section of the cortical commissures, whereas a light-dark discrimination would be mediated by subcortical visual centers, since it can be transferred between the hemispheres after section of the cortical commissures but not after section of the subcortical commissures. On this line of reasoning, one would expect that a section of the subcortical commissures leaving the corpus callosum intact has no

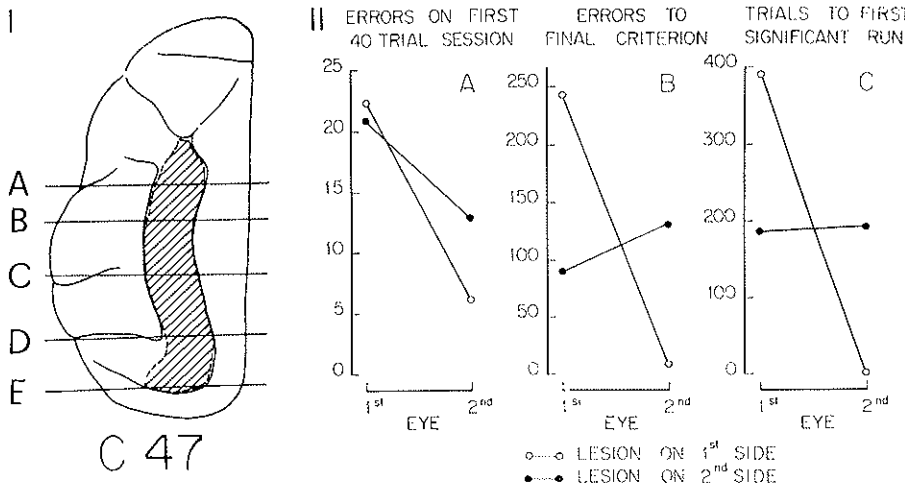


FIG. 3. Effects of cortical lesions on interocular transfer in split-chiasm cats. I shows a case of unilateral suprasylvian lesion. II shows scores for interocular transfer as in Figs. 1 and 2. Note that learning with the eye on the injured side is greatly retarded compared with learning with the eye on the intact side. Transfer to the eye on the intact side is successful, but there is no transfer in the opposite direction (from Berlucchi *et al.*, 1979).

effect on interhemispheric transfer of visual pattern discriminations, and indeed a normal interocular transfer of pattern discriminations was observed in split-chiasm cats with a section of the posterior and tectal commissures, but with intact cortical commissures (see Berlucchi *et al.*, 1978 b). Yet the simplistic allocation of visual pattern discrimination to the cortex and of brightness discrimination to subcortical visual centers such as the superior colliculus (see e.g. Marquis, 1932, Klüver, 1942) is hard to reconcile with several lines of evidence, which argue instead for an interaction of cortex and subcortical centers in the mediation of all kinds of visually guided behavior (see Berlucchi *et al.*, 1972; Sprague *et al.*, 1977).

Recent work by Antonini *et al.* (1978, 1979 a) indicates that section of the corpus callosum affects the cross-midline transmission of visual information not only at the cortical level but also at the level of the superior colliculus, thus indirectly suggesting a possible participation of this subcortical center in the callosum-dependent interhemispheric transfer of visual discriminations. Recordings from the anterior portion of the superior colliculus in split-chiasm cats showed that most neurons had a receptive field in both eyes (Antonini *et al.*, 1978). While the receptive field in the ipsilateral eye could be mediated by uncrossed retinotectal projections, the receptive field in the contralateral eye depended on an indirect across-the-midline connection between that eye and the superior colliculus used for recording, since all direct connections had been severed by cutting the optic chiasm (Fig. 4). The simplest hypothesis, i.e. that these indirect connections run in the commissure of the superior colliculi, was disproved by the finding of a persistence of binocular responses in the SC of split-chiasm cats with a section of such commissures. On the contrary, responses to visual stimulation of the contralateral eye were virtually absent in the SC of split-chiasm cats with intact tectal commissures, but with a section of the posterior two-thirds of the corpus callosum (Fig. 5; see Antonini *et al.*, 1979 a).

Similarly, in cats with a unilateral optic tract transection a callosal section caused the disappearance of visual responses of neurons of the superior colliculus on the side of the severed optic tract. When present, these responses obviously must depend on the optic tract projecting to the other side of the brain, and on a cross-midline connection conveying visual information to the superior colliculus on the deafferented side. The corpus callosum proves to be essential for this indirect conveyance of visual information to the superior colliculus deprived of its direct retinal input (Antonini *et al.*, 1979 b). It is not known whether visual

information is transmitted from the cortex to the superior colliculus via the uncrossed corticotectal projection (Kawamura *et al.*, 1974), or whether it also travels directly from the cortex of one side to the opposite superior colliculus via an uncrossed corticotectal pathway traversing the midline in the callosum (see Powell, 1976). In any case, these findings

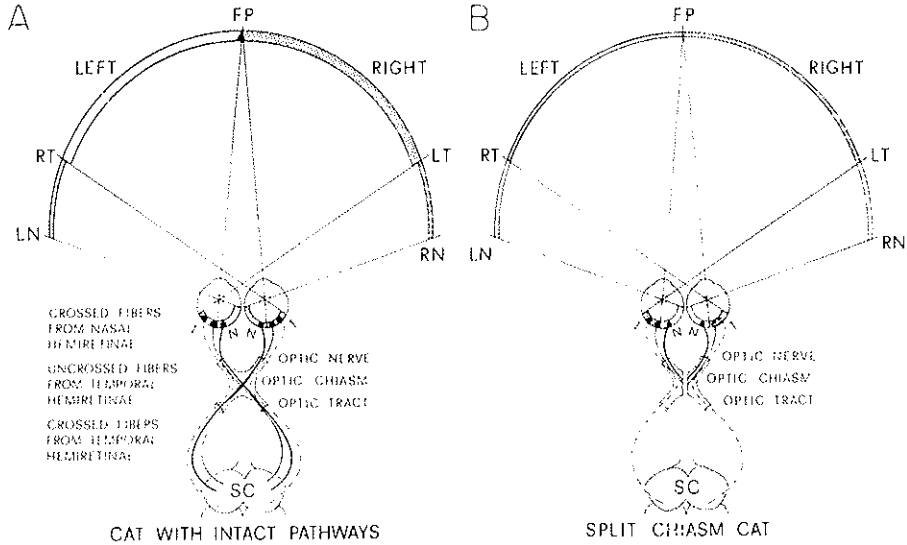


FIG. 4. Connections between the retina and the superior colliculi in normal and split-chiasm cats. In normal cats (left) each nasal hemiretina projects to the opposite superior colliculus, whereas each temporal hemiretina projects to both ipsilateral and contralateral superior colliculus. After splitting of the optic chiasm (right), each superior colliculus receives a direct input only from the ipsilateral temporal hemiretina. The nasal hemiretinae are deefferented, and the representation of each temporal hemiretina on the contralateral superior colliculus can only occur via indirect cross-midline connections. RT, LT: peripheral limits of the field of the right and left temporal hemiretina; RN, LN: peripheral limits of the field of the right and left nasal hemiretina; FP: fixation point; SC: superior colliculus (from Antonini *et al.*, 1978).

make it clear that a callosal section in split-chiasm cats restricts visual information to the half brain ipsilateral to the eye receiving the input, both at cortical level and at the level of the superior colliculus. The absence of interocular transfer of pattern discriminations may thus be the consequence of this complex cortical-subcortical disconnection, rather than the result of a pure cortical disconnection, in accord with an earlier suggestion by Thompson (1965). Conversely, a recent reexamination of interocular transfer of brightness discrimination in split-chiasm cats has

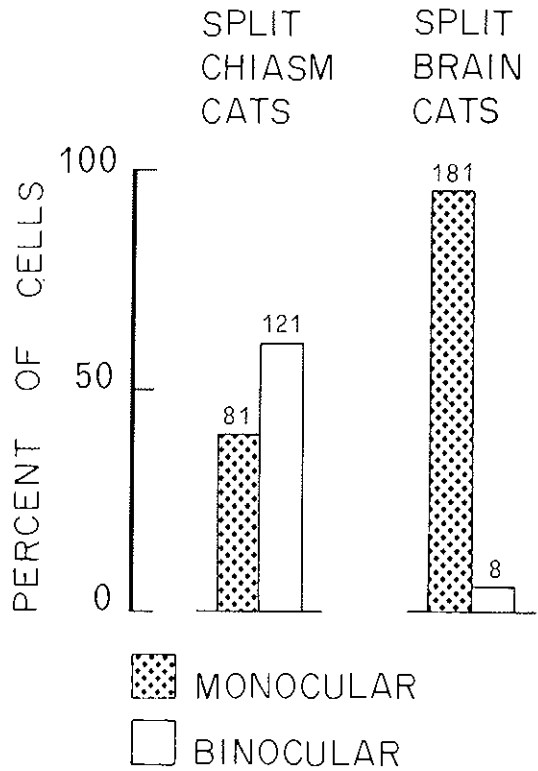


FIG. 5. Monocularly and binocularly driven cells in the superior colliculus of split-chiasm and split-brain cats. Monocularly driven cells receive an input only from the ipsilateral eye. Note that virtually all cells in the superior colliculus of split-brain cats are monocularly driven, whereas in the split-chiasm cats there is a majority of binocularly driven neurons (from Antonini *et al.*, 1979a).

led to the conclusion that the posterior part of the corpus callosum is the principal route also for this task (Peck *et al.*, 1979). It can be concluded that the subcortical commissures play a minor role, if any, in visual interhemispheric transfer in the cat, since the transmission of visual information across the midline to both cortical and subcortical visual centers is effected by the corpus callosum.

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DISCUSSION

WEISKRANTZ

The inferotemporal neurons in the monkey have been claimed by Gross in fact to have very specific but very complex trigger features, such as a monkey hand. I do not know if there is anything at all comparable in the cat.

BERLUCCHI

While it is true that Gross has described very specific responses to complex objects for inferotemporal neurons in the monkey, it is also true that these neurons appear to respond, although in a much weaker way, to simple stimuli presented throughout their receptive field. It is possible that neurons in the suprasylvian cortices of the cat are also specifically sensitive to complex visual stimuli, but nobody has as yet shown this convincingly.

ROBERTS

Would you care to comment on the work of Daniels and Pettigrew in which they changed the response characteristics with picrotoxin or bicuculline, substances that block postsynaptic efficacy of GABA, the major inhibitory transmitter?

BERLUCCHI

The callosal connections between areas 17, 18 and 19 appear to match the visual input from the thalamus to the cortex in a very specific spatial pattern. Only those neurons which have receptive fields adjoining the vertical midline of the general visual field project to or receive from the corpus callosum. These connections by way of the corpus callosum are very precise, and I submit that they are involved in binocular fusion and stereopsis along the midsagittal plane, rather than in the interhemispheric transmission of learned visual information. I suspect that receptive fields mediated in these areas by the callosal input may be easily modified by applying picrotoxin and bicuculline, as these drugs have been shown to have influences on response properties of cortical neurons in general. However, I don't know of any specific experiment on this problem.

FAMBROUGH

This is probably a naive question, for information only. What is the fate of the axotomized cells when you cut the commissures? And what is the fate of the endings which were on those cells and so on back through the system? Could there be rearrangements and reconnections, death of certain elements?

BERLUCCHI

There is no doubt that a callosal section must produce both anterograde and retrograde degeneration in the cortex, and that these processes must involve cell loss, gliosis and general biochemical changes in the cortical tissue. However the electrophysiological effects of callosal sections that we have described in the visual cortex and the superior colliculus are present a few minutes after the section, and are not substantially changed as long as one year after the section. I also don't believe in the possibility that sectioned callosal axons regenerate in the cat, at least in the adult state.

NELSON

I had a question about the projection of these areas outside of 17, 18 and 19. Do they go across to homologous areas on the other side?

BERLUCCHI

There are callosal connections between homotopical areas and heterotopical areas. For example, neurons in area 17 project to contralateral areas 17, 18, 19 and lateral suprasylvian. Some of these connections may occur between neurons with receptive fields having the same organization, while other connections may link up neurons whose receptive fields show different degrees of complexity. As an example, a "simple" cell in area 17 may project to a "complex" cell in the same area or in areas 18 or 19 in the opposite hemisphere. Such a projection would bave the function of extending the receptive field of the recipient cell across the vertical midline of the visual field.

NELSON

It seems a little odd that the projection from an area that has performed a complex processing function goes to an area that has essentially the same organization. That is, there would appear to be no further processing in this step of the hierarchical or sequential system.

BERLUCCHI

No, but you see, in area 17—I simplified things a lot, but you know that among the cells in 17, 18 and 19 you have different degrees of receptivity complexities, although the receptor fields are specialized for form and orientation. And one possibility which has been indirectly supported is that you may have a simple cell in area 17, a simple cell here (drawing on blackboard) projecting to a complex cell in area 17 on the other side, and according to this hypothesis the complex cells receive many inputs from many simple cells. This would simply extend the receptive field of a complex cell across the vertical meridian of the visual field. In other words, this cell would have a complex field with a portion which is transmitted from the other side of the brain, from a cell in area 17. This is what I suggested a long time ago.

ECCLES

Are those connections from 17 to 17 monosynaptic? I thought that there were no direct pathways from 17 to 17.

BERLUCCHI

Contrary to Flechsig's principle, which stated that primary sensory areas of the cortex had no callosal connections, it is now known that there are callosal connections for that part of area 17 which represents the vertical meridian of the visual field. In many animals, including cat and monkey, this callosal portion of area 17 is at the border with area 18.

ECCLES

So it is just the meridional field which in fact gets the crossed innervation. That makes good sense because you want to have correlation there, but you do not want to be correlating a point you see there to a point you see there. I understand now.

ROBERTS

I was under the impression that the projection was largely to the spiny stellates rather than to the pyramids directly and that it was usually in lamina IV. Am I correct? Could it be that the release of pyramidal neurons in laminae III and V, the simple cells, for action would then be followed by release or activation of the complex and/or hypercomplex cells (pyramids in other laminae), and that direct monosynaptic transcallosal projections may take

place not only on the dendrites of the pyramidal cells but, even more importantly, onto interneuronal local circuit elements which regulate activities of the pyramidal neurons?

BERLUCCHI

The relation between neuronal morphology and type of the receptive field in the visual cortex is not known in detail. It now appears that both stellate and pyramidal cells may have a "simple" receptive field. It is known, however, that although the majority of cells in the visual cortex which project to the corpus callosum are pyramidal, some callosal fibers can also originate from stellate cells. Callosal fibers are known to terminate both on stellate and pyramidal cells.

ROBERTS

Certainly pyramids project across, but are the cells to which they project directly largely local circuit stellates, or the pyramidal neurons, or both?

BERLUCCHI

Certainly there are callosal neurons ending on pyramidal cells, I think—at least in the cat.

NELSON

Now I think it is highly appropriate in a form such as this, where I think all of us have felt the difficulty of spanning the range from the molecular to the bebavioral, that the final word should go to Professor Eccles, wbo I think has grappled with that as well as anyone.

ECCLES

I am sorry to disappoint you so, but there is not going to be much grappling. This is not the place nor the time for grappling. I think it is more like the time for relaxing. I am very happy indeed to be bringing this excellent symposium to a conclusion. We must start by thanking the Pontifical Academy of Science for making this occasion possible; and then we must also thank the Academician Rita Levi-Montalcini for asking us to come along when she was entrusted with the task. She selected us. We must all agree she has done pretty well and we must thank her for this magnificent occasion.

Then I would like to remind you that in 1964 there was a meeting in this same room on Brain and Conscious Experience that I organized. Four of those participants are here: Roger Sperry, Giuseppe Moruzzi, Carlos Chagas and myself. And now the Academy has done a very nice thing by giving us the Proceedings of the 1964 Congress. Of course, projecting into the future, no doubt there will be another neuroscience conference in 1992, fourteen years further on, and those attending will be presented with a copy of the transactions of this conference. You have to remember that Rome is eternal and we are just at the beginning of these neuroscience conferences of the Pontifical Academy.

Now, this conference is on Nerve Cells, Transmitters and Behavior. I thought I knew something about nerve cells. After all, I have played around with nerve cells all my life, and I got into much controversy about transmitters at one time. But you know that subject has got out of hand altogether with its tremendous progress. I also worked on some of these other problems, like nerve muscle transmission. It is amazing how the subject has moved from these early days. I can still understand it pretty well—at least I persuade myself I can! I can even appreciate the subtleties of the techniques that are used. The techniques of course have progressed fantastically since those days. More power to people who can use these techniques appropriately in all their subtlety. I would suggest, though, that this is only the beginning of the story.

We may think we have done pretty well, but I believe we are at a primitive level still in understanding the brain: how it is all put together and how it all works. Do not think that you are going to solve all the problems in the next few years. The important thing is we are making progress. This is evident. But I do not see any finality about it. I think we have to be very humble before the immensities of the problems. A simple example: although we have nerve growth factor, which does amazingly well with the sympathetic system, we cannot explain how the whole brain grows all these selective connectivities by the process of nerve cells sprouting axons. These sprouts seem to move in a miraculous way to get to their targets. How can this be explained? Ontogeny is a fantastic process and we are only at the very beginning, but I believe that what we have been talking about at this conference is going to help in attaining these insights. We have the unexpected new discoveries coming up like that developed by Dr. Kosterlitz with the enkephalins. A few years ago these were unknown and suddenly we are presented by a new kind of operation in the nervous system. This is certainly going to grow and be of the greatest importance to the future. I have hardly mentioned all the excellent work that was presented. I was par-

ticularly attracted by the neuromuscular work, which was a field I played around with a long time ago. Things happen these days which were completely inconceivable to our simple minded views when in several laboratories it was found out how acetylcholine works as the synaptic transmitter and about the end-plate potential. But I do not feel too disheartened because I know that you also are going to be overpassed very much in the future and you will of course overpass yourselves.

In the last section of the conference we have been given an account of a very small fragment of behavior. We would all recognize that you cannot cover behavior in a day, but some most interesting investigations have been described. The human brain or the primate brain can be understood up to a point. Of course the mystery is the greatest in this last part of the discussion.

Another aspect of the meeting is the background given by this lovely meeting room. There is nothing like it in the world. Maybe you have not noticed. Have you been so intent on the program that you have not looked around as I have at the frescoes? I recommend them to you. These frescoes are most interesting pictures. You can look at the beautiful ladies that Tiepolo could have painted, for they are in the style of Tiepolo, though painted earlier I think. These frescoes reveal a fascinating way of filling a ceiling with beautiful forms: all kinds of strange animals; lots of birds and "putti" and landscapes. There is something for every taste in the ceiling! The only difficulty is that the lights have been off too much of the time so we can look at the slides. This surely is a wonderful conference chamber. Then you can walk outside. And what better outside could you get than here, with the marble seats around and the beautiful court-yard, and always the sun?

I think you know it has been a superb occasion, Rita, that you have given us. I am quite sure of one thing, and that is: every member of this conference will never forget it and will be eternally grateful to you for the occasion. Thank you very much.

LEVI-MONTALCINI

First of all I wish to express my warmest thanks to Sir John for his far too flattering and generous remarks, as well as for his magnificent presentation and most active participation in the discussions of all sections. At the same time I wish to express my gratitude to the President, Prof. Carlos Chagas and to the Director of the Pontifical Academy, Father Enrico di Rovasenda, for having invited me to organize this Study Week and, most of all, for their invaluable help in planning the program and making this week so enjoyable for all of us.

As I mentioned in the introduction, it was with very much perplexity that Prof. Chagas and myself launched such an ambitious project as to devote this conference to the analysis of so many aspects of Neurobiology, from the nerve cell to brain function. As it is the rule in all these conferences, very few of the questions that were raised received an answer and the number of problems which were touched upon, were only a very small fraction of those that are faced everyday by newcomers, by experts and by old-timers as the speaker. Hopefully the organizer of the next Study Week in Neurobiology which, according to Sir John, should take place in this same room fourteen years from now, in 1992, may have at hand the answers to some if not all the questions which have been raised in these days. This optimistic prediction is supported by the evidence of the ever-growing number of scientists who engage in this field of biological sciences and hy the remarkable advances which are taking place at an increasing speed both in the technical as well as in the theoretical approach to problems of brain structure and function. It is in this hope that I adjourn the Conference, thanking all the participants for their presentations and contributions to the discussions.