PONTIFICIAE ACADEMIAE SCIENTIARVM SCRIPTA VARIA
59

WORKING GROUP

ON:

DEVELOPMENTAL NEUROBIOLOGY OF MAMMALS

June 3-7, 1985

EDITED BY
CARLOS CHAGAS and RAFAEL LINDEN

PONTIFICIA
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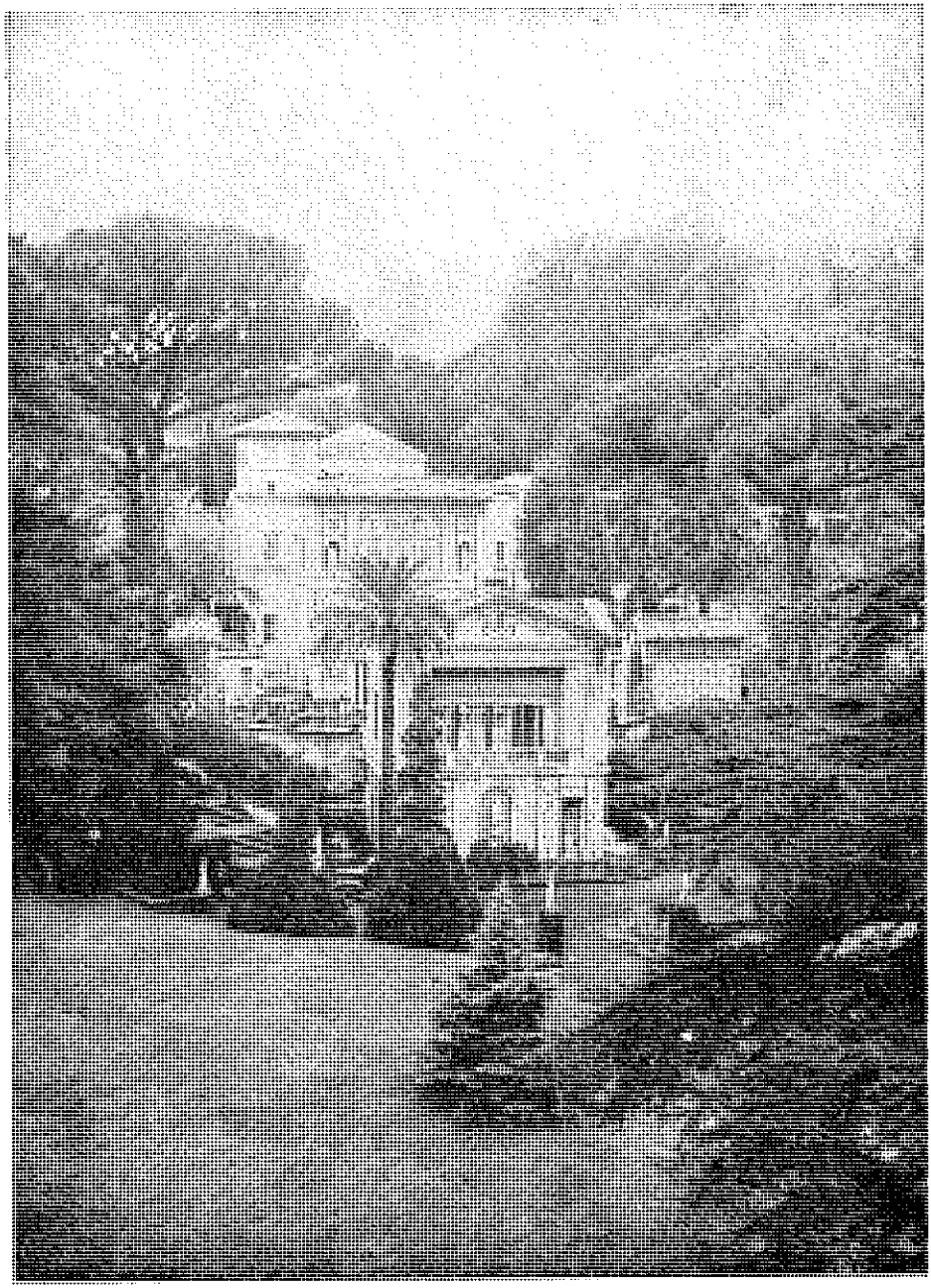
EX AEDIBVS ACADEMICIS IN CIVITATE VATICANA

MCMLXXXVII



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ISBN 88-7761-000-X

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I wish to express my thanks to Professors Robert Lent and Rafael Linden, who organized the meeting. My gratitude goes also to the many participants who contributed to guarantee the success of the Working Group. I wish at the same time to express my thanks to Father Di Rovasenda, O. P., former Director of the Chancellery of the Pontifical Academy of Sciences, and his Associate Director, Reverend R. Dardozzi, its present Director, and to Mrs. Michelle Porcelli and Gilda Massa, without whom it would have been impossible to hold the meeting. Thanks are also extended to Mr. Silvio Devoto for the help he gave during the five days in which the meetings were held.

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SCIENTIFIC PAPERS

The opinions expressed with absolute freedom during the presentation of the papers and in the subsequent discussion by the participants of the Working Group — although published by the Academy — represent the points of view of the participants and not necessarily those of the Academy.

POSTNATAL NEUROGENESIS AND THE FORMATION OF NEURAL CONNECTIONS IN THE VISUAL SYSTEM OF A MARSUPIAL

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INTRODUCTION

About half a century ago, opossums were proposed as choice candidates for studies of neurogenesis on account of the marked immaturity of their Central Nervous System at birth (see [46] for a review). Since that time, progresses on surgical techniques have made intra-uterine manipulation of eutherian mammals more viable and have limited somewhat the strength of that argument. Furthermore, there are some difficulties associated with the breeding of these feral animals in captivity, particularly in confined environments. Our choice of the opossum *Didelphis marsupialis* as a model for studies of development in the visual system was, thus, determined by the following considerations. 1. A significant body of data on the anatomic and functional organization of the opossum's visual system has been obtained in our department. This has allowed a fruitful interaction, useful for the understanding of neural development and plasticity and of visual processing in this animal [62] that could, hopefully, be extended to the Mammalian class. 2. Opossums are multiple ovulators, giving birth to litters of up to 11 pups which develop at an apparently homogeneous pace until the time of eye and mouth opening (55-70 days), when they start to leave the marsupial pouch for short periods. This homogeneous litter development allows accurate comparisons among littermates employed for studies of normal and abnormal development. 3. The late occurrence of eye opening, a reliable index of functional maturation in several mammals, argues for a protracted period

of visual system development during pouch life. This should allow a greater resolution of developmental events than could be obtained in eutherian mammals with brains of similar size and/or complexity. 4. Our studies have, indeed, revealed that developmental stages occurring in the opossum's early postnatal life correspond to embryonic epochs in the development of the visual system of altritial mammals such as some rodents. Postnatal development of the opossum's visual system has, thus, a different connotation from that applied to eutherians.

How far advanced is the development of the visual system during the climb to the pouch? A starting point for this analysis would be the establishment of proliferation schedules for some components of the primary visual system such as the superior colliculus (SC) and the retinal ganglion cell layer itself. This could provide a framework for the interpretation of events occurring during the formation of retinocollicular projections and for the changes induced by lesions inflicted at different developmental stages.

The timing and features of these developmental events will be described as well as some features of glial cell differentiation in the SC and along the retinofugal pathway which may have a bearing on the stabilization of the pattern of retinocollicular projections.

The Organization of the Opossum's Ganglion Cell Layer and Retinocollicular Projections

Before considering our developmental work, it may be helpful to briefly review the organization of the retinal ganglion cell layer and of the retinocollicular projection in the opossum *Didelphis marsupialis* (subspecies *aurita*).

The opossum retinal ganglion cell layer consists of projection cells and displaced amacrines distributed at low density in an area of about 130 mm^2. Estimates of the number of ganglion cells identified by cytologic criteria defined after optic nerve or tract section and by horseradish peroxidase (HRP) retrograde labeling from the mesodiencephalon indicate an average value of 114,000 (SD = ± 22,000) (J.N. Hokoç, personal communication), thus, higher than that obtained from Nissl preparations [26]. These cells are distributed along a smooth center-to-periphery density gradient, with peak values centered at about one-third of the distance from the optic nerve head to the temporal rim of the retina [26, 60]. The peak density of ipsilaterally-projecting cells

is located slightly temporal to this *area centralis* [21] in analogy with other nocturnal animals [14, 18].

As in all non-primate mammals so far studied [68], the opossum's temporal retina gives rise both to uncrossed and crossed retinocollicular projections [44, 48]. Ipsilateral retinocollicular projections arise from 50% of the whole complement of temporal ganglion cells projecting to the SC [48], a proportion much higher than that observed in rodents [14, 18]. It is unclear whether all cells of the nasal retina project to the SC but virtually all of those that do project are destined to the contralateral nucleus [48].

There exist some differences in the pattern of radial distribution of retinocollicular projections in the opossum as compared to eutherians and diprotodont marsupials which are, actually, rather favorable for visuotopic studies in normal and precociously-lesioned animals. Uncrossed and crossed projections are complementarily-distributed in laminae occupying, respectively, most of the *stratum zonale* and the *stratum griseum superficiale* [10, 50, 61] (Fig. 1). This region corresponds to about 30% of the SC surface area and has been denoted the *direct binocular region* by a combination of criteria derived from anterograde tracing or degeneration and electrophysiological recording [44, 59, 61, 71]. An additional uncrossed innervation is represented by ill-defined clusters of label in upper *stratum griseum intermediale*, also restricted to the direct binocular region [50]. Rostrally and caudally to the *direct binocular region* lie divisions of the SC where crossed projections are distributed from the sub-pial level to the lower reaches of *stratum griseum superficiale* but arise, respectively, from the temporal (*rostral pole*) and nasal retina (*caudal region*).

Some Aspects of Postnatal Cell Proliferation in the Opossum Superior Colliculus

The occurrence of postnatal cell proliferation in the SC was first tested by injecting pouch young 4 or 7 day-old (PND4, PND7) with the DNA precursor thymidine, labeled with tritium (^{3}H-T), sacrificing them 1.5 hours later and processing the brains for autoradiography. We found no uptake within the collicular plate itself, but many nuclei within the upper part of the ventricular, pseudostratified epithelium appeared labeled. In additional animals that received a pulse of ^{3}H-T at PND5

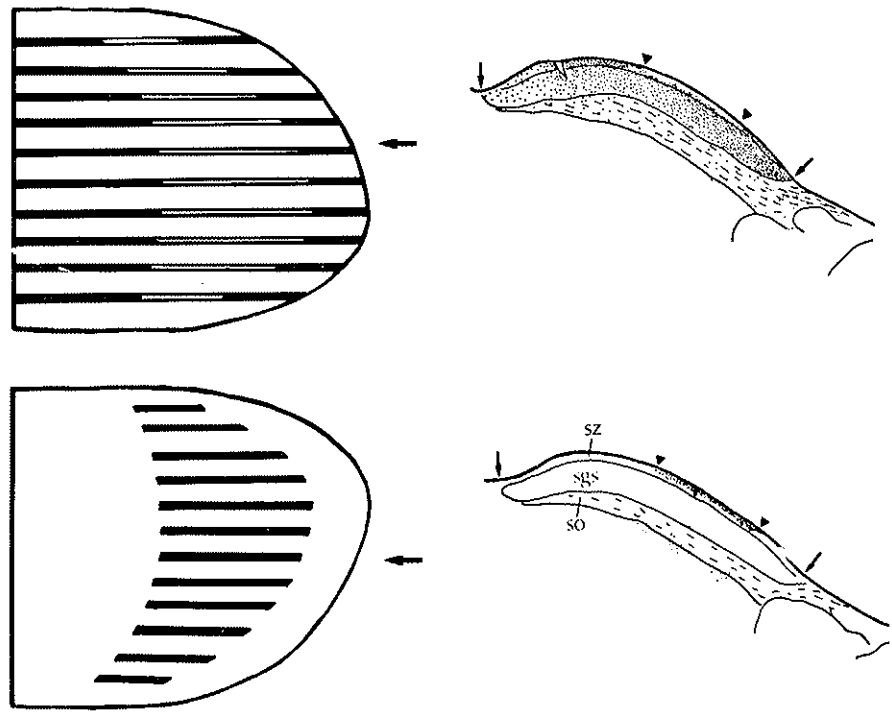

FIG. 1. Drawings of the superior colliculus (SC) of the opossum, contralateral (upper) and ipsilateral (lower) to the eye injected with HRP. Rostral is to the right. Left: Dorsal views reconstructed from parasagittal sections after alignment of the caudal border. The direct binocular region (DBR) is shaded on the ipsilateral SC. Arrows indicate the levels corresponding to the sections shown on the right-hand side. Right: Parasagittal sections showing the radial distribution of retinocollicular projections. Arrows indicate the limits of the SC and arrowheads those of DBR. Abreviations: sz, sgs and so: *strata zonale, griseum superficiale and opticum.*

or PND7 and were allowed 24 hours survival the labeled nuclei extended closer to the ventricular border but very few had migrated away from the outside border of the ventricular zone.

In order to determine that the labeled cells included neuron precursors, we followed the usual procedure of pulse-labeling immature specimens (2 to 16 days old) with ^{3}H-T and allowing long survivals. The animals were sacrificed at about the time of eye-opening (55 to 70 days). Particular attention was paid in the autoradiographic analysis

to verifying whether neurogenesis declined steadily from birth and whether the general inside-out gradient described in other mammals (rat [2, 5, 53], rabbit [54], monkey [12], hamster [16]) was also present in the opossum SC.

A non-monotonical curve of postnatal neurogenesis for the SC was established (Fig. 2) with very low levels soon after birth, a peak by the end of the first week and a return to negligible figures after the 10th day [11]. The possibility that such a curve was generated by variations in the availability of ^{3}H-T is readily dismissed by the following obser-

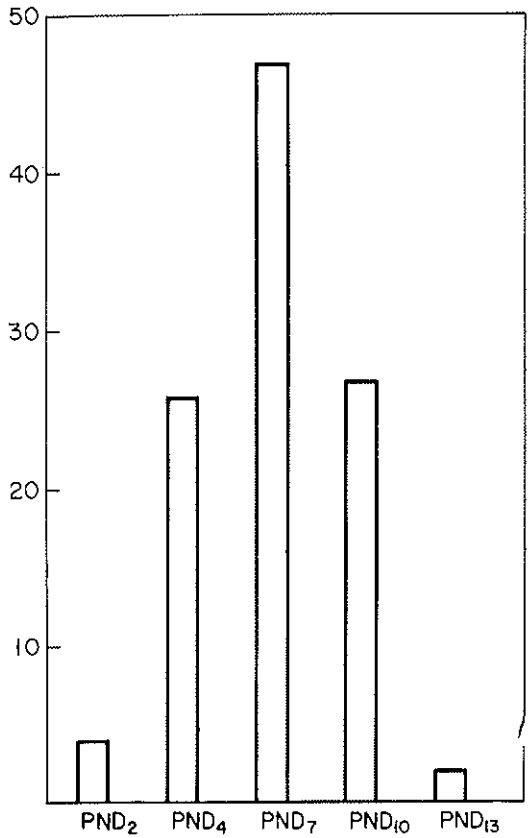

FIG. 2. Number of heavily-labeled neurons per section in the superior colliculus of opossum injected with a pulse of ^{3}H-T at postnatal day (PND) 2, 4, 7, 10 or 13. Observe that postnatal neurogenesis rises to a peak by the end of the first week.

vation. Soon after birth, the mesencephalic tegment contains a higher density of heavily-labeled neurons than the SC, including the monoaminergic neurons of the substantia nigra and ventral tegmental area, the genesis of which cease before or at peak production of neurons for the SC in rat and monkey [1, 2, 12, 42]. These neurons are no longer labeled at the peak of postnatal collicular neurogenesis in the opossum.

Preliminary studies of neurogenesis in the brushtailed possum, an Australian marsupial with a much longer gestation period (17.5 days) than the opossum (12.75 days) indicate that numerous collicular neurons are labeled by ^{3}H-T injection at 9 days, fewer at 12 days and almost none at 39 days postnatal [64]. Furthermore, neuron production for the possum's visual cortex has not started at 12 days and is destined to layers III-IV at 39 days. We have also observed that in the final stages of collicular neurogenesis (10-13 days), only infragranular neurons of the opossum's striate cortex are produced.

Postnatal neurogenesis for the SC proceeds according to an inside-out gradient, thus recapitulating most of the sequence in other mammals. It also tends to proceed from rostral to caudal in all collicular layers with an additional latero-medial vector in the superficial layers [11] (Fig. 3). A rostrolateral-to-caudomedial gradient has been suggested by inspection of autoradiographs of pulse-labeled material in the rat [53] but has not been confirmed by quantitative analysis in the monkey SC [13], although there is an agreement in the finding that the latest-labeled neurons are found in the caudo-medial edge of the SC [1, 5, 12, 53]. It should be noted that even in the opossum the establishment of tangential gradients required extensive sampling and, in the case of the latero-medial component, separate analysis of the 3 sets of layers.

Since we have sacrificed our animals before they reached adulthood, it may well be asked whether our labeled neurons are a representative sample of a "permanent" collicular cell population [3]. This question is indeed pertinent since it has been reported that caudal SC and nasal ganglion cell layer have significantly more degenerating profiles than the remaining collicular and retinal regions in 6-7 days old rats [15]. It is irrelevant for our discussion that the authors judged this to be the peak of cell death in both structures while quantitative studies indicate that the bulk of ganglion cell loss occurs prior to day 6 postnatal [55, 56]. If it is admitted that there is a regional correspondence between death of collicular and ganglion cells and cell death persists longer in nasal

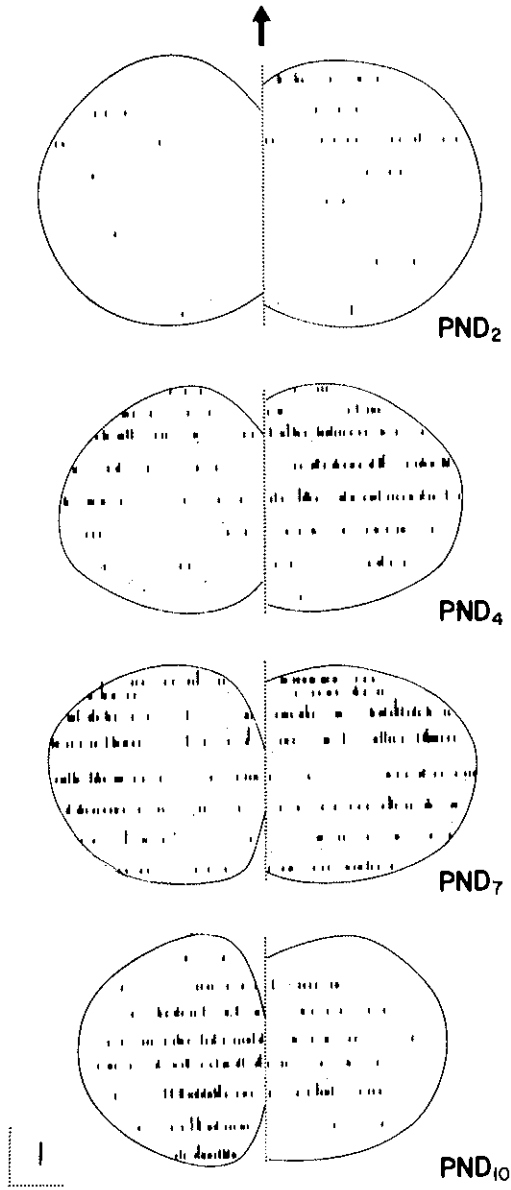

FIG. 3. Schematic dorsal views of the outlines of superficial layers (left) and remaining layers (right) of the superior colliculus (SC), projected upon a horizontal plane, showing counts of heavily labeled neurons (solid bars) in opossums injected with a pulse of ^{3}H-T at postnatal day (PND) 2, 4, 7 or 10 and sacrificed at from 50 (PND10) to 70 days (PND2). Observe that there is an inside-out gradient of neurogenesis in the SC and a rostrolateral-to-caudomedial gradient in the superficial layers.

retina, the use of young pups could bias our results towards a steeper rostro-caudal gradient of labeled neurons.

We have not evaluated ganglion cell numbers in the developing *Didelphis marsupialis*, but Kirby and Wilson recently reported that the bulk of axon loss in the optic nerve of *Didelphis virginiana* occurs from day 27 to day 36 postnatal and that the countings stabilize at adult mean value between tbe days 50 and 59 [36]. Since there is quite a good correlation between their results and our data on the restriction of retinocollicular projections [50], as well as on the onset of myelination in the optic nerve [45] it is quite reasonable that several developmental events including cell death are similarly timed within the genus *Didelphis*. Nevertheless any substantial change in the pattern of neurogenesis that we have described would only apply under very extreme conditions such as catastrophic cell loss restricted to the caudal third of the SC and occurring at the very end of the period of ganglion cell death. We found virtually no pyknotic nuclei either in serial paraffin sections or in semi-thin sections at 3 different collicular levels at PND50, PND56 or PND63.

Is the tangential gradient of labelled cells in collicular superficial layers generated by collicular cell death, determined by ganglion cell differentiation, or is it just an expression of an intrinsic gradient in the withdrawal of precursors from the mitotic cycle?

During the final stages of collicular neurogenesis, say, at PND7 and later, the distribution of mitotic figures in the ventricular zone should give a reasonable approximation of the strength of neurogenesis since undifferentiated precursors would go through few mitotic cycles. At these epochs there is indeed a shift from lateral to medial localization of the majority of mitotic figures. Furthermore, these are found more often caudally as collicular neurogenesis approaches its end. These findings do not exclude the possibility that neurons generated at a given day could die just because they resided in the "wrong sector" of the SC. Counts of ^{3}H-T-labeled, degenerating neurons in developing animals are necessary to solve this question.

With regard to a presumptive action of ganglion cell differentiation on the generation of gradients of ^{3}H-T labeling in superficial collicular layers, a set of temporal and spatial requirements includes earlier "birth-days" of a few ganglion cells as compared to those of neurons of the superficial SC, early outgrowth of optic axons so as to arrive in time at the mesencephalon and some sort of communication channel between optic axons and ventricular cells. The usually-invoked requirement of

topographic matching during the genesis of retinal and tectal cells [22, 33] may possibly be waived since it is well-known that mammalian optic fibers may occupy temporarily sites of the SC from which they withdraw during the course of maturation. Nevertheless, some preliminary data on the tendencies towards spatial-temporal patterns of ganglion cell genesis will be commented upon in the next section.

Postnatal Differentiation of the Opossum's Retinal Ganglion Cell Layer and Early Development of the Retinofugal Pathway

At PND2, i.e., the 15th postconceptional day, the neural retina still consists of a pseudostratified epithelium, except in the region adjacent to the posterior pole (Fig. 4A). Autoradiographs of specimens injected with ^{3}H-T 1-2 hours before sacrifice show a tier of labeled nuclei in the vitreal half of the ventricular zone (Fig. 4B). Few unlabeled cells are found between the labeled nuclei and the marginal zone, suggesting that few ganglion cells (and displaced amacrines?) have left the mitotic cycle and completed the migration away from the primitive ventricular surface. Additional observations on incorporation of ^{3}H-T were obtained at PND5 and PND9 when labeling of the ventricular zone still extended from one edge of the *ora serrata* to the other. From PND9 to PND10 the internal plexiform layer can be recognized in a limited, central extent of the retina (Fig. 5).

Our observations in long-survival, adult animals are not sufficient for an accurate analysis of gradients although there is a general tendency for a center-to-periphery sequence in the final distribution of labeled neurons in the ganglion cell layer (S. Allodi, J.N. Hokoç and L.A. Cavalcante, in progress.) Ganglion cells labeled at PND2 have as their eventual destination an oval area extending symmetrically from the optic nerve head to about one-third of the way to the *ora serrata*. The final distribution of ganglion cells labeled with ^{3}H-T at PND5 or PND7 extends to almost all the retina and even those labeled at PND10 are not restricted to the extreme periphery (Fig. 6).

The distribution of ^{3}H-T labeled neurons in the ganglion cell layer of adults injected at PND1 seems relatively wide as compared to the apparently small number of postmigratory cells at about the time of injection (actually PND2). It is possible that postmitotic migration takes a while to start and/or to be completed. On the other hand, axon

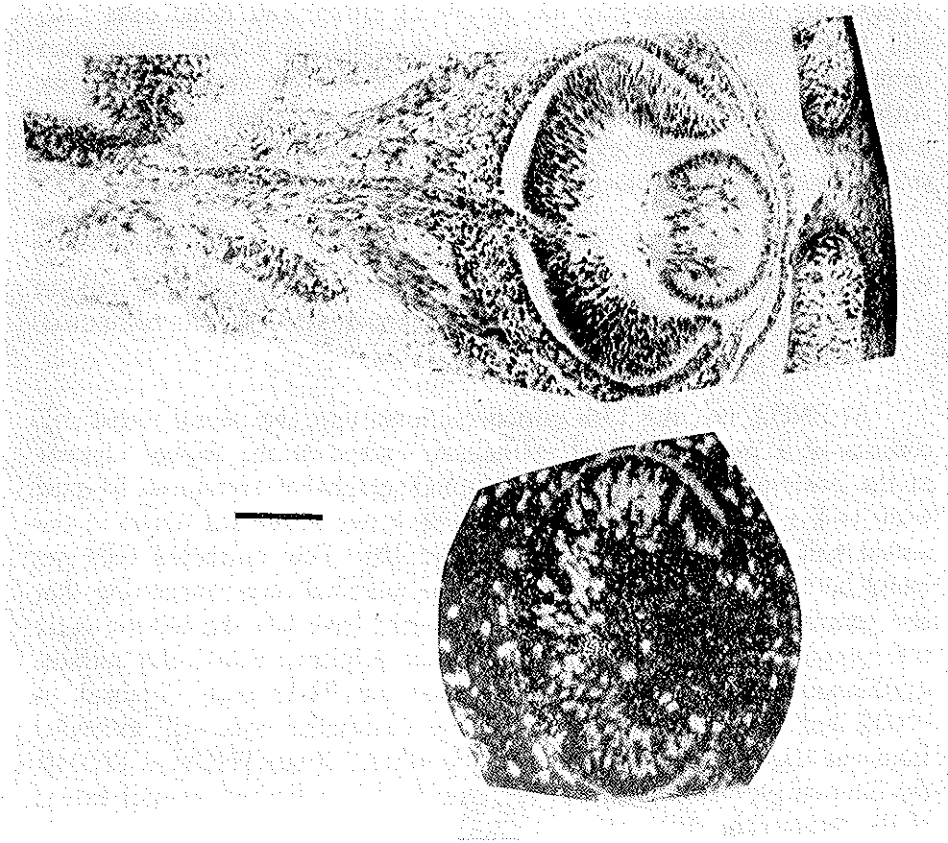

FIG. 4. Opossum's retina at 2 days postnatal. Above: Hematoxylin-eosin stained section passing through the retina and floor of the diencephalon of a pouch young opossum, injected with ^{3}H-T two hours before sacrifice. Section is oblique with midline slightly caudal. Below: Radioautograph of an adjacent section showing the thick ventricular zone. Calibration bar = 100 μm.

outgrowth may occur very rapidly since intra-ocular application of horseradish peroxidase (HRP) at PND3 results in heavy labeling of fibers at the chiasma [50]. In fact, fibers may have reached the chiasma earlier than that since PND3 was the earliest stage at which successful HRP labeling was obtained.

An unexpected finding was the apparently slow progress of the wavefront of laheling from PND3 to PND7. Both at PND5 and PND7, laheled fibers were detected in the hypothalamic but not in the thalamic

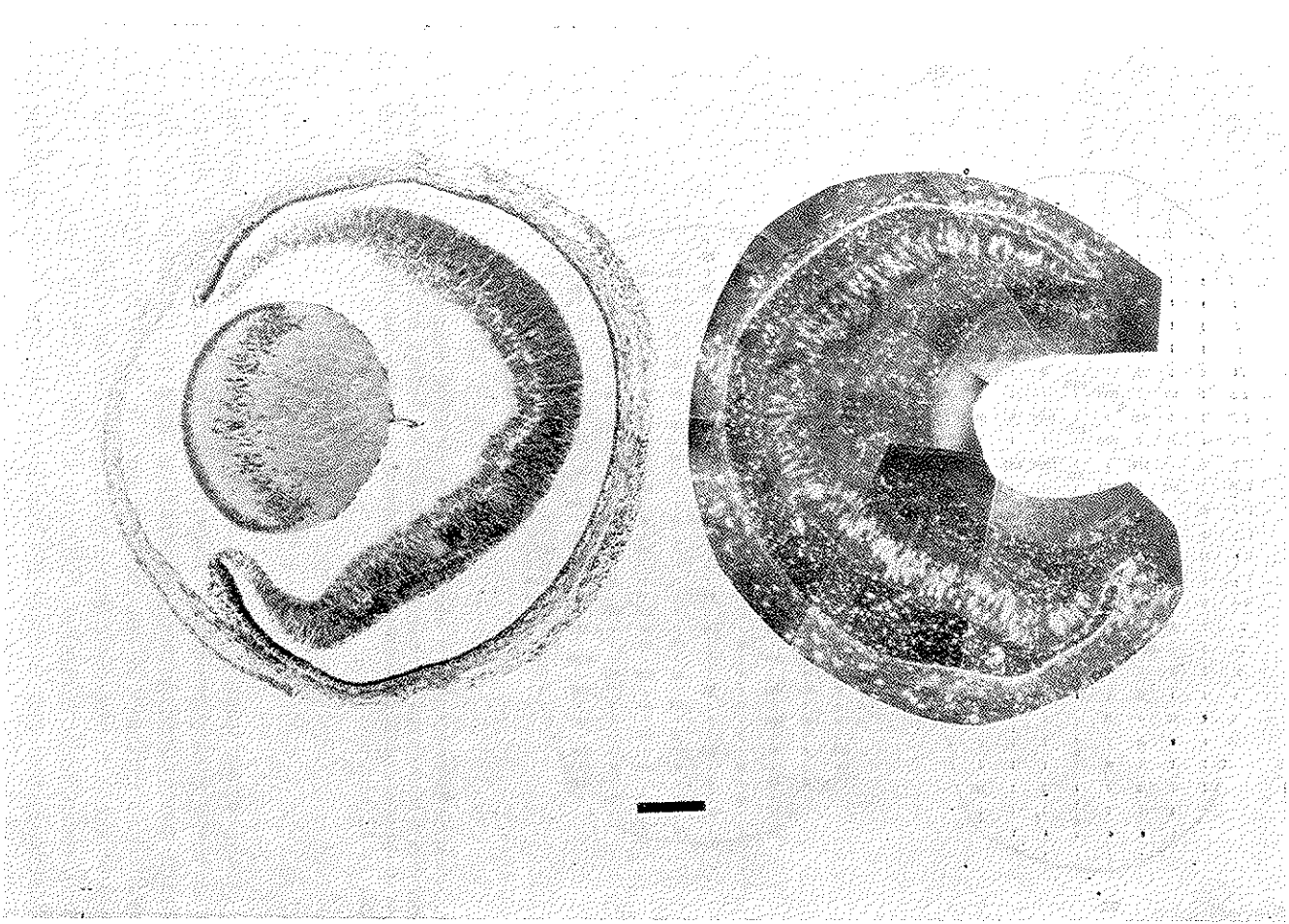

FIG. 5. Opossum's retina at 9 days postnatal. Left: Hematoxylin-eosin stained section passing through the center of the eyeball of a pouch young injected with ^{3}H-T two hours before sacrifice. Observe that the inner plexiform layer appears in central portions of the retina. Right: Radioautograph of an adjacent section showing that the ventricular zone extends to the entire tangential extent of the retina: Calibration bar = 200 μm.

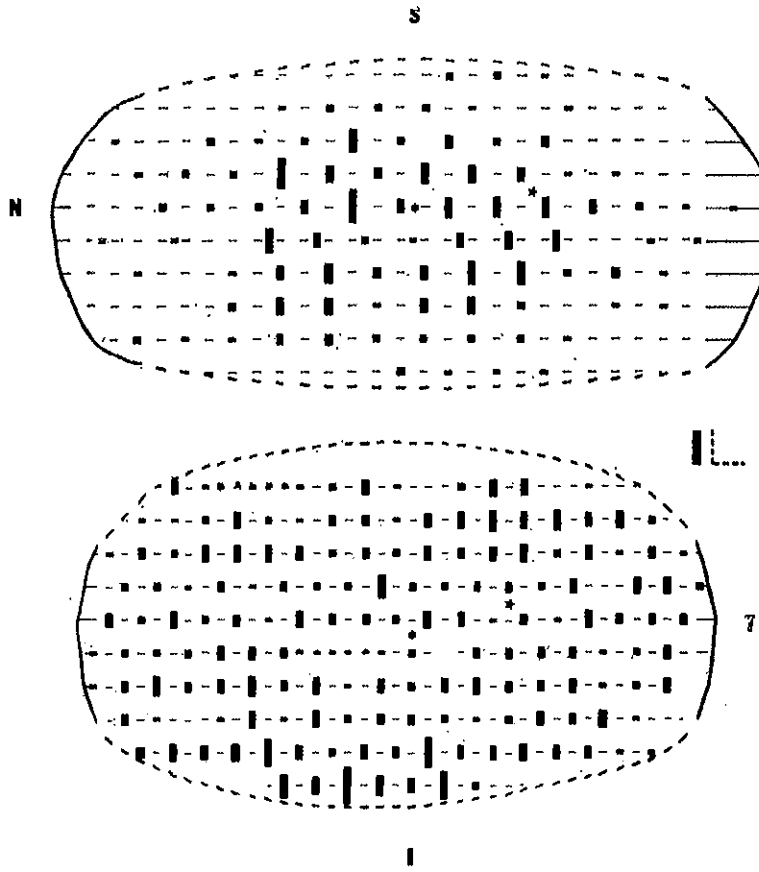

FIG. 6. Schematic representation of the distribution of retinal ganglion cells generated at postnatal days 1 (above) and 10 (below). The star and the asterisk indicate the approximate locations of the *area centralis* and nerve head, respectively. Scales: Interrupted line = 1 mm, filled bar = 10 labeled cells.

level of the optic tract. To miss the wavefront of label in growing fibers is easily understandable if their number remains nearly stationary since their path becomes more dispersed as they go further beyond the chiasma. This probably is not the case since in *Didelphis virginiana* the number of axons in the optic nerve rises from 24,000 (slightly over one fourth of mean adult values) at PND5 to 87,000 at PND9 [36].

Several factors may account for the apparently slow progress of the wavefront of labeling. First, it is possible that fibers take divergent

paths into the main optic tract, the accessory tract or even into the nerve of the opposite side. Second, it is also possible that the migration of ganglion cell perikarya becomes more and more delayed and many remain in a more sclerad position although their axons have exited from the eye. The access of HRP to these perikarya may be impaired and, consequently, their intact axons although present in the nerve would not carry the enzyme. Finally, it is possible that HRP leaks from the axon growing tip and the content of the label falls to subthreshold levels in the most immature fibers.

We have circumstantial evidence for the transfer of HRP from immature retinal fibers to non-neuronal cells situated in the vicinity of the chiasma [49]. In animals receiving intra-ocular applications of HRP at PND3, PND5 or PND7, we have found that the enzyme filled processes running nearly perpendicular to the chiasma and leading to cells in the adjacent wall of the third ventricle (Fig. 7). The somata of such

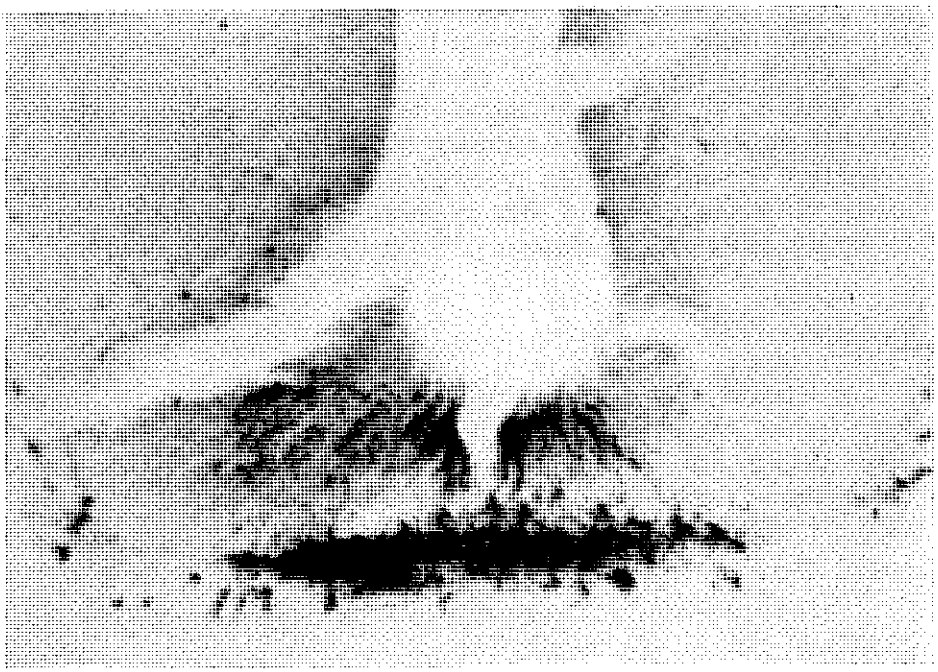

FIG. 7. HRP-labeled « ependymal cells » and radial processes in the vicinity of the optic chiasma in a 5 day-old opossum that received an eye injection of the enzyme 6 hours before sacrifice.

cells are not clearly delineated by the lahel but they certainly are not "elliptical, with their long axis parallel to the orientation of axons" as the presumptive macrophages lying in the optic chiasma of neonate hamsters [41]. Since similar cells appear labeled in the SC as retinal fibers are visualized there, we shall return to a discussion on their identity and possible functions after a description of the late transformations of the retinocollicular pathway. They should be taken, for the moment, just as indicators of loss of HRP from retinal fihers at a time when their numbers are increasing progressively.

There is some uncertainty on the time of arrival of significant numbers of retinal fibers to the SC but crossed axons are clearly visualized as far as the collicular caudo-medial edge at PND10. There is, thus, some overlap between the time schedules for neurogenesis for the superficial layers and the arrival of optic fibers. Since the first fibers are detected at just sub-pial levels [9, 13, 50] prospective target neurons generated at PND10 (or PND7) may have to migrate through retinal afferents. It remains to be determined whether these afferents do not actually arrive at the SC much earlier than detected by current tracing techniques. If they do, the temporal requirement for an action of ganglion cells on neurogenetic gradients of the SC could be fulfilled.

With regard to a morphological substrate for the communication between precursors of retino-recipient neurons and optic fibers, it is interesting to note that radial processes leading to periacqueductal cells appear labeled with HRP as soon as retinal fibers are visualized in the SC by the histoenzymatic reaction.

Transformations of the Retinocollicular Pathway

A series of changes can be followed in the distribution of anterograde tracers transported from the developing retina to its central targets. These changes have usually been considered as correlates of 3 types of developmental events:

1) Sequential invasion of target sites by crossed and uncrossed retinal fibers [6, 9, 17, 20, 23, 43, 50, 65, 66, 69].

2) Exuberant distribution of fihers from both eyes [9, 20, 23, 35, 38, 43, 50, 57, 65, 66, 69, 74].

3) Formation of heterogeneities in the distribution of crossed and uncrossed fibers within a given target [9, 23, 35, 43, 50, 57, 65, 66, 69, 74].

A delayed development of ipsilateral with respect to contralateral retinal projections was first proposed by Currie and Cowan [17] on the basis of countings of grains in autoradiographs of the thalamic neuropil in *Rana pipiens* after eye injection with tritiated proline. Early autoradiographic studies in postnatal opossums [9] and hamsters [20, 69] suggested the same sequence in mammals although it was recognized that factors such as low blood-brain barrier to proline could impair the detection of early ipsilateral projections. It should be noted, in passing, that the developing retino-hypothalamic projection of the opossum did not seem to conform to the postulated pattern [8] but this may be due to bilateral branching of the early-arriving fibers.

The view that there is a delay in the formation of the cat's uncrossed retinogeniculate projections has been recently challenged [74] since bilateral labeling was found both in the thalamus and the tectum after a unilateral eye injection of HRP in the earliest stage examined in both studies [66, 74]. Since the imbalance between the numbers of ipsilaterally-versus contralaterally-projecting cells is more severe in the opossum (ipsilateral/contralateral about 1/9 [21]) than in the cat (at adulthood) [72], a discussion about a developmental delay in the establishment of ipsilateral retinocollicular projections in the former may seem an idle one. There seem, however, to be some differences between the maturational stages at which we found unequivocal evidence for labeling at ipsilateral collicular levels in the opossum (PND15) and the earliest stage examined in the cat (E39). Axon counting in the cat's optic nerve reaches a maximum (about 3.5 times mean adult values) [76] at E39 while maximal counting was obtained in *Didelphis virginiana* after the 3rd week [36]. It should, however, be granted that axon counting is probably higher at PND15 than in adult opossums since it rises from 80% of adult values at PND9 to over twice these values by the end of the 3rd week.

Recent work on the genesis of ganglion cells in the mouse [19] may offer an explanation for the delayed recognition of uncrossed projections in the earliest stages [6, 23, 50]. Ganglion cells giving rise to uncrossed projections start forming as early as (nasal) contralaterally-projecting cells at the 11th embryonic day (E11) and their formation ceases at E16 while that of contralaterally-projecting cells extends to E18. Assuming that the proportions of the two populations by the end of the proliferation period bear any resemblance to those found at adulthood (contralateral: ipsilateral about 20:1), it would take three fourths

of the neurogenetic period to generate one population, say, about 10 times smaller than the other, formed throughout this period. Unless an early, very sharp proliferation step accounts for the formation of most ipsilaterally-projecting cells, their axons would be slowly added to the optic nerve and could be transiently found in a proportion smaller than that observed at adulthood.

It is interesting to note that the above interpretation can explain the presence of an occasional ipsilateral fiber accompanying the crossed axons in the opossum's optic tract before these reach the thalamic level (PND5-7) and some solitary fibers in the ipsilateral SC simultaneously with the development of crossed axons throughout this structure (PND10). Unequivocal evidence for invasion of the SC by ipsilateral retinal fibers was only found at PND15.

Would a late arrival put uncrossed fibers in a disadvantageous position in the competition for terminal space? If this were true one might expect wide differences in the distribution of uncrossed fibers in animals submitted to uniocular enucleation prior to or after the invasion of an ipsilateral target by retinal fibers. At the present time, there is no indication that a particular advantage could be gained by prior removal of the prospective competitors from the other eye. Méndez-Otero and coworkers [51] found indeed small differences in one-eyed opossums enucleated from 5 to 10 days (early-enucleates) or from 15 to 34 days (late-enucleates). Late-enucleates show a nearly homogeneous distribution of HRP reaction product throughout the entire rostro-caudal extent of the superficial layers [39, 40, 51]. Early-enucleates showed such a distribution in the caudal three-fourths and a banded complex in the rostral fourth [51]. By this term, there is meant a sequence of an anterior band of very high laheling density and a posterior band, still contained in the rostral fourth of the SC, of very low labeling density, both encompassing the entire medio-lateral extent.

Low density regions have been described within the territory of the enlarged retinocollicular projection in hamsters with one eye enucleated at birth [35], but seem to be preferentially located in caudal colliculus. An absence of the ipsilateral projection from the remaining eye was proposed to occur in mice enucleated (at E12-E13) before optic fibers advance past the prospective optic chiasma [24]. Although this has not been confirmed by retrograde labeling of ganglion cells, the number of ipsilaterally-projecting elements is actually lower than in normal animals [23]. It is possible that the removal of one eye before a sufficiently

large contingent of fibers has entered the optic chiasma may cause more fibers from the remaining eye to deviate towards the opposite side, to fill preferentially vacated spaces in more proximal targets or to fight less successfully against competitors originating in other brain regions. One source of afferents that deserves close examination is the parabigeminal nucleus that projects heavily to the region of vertical meridian representation in the opossum's SC [47] and the neurons of which are formed before PND5 (L.A. Cavalcante and C.E. Rocha-Miranda, unpublished results). Although the striate cortex is a possible competitor, the formation of its infragranular neurons is still under way by PND10 so that its entrance into the scene may occur at a slightly later stage.

A simple explanation for the vacant rostral region in the SC of early-enucleated opossums is that those (rostral) collicular cells that were generated earlier die because of a prolonged period of non-afferentation (enucleation at PND5) or because of deafferentation (enucleation at PND10) and the space they occupied is filled by cells migrating from the subjacent levels. Cells destined to the caudal SC are generated later and may be rescued by the providential arrival of uncrossed fibers.

In contrast to the notion of a sequential invasion of target sites by crossed and uncrossed retinal fibers, there is no dispute that there is a stage of considerable spatial overlap in the distribution of fibers from both eyes in all mammals [9, 20, 23, 35, 36, 38, 43, 50, 57, 65, 66, 69, 74]. We have chosen to reanalyze the development of binocular segregation of retinocollicular projections since we had not detected in our previous work with autoradiography [9] the extensive tangential overlap of crossed and uncrossed fibers observed in other developing mammals by HRP histochemistry.

With HRP histochemistry we observed, as others have done [6, 36, 38], that ipsilateral fibers grow to the very end of the SC [50]. Although the first fibers are found at sub-pial levels (see also 13), they rapidly come to also run dispersed through the prospective *stratum griseum superficiale* without condensing into a *stratum opticum* (Figs. 8 and 9). A remarkable feature was the coarse texture of grains of HRP (TMB) reaction product and their clear tendency to be linearly oriented along the rostrocaudal axis of the SC. The rows of granules can be easily equated with the presence of poorly-arborized fibers but what can be deducted from the large size of the grains? A reasonable explanation is that growth cones *en passage* are issued from the fiber shaft. The large size of growth cones could allow the accommodation of large quantities of label while their proximity to the shaft would determine the formation

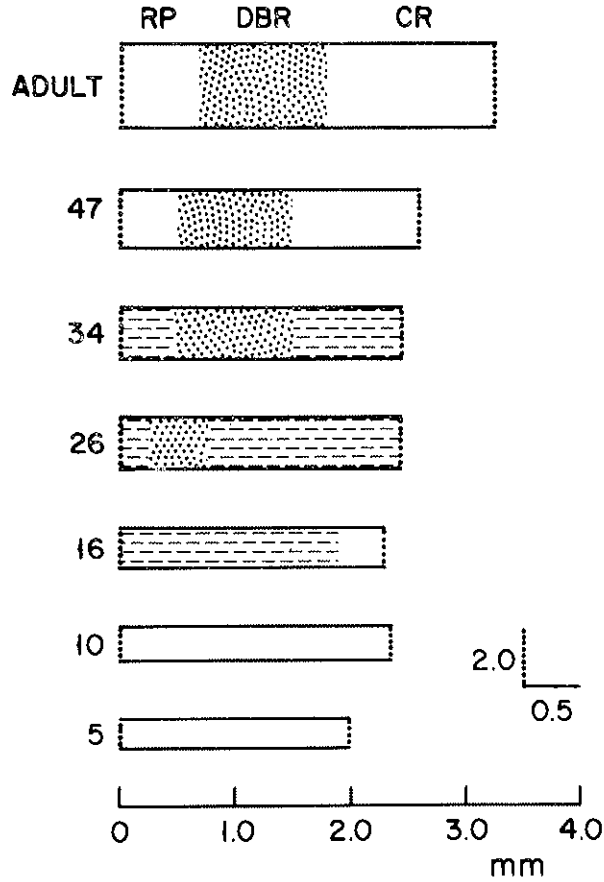

FIG. 8. Schematic representation of the development of the uncrossed retinocollicular projection in the superficial gray. The length and height of the rectangles indicate the length and width of the SC, respectively. Numerals at the left indicate days after birth. Dots and interrupted lines within the rectangles indicate the arrangement of grains of HRP reaction product. Observe the regionally-selective development of an adult-like pattern of labeling. Observations: RP = rostral pole, DBR = direct binocular region, RC = caudal pole.

of large grains of reaction product without distorting their linear orientation.

The transformation from this pattern to a restricted, adult-like distribution of the label in the ipsilateral SC takes almost 2 weeks (from later than PND22 to PND34). The hypothetical events involved in this transformation will be stated first, followed by the evidence derived

from anterograde tracing. Although these hypothetical events will be described in a given order, we have no indication that they occur in a temporal sequence.

1) Ipsilateral fibers arborize extensively in a sub-pial region corresponding in rostro-caudal coordinates to the location of the future direct binocular region. This profuse arborization does not occur in rostral or caudal collicular poles, although it follows a rostro-caudal gradient within the prospective direct binocular region (Figs. 8 and 9).

2) Fibers that either failed to arborize or were late arrivals (too

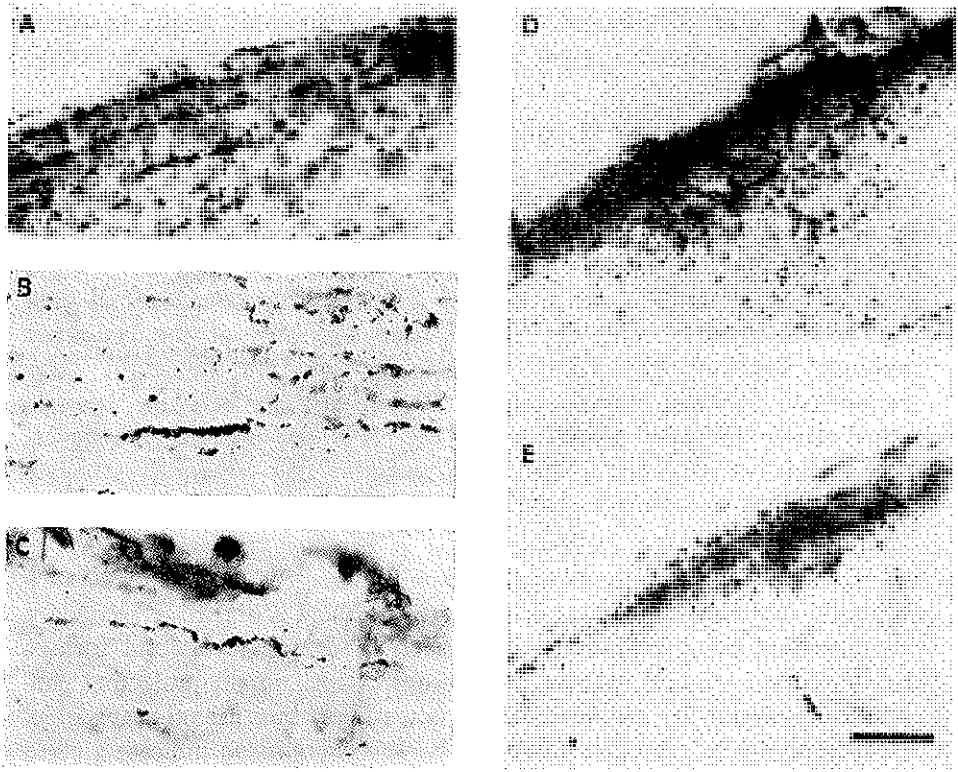

FIG. 9. Changes in the arrangements and texture of grains of HRP (TMB) reaction product in the superior colliculus (SC) ipsilateral to an eye injection of the enzyme at 2 developmental stages or at adulthood. A was taken from the rostral third of the SC at 17 days. Observe that the size and pattern of distribution of grains is more similar to those found in the *stratum opticum* (B) and in some remnant label in the caudal SC (C) of 34 day old animals than in corresponding regions at 34 days (D) and in the adult (E).

"young") retract from superficial planes of the SC. Occasional poorly-arborized fibers persist for a while near the collicular caudal end.

3) Fibers that constitute a minor component of the ipsilateral retino-collicular projection arborize profusely in discrete patches reaching the prospective *stratum opticum*. All the patches are found within a territory, the boundaries of which are in register with those of the prospective direct binocular region.

A selective arborization of ipsilateral fibers within the region receiving this projection in the adult is deduced from the fact that only there the grains of reaction product became very small and assumed a diffuse distribution, mostly within a narrow superficial tier (Figs. 8 and 9). There was no stage in which they appeared within the collicular poles to disappear later. By contrast, the large, linearly-oriented grains appeared throughout the collicular extent to disappear altogether from the caudal half.

There is some degree of resemblance between a selective arborization of uncrossed fibers in the direct binocular region of the opossum SC and some events in the development of the cat's visual callosal projections [30]. In the neonate cat, neurons situated in prospective acallosal areas (e.g. medial area 17) send axons to the white matter of the opposite cerebral cortex but only those axons proceeding from a prospective callosal area (e.g. 17/18 border) enter the gray matter and only at the restricted tangential locations where they will also be found in the adult. The resemblance between the two systems does not go very far because retinal axons do enter regions where they will not arborize. However, some ultrastructural work in the rat SC suggests that temporary synapses are not formed by wrongly placed fibers [34; but see 7].

The next question to be answered is whether the development of arborizations is temporally related to axon elimination. The disappearance of poorly-arborized axonal segments could be merely due to a secondary cell migration causing fibers to be pushed to deeper levels and form a clearly defined *stratum opticum*. Countings of ipsilaterally-projecting ganglion cells would be the most informative data but are not available. Axon countings in the optic nerve of *Didelphis virginiana* indicate a close correlation between the time courses of axon elimination [36] and restriction of ipsilateral projections [50]. Countings of 2.5 times the mean adult value are obtained from the end of the third week to PND27, falling to half of this maximum by PND36 [36].

The mere fact that axon elimination and the redistribution of the

anterograde tracer are temporally related should not be reduced to a common denominator, the regression of portions of axonal arbors of degenerating ganglion cells [15, 32, 37, 55, 56, 75]. It is possible that the elaboration of axonal arbors of some ganglion cells starts earlier but follows a more prolonged time course than the degeneration and death of other cells. In other words, the onset of the profuse arborizations of the axons of some ganglion cells, crossed or uncrossed, would gradually create multiple sites of target deprivation for other cells. These would rapidly die as soon as this deprivation reached a critical level. The more prolonged persistence of poorly-arborized, uncrossed fibers at superficial levels of the caudal SC may be related to the late arborization of crossed fibers from the peripheral nasal retina. Studies of the development of arborizations of axons arising from limited regions of the retina are needed to settle the question of the correct sequence of axonal arborization versus elimination.

The last component of the uncrossed retinocollicular projection to show conspicuous transformations is that situated deeply. Patches of labeling reaching to the upper stratum griseum intermediale are still sharply defined after the 6th week but are recognized with difficulty by the time of eye opening (PND60) [50]. By contrast, the laminar segregation of crossed and uncrossed projections is still incipient by the end of the 6th week and becomes increasingly sharp in older animals. Both changes seem related to the expansion of the neuropil but other factors may be involved.

Glial Cells and the Maturation of Retinofugal Projections

It has been mentioned elsewhere that the anterograde transport of HRP along immature retinocollicular fibers may be accompanied by the labeling of radial processes leading to cells forming the walls of the brain ventricles at hypothalamic or mesencephalic levels. This was a fortuitous finding during our analysis of the growth and restriction of retinocollicular projections [50] and appeared limited to the first two weeks of postnatal life. Control studies of the optimal survival period after intra-ocular injection of HRP showed, however, that the labeling of the acqueductal ependyma outlasted the most obvious transformations of the retinocollicular projections provided that survival after eye injection was increased with age at injection [49]. A survival period of 3 days is sufficient to label heavily periacqueductal cells if injection is done at PND20

but 5 days are required for comparable labeling if done at PND45 (Fig. 10). After the onset of myelination in the optic layer (50-55 days) [45] no labeling of periacqueductal cells was obtained within a survival period of 9 days.

Some entrance of HRP into the blood circulation probably occurs when the enzyme is topically applied to or injected into the eye. In fact, we have occasionally seen in the brains of pouch youngs, HRP-labeled cells with similar shape and localization to those found labeled by blood-borne HRP in the brains of neonatal hamsters [41]. The intensity of labeling in these cells situated, for instance, within the internal capsule is bilaterally symmetrical in contrast with the labeling of periacqueductal cells after an eye injection.

Which are the possible routes for HRP to gain access to the processes of the cells in question? Periaxonal diffusion along intercellular channels to the optic chiasma [67] perhaps ougbt to be considered in early post-natal life. It is, however, unlikely that such a route, prolonged to the SC, would remain open until PND30-45.

Intra-axonal diffusion, rather than membrane-bound transport of HRP might be significant in immature animals either by intrinsic reasons or consequent to axon damage by mechanical, osmotic or chemical trauma associated with the injection of HRP and vehicle. Irrespective of diffusion or transport, HRP is selectively accumulated by cells with radial processes but not by local neurons. Wbat is the identity of such cells and what is the significance, if any, of this selective uptake and accumulation?

Since the HRP molecule is relatively small (40,000 daltons) its endocytosis is not sufficient to characterize a phagocyte without the simultaneous internalization of large particles or the presence of cell debris or, at least, large vacuoles in the cytoplasm. Ultrastructural evidence suggestive of axonal phagocytosis has been found for two types of profiles, "gitter cells" of the cortical white matter [29] and astrocytic processes of the spinal ventral horn [63] of the neonate cat. The endocytosis of HRP is exhibited by a variety of cells including ramified microglia, "gitter cells" or macrophages clustered in the white matter, fibrillary astrocytes, pericytes and cells of the telencephalic subventricular zone [28, 32, 41, 70]. Bergmann glia [25] and the ependymoglia [52] of amphibians endocytose topically-applied HRP only when damage to the pia is prevented or minimized. This is perhaps the reason why endocytosis of HRP by radial glial cells is questioned by many workers [28, 70].

Periacqueductal cells of the developing opossum have in common

FIG. 10. HRP-labeled periacqueductal cells in a 23 day old opossum that received a unilateral eye injection of the tracer 3 days before sacrifice. Side ipsilateral to the injected eye to the left. The heavy labeling of the somata on the contralateral side appears out of focus to allow clearer visualization of the processes.

with the ependymoglia of the amphibian optic tectum the capacity to accumulate HRP anterogradely transported by retinal fibers [73]. The suggestion that they are radial glial cells is, therefore, quite reasonable although immunocytochemical testing has not been performed.

The most relevant point is whether this transcellular transfer of a glycoprotein is a clue to interactions between developing fibers and radial glia. Could the same cell type be involved with such diverse developmental events, as neuronal migration [58] and phagocytosis of afferent fiber endings? If endocytosis of HRP is a clue to a phagocytic activity, the loss of axonal endings would happen throughout the phases

of axon production (up to PND27) and axon elimination (from PND27 to PND50) in the optic nerve of *Didelphis* [36].

There is no compelling argument either against a continuous loss of afferent axons [75] or against multiple roles being played by radial glia at different developmental epochs. For instance, they may exhibit changes similar to those displayed by reactive astrocytes *vis-à-vis* damage to adjacent neurons [4].

A possibility that may deserve examination is that there is a bi-directional transfer of molecules between radial glia and optic fibers. Terminal competition could possibly involve a long-lasting fight for a limited supply of a glial factor instead of or in addition to factors related to target neurons.

ACKNOWLEDGEMENTS

I would like to thank my colleagues S. Allodi, P.C. Barradas, E. Carvalho-Dias, J.N. Hokoç, A.B. Martinez, R. Méndez-Otero and C.E. Rocha-Miranda for permission to quote unpublished results. Thanks are also due to C.E.L. Esbèrard for developing and maintaining our opossum colony. Financial support was provided by CNPq (Proc. 40.5917/83), FINEP (41.85.0245.00) and CEPG/UFRJ. Permission to reproduce Fig. 8 from *Developmental Brain Research* is gratefully acknowledged.

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ON THE DEVELOPMENT OF NEURONAL ARBORS

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INTRODUCTION

To attain a high level of connectional specificity in the brain, axons from a group of neurons must not only reach their target zones, but once there, must solve the problem of where and how to terminate within these regions. In a recent study of retinofugal axon morphogenesis, we have shown that during the phase of elongating toward their targets and the phase of ramifying therein, axons of retinal ganglion cells exhibit specific and distinct growth characteristics. The differences imply that the cell is exhibiting different *modes* of growth, which we refer to as the *elongation* and *arborization modes* [64, 65, 141].

Much information is available with regard to possible regulatory controls on axon growth during the elongation mode. The data have been used to argue for specific strategies which may be utilized by the axon in reaching its target. On the other hand, relatively little is known about factors that might orchestrate developmental transformations of axons in the arborization mode. In this paper, we review selected studies on axonal and dendritic growth, concentrating on the intermediate stages of axon morphogenesis — i.e., maturational events that take place *after* target-directed elongation has already occurred. We begin with a classification of what appear to be distinct phenomena in the developmental transformations of end-arbor structure, emphasizing studies on mammalian CNS (See ref. 113 for review of PNS work). We next summarize some basic factors or rules which may underly these phenomena. In a final section, we review some theoretical work on arbor formation and present preliminary studies on the potential of computer simulation in testing the

relevance of growth and interactive parameters related to neuronal arbor development.

Various Kinds of Exuberance in Axonal Development

Most reviews of axonal growth have emphasized mechanisms of selective fiber elongation and synaptogenesis. Descriptions and analyses of branching pattern development, at the single axon level, are not as numerous. Inferences about axon arbor maturation have been based, for the most part, on studies of *populations of axons*. Many of these have supported the idea that axons grow directly to and ramify at their proper locations within terminal regions, relying on chemical signals (e.g., ref. 154) or "mechanical" factors and timing of axon arrival (e.g., refs. 53, 114) for appropriate targetting. However, recent studies of axon growth reported in the literature have stressed transformations involving branch loss: the phenomena of transient "exuberant projections" [54, 55]. Several types of exuberance can be discerned, and are outlined below.

1) *Anomalous projections of axon collaterals that are subsequently withdrawn.* In young animals, axons of neocortical cells project, via the callosum, to target zones which are acallosal in the adult [18, 54, 55, 57, 60,, 103]. In mature animals, many of these cells may have no callosal connections at all. Transient projections from tbe auditory cortex to visual cortical areas in the kitten are also lost with further development [56, 58]. The shaping of the mature pattern of these projection fields has been shown to occur as a result of axon collateral withdrawal, rather than of cell death. Similarly, neurons in the immature occipital cortex extend axons into the cerebellum and spinal cord, collaterals of fibers projecting to the midbrain. As development proceeds, axons extending beyond the pontine grey are lost [28, 155].

In the hamster, developing retinofugal axons innervating the lateral geniculate body transiently extend beyond their target to invade the lateral margins of the ventrobasal nucleus (Fig. 1). Moreover, fibers of retinal origin reaching the caudomedial margin of the superior colliculus continue over the surface of the inferior colliculus (Fig. 1), only to be partially removed in older animals (see also refs. 43, 62, 70). Such specific but "anomalous" projections in immature animals may represent a kind of partial recapitulation of the evolutionary history of the species [30, 138].

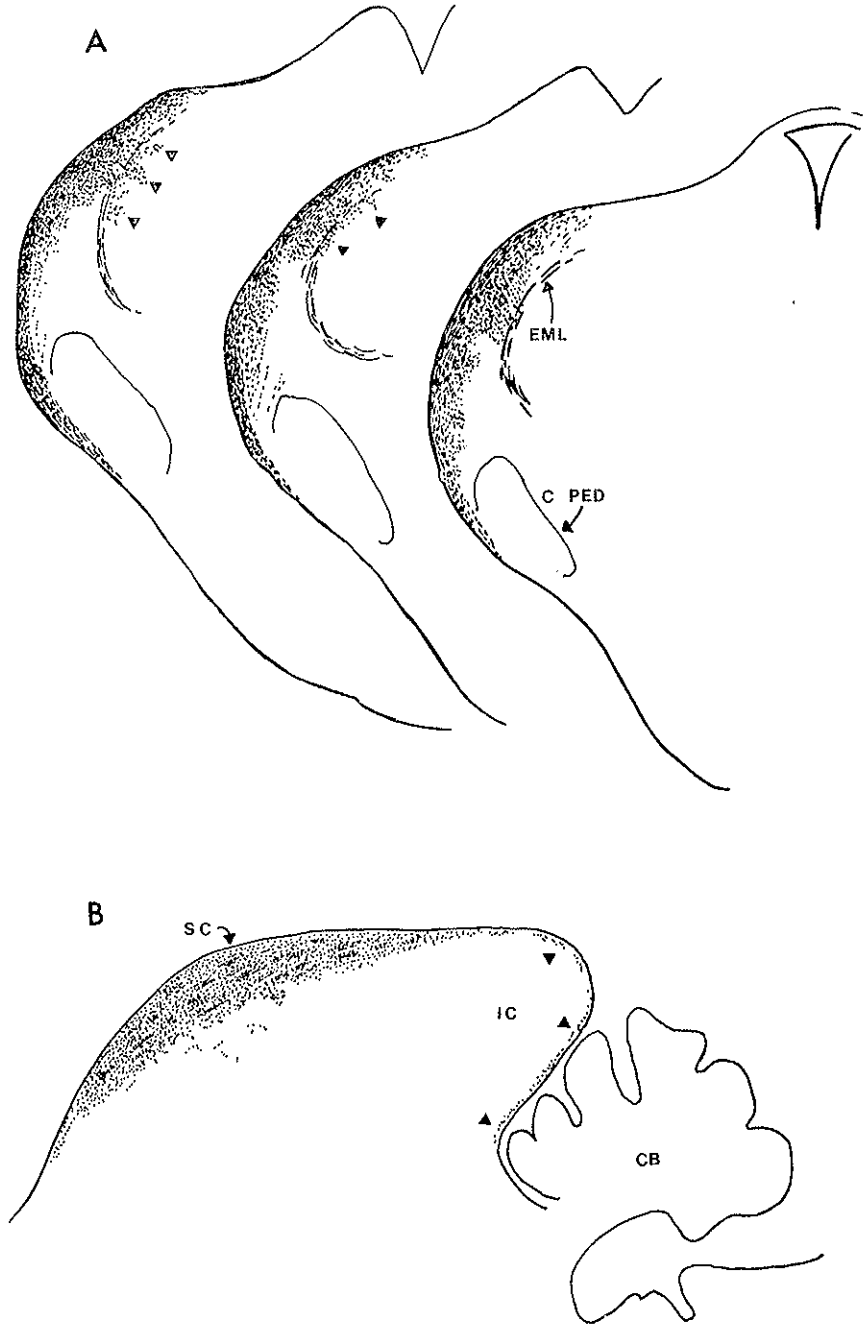


FIG. 1. Charts from brain sections of 3-day-old hamster pups in which one eye had been injected with HRP. Tissue was processed for HRP histochemistry using tetramethyl benzidine as the chromogen. *A*) Transverse sections through the lateral geniculate body, showing extension of contralateral retinal projections through the external medullary lamina into lateral margins of the ventrobasal nucleus (arrowheads). At this caudal level of the LGBd, such an extension is transient. *B*) Sagittal section, showing retinal projection over the surface of the inferior colliculus (arrowheads).

2) *Initial development of axons in a target area where a topographic map forms.* Axons from the hamster's retina extend through the entire superior colliculus by the 13th day after conception. After a delay of two days (a "waiting" period), the axons begin to sprout multiple collaterals which are distinct but not very complex. The early widespread branching is followed by a focalization of the end arbor, as one or a few collaterals are augmented and others lost [141; see also ref. 126]. Axons in the hamster's visual cortex show a similar progression at about the same time [Figs. 2-4; refs 97, 99]: the widespread rudimentary branching occurs in the intermediate zone below the developing cortical plate [cf. ref. 56].

In the retinocollicular as well as geniculocortical systems, we have shown that the period of axon arbor focalization is temporally correlated with the sharpening of the topography of the connection [97, 98, 140]. In fact, an initially diffuse projection is a common prelude to the formation of precise, point-to-point connections between two groups of neurons — not only during development [87, 102, 126, 164] but also during regeneration [22, 45, 89, 132, 158, 163]. In maturing systems, although the role of axon collateral loss in the formation of topographically specific connections has been well documented, cell death may also be a major contributing factor in the creation of an accurate map [23, 102].

3) *Transformation in a focalized arbor.* After topographically organized axons have established their terminal ramifications in target regions, an end arbor may spread over a relatively larger territory than in the mature form. With the influence of physiological activity, these end arbors shrink in territory as their density continues to be augmented. Such a phenomenon has been reported for the visual system of several species [21, 80, 81, 82, 117, 131, 161]. *Late focalization* is observed for X cell axons in the lateral geniculate body of the cat and not for Y cell axons. This difference may explain some alterations in physiological properties of geniculate cells, apparently due to an abnormally wide distribution of X type axons, as a consequence of visual deprivation [42, 159].

4) *Development of laminar specificity of axonal arbors.* Just as remarkable as the selection of distinct tangential loci by rapidly forming axonal arbors is the laminar specificity many of them display. A dissociation between tangential and laminar organization of developing axon systems has been demonstrated by Rakic [118] in the geniculocortical projection of primates. Unilateral eye enucleation in fetal monkeys results

in the failure of segregation of cortical afferents from the geniculate into ocular dominance columns. In these same animals, however, the laminar separation of the afferents to cortical layers 4A and 4Cβ (from the parvocellular geniculate laminae) and to cortical layer 4Cα (from the magnocellular geniculate laminae) develops without disruption. This observation indicates that the two phenomena are under different regulatory controls.

In the lateral geniculate body of the cat, optic-tract axons form their telodendritic arbors in precise laminar formations [12, 160, Tello, cited in ref. 121]. In a recent review, Shatz and Sretavan [143] have cogently argued that binocular interactions between retinal axons, in addition to interactions between fibers from the same eye, are necessary in establishing these precisely targeted projections to the lateral geniculate body.

Developing optic-tract afferents to the midbrain tectum of mice display another type of phenomenon [33]. In mature animals, retinofugal axons enter the SC through the optic fiber layer, subjacent to the layer of cells among which their terminal arbors form. During the period of initial ingrowth, however, optic tract axons are found near the pial surface and also scattered in tiny bundles throughout the superficial grey layer. As arborization proceeds, the superficial bundles disappear, apparently as a result of degenerative events. Remaining afferents from the retina continue to mature and form two major types of end arbor with distinct laminar specificity [44, 48, 129]. Moreover, axons from the visual cortex project mainly in the deeper of the two sublaminae. The laminar specificity of the corticotectal projection is dependent on tbe presence of the retinocollicular axons. Removal of one eye early in life results in a disruption of the laminar organization of corticotectal axons in the contralateral SC [139, 169].

Comment. The various types of "exuberance" of developing axons noted above can be related to the two modes of growth discussed earlier. *Exuberance of axons in the elongation mode of growth* can cause what appear to be anomalous projections far from the normal targets, destined to be removed by collateral elimination. The recent work of Innocenti [56] lends credence to the notion of exuberance in the elongation stage: exuberant callosal axons remain in the white matter, and only enter more superficial cortical layers (i.e., arborize) in regions where the stable adult projections will form. More study of this type of phenomenon is necessary to determine whether such excess collaterals display *any* rudimentary arbors before their disappearance.

Exuberance in the arborization mode can be of several types: a) Axons form transient, rudimentary arbors at more positions along their course within a target area than will be maintained as the topographically correct endings become augmented (e.g., retinofugal axons with widespread branching in the SC,

or neocortical axons in area 17 — see Figs. 2-4). b) Axons penetrating a target zone form transient branches outside their normal laminae of termination (e.g., retina to VB, or contralateral retina to all layers of the LGBd). c) Some axons which are forming focal end arbors show a transient spread, tangentially, beyond their mature territory; thus, their overlap with neighboring end arbors goes through a supernormal stage (e.g., in ocular dominance columns).

The usefulness of this dual classification of the phenomena of exuberant axon growth requires further experimental documentation. Such a schema implies that neurons use distinct growth strategies while exhibiting the two types of events. Observations in mutant mice provide support for this dissociation: Retinal axons of reeler mice follow abnormal trajectories in the superior colliculus (i.e., exhibit errors of elongation) while their pattern of terminal arborization remains normal [44].

If supported by further evidence, this kind of a differentiation could help sort out various mechanisms of regeneration. For example, regeneration of adult mammalian CNS neurons, through peripheral nerve grafts, appears to involve massive growth of axons in the elongation phase, whereas arborization by these regenerating fibers, after tbey have re-entered the CNS, is severely limited [25].

It should be noted that phenomena of "exuberance of cell number", demonstrated by findings of cell death during brain development, should be distinguished from those in the above classification. Although one might be tempted to call the anomalous, transient projection of retinal axons to the *ipsilateral* LGBd an "exuberance" of elongation, since the axons are on the wrong side of the brain, these axons arborize and form synapses as well [17].

Dendritic Arbor Formation

Two types of phenomena have been described for dendritic arbor formation. In the first, a postmigratory neuron develops progressively more complex dendritic extensions. Such a sequence has been reported for several different cell types [1, 4, 19, 157]. On the other hand, as noted for many axonal systems, an initial exuberance of neuritic extensions coupled with a subsequent regression is also a commonly observed phenomenon in studies of dendrogenesis: e.g., the Purkinje cells of the cerebellum [121, 130], neurons of the mammalian and avian auditory system [66, 93, 148], and ciliary ganglion cells [76]. The formation of excess dendritic spines on immature neocortical neurons [83, 93, 110] is another form of exuberance in dendritic ontogenesis.

There is, at present, no consistent explanation for why certain cells undergo such remarkable developmental transformations in dendritic arbor whereas others exhibit a simpler progression of dendritic branch growth. It is possible that a variety in such dendritic growth phenomena, as well

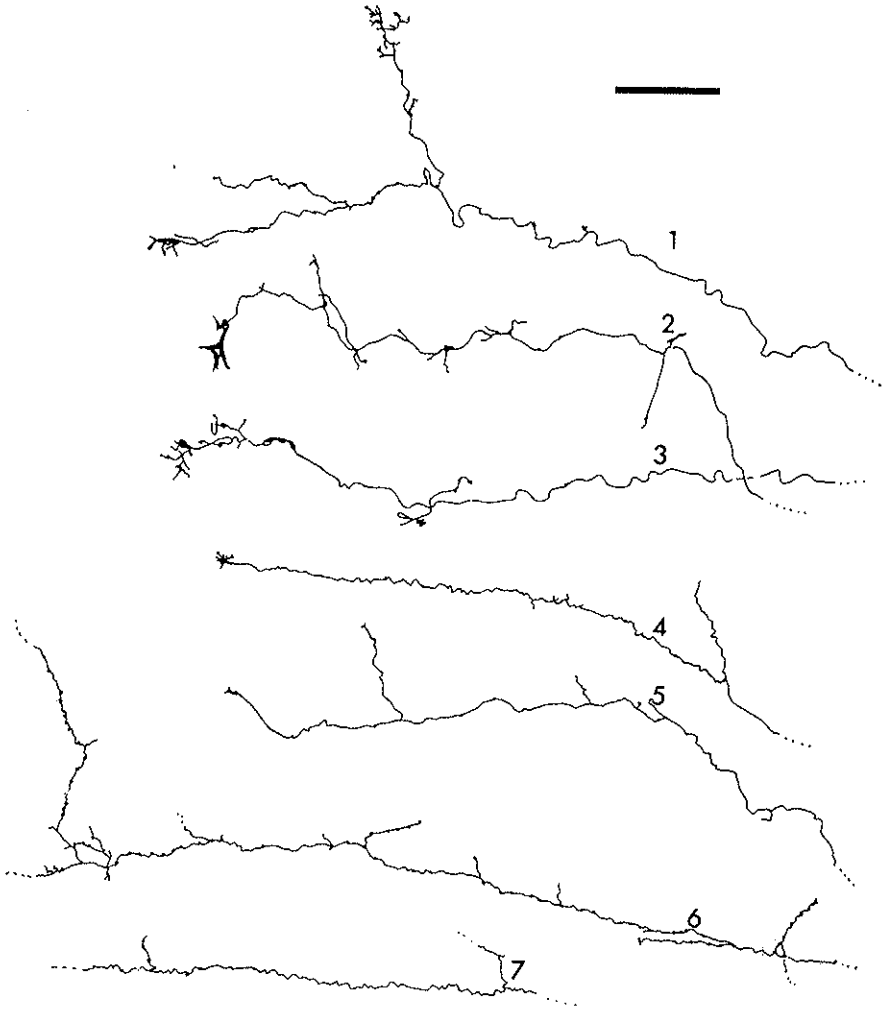


FIG. 2. Camera lucida drawings of axons in neocortical area 17 of hamsters with local injections of HRP in, or just lateral to, visual cortex. Tissue was sectioned transversely and processed for HRP histochemistry using diaminobenzidine (DAB) as a chromogen. Axons 1-5 are from animals on the day of birth (P0) through P2; axons 6 & 7 are from animals at P4 & 5. Medial is to the left. The horizontal axon trunks are in the intermediate zone or deep part of the cortical plate. Note the widespread but rudimentary branching which precedes early focalization of the end arbor. Growth cones are prominent on some of the axons. Scale bar: 20 μm for axons 1-3, and 50 μm for axons 4-7. (Redrawn from ref. 97).

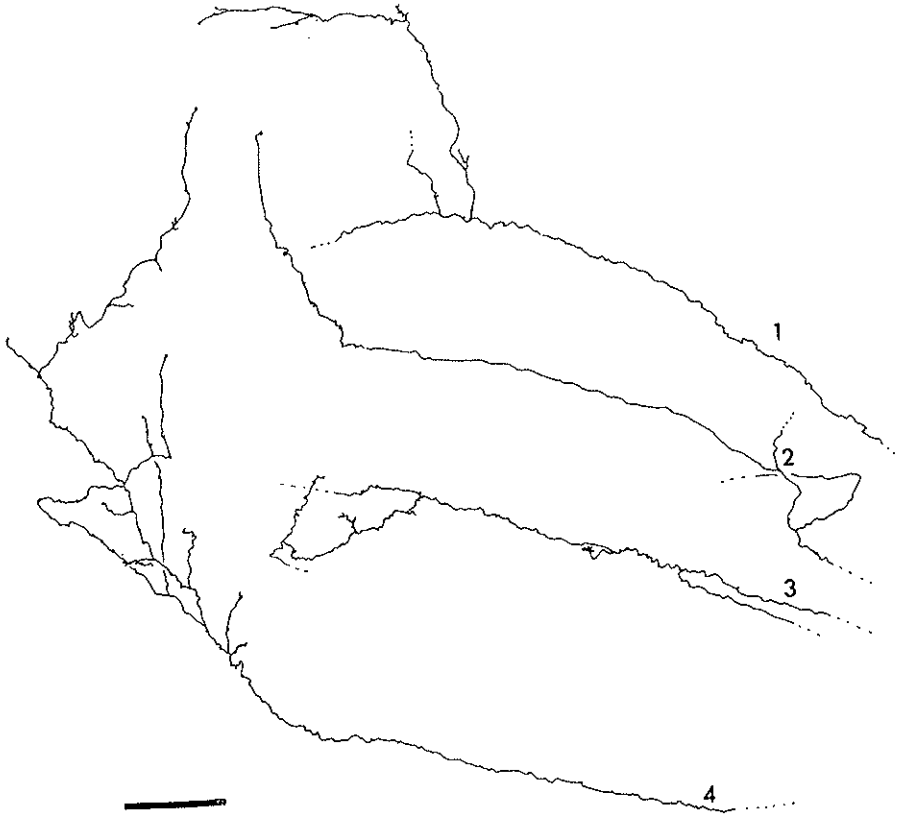


FIG. 3. Axons from striate cortex processed and displayed as in figure 2. Hamsters were aged P0-P2 (axons 1-3) and P5 (axon 4). Focalization is beginning, especially in axon 4, but some exuberant collaterals are still evident, and in some cases the axon trunk extends beyond a developing focal arbor (axons 1 and 3). Scale bar = 50 μm. (Redrawn from ref. 97).

as various degrees of cell death in different neocortical areas [36] may accompany a surprisingly uniform course of synaptogenesis across the cortex, with an overproduction of synapses followed by a gradual loss [119].

EXTRINSIC AND INTRINSIC DETERMINATION OF ARBOR FORMATION

Numerous reports have documented the involvement of afferent input in sculpting mature dendritic arbors. Descriptive studies demonstrate

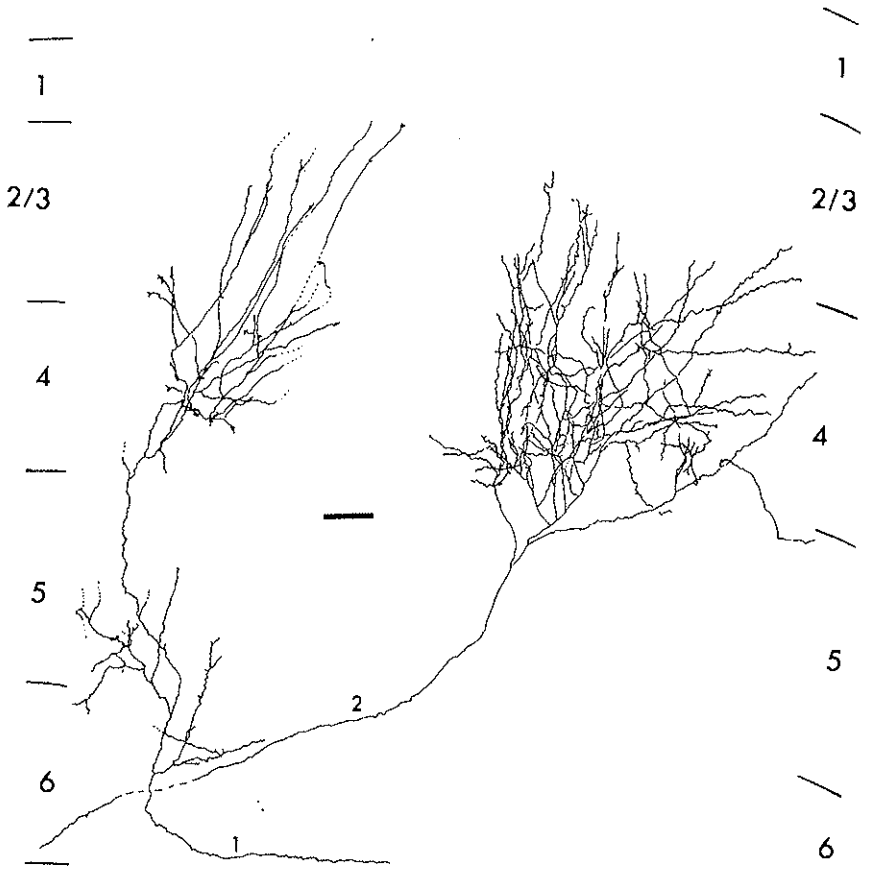


FIG. 4. Axons from striate cortex processed as for figure 2. Hamsters were 12 days old and adult (axons 1 and 2, respectively). Orientation is as in figures 2 and 3 except medial is to the right for axon 2. Axon 1 has at least 10 growth cones in its expanding end arbor. Large numbers at left and right indicate neocortical layers. Scale bar = 50 μm. (Redrawn from ref. 97).

a high degree of synchrony in the anatomical development of incoming axons and of the target cells [66, 92, 94, 109, 115]. Incoming immature axons form synaptic contacts on the transient, exuberant somatic processes [67, 74, 76] and thus are in a position to influence postsynaptic neurons. These observations strongly suggest that interactions between axons and target cells are active in shaping morphological development.

A second line of evidence involves the removal of afferents and

subsequent examination of target dendrites. Such studies include removal or alteration of afferents by genetic perturbations [24, 111, 120, 152] or by experimental manipulations [2, 10, 109, 153, 156, 167]. Results from the above studies support the conclusion that dendritic arbors are, in many cases, severely affected by absence or alterations of afferent input. Moreover, morphological studies have also provided evidence for competition among the dendrites of retinal ganglion cells [108] and cerebellar Purkinje cells [140] for exclusive occupancy of neighboring domains.

In addition, however, a fundamental determinant of the characteristic shapes of many neuronal cells and their dendrites is apparently derived *intrinsically* [116, 153, 168]. Tissue culture experiments support this statement. Neurons grown in dispersed cell culture have shown a striking morphological resemblance to their *in vivo* appearance [4, 16, 75].

Even stronger evidence for genetic determination of neurite branching patterns has come from studies of neuroblastoma cells [150, 151]. Sister cells grown *in vitro* have very similar neurite branching patterns, in contrast to cells that are unrelated by lineage. Disruption of the neurites by colchicine treatment is reversible: after removal of the colchicine, each cell re-establishes a pattern of neurites similar to its earlier one. Thus, even when neurites are growing in isolation in homogeneous environments, characteristic branching patterns can be established.

Evidence of the importance of *extrinsic influences on patterns of axonal termination* abounds. Included are examples of target influences on end-arbor shape, on the topographic distribution of axonal arrays, and on the survival of afferent axons. Such effects can be illustrated from our work with the visual system of the Syrian hamster. At the time of birth, retinofugal axons have invaded the superior colliculus but have scarcely begun to arborize. If the superficial layers of the SC on one side are destroyed at this stage, axons from the retina re-invade and terminate in the area of damage. In addition, optic axons form an abnormal decussation, crossing the tectal midline to invade the intact superficial grey layer on the opposite side [37, 135, 136, 149]. The conditions of axo-axonal competition are drastically abnormal for the decussating axons: They invade territory already occupied by normally projecting axons from the other eye, and must compete for space there (the two populations segregate to a considerable degree in a few days time). As a consequence, axons from the wrong eye squeeze most of their terminals into a narrow medial

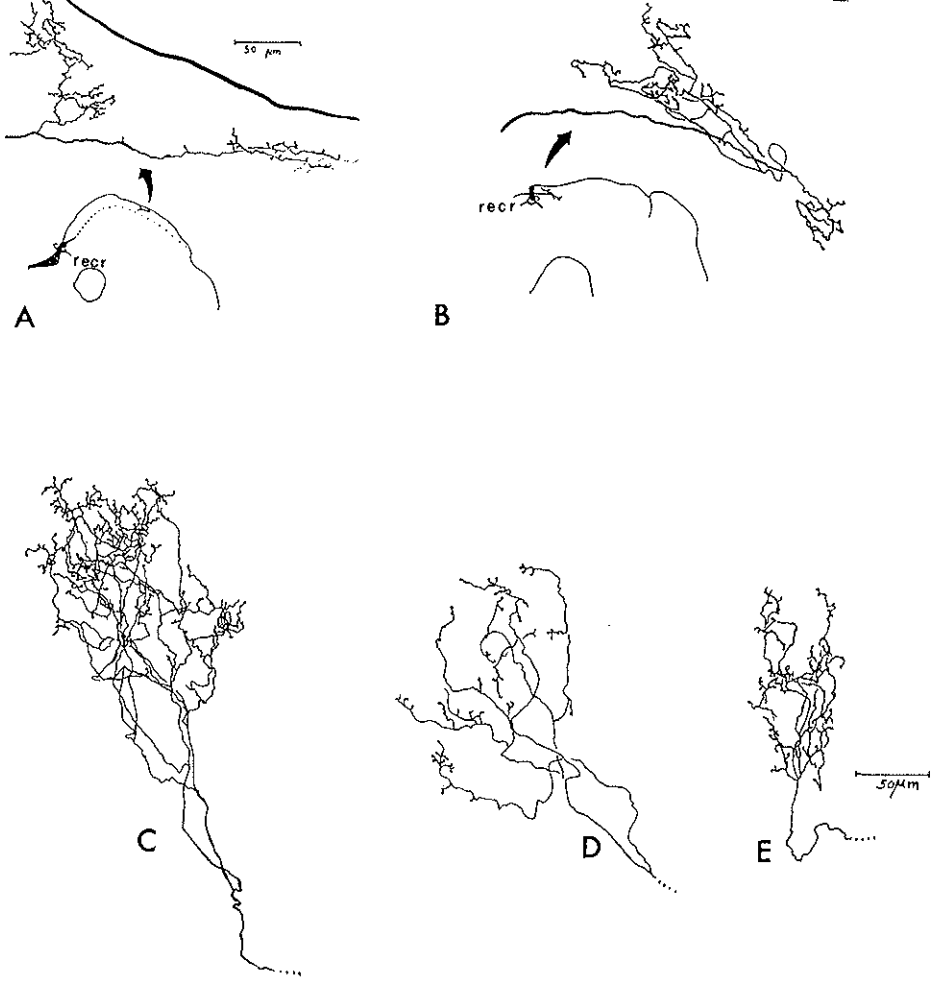


FIG. 5. Camera lucida drawing of axons traced from animals with local injections of HRP in the brachium of the superior colliculus. Tissue was processed for HRP histochemistry using DAB as a chromogen. Abbreviation: *recr*, bundle of optic-tract axons recrossing the midline into the superficial gray layer of the wrong side. (Redrawn from ref. 125).

A) Axon from an abnormal, recrossing projection in a hamster which had suffered ablation of the left tectum and removal of the left eye at birth. Low-power view shows that this axon terminated in the lateral portion of the intact SC, where the retinal projection generally becomes sparse and very superficial in such cases. The camera lucida tracing was from two adjacent transverse sections. The axonal arborization gave rise to two small terminal fields separated by approximately 10 μm. Cf. normal axons (C and D).

B) Axon that crosses the tectal midline abnormally in hamster with neonatal unilateral tectal ablation. The high-magnification drawing was reconstructed from the section in the low-power view plus two adjacent sections. The lateral margin of the terminal distribution (extending diagonally from upper left to lower right) is along the edge of the terminal zone of the recrossing projection, which abuts a projectoin from the other eye.

C) Reconstruction of a type U retinofugal axon terminating in the upper part of the superficial gray layer of the SC of a normal hamster, from 4 adjacent sections cut sagitally at 70 μm. Caudal is to left. (From ref. 129).

D) Reconstruction of terminal arbor of type U axon in hamster SC (sagittal view). This axon has the least extensive terminal field of 16 axons of this type reconstructed from normal hamsters.

E) Similar reconstruction of axon in superficial gray layer of hamster SC, showing the least extensive field of 13 axons reconstructed from hamsters with neonatal lesions of the more caudal part of the same layer. Statistical comparison of the anteroposterior extent of normal axon arbors and arbors in the partial tectum lesion cases shows that the compression of the latter arbors is a significant trend ($p<.01$, Mann-Whitney U test).

strip of tectal tissue [37, 127]. By contrast, if the competition is eliminated by removal of the eye contralateral to the intact colliculus, the abnormally crossing axons have access to more than the normal amount of target space, especially in the more lateral tectum, which is reached by fewer axons.

Under such conditions (retinofugal axons have entered the tectum along an abnormal trajectory, exhibit considerable disorder among themselves, and encounter greater or less crowding than normal), terminal ramifications of individual axons are altered. We have found arbors that are extended over an abnormally large territory but the total number of varicosities (terminal boutons?) on each arbor is not correspondingly supernormal (Fig. 5; refs. 125, 142). Thus, the effect of the environment on axon arbor shape is undeniable, but distinguishing the relative roles of intrinsic and extrinsic determinants will require much further investigation.

Neonatal ablation of the caudal or rostral portion of the superficial grey layer prior to arborization of the axons results in an increased crowding of retinal axons in the remaining SC. Such a paradigm was first applied to the mammalian visual system in our laboratory [63, 68, 139]. This kind of manipulation results in the formation of a compressed representation of the retina in the remaining tectum. Individual end-arbors are compressed as well [125, 128; see Fig. 5]. Electrophysiological mapping studies of such cases revealed that the compression was not a simple linear one, but included systematic deviations from linearity [35]. Anatomical observations also uncovered isolated areas of discontinuity in the maps [63].

On the other hand, despite the compression of individual arbors, the reduced tissue volume cannot support a normal number of innervating axons. In fact, there is a reduction in optic tract size and a loss of retinal ganglion cells proportional to the reduction in tectal size [165]. Thus, altered conditions of competition can affect the very survival of axons and cells (a phenomenon that varies markedly with the age of the animal at the time of the lesion — see ref. 107).

From these and many other studies, one can strongly conclude that growing axons are affected by *extrinsic* factors in their competition for arbor formation, and that these factors influence arbor shape, specific sites of arbor location (details of map formation) and the survival of the axon itself (see also reviews in refs 61, 84, 113). Much less work has been focussed on revealing the nature of *intrinsic* determinants of axonal arbor shape (see refs. 72, 78). Nevertheless, there is no doubt that both

intrinsic and extrinsic factors and their interaction will have to be clarified before we can comprehend how the multiple distinct species of axonal trees can develop in the rich forests of central neuropil. To approach this important task, we begin by listing major classes of ontogenetic "rules" supported hy current evidence.

Multiple Rules of Axonal Growth and Arbor Formation

Many rules of neurite growth have been proposed in the literature. We discuss below some of the ones most relevant to axon arbor formation, concentrating on those which can be specified at the cellular, if not the molecular, level.

Specification of cellular rules which govern competitive interactions among arborizing axons has benefited from a wide range of experimental approaches (including both tissue culture and *in vivo* work on CNS and PNS) as well as from theoretical studies. Commonly cited rules, or classes of rules, are: a) *cell-cell adhesivity*, involving various degrees of affinity between cells, and b) *trophic effects*, in which chemical factors necessary for cell survival are taken up at target sites, or in which the molecular environment has a local or global influence on the vigor of growth. Less frequently mentioned but nonetheless important rules involve c) dynamic consequences of *axo-axonal contacts* resulting in redirection or inhibition of growth. Two kinds of quantitative limitations on synaptogenesis (and thus on arbor formation) include d) *conservation rules*, in which the growth vigor of an axonal arbor is diminished as it approaches a certain limit in number of its terminals, and e) *saturation rules*, in which target cells accept only a certain number of synaptic contacts, thus regulating synaptic, and therefore afferent arbor, density. To this list of rules must be added f) *intrinsic controls* on patterned branch formation. [Note: The term "chemoaffinity" [154] generally refers to selective cell-cell adhesivity. The broader "chemospecificity" applies to selective effects involving either of the first two types of rules (*a* and *b*), and it may he relevant to special axo-axonal contact reactions (rule *c*) as well.]

Evidence for *a*) *cell-cell adhesivity* — which can vary in strength depending on cell identities — comes mainly from tissue culture studies (reviewed in refs. 77, 78, 113). Adhesivity refers to the relative "stickiness" between cells and substrate at their contact points. How it is expressed *in vivo* is not very clear: it could affect the average duration of contacts, as well as the force needed to separate cell and substrate.

This factor has played a major role in theories of retinotectal specificity [38, 39, 40, 154], and has received special attention with the discovery of specific cell-adhesion molecules, designated as N-CAM [32, 124, 144, 162]. When axons begin to arborize, they must leave their fascicles and grow on other cell surfaces (glial or neuronal). *In vitro* studies have demonstrated that axons exhibit a preference for growing on more adhesive substrates, and that the amount of axon branching is directly correlated with the adhesivity of the substrate [77]. Thus, for axons maturing in the brain, a shift in relative adhesive forces could induce fibers to defasciculate, enter a target and begin arborization therein [144]. Such adhesive forces could be mediated via specific molecular markers expressed transiently on the surfaces of growing neurites [46].

In tissue culture, axon branches arise from bifurcations of the growth cone, a result of greater adhesive attachment of the lateral than of the central regions of the growth cone [13, 170]. *In vivo*, growth cones occur at the tips of immature axons and are probably the site of initiation of terminal ramifications. These specialized tips on growing fibers exhibit morphological variations depending on whether the axons are growing in a fasciculated fiber tract or arborizing among postsynaptic cells [86]. On the other hand, branches also occur along the trunk of an axon. For instance, during development, retinogeniculate collaterals commonly arise from varicosities on axons that continue caudally to innervate the SC [64]. It is possible that, *in vivo*, these varicosities function as *en passant* growth cones.

The role of *b*) *trophic factors* in both axonal and dendritic arborization has long been appreciated, but mechanisms of action at the cellular and molecular level remain a major experimental issue. A detailed discussion of trophic agents is beyond the scope of this review (see refs. 5, 7). A common assay in the study of effects of trophic factors has been the observation of increased growth of neurites in culture. Thus far, analyses of neurite growth in response to growth-promoting chemical substances bave not distinguished between axon elongation and axon arborization (see, however, ref. 166). Nevertheless, it seems clear that such substances could affect both the direction of outgrowth of branches and the vigor of growth.

The role of *electrical activity* in modulating such trophic effects is becoming increasingly clear [133]. Interest in this area has focussed on the role of synaptic activity in selective stabilization of presynaptic terminals and the consequences of such stabilization in sbaping of afferent

arbors. Activity-mediated influences on the focalization of an initially widespread axon arbor (termed "late focalization" above) are implicated by findings on the development of ocular dominance patches or topographic maps [11, 34, 90, 91, 123, 132, 134]. Patterned activity can also affect the form of dendritic arbors [47, 167]. Furthermore, other effects of small electrical currents, which may be generated by potential differences across the primitive neuroepithelium as well as by action potentials, are being revealed in recent tissue culture studies of growth cone dynamics [41, 106].

The role of *c*) *inter-axonal contact reactions* in growth dynamics was examined by Dunn [29], who described a "contact inhibition of extension" in non-fasciculated axons growing in explant cultures of dorsal root ganglia. When a growth cone encountered another axon, it retracted, changed direction and continued its growth. Such dynamics could simply be the result of greater adhesion between axon and substrate than between axons. However, at present we must consider the possibility that there are additional, intrinsic changes in the growing axon not fully explained by adhesion mechanisms. Possible effects of contact different from those described by Dunn are not difficult to imagine. For example, an axon touched by another axon could inhibit growth of new collaterals in the vicinity of the contact.

Direct encounters between neurons are made by filopodial extensions from the growth cones and from the shafts of growing axons. These filopodia or "microspikes" show lengths of up to 120-150 μm in tissue culture [101] but maximum lengths of 6-20 μm are more common [3, 14, 101]. Therefore, the "interactive cross section" of a growing axon *in vitro* is probably 12-40 μm. An axon could thus contact another axon (or neuron) whose shaft was up to 80 μm distant, or, more rarely, up to 300 μm away. In light- and electronmicroscopic studies of filopodial contacts and selective adhesion in the grasshopper embryo, microspike lengths of 30-50 μm are cited [46]. It should be noted that all of these observations of microspike length probably involve axons in an elongation, rather than in an arborization, mode of growth.

d) An intrinsic limit to the number of synaptic contacts which an axon can form [104], or the "*conservation rule*", is supported by the observation that a decreased axonal arbor at one locus results in promotion of arbor growth in other regions — phenomena which have been called "pruning effects" [26, 27, 135; see below]. Conservation rules of this

type have been verified in recent tissue culture studies [147]. Such constraints imposed on axon growth may be manifest as follows: The "competitive growth vigor" of an axon would decrease as a function of the number of terminals formed [141]. Thus, formation of a larger terminal arbor would reduce an axon's ability to compete for further terminal space [26, 27]. On the other hand, restricting arbor development in one target region would make the axon more vigorously competitive in other terminal regions [135]. Modulation of this factor, e.g., by contact with chemical factors, by electrical activity, or by degree of maturation, appears likely although such modulation could work via other growth rules also. At the cellular level, competitive growth vigor may be reflected by axon elongation rate, filopodial length and speed of action, degree of reaction to contact by other axons, or a combination of such properties.

e) "*Saturation rules*" are equally important for the control of arborization dynamics: synaptic density appears to be regulated by target cells in the CNS [20, 95, 96]. Thus far, little is known about such regulation. Does the cell regulate the maximum number of contacts by producing a limited amount of trophic factor or factors released at synapses, or are there other constraints for such regulation, e.g., spacing between adjacent contacts? Do all neurons limit the *total* number of synapses on their surface? Or is each afferent type regulated separately? It is possible that a combination of conservation and saturation rules may govern the degree of plasticity in a system. For example, if the maximum number of terminals possible for an axon is greater than the upper limit of synapses allowed by the postsynaptic cells, such an axon would be likely to exhibit sprouting of collaterals when adjacent terminals were removed or diminished [137].

f) *Intrinsic control of neuritic branching*, discussed earlier, must interact with the other influences listed above. Exactly how nerve cells can control their branching pattern is unknown. Alterations in the shape of the growth cone precede branch formation. These alterations appear to depend on filopodial adhesion to the substrate — extrinsic factors [13, 77] — and on the state of polymerization of axonal microtubules — intrinsic regulation. Letourneau and his collaborators [79] have shown that taxol, a drug that increases microtubule stability, results in a significant reduction in the likelihood of growth cone bifurcation, thus leading to decreased branching. It is also noteworthy that there are changes in the amounts of specific proteins in the fast phase of transport as the axon shifts from elongation to arborization [6].

Additional factors: In the above formulations of growth rules, other considerations have been ignored: e.g., how straight do axons grow, and how does this vary with axon type and with substrate [71]? How does the speed of axonal or dendritic extension vary with cell type, time and substrate? How do "mechanical" factors, often cited in discussions of axonal *elongation* [31, 113, 145, 146], influence the pattern of arbor formation? Such factors include guidance effects of substrate "channels" and of aligned fibrillar structures. Answers to these and other questions must be addressed for a comprehensive understanding of neuronal arbor formation.

Evidence in Search of Theory

The above review indicates that we must contend with a host of simultaneously acting rules which govern active cellular interactions during neurite growth, and especially during axonal arborization. *A list of such rules does not constitute an adequate theory, since we do not know what it predicts for a sizable group or groups of nerve fibers.* To predict specific events of axon arbor formation, two kinds of further information are needed: 1) experiments which include assessment of quantitative details that will aid an evaluation of how each rule acts, i.e., how strong each interaction is, and its precise role for different cell types; and 2) the ability to simulate accurately the rule-bound growth of sizable populations of simultaneously growing neurites.

Some promising directions for meeting these needs can be briefly stated. Descriptive studies of developing neurites are becoming more numerous; attention to quantitative detail in such studies will be increasingly relevant (e.g., information on growth rates, filopodial lengths and frequency as a function of position, branch and terminal numbers, three-dimensional shape parameters including angles of bifurcation, and population distributions at closely spaced intervals of time). *In vitro* studies can be used to examine the cellular basis for fundamental growth rules and provide further quantitative data, e.g., on relative strengths of adhesive forces [69]. *In vivo* analyses of experimentally manipulated animals, including studies of neuronal transplants in developing mammals [85, 88] can furnish new information, e.g., on hierarchies of selectivities in the formation of connections.

The second need noted above can be met only by large-scale computer simulations. Computer simulations have been shown to be of great utility

in modeling the growth of dendritic trees of cerebellar Purkinje cells [8, 130]. Simulation methods have long been used in attempts at modeling the development or regeneration of topographic order in the retinotectal connection [38, 39, 40, 51, 105, 112, 171, 172, 173]. Axonal endings and tectal cells in the latter simulations have been represented as points or regions, initial conditions have been specified and sorting algorithms used to move the endings. The algorithms are based on postulated rules of cellular interaction. Such modeling has demonstrated that *different* sets of rules can generate the *same* topographic map. Some of the models, however, fail to predict the effects (as observed in the laboratory) of specific experimental manipulations [40]. Recently, Fraser [39] has extended his model, using it to make specific predictions which have been empirically tested (e.g., in studies of the effects of anti-N-CAM antibodies on the formation of retinotectal topography).

The stage is set for developing a simulation facility which combines a) the generation of anatomically realistic tree structures displaying the entire course of developmental changes, and b) large numbers of both pre- and post-synaptic elements. We need the ability to include in the simulation each and all of the types of intrinsic and extrinsic rules discussed above, acting simultaneously in large, growing "forests" of neuronal processes. Some progress has been made in this direction [73]. We are developing a large-scale simulation program at M.I.T. which is designed to answer this need (Davis and Schneider, unpublished).

In initial testing of this general purpose modeling facility, we have demonstrated the ability of the program to simulate tree growth in the manner done first by Honda [49] in theoretical botany, but with some important extensions. Honda's initial report included a series of simulations demonstrating that various arboreal shapes can be generated, in 3 dimensions, by linear growth constrained by simple rules. Applying Honda's rules to "axon growth" in our program, we have generated the arbors shown in Fig. 6. The parameters specified, and varied to get different structures, were: 1) the ratio of branch lengths from one order of branching to the next, and 2) the angles of the two daughter branches, measured in a plane determined by the vertical and the parent branch. Given particular values for these parameters, the program always results in the same branching structure. In biological terms, for a given "genotype" the same "phenotype" is consistently produced. We have also been able to generate "Honda trees" with random variations of specified param-

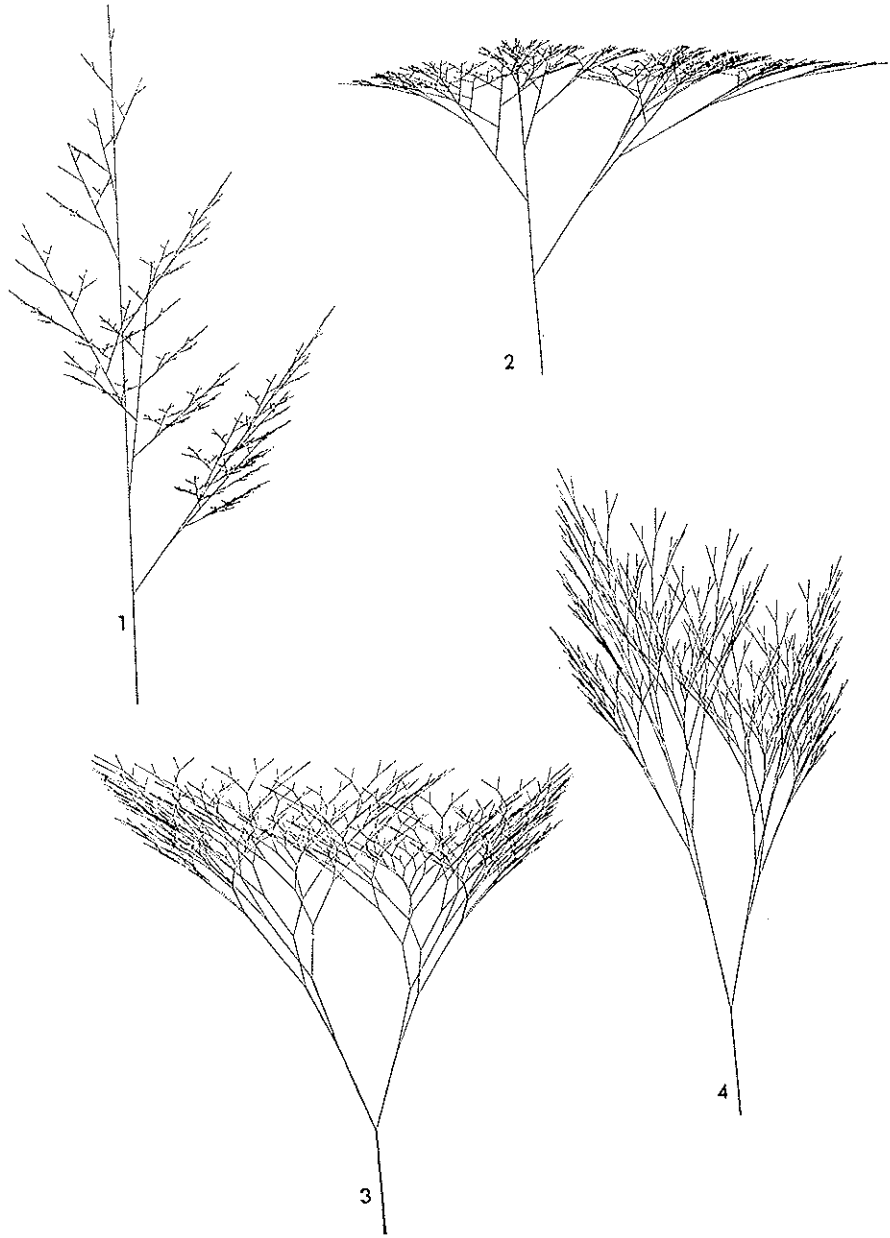


FIG. 6. Three-dimensional tree structures, shown in side-view projections, produced by computer simulation of development using the algorithm of Honda [49], with parameters chosen to match four of his published results. Each simulation was continued through nine orders of branching. Three basic Honda parameters were specified: 1) the ratio of segment length at successive orders of branching and 2) and 3) the angles of the two daughter branches at each bifurcation. Program by Davis and Schneider, developed on an IBM mainframe computer; these and other simulations were run on an Encore Multimax computer.

eters, resulting in a greater amount of "biological" variability of the tree shapes determined by the means values (Fig. 7).

These computer-generated trees have a strong resemblance to the branching structures of real trees or neuritic arbors. However, their production by simple linear growth, without interactions, has limitations with regard to both the range of structures that can be generated and the resemblance to natural growth of either neurons or plants. Therefore, Honda *et al.* [50] extended their program to include: a) competition among the branches of a developing tree — branches growing in close proximity to other branches were inhibited — and b) a simulated flow of nutrients from the trunk, which was influential in determining branch survival.

The latter idea, augmented by Borchert and Honda [9], is of special relevance to the growth of axons. These authors made the assumption that the flow of nutrients increased with time, and was distributed with a specific asymmetry at each branch point. However, when the amount of nutrient in a given branch dropped below a critical value, growth of the branch was not only arrested, but death of the branch ensued. This resulted in a selective loss of the lower branches of the tree as growth proceeded (Fig. 8A). Effects of pruning the growing tree were also simulated. Results showed specific alterations in the shape of the developing tree (Fig. 8B). Despite the simplifications made in the assumptions, results of such simulations bear a remarkable resemblance to the pruning effects described for growing axons [27] and trees [15].

Finally, we have preliminary results from simulations of axo-axonal contact reactions as seen in tissue culture [29]. A small number of simulated cell bodies (about 100) were distributed randomly in a circular region. The initial growth of each axon was in a randomly determined direction. Using an algorithm representing "contact inhibition of extension", the program generated plots of "neurite outgrowth" in simulated explant cultures of dorsal root ganglia. In the initial simulations, application of the contact reaction rule to the growing axons resulted in many of them becoming "trapped" within the circle; the minority that escaped the perimeter of the circular zone came mostly from cells in the outer regions of the zone. We next placed a larger number of cells (309 "neurons") in an annular region and added the further specification that as the axons began their growth, in random directions, those that grew inward should be eliminated. (This was done because the probability of such axons re-emerging appeared to be very small, so computer time could

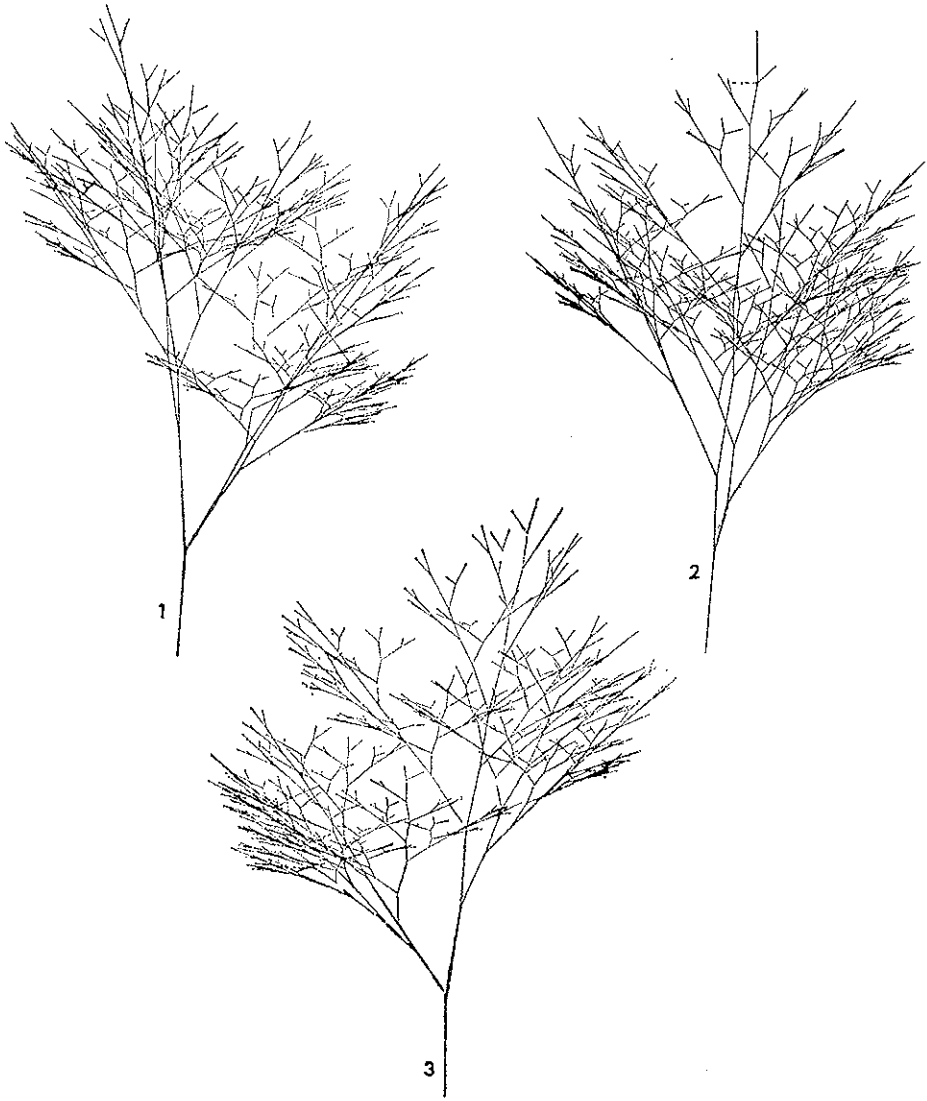
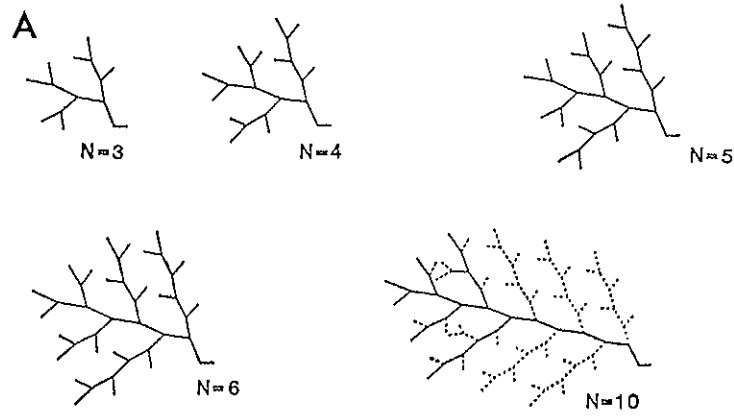


FIG. 7. Results of three additional simulations of tree growth using a single set of parameter values with the Honda [49] algorithm. However, in these simulations, the parameters which determinated the branching angles and branch segment lengths were treated as the mean values of random distributions, with standard deviations of 20% of the means. This use of randomization results in « biological » variability in the phenotypic expression of the same « genotype ».

be saved by eliminating them.) Results of such a simulation are shown in Fig. 9. The initially *randomly directed* axons have become sorted into a pattern of radial, non-fasciculated growth. The pattern bears a resemblance to the "sunburst" pattern so typical of ganglion explant cultures — an outcome which could not have been obtained without the inclusion of



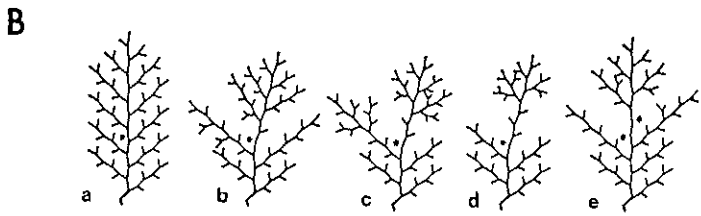


FIG. 8. Two-dimensional simulations of tree growth by Borchert and Honda (ref. 9; their figure 11). *A*) Successive stages of growth of a tree with simulated flux from the roots which is distributed asymmetrically at each branch point according to a specific algorithm based on botanical observations. Branches fail to survive when the flux falls below a specified minimum. Although the total flux increases with time, lower branches are dying (dotted lines) when the tree has reached 10 orders of branching (N=10). *B*) Simulated regeneration of a growing, branching structure after pruning. *a*) Non-pruned tree of 12 orders. *b-d*) The leader was pruned at the same point (*) at times when the tree had developed 7th, 8th, and 9th order branches, respectively, and then each simulation was continued until 12 orders of branching were attained. The uppermost remaining lateral branch replaces the lost leader (note slight tilt off the vertical for upper part of the tree), but lower laterals may also temporarily increase in vigor. *e*) Pruning of two lateral branches (*), at times when the tree had developed 7th and 8th order branches, causes increased vigor of leader and lower laterals. (See original publication for further details).

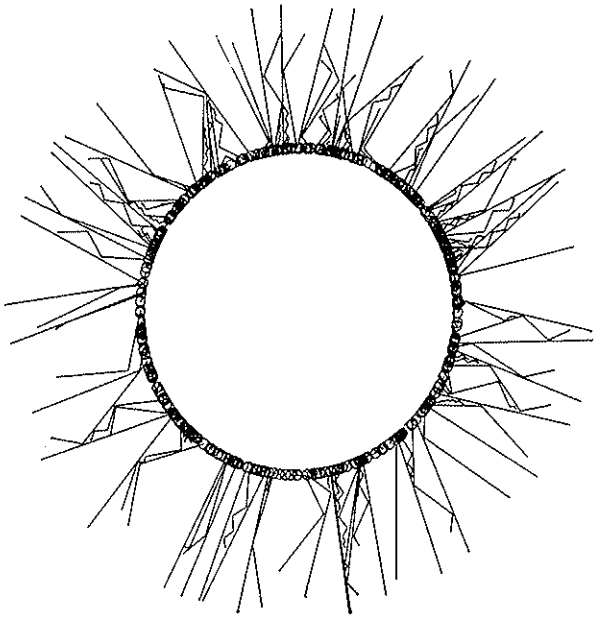


FIG. 9. Simulated explant culture of a dorsal root ganglion, using a « contact inhibition of extension » rule based on the observations of Dunn [29]. 309 cells were distributed randomly around the edge of a circular region; initial directions of growth of a single axon from each cell was randomly determined, but those that grew inward were « killed » since it had been determined that their probability of re-emergence was small. Algorithm designed by Schneider and Davis; simulation program of Davis and Schneider.

a sizable population of cells all following the same rule. The discovery of this kind of "collective phenomenon" [52] is a major goal of our computational studies, for such phenomena are frequently not predictable without simulation.

We plan to extend such simulations of tissue culture phenomena, exploring the effects of a greater variety of postulated growth rules. Subsequently, we will be able to attempt simulations of more complex three-dimensional events based on *in vivo* phenomena. Such an approach can provide a powerful combination of the use of empirically obtained data and theoretical validation of conclusions derived from these data. Multiple rules can be tested for their relevance to the growth of axon arbors, and their parameters varied as guided by both empirical data and simulation results. Large populations of neurons can be included, their size limited only by the memory and speed of the computer. The necessity of obtain-

ing and classifying detailed anatomical observations to support such an endeavor cannot be underestimated, for the impact of such an approach is inextricably linked to the availability of experimentally verified and quantitatively accurate information. We believe that in this way theory may become more comprehensive in dealing with the complexity of the issues we are facing.

ACKNOWLEDGEMENTS

Support for the laboratory research findings included in this review was provided by the NIH, grants EY00126, EY05504, and EY02621. Development of the computer simulation program has been supported by a Biomedical Research Support Grant to M.I.T. and by the Whitaker Health Sciences Fund. We would like to express our gratitude to the Encore Computer Corporation for allowing us to use one of their Multimax computers for the simulations and resulting plots shown in figures 6, 7 and 9. We thank L. Collier for help with manuscript preparation.

ABBREVIATIONS

CB = cerebellum
CNS = central nervous system
C PED = cerebral peduncle
EML = external medullary lamina
IC = inferior colliculus
LGBd = dorsal nucleus of the lateral geniculate body
PNS = peripheral nervous system
SC = superior colliculus
VB = ventrobasal nucleus

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CEREBELLAR SYNAPTOGENESIS AND THE ORGANIZATION OF AFFERENT PROJECTION MAPS

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INTRODUCTION

The cerebellum, especially the cerebellar cortex, is one of the central regions in which ordered organizational patterns are most obvious. This broad zone is composed of five different neuronal populations, each repeating a specific pattern in a monotonous manner along the numerous folia, conferring to the cortex a stereotyped tridimensional geometry. However, in spite of this apparent homogeneity, the cerebellum is in reality a very heterogeneous structure, due to the presence of biochemically different categories of Purkinje cells (PCs; see in [11]) and, more importantly, to its longitudinal-zonal organization, determined hy the successive apposition of structurally and functionally distinct longitudinal strips [31, 45]. The specificity of each of these cerebellar compartments results from the precise pattern of its afferent and efferent connections and reflects the organization of the cerebellar projection maps.

Like the rest of the vertebrate central nervous system, the cerebellum develops through a series of early cellular events — neuronal proliferation and cell commitment, neuronal migration, and neuronal segregation — which follow strictly defined kinetics. Later on, by means of neuronal differentiation, mainly neurite outgrowth, precise categories of neurons, located in different parts of the brain, succeed in approaching each other and in establishing synaptic connections. Synaptogenesis appears, therefore, to be a relatively late event in the ontogeny of the nervous system.

The factors regulating the trajectories followed by specific bundles

of growing axons are not fully understood, but timing, physical proximity and cell adhesion are considered to be important for tbe inner organization of these bundles [6, 18, 40]. The final distribution of a projection can be envisaged as the result of the confrontation of the inherent topography of the axonal bundle containing the projecting fibers with the topographic distribution of its respective cellular targets. If such is the case, it is likely that the mechanisms underlying synaptogenesis would be necessary for projection map organization, and the regressive events implied in the selective stabilization of synapses [10] would probably play an essential role in refining the definitive topography of these maps.

Accordingly, the present chapter reviews recent work done in our laboratory in collaboration with Drs. Arsenio-Nunes, Bourrat and Wassef on the postnatal formation of olivocerebellar and spinocerebellar projection maps and on the inner zonation of cortical PCs in late rat embryos. Moreover, it will correlate the sequential stages in the development of the cerebellar maps with the respective stages of differentiation of the postsynaptic target neurons, especially as concerns synaptogenesis. The aim of this work is to provide some insight on the mechanisms affecting the acquisition of the cerebellar longitudinal-zonal organization.

Analysis of the immature organization of the projections was based on system tracing by means of autoradiography and peroxidase techniques. The study of PC grouping used immunohistochemical techniques with antibodies which selectively stain this entire neuronal population in the adult cerebellum. Synaptogenesis was analyzed in postnatal rat cerebellar vermis prepared for routine electron microscopic observation.

Olivocerebellar Projection Maps

In the adult rat [9, 15], as in the cat (studied more in detail; see references in [8]), the olivocerebellar projection is organized in such a way that neurons in restricted "sectors" of the inferior olivary complex innervate corresponding sagitally aligned strips of cerebellar cortex, providing specific projection maps. The latter are organized according to the following general principles. The projection is entirely crossed and its fibers decussate at the interolivary commissure. The caudal half of the medial accessory olive (MAO) projects almost exclusively to the vermis (sagittal zones A_1-A_2 of Voogd [45]), whereas its rostral half projects to the flocculus, to the paraflocculus, and to a restricted region of the intermediate cortex

(sagittal zone C_2). The ventral and dorsal lamellae of the principal olivary nucleus (PON) supply innervation mainly to the hemispheric cortex (sagittal zones D_1-D_2). The caudal part of the dorsal accessory olive (DAO) projects to the lateral region of the anterior vermis (sagittal zone B), whereas its rostral half projects mainly to the intermediary cortex (sagittal zones C_1-C_3).

One of the main goals of our previous work [43] was to establish whether the broad topographic pattern described above (which gives only a rough idea of the fine neuron-to-neuron organization of the adult olivocerehellar projection) is influenced by synaptogenic mechanisms or not. Electrophysiological studies carried out in rat pups [12, 34] indicate that synaptogenesis between climbing fibers (the termination of all olivary axons in the cerebellar cortex) and PCs is a long, complex process, which starts on the second or third postnatal day and stops towards the end of the second week. The first functional synapses to be identified were recorded in 3-day-old animals (P3) [12, 34]. Although of longer duration, these climbing fiber responses are already very similar to adult ones. However, they are not all-or-none in nature, as in the adult cerebellum but are graded in parallel with increasing stimulus intensities [13, 14, 25]. By P15, the responses acquire their all-or-none adult character. The grading of early responses has been considered as demonstrating that during a transient period, PCs are multiply innervated by climbing fibers. Analysis of intracellular recordings as these synapses mature [14, 25] has allowed the determination of the evolution of multiple innervation. The peak of multiple innervation is reached at P5; from P7 to P10 an abrupt decrease occurs, followed by a slight decrease between P10 and P15. At this age, all PCs are monoinnervated.

Topographic pattern of the olivocerebellar projection in rat pups

In order to evaluate the presumptive influences of both synaptogenesis and regression of the multiple innervation on the establishment of the olivocerebellar topography, we analyzed [43] their organization in newborn rats (P0), before the onset of synaptogenesis and up to five days (P5), when multiple innervation is maximal. Neuroanatomical tracing results led to the following conclusions:

i) Olivocerebellar fibers reach the cerebellum during intrauterine life, since at birth these fibers are already present in the cerebellum.

At this age the small dimensions of the brain and its high water content make it difficult to restrict tracer injections to a single cerebellar lobule or to a definite olivary subnucleus. This situation allows only a rough estimation of the degree of organization of the projection, but it is sufficient to determine the distribution of olivary fibers within the vermal, intermediate and hemispheric cortical regions. Thus, the vermal cortex receives olivary projections from neurons located in the caudal half of the contralateral MAO; the intermediate cortex is innervated mainly by neurons in the contralateral DAO and to a lesser extent by neurons in the contralateral rostral half of the MAO. Finally, the hemispheric cortex receives most of its olivary projection from the contralateral PON and from the contralateral rostral half of the MAO. This highly organized distribution is already similar to that which characterizes tbe adult olivocerebellar projection.

ii) In newborn rats, the bulk of the olivocerebellar fibers are arrested in the prospective white matter (Figs. 1 and 2) and only a few have invaded the overlying gray matter. Therefore, these afferent axons reacb tbeir proper cerebellar territories before their appropriate targets, the Purkinje cells, have matured (see below). As in other systems, like tbe visual pathway [36], the arrival of a projection to a target structure is asynchronous to its definitive distribution to cellular targets.

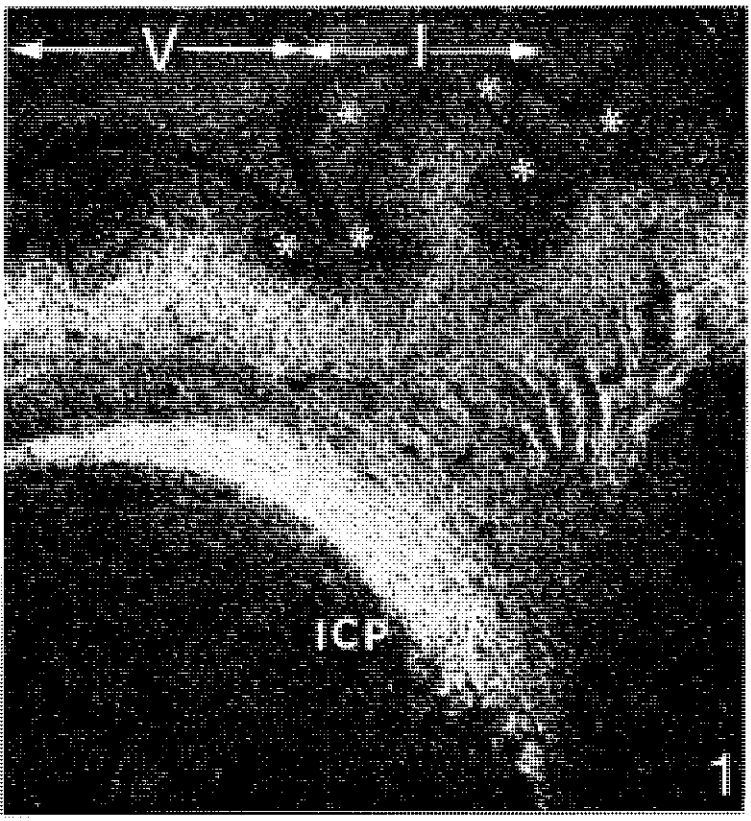
iii) By tbe fifth postnatal day the olivocerebellar fibers have moved from the prospective white matter towards the interface between the molecular and granular layers (Fig. 3), where Purkinje cells arranged

PLATE I

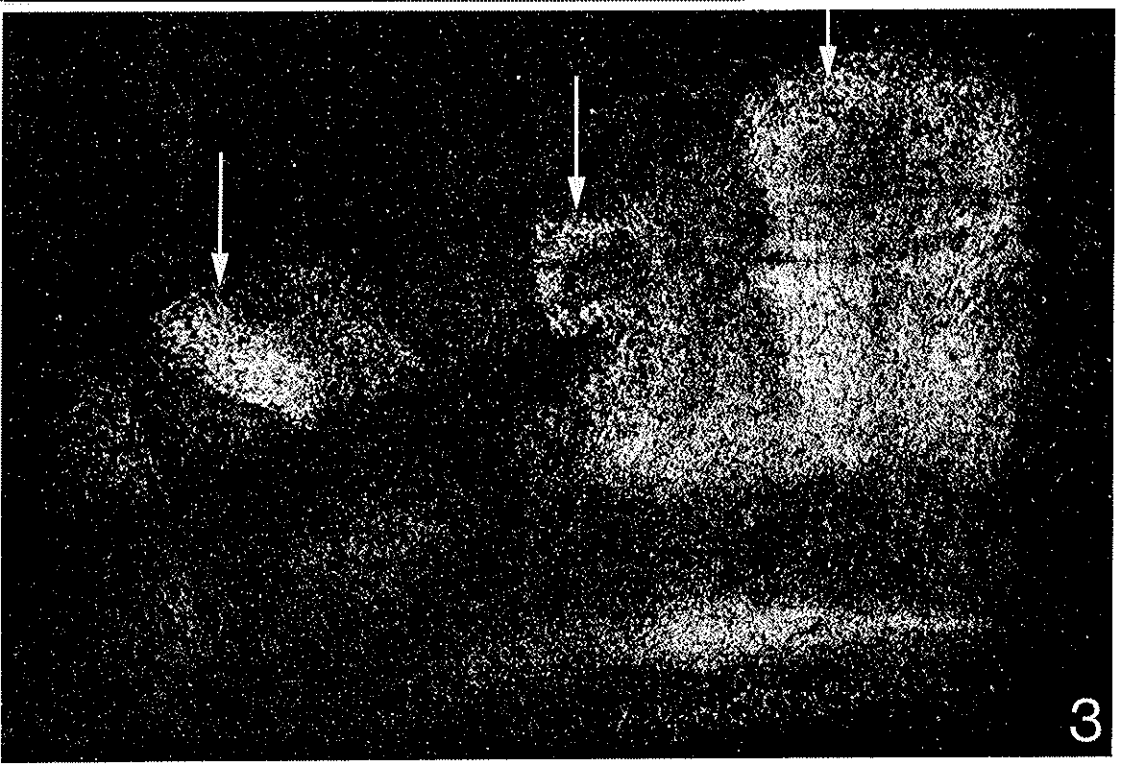
FIG. 1. Darkfield autoradiogram of a rat cerebellum (R4-PO-1) injected on the day of birth with ^{3}H-leucine, partially involving the caudal half of tbe contralateral olive, and fixed 20 hours later. The labeled olivocerebellar fibers reach the cerebellum through the inferior cerebellar peduncle (ICP) and remain in the prospective white matter. Within the latter, the density of the autoradiographic reaction is higher in the vermis (V) than in the region of the intermediate cortex (I). The silver grains do not involve the superficial cortical gray matter (asterisks). X 72.

FIG. 2. Brightfield micrograph of a fragment of the same section illustrated in Fig. 1. Note that the zone devoid of silver grains in the first figure corresponds to the cortical gray matter. The latter is composed of a broad region (between arrows) containing scattered perikarya of PCs and a thinner region corresponding to the nascent molecular layer. X 72.

FIG. 3. Darkfield autoradiogram of the cerebellum of a 5-day-old rat (R1-P4-5), which had received an olivary injection mainly affecting the most caudal regions of the contralateral olive 20 hours before fixation. Note the presence of sagittal strips (arrows) containing a high density of silver grains. In these strips, labeling reaches the most peripheral zones of the cortical gray matter, immediately under the external granular layer. X 72.


V
I
ICP
1


2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themselves into a monolayer. Although a more precise analysis of the topography of the olivocerebellar projection in these older rats is possible, no changes in the organizational pattern were observed. In P5 rats, it is identical to that of adult animals.

Concomitance between maturation of Purkinje cells and gray matter invasion of olivocerebellar axons

As already mentioned, the autoradiographic reaction consecutive to anterograde labeling of olivocerebellar fibers shows up in the prospective white matter of newborn rats, whereas it is located at the PC layer and the nascent molecular layer in 5-day-old animals. Indicative of the gray matter invasion by the olivocerebellar axons, this shifting takes place simultaneously to an essential phase in the maturation of PC dendritic arbors.

We studied this process of PC differentiation in the rat by means of an *in vitro* approach [43]. Newborn and 5-day-old rat pups were anesthetized with ether, and their cerebella were rapidly removed after decapitation and placed in cold Ringer solution (around 5°C), bubbled with a mixture of 92% O_2 and 8% CO_2. Fresh 600-800 μm vibratome and/or handmade slices, cut in the sagittal plane were obtained. After insertion of a crystal of horseradish peroxidase (HRP) into the central medullary zone, the slices were incubated in the oxygenated Ringer solution for 1-3 hours. The slices were then fixed for 30 minutes in buffered 2.5% glutaraldehyde solution, soaked in 20% sucrose, cut on a freezing microtome and reacted for HRP. This method [27] enabled a retrograde filling of PCs whose axons had been lesioned during the implantation of the HRP crystal, giving these neurons a Golgi-like appearance.

PCs in newborn rat cerebellum exhibit different typologies, hut most of them have an elongated shape and are characterized by long, thin dendrites with nearly smooth contours (Fig. 4). These immature PCs, haphazardly distributed into a three- to six-cell-deep layer, are polarized, with most dendrites emerging from the apical pole and the axon from the basal pole. Most of them have only apical dendrites, whicb either ascend towards the external granular layer or bifurcate into two asymmetrical branches also oriented towards the cerebellar surface. Some of these PCs have, in addition, basal dendrites (Fig. 5); more rarely, they are multipolar (Fig. 6), with dendrites spreading in a tapering manner. These immature forms, which hardly resemble mature PCs,

have been reported in several other species, such as the mouse [17], the opossum [24] and even man [26, 49].

PCs in P5 rat cerebellum change appearance dramatically. Instead of being randomly distributed, they are aligned in a single row, demarcating the border between the molecular and the granular layers. Their perikarya are less clearly polarized, due to the regression of the long apical dendrites and to the outgrowth of numerous thin somatic processes in all directions, which gives these neurons a disoriented aspect (Figs. 7 and 8). The typical stout and irregular shape of these immature PCs was first described by Cajal [37] and later [38] called the "phase of the stellate cells with disoriented dendrons".

Autoradiographic data on the anterograde organization of the immature olivocerebellar projection clearly show that the transition between the two phases of PC dendritic maturation coincides with the arrival of the climbing fibers in the proximity of the PC perikarya. A similar simultaneity has been observed in the opossum [24] and in human cerebellum [26].

As is often the case in developmental biology, the concomitance of two distinct but apparently related events raises the question of their presumptive mutual interactions. Climbing fibers represent the earliest and the functionally most important afferents to PCs. Does their arrival induce the dendritic transformation of the PCs, or, on the contrary, does this transformation initiate the attraction of the olivocerebellar fibers arrested in the subcortical medullary zone?

Experiments carried out in our laboratory during the last few years have provided an answer to this question. Since the large majority of climbing fibers reach the cerebellum through the inferior cerebellar peduncle, unilateral pedunculotomy in newborn rats will deprive most PCs, in the pedunculotomized hemicerebellum, of their climbing fiber inputs before these fibers are translocated from the white matter to the cortical gray. Axotomized olivary neurons suffer a fast retrograde degeneration, which ends with the complete destruction of the contralateral olive [3, 42].

The remaining olivary complex develops a compensatory sprouting that will supply climbing fiber inputs to only a minority of the deprived PCs [3]. Therefore, the dendritic arbors of the vast majority of PCs in the pedunculotomized hemicerebellum must grow in the absence of climbing fibers. Under these conditions, PCs succeed in forming spatially oriented dendritic arborizations, although with a reduced number of

branches, and in reabsorbing the immature somatic processes [3, 42]. Moreover, heterotopic transplantations of cerebellar primordia taken from E14 rat embryos and placed in adult rat brain [2, 22] result in highly organized minicerebella, some of them isolated from the host brain. In this instance, in spite of the total absence of climbing inputs, PCs nonetheless develop spatially oriented dendritic arbors and lose their immature somatic filopodia. These experiments indicate that climbing fiber inputs are not essential for PC maturation, and suggest that the early transformation of the PC dendrites from fusiform to stellate is an inherent property of these neurons. Therefore, the correlation between the results obtained with axon tracing methods and those concerning the development of PC dendrites suggests that olivocerebellar fibers remain at the medullary axis, waiting for the appropriate maturation of their postsynaptic targets. The regressive events taking place during the formation of the PC dendrites may be considered as signaling the end of the waiting period and the beginning of the invasion of the cortical gray matter by the olivocerebellar fibers.

Ultrastructural differentiation of Purkinje cells and climbing fiber synaptogenesis

Electron microscopic examination of the gray matter of the vermal cortex in newborn rats (PO) discloses the presence of numerous neuronal

PLATE II

FIGURES 4-8. Golgi-like appearance of Purkinje cells from postnatal rat cerebella. These neurons have been retrogradely filled with HRP by means of "in vitro" slices.

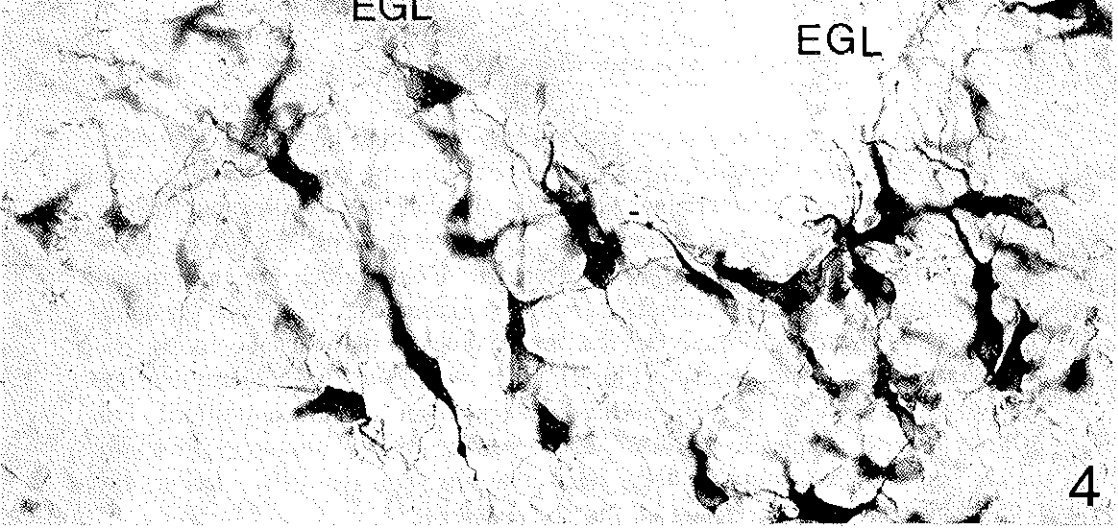
FIG. 4. Newborn rat cerebellum. Purkinje cells are randomly dispersed in the cortical gray matter, under the external granular layer (EGL). They are characterized by a fusiform appearance and by smooth contours. Long apical dendrites reach the EGL without penetrating it. X 500.

FIG. 5. Purkinje cell with apical and basal dendrites. Newborn rat. X 780.

FIG. 6. Purkinje cell with multipolar dendrites, spreading mainly in the upper region of the cortical gray matter. Newborn rat. X 800.

FIG. 7. Five-day-old rat. The Purkinje cells have attained their final position at the interface of the molecular (ML) and granular (GL) layers. The apical and basal dendrites have been transformed into long, thin processes which emerge from the cell bodies in all directions. These Purkinje cells are in the "phase of the stellate cells with disoriented dendrons". X 780.

FIG. 8. Five-day-old rat. Higher magnification of a Purkinje cell in the "phase of the stellate cell with disoriented dendrons". X 1250.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EGL
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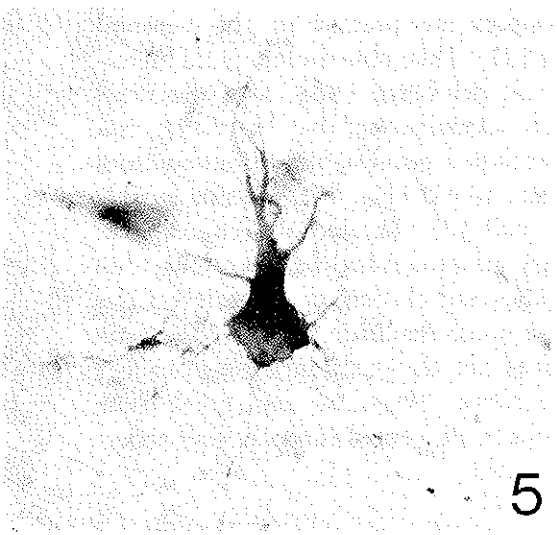
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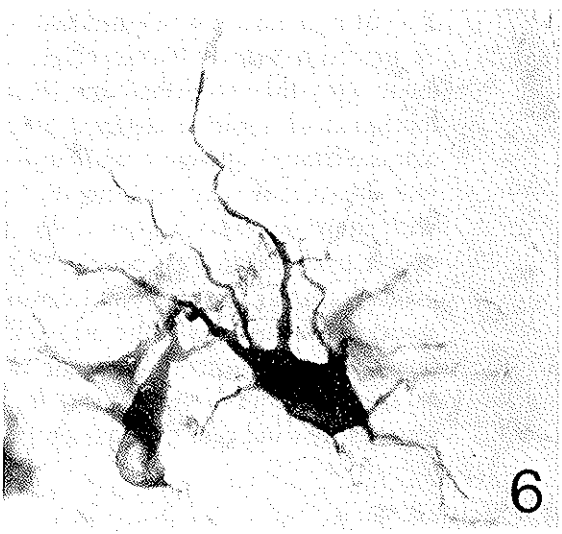
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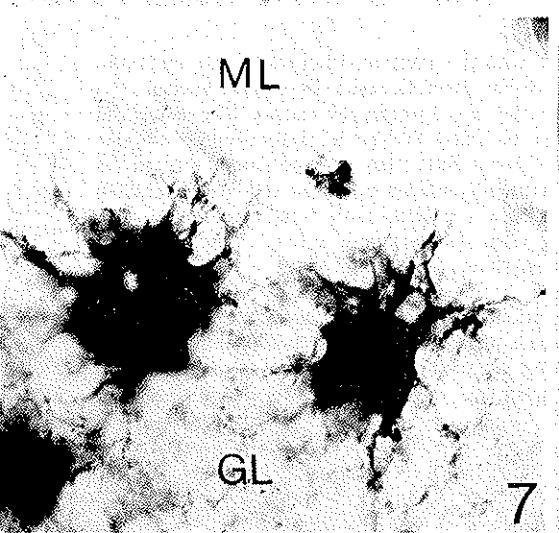
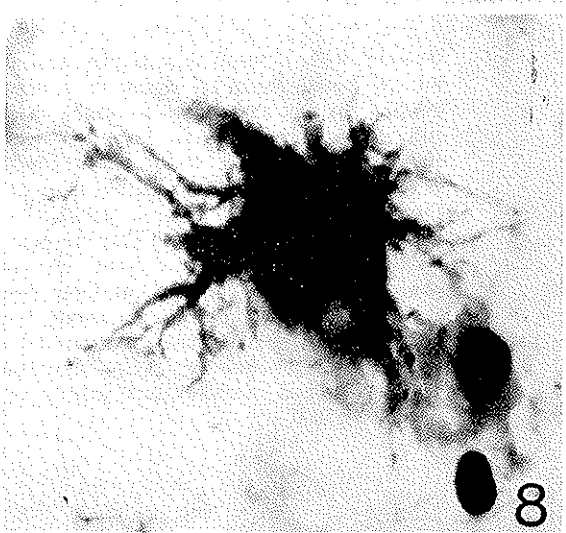

ML
GL
7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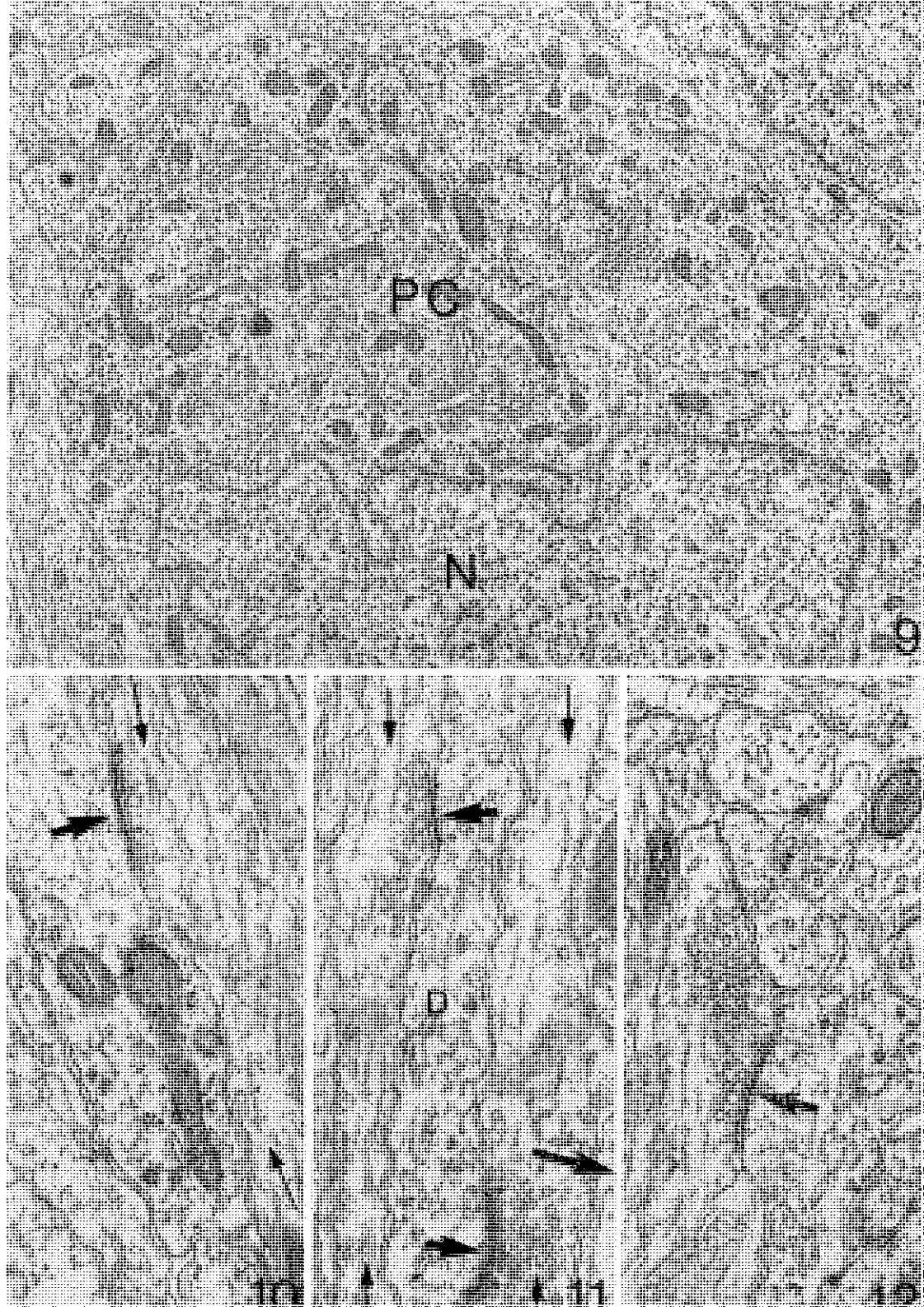
perikarya, dispersed between the external granular layer and the deep medullary zone. None of these neurons exhibits the complete set of cytological features characterizing adult PCs; neither the hypolemmal cistern nor its association with mitochondria [33] are present. However, some of the cell bodies have somewhat excentric nuclei, which are invaginated by abundant secondary folds, filled with cytoplasm (Fig. 9). These invaginations are located primarily at the upper pole of the nucleus, facing the origin of the apical dendrite (Fig. 9), as in adult PCs [33]. These neurons are considered to be PCs in the fusiform stage of differentiation. For our present purposes, the most salient features of the immature PCs are: i) the smooth contour of their perikarya and stem dendrites, and ii) the absence of synaptic inputs at the somatic level.

Morphologically identifiable synapses are present in the neuropil of newborn vermal cortex (Figs. 10-12). This observation is in agreement with that published by West and del Cerro [48], who described formation of synaptic contacts as early as embryonic day 19 (E19). Immature synaptic contacts with well-developed postsynaptic differentiations but almost inexistent presynaptic "active zones" (Fig. 10), are side by side with more mature synapses (Figs. 11 and 12). In coronal sections, most of the synapses are "en passant". They are established between long, thin axons (containing numerous microtubules and clusters of synaptic vesicles associated with presynaptic grids) and smooth dendritic profiles. The axon does not form varicosities, since it does not obviously increase in caliber at the segments bearing the "active zones" (Figs. 10 and 11). These observations indicate that synaptogenesis in P0 vermal cortex is very active.

PLATE III

FIGURES 9-12. Electron micrographs from the vermal cortex of 4-hour-old (P0) rat pups.
FIG. 9. Apical perikaryal region of a presumptive Purkinje cell (PC). The nucleus (N) exhibits the typical indentations which characterize this category of neuron. The cytoplasm still shows some immature features: absence of Nissl bodies, a large number of free polyribosomes, absence of the hypolemmal cistern. Note that the perikaryal surface is smooth and devoid of filopodia. No synaptic terminals impinge upon the cell body. X 14000.
FIG. 10. Early stage in synapse formation between a passing axon (small arrows) and an unidentified dendritic profile. The presynaptic axon contains only a few synaptic vesicles, forming an immature active zone. The postsynaptic dendrite exhibits a well-developed postsynaptic differentiation of the Gray type I (large arrow). X 40000.
FIG. 11. In the neuropil, more mature synaptic complexes (large arrows) can be found between passing axons (small arrows) and unidentified dendrites (D). X 40000.
FIG. 12. This electron micrograpb illustrates a mature synaptic contact (arrow) between two unidentified profiles. These mature synapses are rare in the newborn rat cerebellar vermis. X 31000.


PC
N
9
10
D
11
12

But since the partners involved in the early synapses cannot be identified by merely cytological criteria, it is impossible for the moment to correlate this synaptogenesis with any of the known systems of cerebellar circuitry.

During the first three postnatal days (P1-P3), the shape of the PC perikarya gradually changes. By P3, a few long, slim processes begin to emerge from the cell bodies. However, in the present study, we have not succeeded in finding axon terminals which synapse on these processes. The situation changes completely in five-day-old rats. Although the hypolemmal cisterns are still undifferentiated in P5 rats, the PCs are easily recognizable by the numerous filopodia emerging from the cell bodies. The majority of filopodia are thin and convoluted and can be followed only for short distances in single sections (Fig. 13). Some of them are much thicker and longer and are irregularly shaped (Fig. 13). Presynaptic axon terminals are always present in close vicinity to the PC perikarya, they are often enwrapped by filopodia, and some of them establish synaptic contacts on the filopodial membrane (Fig. 13). These axon terminals belong to climbing fibers, which form a pericellular nest covering the PC perikarya.

Our electron microscopic analysis has not succeeded in establishing the onset of the climbing fiber-PC synaptogenesis. Most probably, the ultrastructural study of postnatal cerebellum in rats by anterograde labeling of olivocerebellar axons would allow a more precise analysis of these early stages. In any case, the present results have corroborated those obtained with electrophysiological techniques [12, 34]. The absence of perisomatic filopodia and axosomatic synaptic inputs in the PCs of new-

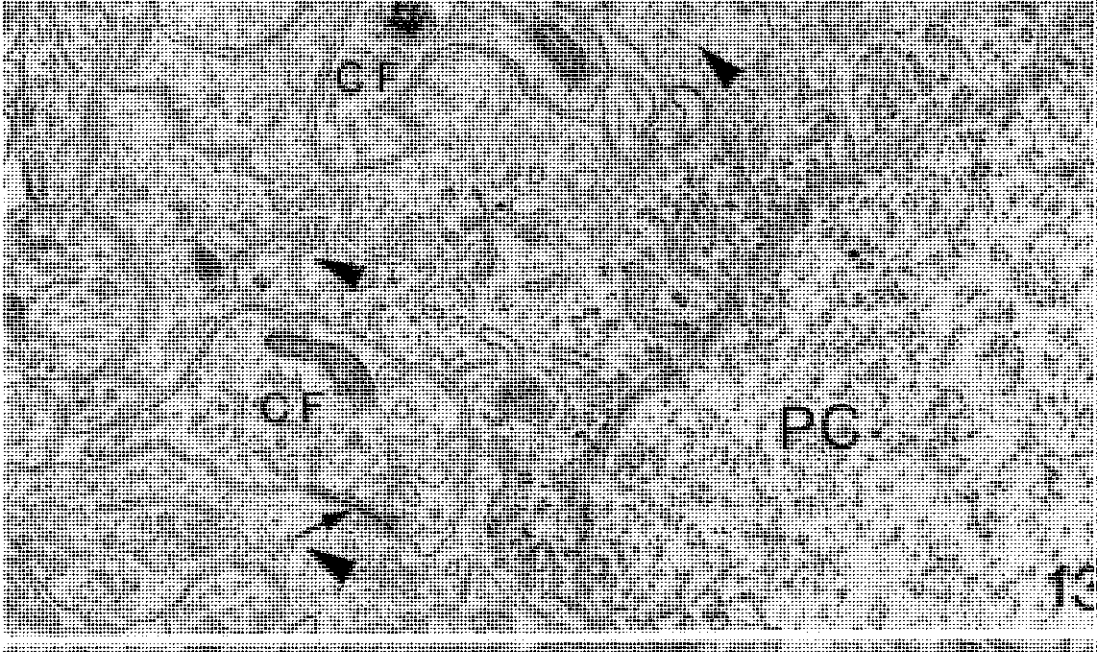
PLATE IV

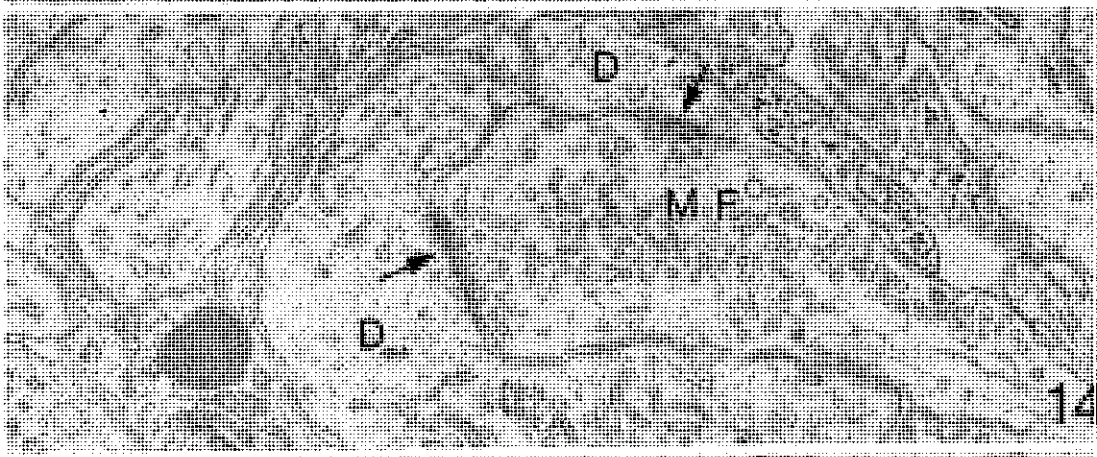
FIGURES 13-15. Electron micrographs taken from the anterior vermis of postnatal rats and illustrating synaptogenic events involving climbing and mossy fibers.

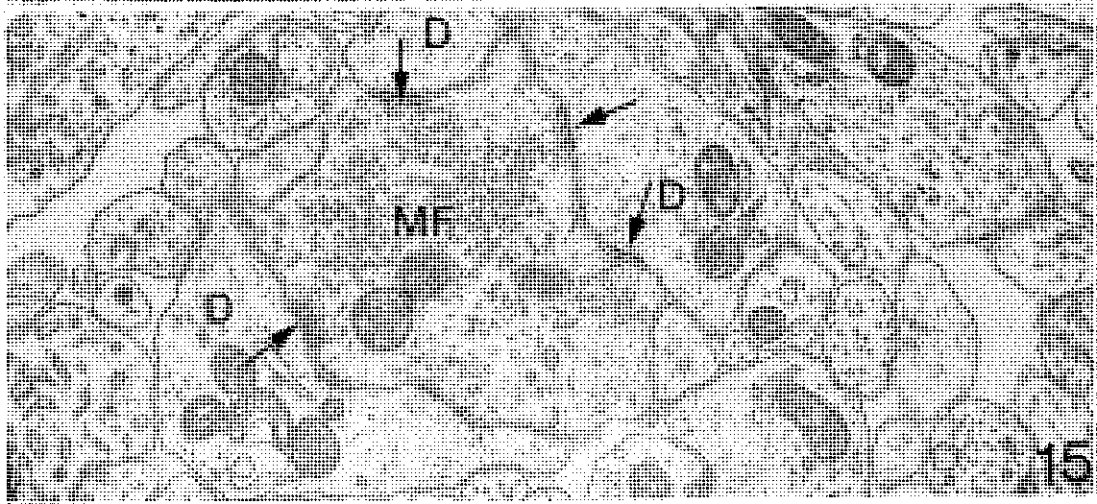
FIG. 13. Purkinje cell (PC) in a 5-day-old cerebellum. The perikaryon of this neuron is no longer smooth, since rather thick processes (arrowheads) emerge from the cell body. Note that axon terminals, identified as belonging to climbing fiber inputs (CF), establish synaptic connections (arrow) with the somatic processes. X 25000.

FIG. 14. Granular layer of the vermian lobule III in a 5-day-old rat. Two dendritic processes (D) almost completely surround a mossy fiber enlargement (MF). Synaptic contacts (arrows) between the axonic and dendritic elements are visible. This arrangement corresponds to that of a mossy fiber synapse in its protoglomerular stage. X 31000.

FIG. 15. Granular layer of the vermian lobule III in a 7-day-old rat. The more complex synaptic arrangement between a central mossy rosette (MF) and the peripheral granule cell dendrites (D) roughly corresponds to that of the beginning of the glomerular stage. The arrows point to the junctional synaptic complexes. X 25000.


CF
CF
PC


D
MF
D


D
MF
D
D

born rats, the progressive appearance of somatic filopodia, and the presence of pericellular nests in five-day-old rats indicate that climbing fiber-PC synaptogenesis is a postnatal event, which must start on the second or third day after birth, and which reaches the culmination of its first stage (the pericellular nest of Cajal [37]) by P5.

Conclusions

The anatomical studies summarized above clearly indicate that olivocerebellar fibers reach their proper sagittal zones before the onset of synaptogenesis between climbing fibers and PCs, and that the projectional pattern remains unchanged at the maximum of the multiple innervation. Therefore, neither synaptogenesis nor the regression of the multiple innervation influences the acquisition of the topography of the olivocerebellar projection. However, our study is not fine enough to provide the final cell-to-cell correlation which characterizes this projection. Most probably, olivocerebellar specificity would be "refined" at the synaptic level within the cerebellar longitudinal zones during the selective processes of synapse elimination and synapse stabilization [10].

Spinocerebellar Topography

The spinocerebellar projection has been studied in various mammals, particularly in cats [7, 29, 39]. Spinal axons end almost exclusively in the vermis, where they are distributed within two distinct areas: i) the anterior target zone, which is the more important and comprises lobules I to V of the anterior lobe; and ii) the posterior target zone, which is restricted to lobule VIII. All spinal fibers terminate as mossy fibers in the granular layer. In the spinal afferents conveying inputs from the hindlimb regions, mossy terminals are segregated into five sagittally parallel zones varying in width from 200 to 480 μm and are separated by terminal-free intervals of 600 to 800 μm.

Information on the development of this projection is scanty. Only recently, Martin *et al.* [28] reported the first experimental study of the formation of the spinocerebellar projection. This work was carried out in a marsupial, the opossum, whose ontogenic calendar differs from that of the rat. Accordingly, spinal afferents reach the cerebellum on the 7th postnatal day and acquire their adult topography on the 50th postnatal

day. The precise stages followed by the intracerebellar spinal axons to achieve their adult pattern were not analyzed.

Most of the electrophysiological information on the development of this projection is limited to rats. Analysis of field potentials evoked by white matter stimulation and aimed at disclosing the earliest signs of synaptic activity have failed to demonstrate functional synaptic transmission between spinal mossy fibers and granule cells before P10 [41]. However, at P7 [35] it has been possible to activate Purkinje cells after limb stimulation by inputs mediated through spinal mossy fibers. These results indicate that synaptogenesis between spinal axons and granule cells is achieved by the end of the first postnatal week.

Formation of the spinocerebellar topography

Anterograde axonal tracing experiments were carried out in rats aged 0, 3, 5, 7 and 30 days [4]. Neuroanatomical markers were injected at the junction hetween the thoracic and the lumbar segments of the spinal cord, involving the last two thoracic segments (Th 12 and 13) and the upper two lumbar ones (L1 and 2). Our results show that spinal afferents reach the cerehellum during fetal life and that, qualitatively, they are definitively organized at the end of the first postnatal week. Therefore, during this week, spinal axons must pass through the various developmental stages necessary to establish the adult spinocerebellar topography. These successive stages can be summarized as follows:

i) The early stage of axonal growth: During intrauterine life, spinal axons grow rostrally in the ventrolateral aspect of the lower brain stem to the inferior and superior cerebellar peduncles. Once in the cerebellum, these fibers enter only those areas containing them in the adult animal, since transient aberrant projections were not detected. There is therefore a close match between adult terminal domains and the "attracting" cerebellar zones for ingrowing spinal fibers.

ii) The intermediate stage or "waiting" period: From P0 to P3, the ingrowing spinal axons remain arrested in the prospective white matter, where they are distributed more or less uniformly. As is the case for olivocerebellar fibers, those emerging from spinal neurons also reach their appropriate territories before the proper maturation of their target cells (the bulk of granule cell proliferation occurs during the second postnatal week; see [1]), and they "wait" in the medullary zone until a distinct granular layer is formed.

to climbing fibers which originate from the inferior olive, mossy fibers are a composite population of which only a part is of spinal origin. However, ultrastructural examination of the immature lobules II and III of the anterior lobe of the vermis, which permits the analysis of mossy fiber maturation in general, discloses indirect evidence of spinocerebellar synaptogenesis. Using this approach [4], we have been able to identify mossy fibers establishing synaptic contacts from P5. They appear as axonal varicosities partially covered by one or two postsynaptic dendrites (Fig. 14) and correspond to the primitive stage of mossy fiber synaptogenesis. By P7, the number of detectable mossy rosettes has increased and maturation has advanced. Here, mossy terminals in the primitive stage and in the cup stage (the most numerous) are intermingled with terminals already entering the claw stage (Fig. 15; see [23] for the definition of these stages). Thus, our results provide indirect evidence that spinocerebellar fibers may start synaptogenesis almost immediately after their invasion of the nascent granular layer, since by P5 primitive mossy rosettes bearing mature synaptic junctions are present in the terminal domains of spinal axons. Comparison of these morphological observations and the electrophysiological results reported above indicates a time lag between the onset of synapse formation and the production of synapse activity, suggesting that synaptogenesis must be rather advanced before global synaptic activity can be detected. More important, the temporal correlation between the columnar organization of spinal axons and the appearance of mossy rosettes with mature synaptic junctions indicates that the process of synapse formation does not interfere with the establishment of spinocerebellar topography.

More direct evidence in favor of the independence of the establishment of spinocerebellar topography and the synaptogenesis between mossy fibers and granule cells was recently obtained in our laboratory by Arsenio-Nunes [5]. In the adult agranular cerebella of either the weaver mouse or the X-irradiated rat, spinocerebellar fibers projecting to their anterior target zone also form alternating strips of high and low density. The disposition of the labeled strips is somewhat different from that observed in normal cerebellum, but five columns can be disclosed. These results suggest that synaptogenesis is not an essential element in the establishment of spinocerebellar topography.

Conclusions

The results summarized above provide the developmental timing of the olivo- and spinocerebellar projections in the rat. Both of these afferent systems reach the cerebellum during intrauterine life. At birth, they are arrested in the prospective white matter and await the appropriate maturation of their respective postsynaptic target neurons.

Olivocerebellar fibers mature somewhat earlier than spinocerebellar. Both projection systems undergo topographic organization from the moment they enter the cerebellum, at a still undetermined fetal age, up to the fifth postnatal day. Moreover, the ultimate broad topography of both projections seems to be attained without any evident synaptogenetic influence.

Transient Biochemical Heterogeneity of Immature Purkinje Cells as a Presumptive Base for Cerebellar Parcellation

The study of cell movements during early cerebellar ontogeny has shown that PCs segregate into discrete cortical clusters after their migration from the ventricular neuroepithelium to the cortical gray matter. This clustering has been observed in several mammalian species, such as the mouse [44], the rat [21], the monkey [20], and even man [16]. Recently, in her cytoarchitectonic analysis of the developing monkey cerebellum, Kappel [20] emphasized the topographical similarity between the pattern of embryonic PC segregation (she recognized nine clusters in each hemicerebellum) and the adult organization of the longitudinal zones containing the olivocerebellar and the corticonuclear projections. It is, therefore, tempting to consider that the embryological clustering of PCs could in some way be related to the longitudinal zonal arrangement which characterizes the input/output organization of the adult cerebellum.

Most of the work on the development of PCs and their ultimate segregation has been done on Nissl-stained material. This approach allows neither the identification of young postmitotic PCs nor the tracing of these neurons during their outward migration toward the cortex. Neuronal cell markers which selectively stain PCs are therefore necessary to provide a full picture of the developmental history of this type of neuron. Antibodies selectively reacting with all PCs in the adult cerebellum are known, and their number progressively increases, indicating that PCs are very antigenic elements. Three of these antibodies have been used by Marion Wassef

et al. [46, 47] to analyze the earliest stages of the differentiation of PCs and their cortical clustering. The three antibodies used in this study were oriented against: i) cGK, cyclic GMP-dependent protein kinase (gift of Prof. Greengard); ii) CaBP, vitamin D-dependent calcium binding protein (gift of Dr. Thomasset); and iii) PSG, Purkinje cell-specific glycoprotein (gift of Dr. Zanetta). The following description will summarize the immunohistochemical results concerning the repartition in the cerebellar cortex of clusters of PCs [46, 47].

The earliest appearance of immunoreactive PCs differs with each antibody. Immunoreactive PCs are first detected in rat embryos at E16 with anti-CaBP, at E17 with anti-cGK, and at E19 with anti-PSG. For our purposes, it is most significant that not all PCs express their antigens simultaneously. Between E16 and P5, there is a period in which each of these markers is expressed in an asynchronous manner. Thus, from E18, it is evident that in the cortex some clusters of PCs are negative for CaBP (Fig. 19). Similarly, at E19, four clusters of PCs are negative for cGK (Fig. 20). Finally, at birth, PC labeling with anti-PSG is restricted to the caudal vermis (Fig. 21) and to three narrow sagittal strips. Each antibody provides a reproducible mosaic of positive and negative PC clusters, varying with age. Although the pattern of the alternating positive and negative clusters differs for each antibody, in some cerebellar regions the clusters have common limits [47]. As in adulthood, all PCs react with all three antibodies by P5.

PLATE VI

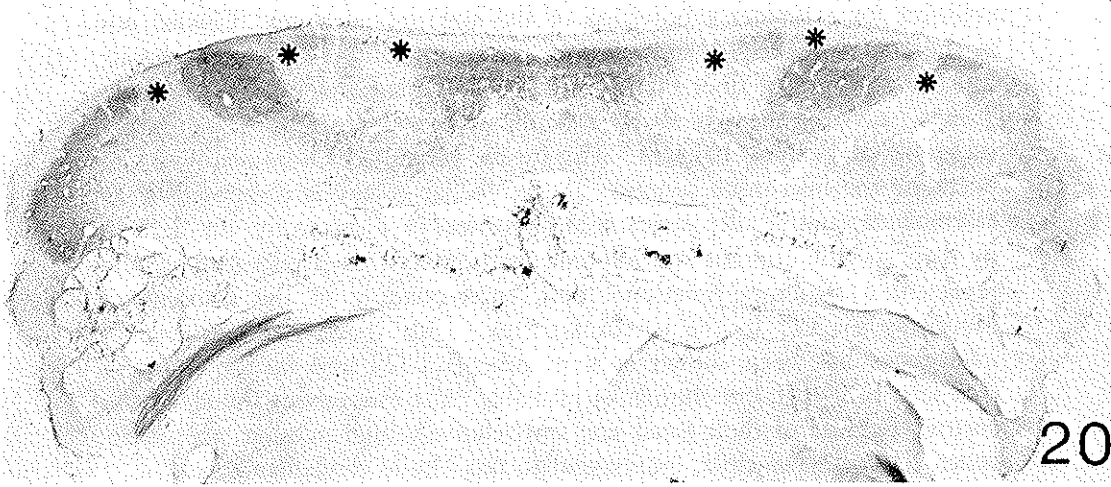
FIGURES 19-21. Immunohistochemistry of Purkinje cells in perinatal rat cerebella. Inner zonation of the cerebellar cortex.

FIG. 19. Frontal section of an E18 embryo stained with CaBP antiserum. This micrograph, taken from the caudal half of the cerebellum, illustrates the biochemical heterogeneity of developing Purkinje cells. The cortical region (except for the most medial part which is devoid of Purkinje cells) contains alternating zones of immunopositive and immunonegative clusters (asterisks) of Purkinje cells. Note the presence of migrating Purkinje cells (arrowheads) and the bundles of immunostaining axons (arrows). X 72.

FIG. 20. Frontal section of an E19 embryo stained with cGK antiserum. The labeling of Purkinje cells is less intense. The cortical distribution of alternating immunopositive and immunonegative clusters (asterisks) also indicates a biochemical heterogeneity of immature Purkinje cells. X 60.

FIG. 21. Sagittal section through the intermediate cortex of the cerebellum of a newborn rat, illustrating the heterogeneity of Purkinje cells as revealed with PSG antiserum. The labeling is restricted mainly to the caudal and anteriorly located folia. X 108.

19



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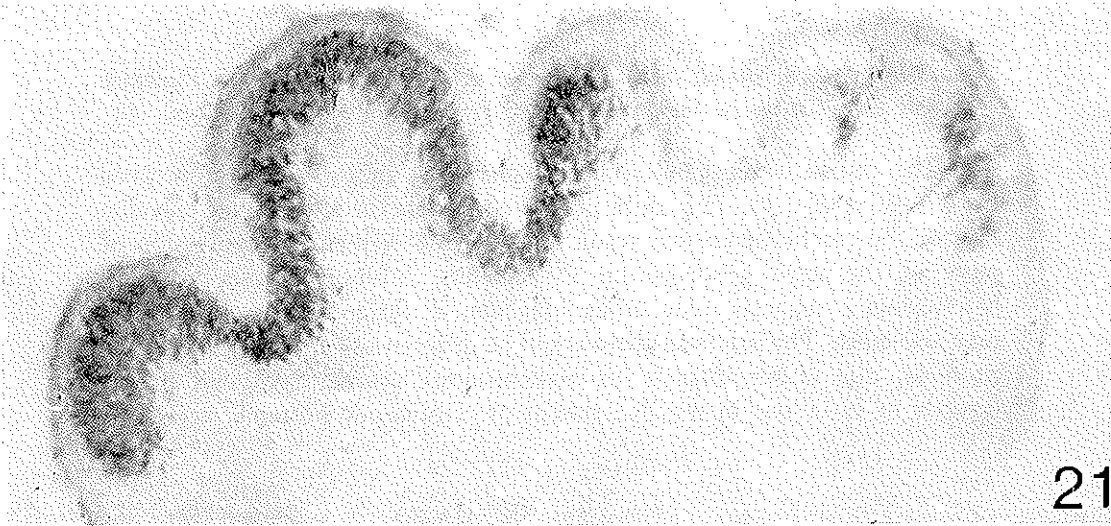

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The "basic cerebellar zone"

The above results would seem to indicate that the synthesis of a whole set of PC proteins is topographically regulated. By definition, a "basic cerebellar zone" is composed of identical PCs. Since there is some overlapping of the clusters obtained with different proteins, it is obvious that the "basic zone" does not coincide with a single PC cluster but results from the intersection of several clusters that are positive or negative for different proteins. From the present immunohistochemical results, the size of a "basic zone" is difficult to evaluate. Sometimes small, it can vary throughout tbe cortex. In any case, Voogd's sagittal zones [45] do not seem to match the "basic zones" as described above.

The fine electrophysiological analysis of Oscarsson [32] has provided evidence for a further subdivision of the longitudinal zones. Carried out in the cat, this analysis showed that according to the functional characteristics of climbing fiber inputs, zone B can be subdivided into five subgroups. Each of them is about 200 μm wide and projects to a different subgroup of Deiter's neurons. This result prompted Oscarsson [32] to postulate the microzone concept: the cerebellar cortex is composed of an assembly of sagittal microzones, 200 μm or less in width in the cat, defined by their specific efferent and afferent connectivity. Each microzone would represent a structural-functional unit, corresponding to the columns of Mountcastle [30] in the cerebral cortex. Until now, the existence of microzones has been shown only in zone B [32] and in the flocculus (see refs. in [19]), but they most probably exist throughout the cerebellar cortex. Because of their small size and relatively high number, the microzones could, in principle, correspond to our "basic cerebellar zones".

Theoretical considerations on the potential interest of the intrinsic Purkinje cell parcellation in the formation of cerebellar projection maps

The transient biochemical heterogeneity of immature PCs observed by Wassef *et al.* [46, 47] could provide the basis for an intrinsic parcellation of the cerebellar cortex, resulting from the differential expression of parts of the same genotype by PC clusters. Moreover, this transient heterogeneity suggests that other biochemical differences between PCs may also exist. For instance, there could be differences in plasma membrane proteins, due either directly to asynchronous protein synthesis or indirectly to variations in the PC content of cytoplasmic proteins, variations

which could influence the posttranscriptional modification of surface components. Thus, the possibility exists that the axons emerging from PCs within a "basic zone" would form distinct bundles provided with specific axolemmal cues.

These two possibilities — inner zonation of the cerebellar cortex and differences in the protein composition of the plasma membrane of PC axons — could be of great importance in the formation of cerebellar projection maps. Although more work is necessary to correlate PC parcellation and sagittal microzones, we can postulate that the former is essential for tbe topographic arrangement of extracerebellar afferent fibers. A tentative explanation could be that the broad topography of spino- and olivocerebellar afferents (observed in early postnatal rats and acquired independently of synaptogenesis) results from the confrontation between two maps: one, issuing from the arrangement of axons within the ascending tracts and the cerebellar peduncles; and the other, related to PC parcellation. In this way, cues on PC axons would indicate their belonging to a precise "basic zone" and would direct the ingrowing specific afferents toward it.

This hypothesis, which attempts to integrate all the experimental data obtained in our laboratory, proposes that PCs are the "organizers" of the cerebellar projection maps. Our present experimental program should help to prove or disprove this assertion.

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STRUCTURE, SPECIFICITY AND DISCONTINUITIES IN THE DEVELOPMENT OF CORTICOCORTICAL CONNECTIONS

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Introduction

In the course of normal development the neocortex gives rise to transitory projections. The earliest evidence came from studies using the retrograde transport of horseradish peroxidase (HRP) to investigate the development of callosal connections of visual areas 17 and 18 in the cat [21]. It was found that at birth callosal axons originate from the entire extent of both areas, while in the adult only neurons near the 17/18 border send axons into the corpus callosum. A large portion of the primary somatosensory area (S1) of the cat is similarly acallosal in the adult but not in the newborn kitten [16].

Comparable results were obtained in other areas and species [4, 9, 25]. In summary, wherever callosal neurons have a tangentially discontinuous distribution in the adult, a continuous distribution was found in development. Maturation involves selective loss of the projections originating at specific tangential locations. The loss is due to axonal elimination without or perhaps with little (and still undocumented) neuronal death (see below).

The retrograde transport studies mentioned above did not determine in detail the topography of the transitory projections. However, these appeared to be between homologous (homotopic) areas or, at least, functionally related areas. The latter was for example true for the transitory projection from S1 to contralateral secondary somatosensory areas (S2) [16]. It was also known from electrophysiological and an-

atomical studies that at least some of the adult callosal connections, in particular those between primary and/or non-primary sensory areas connect corresponding portions of the representations of each sensory modality (for references see [15]).

On these grounds, the formation of transitory projections could be interpreted as a special development strategy destined to producing reciprocal, orderly and discontinuous connections between identical or closely related regions of the brain.

More recently, however, it was found that transitory projections are formed not only between, but also within the hemispheres [17]. Furthermore, transitory callosal and associational projections form between areas belonging to functionally different systems and which later become entirely disconnected. For example, a heavy transitory projection in the cat originates from the auditory cortex and terminates bilaterally into the visual cortex [5, 6, 17, 18].

Other bizarre corticofugal projections are also formed in development, for example, from the visual cortex to the spinal cord or from various cortical areas to the cerebellum [7, 36, 38]. Transitory projections are not restricted to the cerebral cortex but have been described in subcortical structures as well [2, 10, 29].

A precise estimate of the magnitude of the reorganization of neocortical connections was obtained by counting callosal axons [27]. About 70% of the axons present in the corpus callosum of a newborn kitten (79 million) are lost by adulthood (23 million). A quantitatively similar loss of callosal axons seems to occur in the monkey [28].

This enormous and ubiquitous production of odd transitory projection seems to challenge two concepts at the roots of our understanding of the brain and its development. One is the concept of structure: "An organized body or combination of mutually connected and dependent parts or elements" (Shorter Oxford Dictionary, 1974). The question is whether connections in the developing brain have any organization, or structure at all. Alternatively, diffuse or random interconnections might exist among all its parts.

The second concept challenged by developmental exuberancy is that of developmental specificity. A large body of evidence indicates that in development, axons seem to travel selectively to their site of termination. Much of this evidence derives from studies in lower vertebrates and in invertebrates and on systems of connection between the central nervous system and the periphery. The way corticocortical connections develop

makes one doubt that the notion of developmental specificity applies to connections within the central nervous system, at least of mammals.

The work which I will summarize here helps to clarify both the above issues.

Juvenile organization of neocortical connections

In newborn kittens, i.e. at the age when callosal neurons are ubiquitous in the visual areas, some callosal axons have entered selectively the gray matter near the 17/18 border and the other restricted cortical locations where they will also terminate in the adult [13]. Other callosal axons are more widely distributed but they appear not to enter the gray matter to any great extent. A fraction of those axons terminate under area 17 which receives no callosal afferents in the adult.

In order to determine the sites of origin of the axons which at birth have entered the cortex and of those which have not, restricted injections of the retrograde fluorescent tracers Fast Blue (FB) or Diamidino Yellow (DY) were placed separately or in various combinations, in the gray and/or white matter of areas 17, 18 and of other visual areas in 33 kittens aged between 0 and 10 days [18].

Injections confined to the gray matter yielded retrograde labeling in the contralateral hemisphere when placed near the 17/18 border but not when placed in medial area 17 or lateral 18. The distribution of labeled neurons resulting from these injections was similar to that found in the adult. Labeled callosal neurons were restricted to the cortex near the 17/18 border and to parts of areas 19, PMLS and PLLS representing the vertical meridian of the visual field. Only a few labeled neurons were found elsewhere, in particular in those parts of area 17 where no callosal neurons are found in the adult. These few neurons may send a transitory axon into the cortex. They are probably part of those callosal projections whose elimination or stabilization depends on visual experience [23].

Injections spreading to the white matter under area 17 and therefore encroaching upon the transitory axons directed to this area yielded a characteristic but totally different distribution (Fig. 1). Labeled neurons defined an irregularly crescent shaped, continuous territory oriented approximately mediolaterally. This territory extended across areas 17, 18, 19, 21a and the lateral suprasylvian visual areas. In each of these areas, labeled neurons were found not only within the regions labeled by the

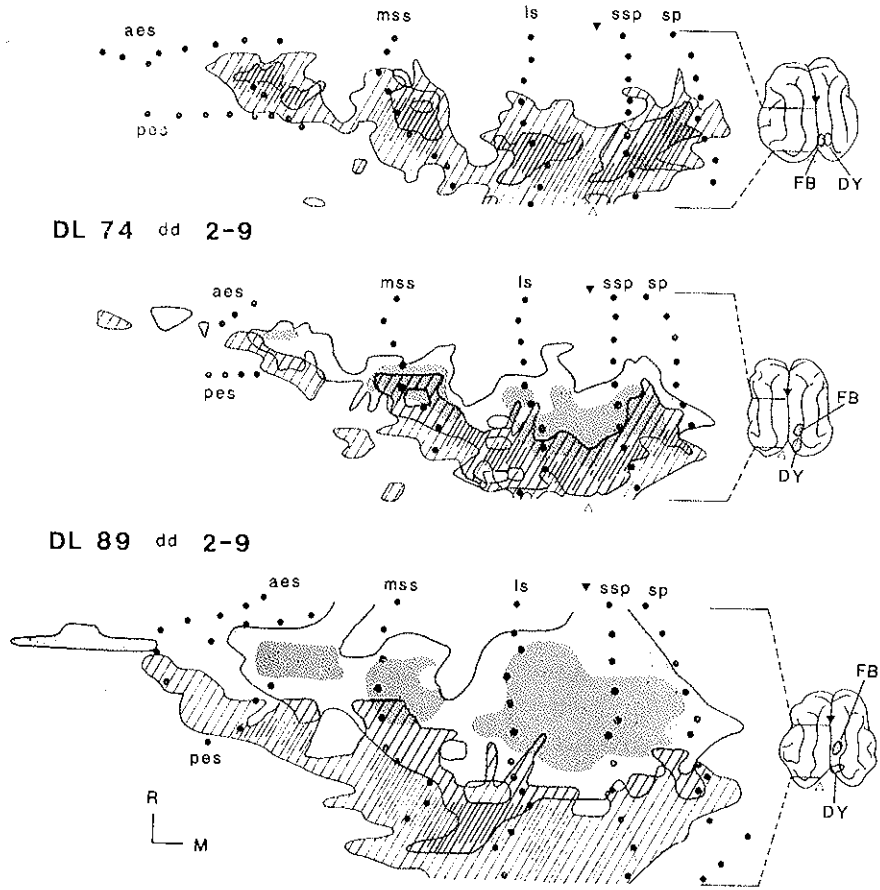

FIG. 1. Flat reconstructions of the occipital portion of the brains of three kittens (identified by code number, age at injection and at death), showing regions containing layer II-VI callosal neurons retrogradely labeled by Diamidino Yellow (DY; hatching) or by Fast Blue (FB; shading). The region reconstructed is between bars on brain insets. The reconstructions are based on hand-drawn sketches of cell distributions. Two arbitrary levels in density of labeled neurons are indicated, i.e. high density (dark shading or hatching) and low density (light shading or hatching). Location of injection sites is marked on brain insets. In DL 76, the FB injection was restricted to the white matter under area 17; the DY injection was in the gray and white matter near the 17/18 border. In DL 74 and 89 all injections were in the gray and white matter near the 17/18 border. Filled and empty triangles point to corresponding positions on the brain surface and on the reconstructions; they denote the axis along which coronal sections were aligned. Dotted lines correspond to sulci, the most important of which are also marked by abbreviations.

Notice that each injection involving the 17/18 border and the underlying white matter labels a continuous, crescent-shaped territory extending from the bottom of the splenial sulcus (i.e. from beyond the medial border or area 17) as far as the ectosylvian sulcus. This territory includes most of the visual areas, probably area 7 and, laterally, the auditory areas. The rostrocaudal position of the labeled crescent varies with that of the injection site along the lateral and postlateral gyri. In the region where the territories labeled by the two injections overlap, few or no neurons labeled by both tracers were found. This indicates that the overlap is probably due to divergence in the projection (neurons located nearby project to rostrocaudally spaced points) not to collateralization of single axons nor to overlap of the regions of tracer uptake.

Abbreviations: aes, anterior ectosylvian sulcus; pes, posterior ectosylvian sulcus; mss, middle suprasylvian sulcus; ls, lateral sulcus; ssp, suprasplenial sulcus; sp, splenial sulcus. R is rostral, M is medial. Calibration bars are 2 mm. (From [187]).

gray matter injections but also outside. For example, labeled neurons were found throughout the mediolateral extent of area 17. Furthermore, the labeled territory extended medially over the limbic cortex and laterally over the auditory areas and it probably also included portions of area 7. None of these areas project to area 17 in the adult.

The rostrocaudal position of the labeled crescent varied with the location of the injection along the lateral and postlateral gyri (Fig. 1). Shifting the injection site laterally, into the other visual areas, also shifted laterally the labeled territory in the contralateral hemisphere. However, not all of the neonatal projections are reciprocal. For example, injections into area 17 labeled both PLLS and the auditory cortex, but injections into either of these latter regions failed to label area 17.

Both rostrocaudally and mediolaterally, the precision of the topography is limited by the extensive divergence/convergence of the projection: neurons located near to each other in area 17, for example, project to loci that are widely separated rostrocaudally or mediolaterally in the contralateral hemisphere.

The simultaneous injections of different tracers at different locations allowed to determine whether juvenile axons branch and to investigate the relative distributions within the cortex of neurons projecting to different targets.

Young callosal and association neurons seem not to have indiscriminately branching axons. They project selectively to one or perhaps a few targets. Selectivity is apparent in the choice of both transitory and permanent targets. For example at birth, neurons in medial area 17 project transitorily either to contralateral areas 17 and 18 or to PMLS but not to both. Furthermore, some of these neurons also project, probably permanently, to ipsilateral visual areas, but only to some of them (see below).

Even more striking is the precise and characteristic differential radial distribution of efferent neurons, including those with transitory projections, before the latter are eliminated. For example in auditory cortex, the neurons projecting transitorily to *ipsilateral* areas 17 and 18, those projecting also transitorily to *contralateral* areas 17 and 18 and those projecting, at least in part permanently [9], to contralateral auditory areas have three different radial distributions [17]. The first type of neuron is in layer II and the upper part of layer III. The second type is found deeper in layer III. The third type occupies still deeper portions of layer III and layer IV.

These results clearly show that the juvenile projections are organized although their organization, especially their topography, is very different from that of the adult. Why this difference? The adult and the juvenile organizations of callosal connections probably reflect different functional rôles. The rôle of adult connections is to match neurons with specific functional properties which, however, they probably do not determine. For example, in areas 17 and 18 the corpus callosum interconnects neurons with similar receptive field positions along the vertical meridian and with similar orientation specificities [1]. Both properties are probably largely due to the specific organization of the thalamocortical projections. The rôle of the juvenile callosal topography is probably that of directing axons of a given class of neurons to the more or less appropriate part of the brain before the information necessary for the precise neuron-to-neuron match is either available or usable. This interpretation implies that the transitory projections, and in general all transitory brain structures, provide the potential for the developmental plasticity of the brain. Indeed, transitory structures can be maintained, and/or structures which are normally maintained can be eliminated when abnormal morphogenetic factors come into play [14]. This interpretation does not exclude that the transitory projections may have other functions. For example in the chick, the transitory retino-isthmic projection may serve to guide axons of the isthmo-optic tract [37].

But what mechanisms are responsible for the juvenile organization? As discussed elsewhere [18], the characteristic differential radial distribution of cortical neurons projecting to different targets suggests that the time of generation of a neuron may restrict, or perhaps even more precisely determine, the choice of where its axon will grow. To what extent the topographical relations that growing axons establish with each other along their pathway may also be important for the topographic organization of immature connections remains to be determined. However, these two mechanisms are not sufficient to explain why *transitory* projections are formed. For this one has to admit that the mechanism directing neurons to a given target (according to their time of generation) may have a limited discriminative power. An analogy could be found in the way an army is drafted: at first all individuals born in a certain year are drafted although some of them will later be discarded. Alternatively, axons growing within the nervous system may have a tendency to overshoot their targets. Critical evidence for this hypothesis may come from identifying the final target of neurons which give rise to transitory

projections. So far, the overshooting hypothesis could explain why some neurons eventually projecting to ipsilateral area 18 originally project to the contralateral hemisphere (see below) and why neurons eventually projecting to the superior colliculus and/or pons originally project to the spinal cord [31].

A different level of interpretation is that the development of cortico-cortical connections recapitulates a phylogenetic sequence (for this concept and its discussion see [8]). The organization of callosal connections in the kitten would correspond to that found in adult, but more "primitive" mammals. It has indeed been argued that the commissural connections of the opossum may be widespread and indeed, more similar to those of newborn kittens than to those of the adult cats and monkeys [8]. However, a comprehensive study of commissural connections in marsupials, using modern techniques, seems to be lacking.

The task of ranking the existing mammalian species in a phylogenetic sequence from "primitive" to "evolved" may appear to be a formidable one. A more modest and sufficient achievement may, however, be to rank the existing mammals according to the evolution of their neocortex or perhaps just of a given cortical area. It would probably be difficult to argue against the statement that the primary visual cortex of rodents is more "primitive" than that of cat or monkey. It is amazing, and in favor of the recapitulation hypothesis that the primary visual cortex of the adult rat receives afferents from the auditory and somatosensory areas [30] just as it does transitorily in the cat [5, 6, 17, 18].

The Transition from the Juvenile to the Adult Organization of Neocortical Connections

The factors determining the transition from the juvenile to the adult organization of cortical connections are incompletely known. Evidence which will not be reviewed here indicates that visual experience is responsible for the elimination and/or stabilization of a small fraction of the juvenile callosal projections [22, 23]. It is plausible, although still hypothetical, that these axons form transitory synapses in the cortex. The largest fraction of the transitory callosal projections, though, appears to be eliminated or stabilized, independent of visual experience.

Several studies have indicated that callosal axons do not grow into the cortex diffusely, but selectively at the locations where they are also

found in the adult [9, 11, 13, 24]. As mentioned in the previous section most of the axons which enter the cortex originate from regions destined to maintain their callosal projections. In contrast, most of the axons which do not enter the cortex originate from cortical regions destined to eliminate their callosal projections. Thus it seems probable that for the majority of the juvenile axons the crucial events deciding their fate occur near the interface between gray and white matter. Presumably, these events involve specific axon-target or axo-axonal interactions. They lead to the selection of axons based on the tangential position of their neurons of origin in the cortex, or perhaps of some other associated property.

A similar mechanism may guide the development of intrahemispheric connections. Most of the transitory association projections to areas 17 and 18 are revealed only by injections of retrograde tracers extending to the white matter under these areas, where most transitory association axons appear to terminate [5].

Since the transitorily-callosal neurons do not die after eliminating their callosal axons it was assumed that they establish permanent projections in the ipsilateral hemisphere [13]. Furthermore, callosal neurons appear to be selective in their early choice of a target, were it a temporary one. Thus, a relationship may exist between a neuron's initial and final choice of its target. In a recent study [20] this hypothesis was tested by injecting areas 17 and 18 in one hemisphere with the retrograde long-lasting fluorescent tracer Fast Blue (FB) during the first postnatal week. During the second postnatal month, i.e. after elimination of most of the transitory callosal projections that originate from area 17, another retrograde fluorescent tracer, the Diamidino Yellow (DY) was injected in contralateral visual areas which are known to receive intrahemispheric projections from area 17. In portions of area 17 which project transitorily through the corpus callosum we looked for neurons labeled both by the early and the late injection. It was found that a fraction of the neurons projecting transitorily to contralateral areas 17-18 later project either within ipsilateral 17 or to ipsilateral 18. None seems to project to other visual areas (Fig. 2).

An explanation for these findings may be that cortical neurons are programmed to project specifically to a given cortical area, irrespective of its side in the brain. In order to test this hypothesis we performed experiments identical to those described above, but area PMLS was injected during the first postnatal week. Neurons projecting transitorily

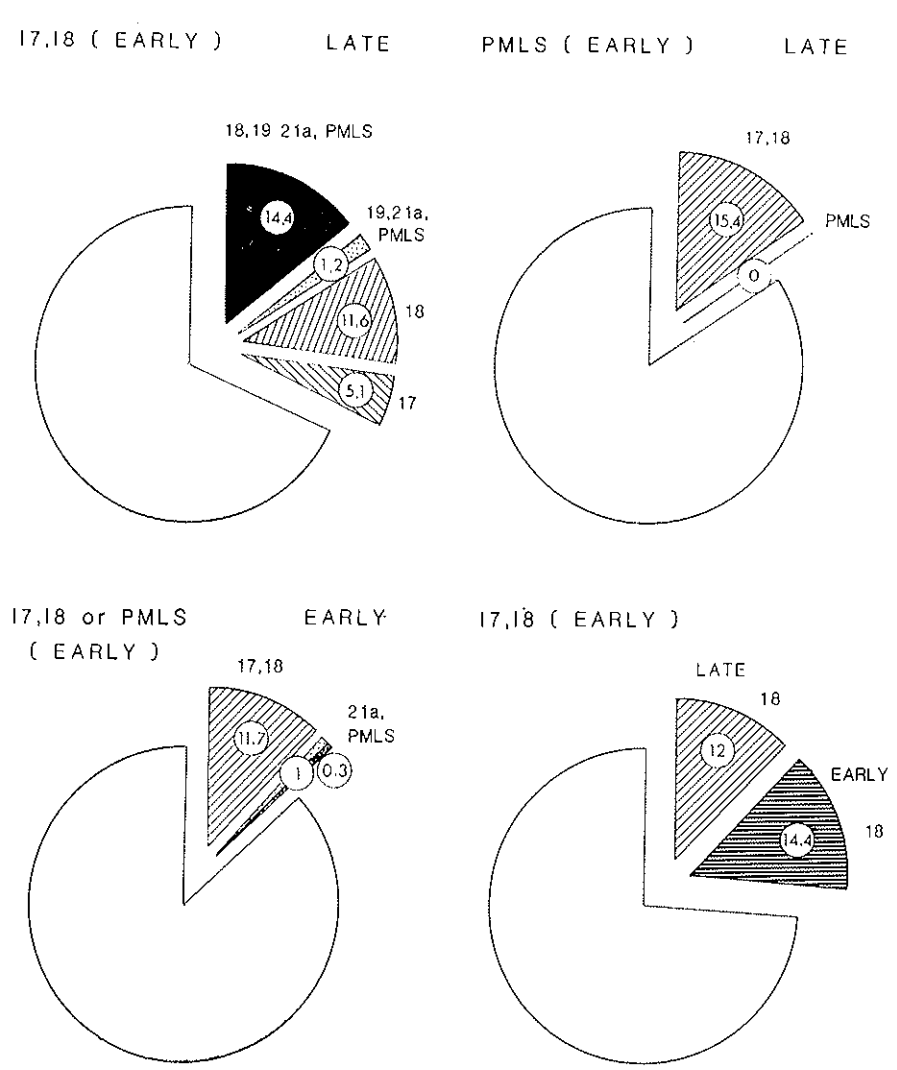

FIG. 2. Diagrams showing which proportions of transitorily-callosal neurons in area 17 (labeled by early FB injections in the contralateral hemisphere) could be relabeled by injections of a second tracer (DY or RL) in different areas of the same hemisphere. Which percent of the FB labeled population is also labeled by the second tracer is indicated by size of sectors as well as by the number on each sector. Top left: FB was injected in contralateral 17 and 18 during the first postnatal week, DY was injected in the different ipsilateral visual areas (in different animals) during the second postnatal month. Top right: FB was injected in contralateral PMLS during the first week, DY was injected in ipsilateral 17-18 or in PMLS (in different animals) during the second month. Bottom left: FB was injected in contralateral 17-18 or in PMLS and DY in ipsilateral 17-18 or 21a-PMLS (in different animals) during the first week. Bottom right: FB was injected in contralateral 17-18 and RL in ipsilateral 18 during the first week (sector marked "early"). In the same animal, DY was reinjected in ipsilateral 18 during the second month (sector marked "late"). (From [20]).

to contralateral area PMLS at birth are a distinct set and differ from those projecting transitorily to contralateral 17-18. The hypothesis of area specificity would have predicted that they should form permanent projections to ipsilateral PMLS. Instead, similar to those projecting transitorily to 17-18, they could be relabeled from ipsilateral areas 17 and 18 but not from PMLS (Fig. 2). It must be stressed that thus far we could relabel only about 15 % of the neurons with transitory callosal axons. A substantial fraction of the transitorily-callosal neurons whose final target cannot be identified may form short intrinsic connections within area 17 (for discussion see [20]).

Probably, at least some of the transitorily-callosal neurons which finally establish projections to ipsilateral areas 17 or 18 at birth have sent an axon collateral specifically to these areas [12, 13]. As also suggested by others [26, 32] these neurons might later eliminate their callosal collateral but keep their ipsilateral collateral. Consistent with this hypothesis, in portions of area 17 destined to become acallosal some neurons can be double-labeled by simultaneous, neonatal injections of FB in the contralateral areas 17-18 and of DY in ipsilateral 18 (Fig. 2). In contrast, no double labeling could be observed after simultaneous, neonatal injections in contralateral 17-18 and in ipsilateral PMLS or in PMLS in both hemispheres. This suggests that the specificity neurons display in their final connections may already be expressed at birth.

In order to test whether the neurons which have bifurcating axons at birth are the same ones which later project to ipsilateral areas 17 or 18, the following triple-labeling experiment was performed. Newborn kittens were injected with FB in areas 17 and 18 and with Rhodamine conjugated Latex beads (RL) in contralateral area 18. This same area was reinjected with DY during the second postnatal month (Fig. 2). Some triple labeled neurons were found in the part of area 17 which is acallosal in the adult. This finding is consistent with the hypothesis that at least some neurons in area 17 may have at birth two axon collaterals, one going through the corpus callosum, which they selectively eliminate, and one to ipsilateral area 18, which they maintain. Nevertheless, these findings fail to rule out the alternative possibility that transitorily-callosal neurons grow a new axon to ipsilateral areas 17 and/or 18 after eliminating the transitory axon to contralateral cortex (for discussion see [20]).

The fact that transitorily-callosal neurons form permanent projections to ipsilateral area 17 or 18 but not to other visual areas is another

example of specificity in the development of corticocortical connectivity. What may the origin of this specificity be?

The comparative analysis of the distributions of neurons labeled in area 17 by early injections in contralateral areas 17 and 18 and of those labeled by later injections in various ipsilateral areas indicates subtle but consistent differences in the radial distributions of the various sets of corticocortically projecting neurons in area 17 (Fig. 3). The bulk of the transitorily-callosal neurons are in layers III and IVab, although a few can also be found in layers II, IVc and VI. There are only small differences in the radial distributions of callosal neurons to areas 17-18 or to PMLS. The latter tend to be less frequent in layers II, IVc and in the deep half of layer IVab. The bulk of the neurons projecting from area 17 to ipsilateral 18 are also in layers III and IVab although a higher fraction of these neurons, than of callosal neurons, is also found in layer II. The other corticocortically projecting neurons are on average more superficial, mostly in layers III and II, depending on the projection.

These findings complement those quoted above in suggesting that radial position may determine where a neuron will send its axon. Radial position, though, is probably important because it reflects the birthdate of a neuron. That the birthdate may be the critical factor is suggested by the finding that in "reeler" mutants and in normal mice callosally projecting neurons have different radial position although they are probably generated at comparable embryonic ages [3].

As further speculations I suggest two possible ways in which age may condition the fate of an axon. Axons originating from a bit of cortex follow different and highly stereotyped routes leading to different targets [19, 33] (and unpublished results). This compartmentalization of axon pathways may be due to cues, in the white matter, differentially guiding the various sets of corticofugal axons. In principle, these hypothetical cues could appear at different times in different parts of the white matter. Time of generation may determine when a neuron will grow its axons and therefore, which white matter cues it will respond to.

There seem to be at least two arguments against a strictly time-based model. First, HRP tracing in the adult indicates that sometimes individual axons in the neocortical white matter take indirect, zig-zagging trajectories towards their targets as if at the time of their growth they had been correcting mistakes in their trajectories (Fig. 4). Second, grafts of fetal cortex into newborn brains should dissociate the normal temporal relationship between the maturation of cortical layers and that of the

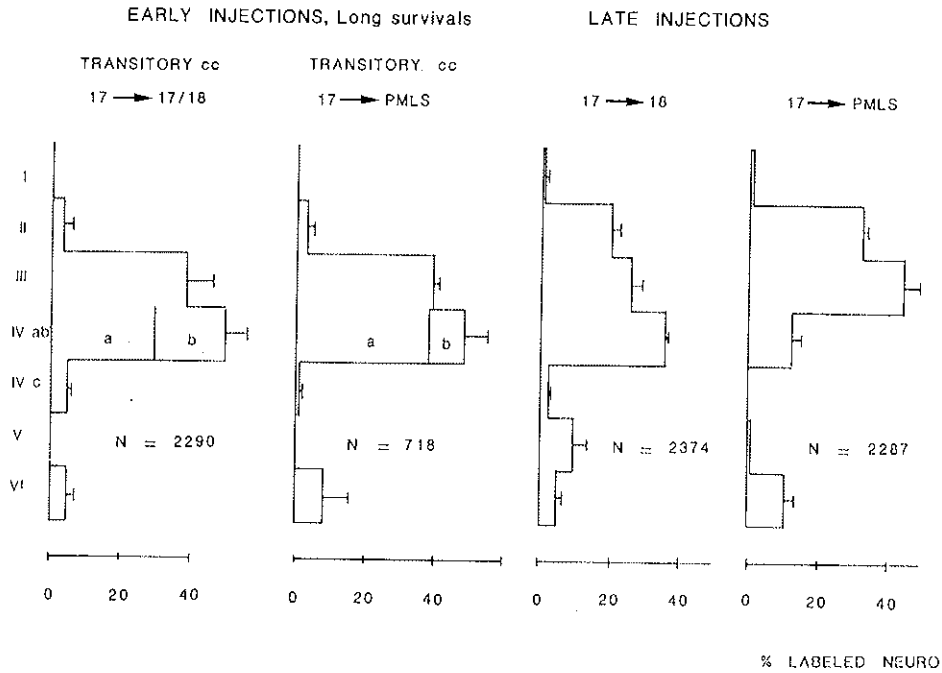

FIG. 3. Histograms showing radial distributions of four sets of efferent neurons in area 17, each defined as projecting to a different target. In each histogram, columns represent the fraction of the total population (number below each histogram); in two of the histograms, the fractions of neurons in the upper and lower half of layer IVab are represented separately. Histograms derive from counts pooled from different animals; bars above each column are standard deviations across animals. The two histograms on the left represent the distributions of area 17 neurons labeled by FB injected in contralateral 17-18 during the first postnatal week; the animals were killed during the second postnatal month. The two histograms on the right represent the distributions of area 17 neurons projecting to ipsilateral 18 or PMLS. Notice that neurons projecting transitorily through the corpus callosum (transitory cc) are most common in layers IVab and III, the same as the neurons projecting to ipsilateral area 18. The neurons projecting to ipsilateral PMLS are located more superficially, with maxima in layers III and II.

white matter. Preliminary results suggest, however, that neurons in the graft form connections appropriate for their time of generation [35].

Therefore, it appears more probable that, in addition or alternatively to the strict temporal guidance hypothesis, neurons generated at different times may also possess unique properties which provide them with different capacities to read directional cues in the white matter. One could, for example, postulate chemical heterogeneity of neurons in the different

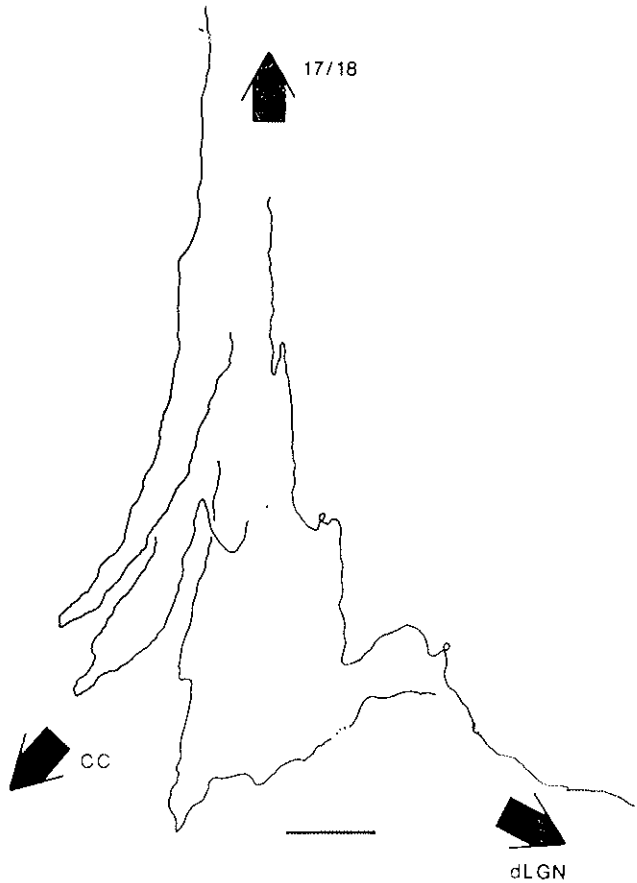

FIG. 4. Bizarre axonal trajectories in an adult cat injected with HRP in the lateral and postlateral gyri (containing areas 17 and 18) in the same hemisphere. Camera-lucida drawings of single, HRP-filled axons in the white matter above the lateral ventricle, i.e. at a location where axons going to the corpus callosum (arrow) and to the dLGN (as well as to other ipsilateral targets; arrow) separate. Some axons, probably originating in the cortex, seem to have experienced difficulties in finding their correct route. Most of the other axons in the same region had straight trajectories, which suggest that the zig-zagging and looping of those illustrated here may not be due to modifications of the geometry of the white matter *posterior* to the axonal growth. Interestingly, the three axons on the left, whose final target seems not to have been the corpus callosum, have grown in the direction of the corpus callosum for some distance, possibly following callosal axons they intersected.

cortical layers. These heterogeneities do indeed exist (Innocenti *et al.*, unpublished) although it is impossible to decide, for the time being, whether they relate to target specificity. Furthermore, the chemical heterogeneity of neurons in different layers may be the consequence, rather than the cause, of target specificity.

Conclusions

The study of transitory projections casts a new light on the notions of structure and specificity in neocortical development. It also affects profoundly our appreciation of the qualities of the process which leads to the development of corticocortical connections. This process is essentially a discontinuous one. During the development of callosal connections at least three steps, controlled by different factors or combinations of factors, can presently be identified. The first step seems to be the choice of a target, or possibly of a pathway, by young neurons. This choice seems to be independent of whether a neuron will establish permanent connections with this particular target, but in some unknown way it relates to a neuron's radial position, or, most probably to its birthdate. The second step seems to involve a mechanism that allows only axons originating at specific tangential positions to enter the cortex and only at specific tangential locations. Axons which do not enter the cortex are eliminated. The third step seems to be activity-dependent, at least in the case of visual callosal connections. Over several weeks, following natural eye opening, visual experience seems to stabilize certain callosal connections, while eliminating some others [23]. It is probable that a Hebbian type of synaptic stabilization/elimination mechanism (for a discussion of the concept see [34]) could explain this late developmental step.

Perhaps the most striking feature of the whole developmental process is that each step seems to organize the connections according to a different project or "Bauplan". These discontinuities in the formation of connections recall the metamorphic development of some lower vertebrates and of invertebrates. It is of course crucial to understand to what an extent this similarity is a substantial one. Especially tempting is the possibility that the transcription of different genetic instructions may be responsible for events characterizing the various developmental steps just as this appears to be a crucial mechanism of metamorphic development.

Acknowledgements

This work was supported by the Swiss National Science Foundation Grant 3.422.0.83. I am grateful to Dr. P. Raymond for comments on the manuscript.

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COMPETITIVE INTERACTIONS AND REGULATION OF DEVELOPMENTAL NEURONAL DEATH IN THE RETINA

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INTRODUCTION

Several characteristics of retinal ganglion cell populations are clearly related to defined functional attributes of the visual system in adult mammals. The number of ganglion cells and a centro-peripheral gradient of cell density are fairly constant within any given species, and set an upper limit for visual acuity [29]. Pathways conveying binocular information essential for stereopsis rely upon the presence of both a nasal retinal area with a crossed projection to visual centres of the brain, and a temporal crescent with an ipsilateral projection from at least part of its cells [74]. Major ganglion cell types defined hy morphological and physiological criteria are organized in regular arrays, the so-called retinal "mosaics", providing effective coverage of the whole retinal area with the dendritic trees of different cell types [82].

The developmental processes through which these features are attained are mostly unknown. Recent studies have emphasized the role of regressive phenomena in neurogenesis [53]. It is the purpose of this paper to evaluate the contribution of natural neuronal death to the development of retinal ganglion cell populations. Evidence for the interpretation that both terminal-axonic and dendritic competition concur for the regulation of cell death in the retina has been gathered from studies of abnormal development, and is currently reviewed. It will hopefully become apparent that natural cell death may be involved in the modulation of some, though not all, developmental processes leading to the maturation of major morphological features of the retina.

Natural Neuronal Death in the Developing Retina

There is now overwhelming evidence that natural neuronal death is an ubiquitous component of neurogenesis [9, 53]. Among an extensive series of studies in many areas of the developing nervous system of amphibians, birds and mammals [53], there is only one reported case of a failure to find such evidence, namely the pontine nuclei of the chick [1].

In the chick retina, it was found that a massive reduction in the number of optic axons occurs in embryonic periods. This was attributed to natural cell death, detected in the ganglion cell layer with the use of both optic and electron microscopic techniques [31, 63].

In mammals, pyknotic nuclei were found postnatally in the retina of hamsters [70] and rats [15]. At least part of those pyknotic nuclei were ascribed to neurones, on the basis of electron microscopic findings of early stages of degeneration [15]. The magnitude of the phenomenon was estimated in the retina of the hamster using two independent methods [69, 70]. In the first study, the clearance rate of the debris was assumed to be the same as in the mouse spinal cord, and the estimate was of 64% cell loss. In the second study, the calculations were based on counts of pyknotic nuclei and of normal cells following enucleation, and the estimate was of 49% cell loss. Neither study, however, did take into account the addition of cells to the ganglion cell layer in the period of neuronal death (see below). Further, no distinction was made between displaced amacrines and ganglion cells nor, indeed, between neurones and glia.

The method of choice for estimating the amount of cell death in a given neural population has for long been considered the counting of normal neurones at successive stages of development. This was done for the ganglion cell layer of the retina of the pigmented rat [60], and it was found that the total population of neurones in this layer increased from the day of birth up to postnatal day 5 (PND 5), and thereafter decreased towards adult values which were attained after PND 10. Following the initial increase, the cell loss in this layer amounted to a minimum of 20%. Counts of pyknotic nuclei were the highest at birth and slowly decreased over the following days, providing direct evidence for natural degeneration even before the total neurone numbers start to decline.

The ganglion cell layer in the rat, however, contains both ganglion cells and displaced amacrines in roughly equal numbers [57]. Numbers of ganglion cells were estimated both from horseradish peroxidase labelling and from counts of optic axons in electron micrographs, for albino [39, 62] and pigmented rats [60]. The estimates showed a 35-55% loss of

ganglion cells (Fig. 1) from the day of birth to PND 3-5 in albinos, or at least to PND 10 in pigmented rats.

The possibility that the reduction in the number of ganglion cells is the result of transdifferentiation into displaced amacrines [26, 27] was investigated in rats [19, 60]. The data failed to provide evidence that any cell would retract an axon that had elongated far enough as to allow its retrograde labelling from HRP injections into the brain. Displaced amacrine cells of the rat's retina, instead, appear to reach their final destination largely by late, postnatal migration through the developing inner plexiform layer. This late migration can explain the fact that the total number of neurones in the ganglion cell layer increases from birth to PND 5 in the rat, while the numbers of ganglion cells are diminishing [60].

The results indicated that about half of the ganglion cells present in the newborn rat retina degenerate over the following one to two weeks. Recent studies in albino rats showed that the loss of optic axons starts before birth [11]. The magnitude of the cell loss is, therefore, likely to be higher than 50%.

During the period of cell death, the vitreal surface of the retina is inhabited by a population of non-neuronal cells scattered among and abutting the walls of blood vessels which form an immature vascular network. These cells have been identified as macrophages with the use of monoclonal antibodies in the mouse retina [32], and by histoenzymatic and functional criteria in the retina of the rat [3, 44]. The presence in the retina of macrophages is probably related to the removal of natural degeneration products, though other functions cannot be discarded.

In other mammals, evidence has been reported of massive axon loss in the developing optic nerve. The cat optic nerve at embryonic day 48 has twice as many axons as the adult optic nerve [85], while the monkey optic nerve reaches a total of 2.85 million axons at embryonic day 69 compared to 1.2-1.3 million axons in the adult [64]. Estimates of total cell numbers in the ganglion cell layer of the cat retina also revealed a massive cell loss in the embryonic period [75].

A recent survey of the postnatally developing retina of the mouse [86] showed the presence of pyknotic nuclei in all retinal layers. Based on the small proportion of degenerating profiles among the normal cells identified in each section of the retina, the author concluded that the cell loss was small. We have, however, estimated the total numbers of pyknotic nuclei in the ganglion cell layer of the retina in mice of different ages from birth

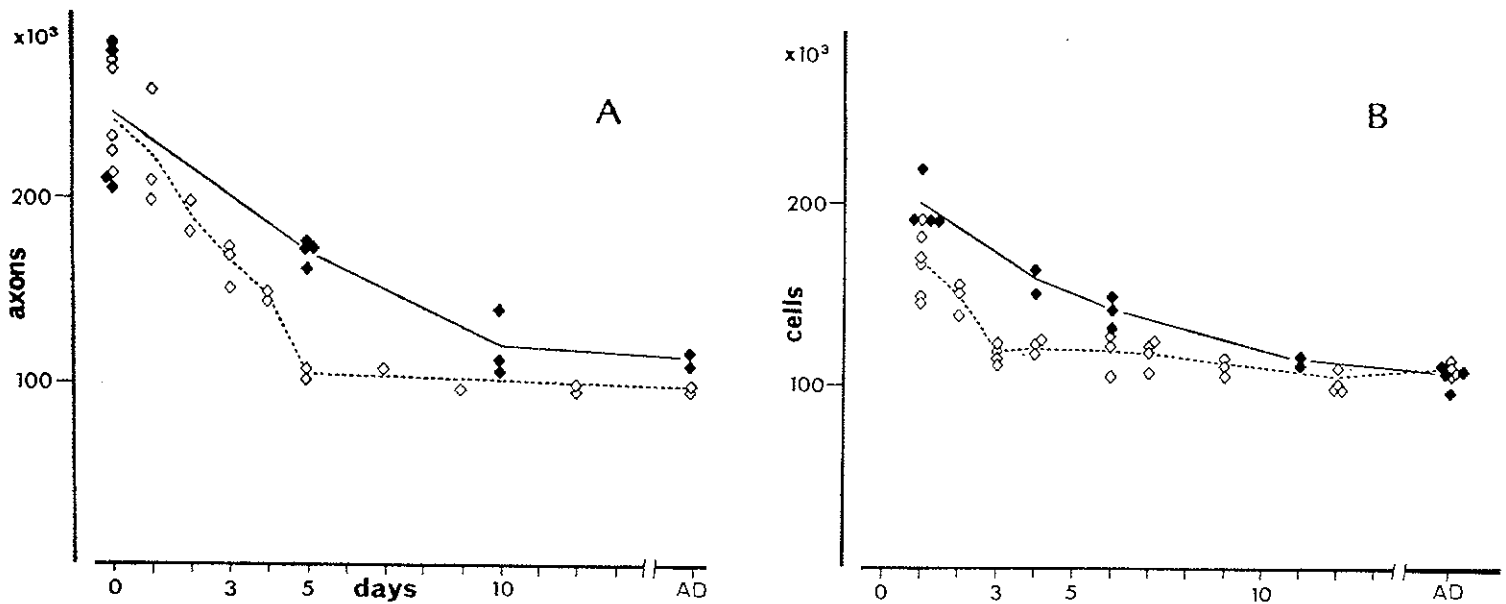

FIG. 1. Estimated numbers of (A) optic axons and (B) retinal ganglion cells in albino (open symbols) and pigmented (filled symbols) rats. The horizontal axis shows the age in days after birth. The day of birth was designated day 0. AD = adult rats. Notice the decrease in the population, which seems to occur at a slightly faster rate in albinos. Data for albinos from [39, 62]; for pigmented rats from [60].

to postnatal day twelve [47]. Our estimates showed that the peak numbers of degenerating cells in this layer are only about 10% lower than the maximum numbers found in postnatal rats [60]. The loss of neurones following birth in the rat is of the order of 10^5 ganglion cells [60]. These data suggest that the postnatal cell loss in the ganglion cell layer of the mouse retina involves several tens of thousands of cells, lest the clearance rate of degeneration products be too different between the two species of rodents. It must be noted that adult mice have only about 120,000 neurones in the ganglion cell layer, including both ganglion cells and displaced amacrines [18, 47], and therefore such a cell loss is proportionally quite high.

Notwithstanding the large differences in the total number of cells, in the duration of the gestational period, and in the degree of maturity of the newborn animal of a given species, a loss of at least half of the ganglion cells is, therefore, a widespread feature of developing mammalian retinae.

Competition Among Axons for Terminal Space and Regulation of Neuronal Death

Several lines of evidence have, in recent years, supported the classical conception that the availability of target space is required for the survival of projecting neurones. Most of the data have been gathered in experiments involving either spinal or cranial motoneurones, and cells of the isthmo-optic nucleus of the chick [5, 53].

Experiments in the chick retinotectal pathway also provided evidence for such a process, given that removal of the optic tectum *prior* to its innervation by developing optic axons greatly increases the amount of ganglion cell death in the retina [31].

Lesions to the tectum of newhorn rodents increase the amount of ganglion cell death in the retina [45, 46, 58, 59, 77]. Such lesions, however, involve actual damage to optic axons, many of which have already reached the tectum on the day of birth [20, 40]. It cannot be excluded that late-developing ganglion cells may degenerate as a result of reduced postsynaptic space [19, 77]. Nonetheless, the most likely explanation for ganglion cell death after tectal lesions in neonatal rodents is still the direct damage to the immature optic axons [58, 59].

The idea that the survival of developing neurones depends on the targets has, however, been extended to the mammalian retinofugal path-

ways. This followed the demonstration that the aberrant ipsilateral projections consequent to early monocular enucleation in rodents arise from an increased population of ganglion cells [38, 50], due to a reduction in the amount of cell death [69].

In newborn rats, the terminal fields of axons from the ipsilateral retina are widespread over the retinal targets [36, 40], and arise from a larger number of cells than in adults [36, 41]. During the first 7-10 days of postnatal life, the projection retracts to occupy its characteristic position [40]. This retraction can be interrupted by monocular enucleation, which removes the crossed projection from the terminal fields widely occupied by the immature ipsilateral pathway [40, 50] (Fig. 2).

An actual increase in the total number of ganglion cells, that is both with crossed and uncrossed projections, has been reported for the remaining retina of hamsters following monocular enucleation at birth [71]. These data supported the hypothesis that the reduced cell death is a major component of the plastic response following enucleation.

Further studies were made with the use of long-lasting fluorescent tracers [10, 34]. The tracers were injected in the superior colliculus of newborn rats, so as to label ganglion cells in the ipsilateral retina. In normal rats, the number of labeled cells declines with increasing survival time after the injection, whereas this number remains high in rats monocularly enucleated at birth. The reduction in the number of labeled cells in normal rats was interpreted as evidence for cell death, as opposed to the axonal retraction demonstrated in other systems [33]. The maintenance of large numbers of labeled cells in enucleated rats was, in turn, interpreted as a result of rescuing cells from natural degeneration [10, 34].

Data from other mammals are consistent with the results obtained in rodents. In monkeys and cats, early monocular enucleation results in the retention of an increased number of axons in the remaining optic nerve [65, 85]. In cats, the increased number of axons matches an increased number of ganglion cells, as demonstrated by retrograde labeling with HRP [4].

The results in several species of mammals, therefore, indicate that cell death among retinal ganglion cells is reduced following the removal of other ganglion cells projecting to the same targets. The crossed and uncrossed terminals in the developing animal initially overlap extensively. Presumably, during this period the axons compete for limited post-

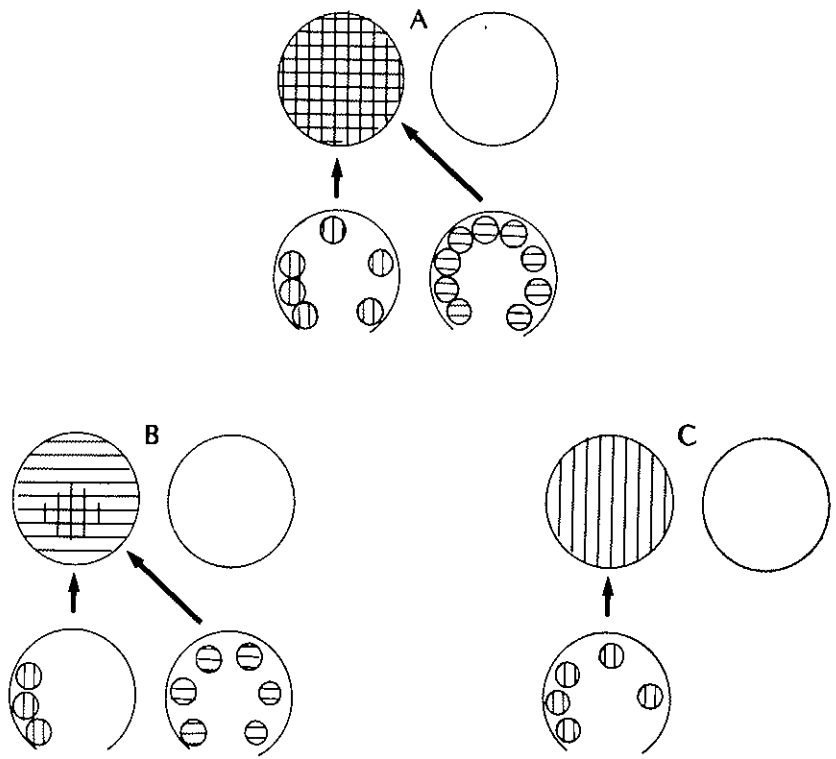

FIG. 2. Plasticity of the ipsilateral retinofugal pathway following early monocular enucleation. In each diagram, both eyes and both sides of the brain are represented as the lower and the upper pairs of circles, respectively. The optic pathways are shown with arrows pointing to the side of the brain at the left of the reader. Ganglion cells (small circles) with crossed projections are shown with horizontal hatching; those with uncrossed projections are shown with vertical hatching. The corresponding terminal fields in the brain are shown with the appropriate direction of hatching. (A) newborn rat, with extensive overlap of crossed and uncrossed projections in the brain. (B) normal adult rat, with its typically restricted ipsilateral projection. (C) enucleated rat, with expanded uncrossed projection from an abnormally large number of ganglion cells.

synaptic space, and the cells eliminated from the immature ipsilateral pathways are those whose axons failed to establish a certain set of connections. There is, of course, no reason to believe that the ipsilateral pathway is the sole loser of such a competitive process. It is likely that cells with crossed projections are also eliminated through binocular competition, though this remains to be demonstrated.

Afferent Control of Neuronal Death: the Principle of Dendritic Competition

While competition for terminal space is a widely accepted principle of neuronal development, similar events that might occur at the afferent side of neurones have hardly been granted a similar status. Developing neurones are highly sensitive to deafferentation [8, 73], but a presumptive role of incoming afferents on the control of natural neuronal death remains disputed.

In several instances, deafferentation has been shown to produce a reduction in the number of neurones. Levi-Montalcini's original findings of extensive cell loss in nuclei angularis and magnocellularis following early otocyst ablation in the chick [42] were extended in the more recent study of Parks [55]. It was shown that the cell loss in nucleus angularis is less extensive than previously reported, but its time course overlaps a small normal cell loss [55].

Early results of reduced cell numbers in the mouse superior colliculus following enucleation at birth [17] were interpreted as a reduction in glial content. The study of Heumann and Rabinowicz [25], however, showed that the time course of the cell loss induced in the dorsal lateral geniculate nucleus following contralateral or bilateral enucleation at birth overlaps the normal cell loss observed in unoperated mice. Recent studies also showed that enucleation in newborn rats affects the pattern of cell death observed in the superior colliculus, during the period of normal cell death [23].

More stringent evidence for the afferent control of natural cell death is available from studies of the isthmo-optic nucleus and of spinal motoneurones in the chick. It was shown that from embryonic day 12 (E12) to E16 in the chick, a wave of neuronal death sweeps over the isthmo-optic nucleus, a midbrain collection of neurones which send centrifugal axons to the eye [7]. This wave of degeneration depends on the peripheral target, since it is greatly enhanced by removal of the eye primordium prior to the arrival of the centrifugal fibres. Nonetheless, lesions made at E12 in the optic tectum, which is the main source of afferents to the isthmo-optic nucleus, also increase neuronal death during the late period of degeneration (from E14 to E16). In their original paper, Clarke and Cowan [7] suggested this to result from a cascading effect: tectal lesions would lead to retrograde retinal degeneration, which would, in turn, deprive the isthmo-optic nucleus of its target. The recent results of a new series of tectal lesions in the chick [6] confirmed the early findings

of increased neuronal death in the isthmo-optic nucleus, but now a number of elegant controls were presented as evidence against a multistage retrograde effect.

Previous attempts had failed to show any contribution of either central or peripheral afferents to natural cell death among spinal motoneurones of the chick [24]. While deafferentation has been shown to produce degeneration, this occurred *after* the main period of natural cell death. Recent results in the amphibian spinal cord confirmed that motoneurone death occurs following sections of dorsal roots made either before or after the period of natural cell death. The time course of the degeneration has not, however, been followed [16].

Recently, Okado and Oppenheim [52] re-examined the effects of early deafferentation upon the survival of motoneurones in the chick. Natural cell death in the lateral motor column of the spinal cord occurs mainly between embryonic day 5(E5) and E10, but extends at least to E14, albeit with a lower rate [52]. It was shown that either spinal transection at cervical and at thoracic levels, or removal of the lumbar neural crest increased the amount of cell death among lumbar motoneurones from E12 to E14. Combined spinal transection *and* neural crest removal, in turn, increased cell death even earlier, well within the main period of natural neuronal death.

The results reviewed above indicate that early deafferentation does, indeed, induce additional cell death cotemporal with the natural neuronal death. The developing neurones appear to be particularly sensitive to deafferentation late in the period of natural degeneration. The results of massive deafferentation following the combined lesions of Okado and Oppenheim [52], however, suggest that afferents may begin to play a sustaining role even earlier.

Cunningham and his associates [14] provided another line of evidence for the afferent control of cell numbers with an elegant experiment in rats. Following prenatal labeling of dividing neurones with tritiated thymidine, they produced hyperinnervation of midbrain structures by retinofugal pathways expanded after neonatal cortical lesions. Counts of labeled neurones in the hyperinnervated nucleus of the optic tract were significantly higher than when the nucleus was normally innervated. The authors suggested that the increased number of neurones might result from diminished cell death, as a consequence of an expanded afferent supply. This experiment is analogous to a highly celebrated experiment of Hollyday and Hamburger [28], in which the early transplantation of a super-

numerary limb in the chick led to reduced cell death among spinal motoneurones, thus providing one of the cornerstones of the principle of terminal competition.

In systems where a number of elements interact competitively, it is expected that the removal of some competitors should benefit the remaining elements. This idea was tested in the retina, following unilateral optic tract lesions made in newborn rats [46]. We took advantage of a particularly useful characteristic of the retina of non-primate mammals, which is the intermixing of neighbouring ganglion cells with crossed or with uncrossed axons. This experiment is represented schematically in figure 3.

The optic tract damage led to pronounced retrograde degeneration of ganglion cells projecting contralaterally from the eye opposite to the lesion. In this same retina, ganglion cells with ipsilateral axons were, therefore, allowed to develop among a diminished number of neighbouring cells. When the ipsilateral projections of this eye were later examined with HRP injections, both the terminal fields and the number of projecting ganglion cells were found to be increased. The crossed projections from the other eye were unchanged. We interpreted the increased number of ipsilaterally-projecting ganglion cells as a result of reduced cell death, related to the removal of neighbouring cells within the retina [46].

The retina contralateral to the optic tract lesion is severely depleted of ganglion cells. The depletion, however, is less severe within the temporal crescent, in which the proportion of cells projecting contralaterally is lower than in the remainder of the retina. In the operated rats, the dendrites of cells located along the border of the temporal crescent had a tendency to point towards the nasal retina (Fig. 4). The dendritic trees looked as if avoiding the areas where the density of remaining ganglion cells was the highest because of the presence of ipsilaterally projecting cells in normal or (see below) increased numbers.

In another experiment we [61] induced retrograde degeneration of all ganglion cells in a peripheral sector of the retina of newborn rats, and subsequently studied the dendritic trees of cells located at the margins of this sector, following HRP labelling. The dendritic trees were found to be strongly biased towards the depleted area (Fig. 4).

To account for these results, we suggested that developing ganglion cells and, probably, other developing neurones are subject to dendritic competition: during development, the growing dendritic trees compete

to receive connections from their afferent supply; cells which fail to receive a certain set of such connections degenerate during the period when neuronal populations are shaped [46, 61].

Other possible explanations for our results will be critically examined in a later section. The experimental data, however, suggest that the survival of developing neurones during the period of natural cell death depends on each cell's success in forming both efferent connections through its axon terminals and afferent connections through its dendrites.

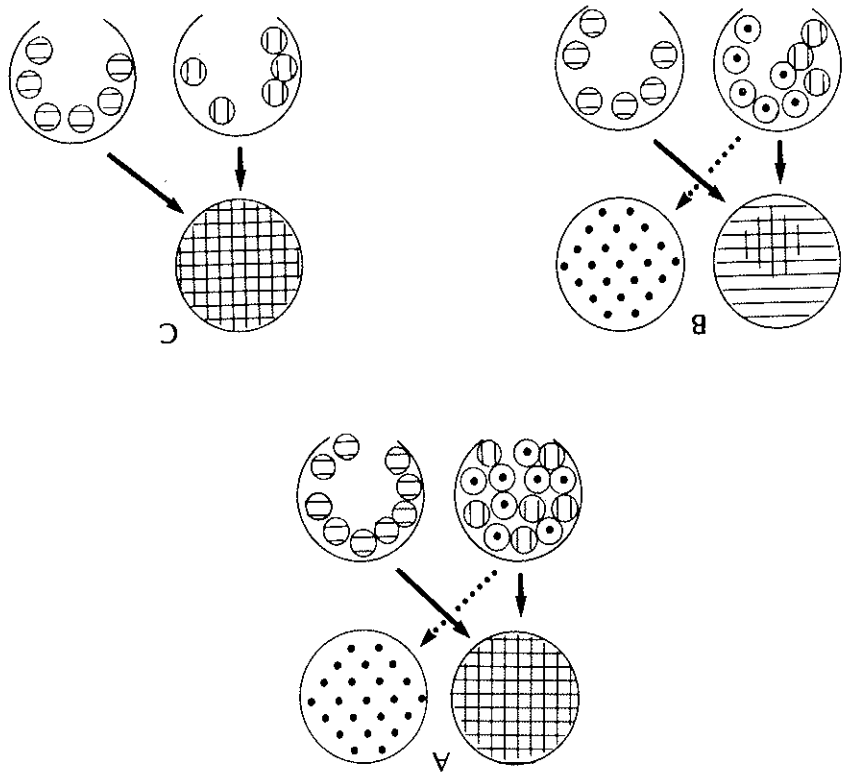

FIG. 3. Plasticity of the ipsilateral retinofugal pathway following early optic tract lesions. The same symbols apply as in Figure 2, plus the crossed projection from the eye at the left of the reader (dots and dotted arrow). (A) newborn rat. (B) normal adult with restricted ipsilateral pathway. (C) following a lesion to the optic tract, the dotted pathway is removed, and the ipsilateral pathway from the same retina is expanded; a larger number of parent ganglion cells project to the brain, leading to extensive overlap with the crossed projection from the other eye.

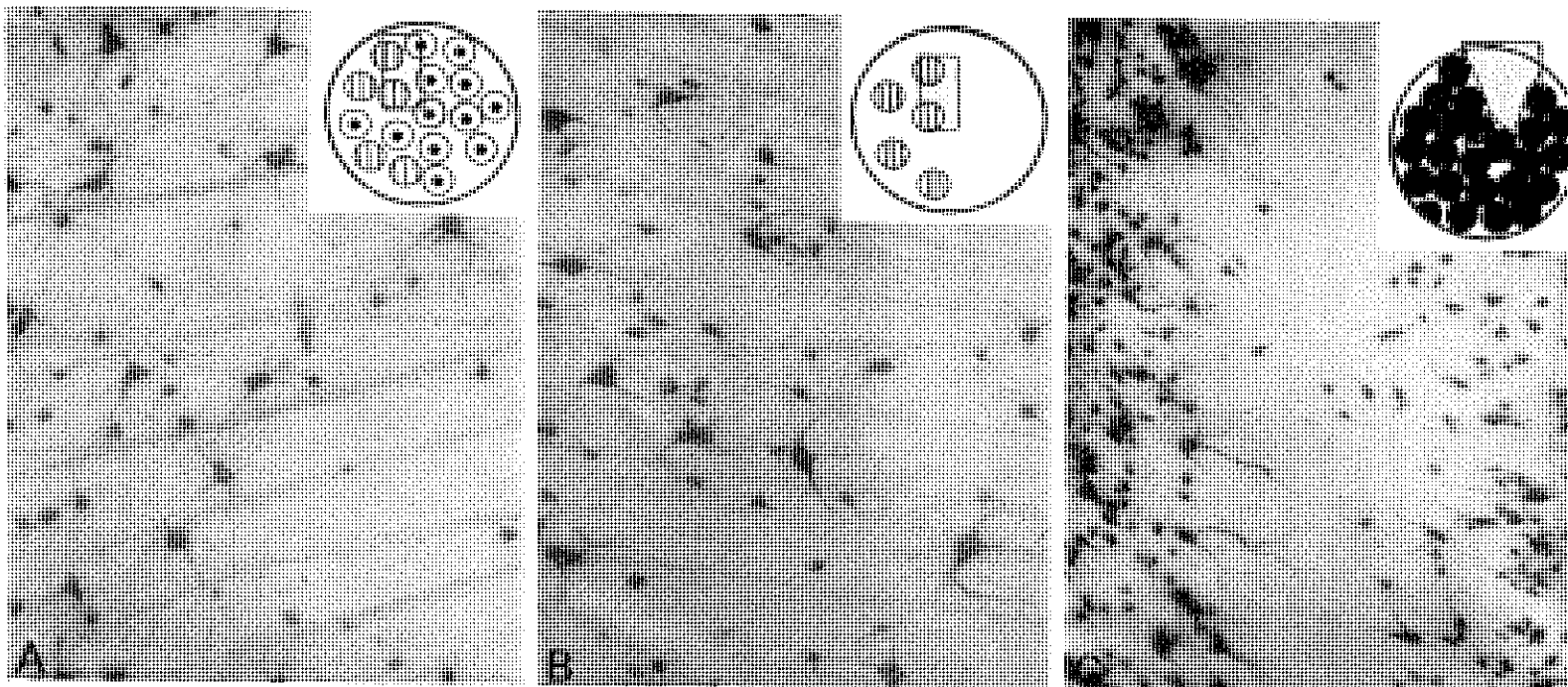

FIG. 4. Dendritic responses to an abnormally low density of neighbouring cells. (A) ganglion cells with ipsilateral axons labeled with HRP, located at the border between the temporal crescent (to the left) and the nasal retina of a normal adult rat; the diagram represents the location of the micrograph with respect to the distribution of cells with crossed or uncrossed projections, using the same symbols as in figure 3B. (B) same as A, for the retina contralateral to a lesion of the optic tract made at birth; the diagram is analogous to figure 3C; notice the dendrites pointing away from the temporal crescent, towards areas of the retina with the lowest density of ganglion cells. (C) ganglion cells with crossed projections labeled with HRP, in the retina of an adult rat given a small retinal lesion at birth; the diagram represents the retina following the retrograde degeneration of cells whose axons were transected; notice the dendrites pointing towards the area depleted of ganglion cells.

Differential Effects and Combination of Experimental Changes in Terminal and Dendritic Competition

Further studies of aberrant retinofugal projections were made in rats following either enucleation or lesion to the optic tract, or both operations simultaneously made at birth. The ganglion cells originating ipsilateral projections were labeled with retrogradely transported horseradish peroxidase [48]. Three aspects were examined: the number and distribution of ipsilaterally-projecting cells, the distribution of cell-body sizes in the population and the naso-temporal division, expressed as the gradient of cell density along the temporo-nasal axis of the retina.

Our results showed that either single or combined enucleation and optic tract lesions lead to a significant increase in the number of ganglion cells with ipsilateral axons in the retina opposite to the lesions, as compared with unoperated rats. The increase was significantly higher in rats with combined lesions than in rats with single optic tract damage. It was also higher, on average, after double lesions than after enucleation alone, but the difference was short of significance. When the increased cell numbers were analysed as a function of retinal topography (Fig. 5), it was found that enucleation had its main effect over the lower temporal areas of the retina, with a decreasing effect towards upper nasal retina. Optic tract lesions, in turn, led to the largest increases in an upper

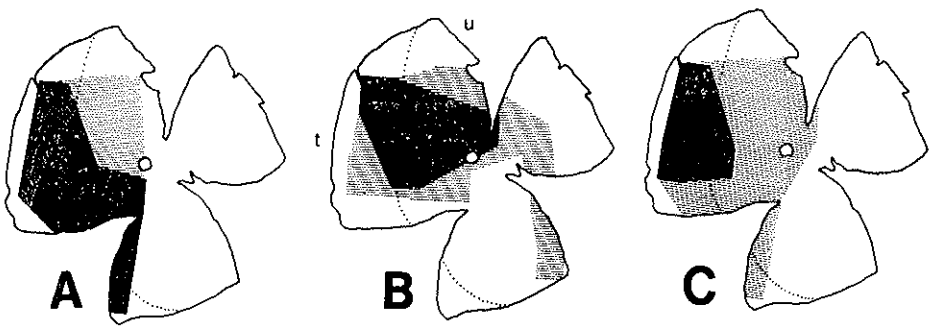

Fig. 5. Distribution of excess neurones rescued in the ipsilateral retinofugal pathway after neonatal surgery. The differences in the average density of labeled cells between experimental and control animals were ranked within each experimental group as high, medium or low. These are shown with dark-shading, light-shading and white in the diagrams, respectively. (A) enucleation, see Fig. 2C. (B) lesion of the optic tract, see Fig. 3C. (C) combined enucleation and optic tract lesion. Dots show the limits of the temporal crescent in this and in subsequent figures. u = upper; t = temporal.

temporal sector mainly outside the temporal crescent, extending nasally towards the optic disk. Smaller effects were found in the remainder of the retina. The distribution of excess cells was roughly similar to the total distribution of ganglion cells in the normal retina [21]. The results of combined lesions were consistent with a composite of a temporo-nasal gradient due to enucleation modulated by the upper temporal predominance of the effects of the optic tract lesion.

Analysis of the cell body size distributions indicated that the ipsilateral pathway of enucleated animals had a higher proportion of small ganglion cells than in normal rats. In contrast, lesions of the optic tract resulted in an increased proportion of large cells, when compared with the controls. The combined lesion produced a mixed effect, consistent with the topographic dependence described above: small cells were favoured among the additional neurones within the temporal crescent, whereas large cells were favoured among cells added in more nasal areas (Fig. 6).

Both the distribution and the cell body sizes of the excess neurones added to the population after enucleation were different from the results of optic tract damage. This supports the hypothesis that the nature of the events leading to the formation of aberrant connections is different in these two cases. The results after combined lesions suggest, furthermore, that the two processes concur to determine the outcome of simultaneous changes in terminal fields and within the retina itself. They failed, however, to discriminate between simple additivity and interaction of the two classes of events.

The distributions of soma sizes were rather homogeneous across different parts of the retina of the rats receiving combined lesions (Fig. 6). This suggests that the differential distributions of large and small cells in the ipsilateral projection from different areas of the retina of normal adult rats may be the outcome of combined terminal and dendritic competition having acted upon a homogeneous distribution in the newborn animal.

The naso-temporal division of the mammalian retina is characterized by a steep gradient of cell density in a median strip of tissue located between the temporal crescent and the nasal retina. Temporal to the median strip, the density of ipsilaterally-projecting ganglion cells is the highest and falls to very low levels in the nasal side of the strip. When the temporo-nasal gradient of cell density was examined in rats following either single or combined enucleation and optic tract lesion, it was found

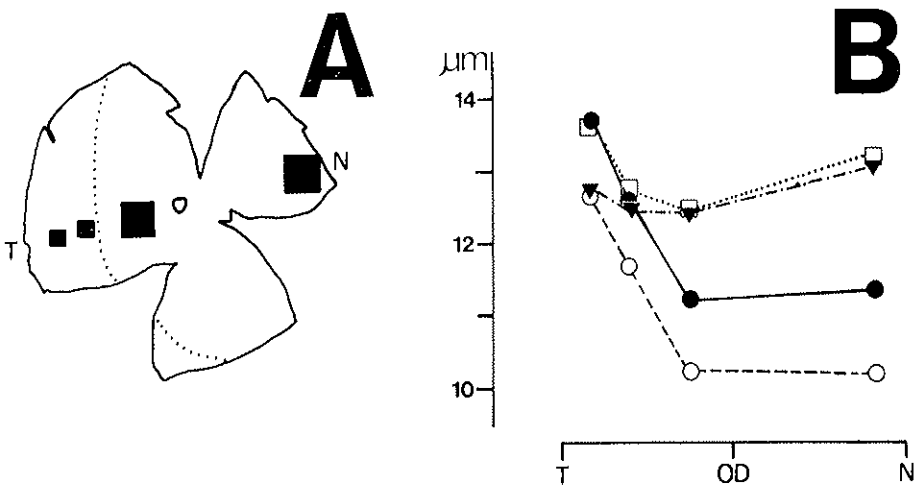

FIG. 6. Soma diameters of ganglion cells with ipsilateral projections at each one of four different locations along a line passing through the optic disk and through the middle of the temporal crescent. (A) the four locations sampled, shown with respect to the temporal crescent. (B) medians of the distributions of soma size as a function of eccentricity from the optic disk. Ordinates in micrometers, abscissae in degrees of visual angle. t = temporal, n = nasal to the optic disk. Filled circles = unoperated; open circles = enucleated; open squares = lesions to the optic tract; filled triangles = combined enucleation and lesion of the optic tract.

that the limits of the temporal crescent remained similar to that of unoperated rats, irrespective of the large and uneven changes of cell density observed in the retinae of operated animals (Fig. 7). The excess neurones added to the ipsilateral projection had, therefore, no effect upon the topographical features of this major landmark of retinal organization.

NATURAL, INDUCED AND REGULATIVE NEURONAL DEATH, AND ALTERNATIVE INTERPRETATIONS OF THE DEVELOPMENT OF ABERRANT RETINOFUGAL PROJECTIONS

The experimental approach based on the population of ganglion cells with ipsilateral axons has mostly been a matter of convenience. Monocular enucleation provides a simple and, nonetheless, highly effective manoeuvre to reduce the afferent supply of all contralateral visual centres; the ipsilateral pathway is, therefore, left with abundant terminal space to innervate, bereft of a major competing pathway. On the other hand, the

retina of non-primate mammals provides a simple model for the study of developmental processes related to cell proximity: cell density can be drastically reduced by the retrograde degeneration of contralaterally-projecting cells caused by optic tract damage, without interfering directly on the remaining ipsilateral population; dendritic tree development, in

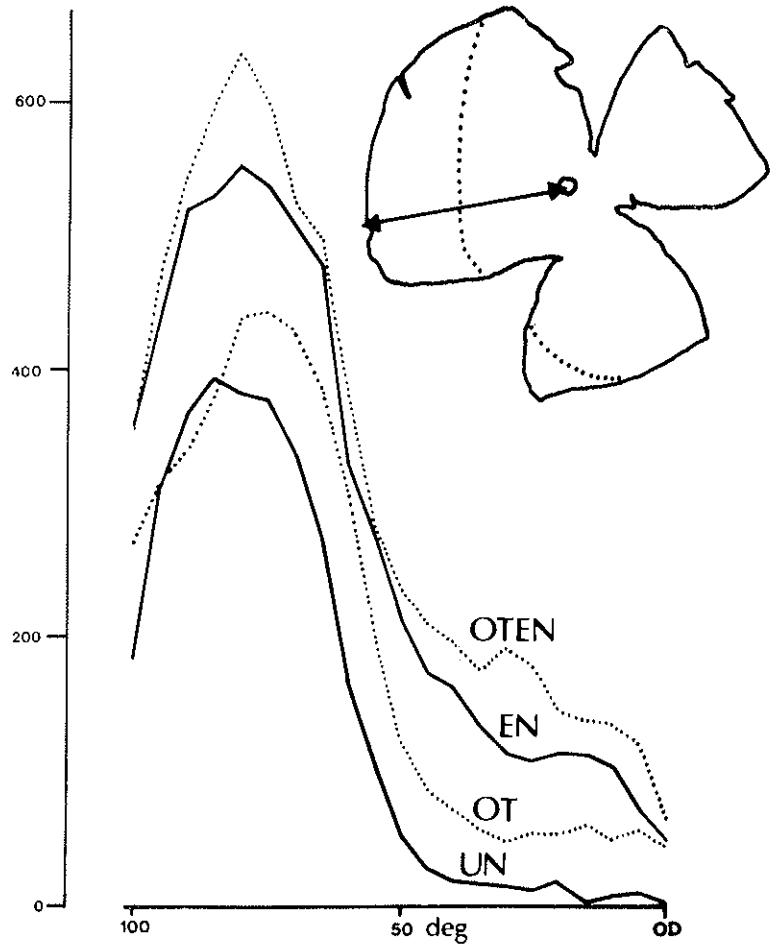

FIG. 7. Naso-temporal division. The graph shows the relative density of ganglion cells with ipsilateral axons along a temporo-nasal axis crossing the optic disk (inset). The figures are the numbers of cells per unit of 25 × 25 degrees. This is an area equivalent to 1 mm² in a whole-mounted retina of 4 mm radius. The horizontal axis shows the eccentricity in degrees temporal to the optic disk. UN = unoperated, EN = enucleated, OT = lesion to the optic tract, OTEN = combined lesion.

turn, occurs basically in the two dimensions that can be analysed directly in whole-mounted retinae.

The results reviewed thus far suggest the following explanation for the control of cell death in the developing retina: during normal development, ganglion cells compete both to form efferent connections and to receive afferent supply; cells overwhelmed by their competitors in either circumstance are bound to degenerate; small cells are particularly sensitive to competition at the terminal-axonic level, while large cells are particularly sensitive to competition at the level of the dendrites; dendritic competition *per se* is more effective for cell elimination in central retina than in peripheral retina; terminal-axonic competition, in turn, is mostly effective in the lower temporal periphery.

There are, however, a number of other hypotheses to account for the formation of aberrant projections, which merit consideration at this point.

An early hypothesis to explain the expansion of ipsilateral projections to visual centres following enucleation was the reactive sprouting of ipsilateral terminals [49]. The demonstration of increased numbers of cells originating this projection, of course, means that sprouting cannot, *per se*, account for the aberrant pathways. It does not, however, preclude that terminals unchecked by competition might well grow and/or arborize more intensively than those subject to competitive interactions with the crossed projection. This behaviour of growing terminals might indeed be expected, but quantitative studies of single axonal arborizations within the visual centres of normal and enucleated animals will be required to assay the contribution of reactive sprouting to the generation of aberrant terminal fields.

Other hypotheses raised to explain the results of enucleation would take into account the increased numhers of cells in the ipsilateral pathway. Studies with both the cobalt filling method and electrophysiological techniques suggested that increased bilateral collateralization occurred as a result of early monocular enucleation [13]. While tbis was later confirmed with the use of double retrograde tracing techniques, it was also shown that the increase in bilaterally projecting axons comprises a very small proportion of the additional cells with ipsilateral axons [38].

It has also been suggested that increased numbers of cells with ipsilateral axons might result from reorientation of either former crossed axons, or late developing axons that would normally cross the midline. Given the new conditions prevailing at tbe level of the optic chiasm after

enucleation, such axons would change their course towards the ipsilateral optic tract [22]. This possibility cannot he completely excluded at present, hut for it to account for the aberrant projections as a whole, it would have to deal with the notable fact that, following enucleation, the total numher of neurones is significantly increased in the remaining eye [4, 71], which strongly supports the hypothesis of reduced cell death.

Alternative hypotheses have also been raised to account for aberrant uncrossed projections following early damage to the contralateral optic tract. Jen and Lund [35] suggested that the expanded retinotectal terminal fields following contralateral hemi-thalamectomy in the newborn rat resulted from reactive sprouting of axons with bilateral projections that had suffered unilateral damage. They based their proposition on a complex series of axon and cell counts that seemed to indicate no change in the population of ipsilaterally projecting cells. This result was, however, not confirmed [46, 48]. Indeed, reactive sprouting either at the level of the optic chiasm or analogous to the "pruning effect" as originally suggested by Schneider [68] and supported hy Jen and Lund [35] has not heen convincingly demonstrated and appears to be quite an unlikely possibility, given the well-documented sensitivity of young neurones to induced cell death following damage to their axons [8, 43].

One hypothesis that would take into account the increased number of ipsilaterally-projecting cells is again the re-orientation of axons at the optic chiasm [72]. It might be argued that late arriving axons, which would he spared by the lesion, might take a course different from their original tendency, given either the presence of degeneration products or the lack of terminal space along their original course.

An experiment in the peripheral nervous system has failed to show evidence for guidance of axons hy degeneration products [84]. Furthermore, should this be the cause of aberrant projections, it might occur in such a way as to allow orientation of developing optic axons *towards* the main mass of degeneration following enucleation, and *away* from it in case of optic tract lesions. If, in turn, reorientation of late arriving axons were the source of additional cells labeled in the ipsilateral pathway, one would expect those to lie mainly in the retinal periphery [51, 67]. Our results, in contrast, revealed a bias towards central retina [48].

Therefore, even though the alternative hypotheses raised above cannot be completely excluded, they seem to require a more complex series of events to account for abnormal retinal development than the

hypothesis of regulated neuronal death. Further studies should be directed toward testing more directly each one of those possibilities.

A further problem concerns the conclusion that terminal and dendritic competition have predominant effects over different cell types, as judged by the cell body size of additional cells found in the aberrant pathways [48]. Lund and co-workers [50] studied cells which project to the ipsilateral superior colliculus and the results showed a general increase in cell body size following enucleation, when compared with unoperated rats. The results are opposite to our findings in the whole ipsilateral population. Unfortunately, they reported having measured cells in normal and enucleated rats matched hy *radial* position, and not necessarily in the same location within the temporal crescent, which was only partially labelled.

It was also reported [22] that the ipsilateral retinogeniculate projection of enucleated albino rats contains an increased number of large cells, when compared with unoperated controls. These data, however, were again obtained from partially-filled temporal crescents. Neither the injections nor the sampling areas were matched between operated and control cases.

In a third study, employing pigmented rats, Jeffery [37] reported an increase in the soma sizes of cells located in the temporal crescent, after neonatal enucleation. The data were obtained from the retinae of two normal and two enucleated rats sampled randomly across the crescent. It should be noted that small samples taken at random tend to lead to larger errors than systematic sampling [83].

The distribution of cell body sizes is not homogeneous among the ipsilaterally-projecting cells of the temporal crescent, in either normal or enucleated rats. There is a normal gradient of cell body sizes from the centre of the crescent towards its nasal border. Also, the upper portions of the crescent contain an obviously larger number of large cells than the lowet parts of the crescent. It is unknown to what extent this may have influenced the results reviewed above. The presence of non-homogeneities in the temporal crescent should, however, be viewed as a major caveat to those studying the experimental changes upon cell body sizes in the ipsilateral pathway.

It might be argued that changes at the level of terminal arbours or of dendritic trees would alter the size of the parent cell body, thus leading to tbe observed changes in soma size distributions. This possibility is difficult to evaluate in the retina, given the existence of various types of

retinal ganglion cells with different, albeit partially overlapping ranges of soma size.

The retrograde filling of cells with HRP in the experiments of Linden and Serfaty [48] did not allow unequivocal identification of cell types, but partially-filled large cells found in the nasal retina following optic tract lesions strongly resemble the alpha-like type I neurones described by Perry [57]. With regard to the ipsilaterally-projecting cells of enucleated rats, it is also hardly to be expected that cells whose axons have been freed of a major competing pathway would undergo shrinkage of their soma. Although the superior colliculus and lateral geniculate nucleus do shrink after contralateral enucleation [36, 76], the absolute size of the aberrant projection field is larger than in unoperated animals, and the individual terminal arbours would hardly be expected to shrink.

It appears, therefore, that although terminal-bound changes in cell body size cannot be disproved at present, the hypothesis of predominant rescue of small cells by reduced terminal competition and of large cells by reduced dendritic competition remains the simplest explanation for the experimental results.

Roles of Cell death in Retinal Development

The following analysis will be made on the basis of three assumptions. First, it will be assumed that the behaviour of the ipsilateral projection after changes either at terminal fields or in the neighbourhood of the parent cell bodies is representative of what happens to any group of retinal ganglion cells in analogous circumstances (e.g., the behaviour of a number of crossed projecting cells following removal of neighbouring cells also with crossed axons). Second, it will be assumed that the competitive interactions discussed in the previous sections occur normally, that is, they are not artifactually imposed by the rather drastic experimental conditions. Third, it will be assumed that the major principles of regulation of neuronal death are similar among different areas of the brain and among homologous neurone types of different species.

The assumptions appear reasonable enough to warrant speculation on the roles natural cell death controlled by both terminal and dendritic competition may play upon the development of retinal ganglion cell populations.

The important demonstration by Clarke and Cowan [7], that neurones of the isthmo-optic nucleus located ectopically or bearing axons that

projected aberrantly died naturally during development, led to the concept of neuronal death as an error-correcting mechanism. This process might eliminate inappropriate connections formed during the course of normal development and, thus, sharpen the patterns of connectivity between areas developing separately [5]. This idea was challenged by Oppenheim [53], on the basis that the proportion of aberrant neurones among those which degenerate naturally in the isthmo-optic nucleus is very low (about 5%). Further studies [54] showed that the prevention of motoneurone cell death in chicks did not lead to detectable errors in the relation between muscle-specific motoneurone pools and their innervated targets. The author concluded that any inappropriateness maintained in this system would be subtle, the major pattern being reached as in normal development [54].

Our analysis of the naso-temporal division in the retina [48] showed that a sharp gradient of density of cells projecting ipsilaterally is maintained between temporal and nasal retina irrespective of the increase in cell numbers attributed to diminished cell death. This implies that neuronal death is not required to establish this major pattern of retinal organization. Either the pattern is already present at birth in the rat, or the trend towards building up the sharp gradient of density is irreversibly determined, independently of the final number of cells. Even the cell addition that follows optic tract lesions, which is maximal in central retina abutting or even straddling the borders of the temporal crescent, had but a very slight effect on the organization of the median strip. This occurred, in fact, when the gradient was measured across the middle of the median strip's dorso-ventral extent; both in upper and, especially, in lower retina a sharp dividing line was left unchanged.

It appears, therefore, that the distribution of cells of the ipsilateral projection, with its characteristic concentration in temporal retina is not significantly sculptured by cell death from an original more homogeneous distribution of cells. The key to development of the naso-temporal division must be searched among the mechanisms leading to the initial ordering of axons at the optic chiasm.

What roles would be, then, left to natural cell death among developing ganglion cells? Many authors have dedicated part of their discussions to this issue, with regard to various populations of nerve cells [5, 9, 12, 53, 63]. It is usually agreed upon that natural cell death is a rather convenient event to allow the quantitative matching of interconnecting cell populations developing separately, through the elimination of excess

neurones in either or both cell groups. With regard to the retina, given the occurrence of cell death in the central targets [23, 25], and the evidence that other retinal neurones die naturally besides ganglion cells [86], one may think of a cascade of natural degeneration leading to the matching of cell populations along a series of connecting areas [60].

While the idea of systems-matching by degeneration [63] has yet to be more extensively quantified, it is consistent with results of experiments dealing with changes in either terminal fields or afferent supplies. The control of cell death by both terminal and dendritic competition might, in fact, ensure that the surviving neurones would be those endowed with either profuse or effective, if not appropriate, afferent and efferent connections [12].

Other features of retinal organization may, however, be dependent on natural cell death. It has been suggested [67] that selective neuronal death at the retinal periphery might play a dominant role on the generation of the gradient of ganglion cell density centred on the area centralis. In the hamster, indeed, the overall rate of cell death is higher in the periphery than in areas of the retina close to the optic disk [70].

The predominance of cell death among neurones located away from the area centralis may be a trivial consequence of the fact that cells of the area centralis are the earliest to be generated [67] and, therefore, likely to be the winners of competition for terminal space.

A recent study [78] of neurogenesis in the cat retina showed that ganglion cells are generated in sequence along a rough spiral from upper nasal to lower temporal retina, pivoted on the area centralis and proceeding away from it. The various types of ganglion cells are produced in temporally distinct, albeit partially overlapping, waves of cell generation. The data also indicated that the latest cells to be born are small [78, 79].

Cell rescue following enucleation in the rat favoured the lower temporal periphery over the remaining retina, and a larger proportion of small cells were rescued than are normally present in the ipsilateral population [48]. These data are consistent with the hypothesis that late-developing ganglion cells suffer a relative disadvantage in the competition for central synaptic sites.

The principle of dendritic competition and, especially, the dependence of retinal plasticity upon topography and cell type following changes either in terminal fields or within the retina [48] add new dimensions to the idea of pattern-generating cell death. It has been shown in cats that the large alpha cells form in the area centralis a smaller proportion of the

total population of ganglion cells than in the periphery [30, 56, 80]; that is, superimposed upon the impressive centro-peripheral gradient of density shared by all cell types, the alpha cells comprise about 2% of all ganglion cells around the area centralis, whereas this proportion rises to 3-5% in the periphery [30, 80]. Optic tract lesions were required to rescue large cells of the central retina in rats [48]. It may be suggested that the pattern of variable *relative* proportions of different ganglion cell types along the centro-peripheral axis of the retina is a consequence of dendritic competition being more damaging to central alpha-like cells than to other cell types.

Circumstantial evidence in favour of the idea of higher sensitivity among alpha cells to dendritic competition stems from the demonstration of minimal dendritic overlap among these cells when separately classified as on- or off-alphas [81], whereas the overlap is larger between adjacent dendritic trees of beta cells [82]. Further, given the high density and large dendritic trees of the remaining cell types [2, 30, 80], it is likely that adjacent cells of these types also have a high degree of dendritic overlap.

We have also suggested that a process of neuronal death controlled by dendritic competition may be involved in the development of retinal mosaics [46]. The rationale for this suggestion was that if, at any moment, a given cell with its dendritic tree overlapped by dendrites of many other cells had a lower chance of surviving than a cell free of dendritic overlap, then regularly spaced cells should, as a rule, have the highest chance of surviving the period of cell death (Fig. 8).

This hypothesis, of course, does not exclude that the growth of a cell's dendrites may be prevented by the presence of abutting dendrites from other cells [81]. Retinal mosaics, however, have as a major feature the regular spacing of cell bodies of each given type [82]. Both mechanisms mentioned above may be, therefore, operating simultaneously during development.

Little can be added, at present, about the presumptive cooperative action of terminal-axonic and dendritic competition upon normal retinal development. Data on the temporal relationships between natural cell death and cell death induced by the removal of either targets or afferents are available only for the spinal cord [52, 53] and for the isthmo-optic nucleus [6, 7]. In both, removal of afferents produced its major effect during the late part of the period of natural cell death, whereas removal of targets had an effect along the whole period of natural degeneration.

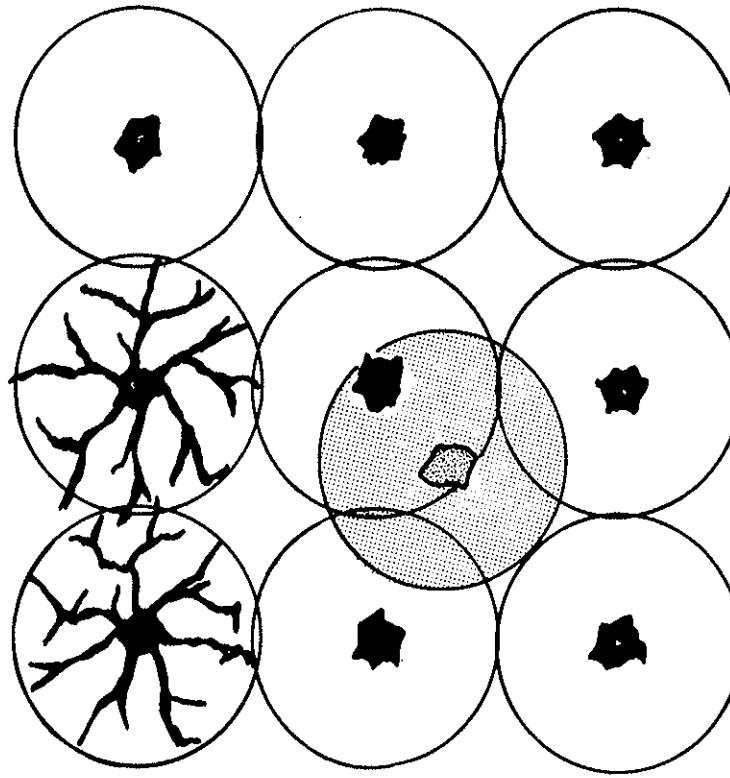

FIG. 8. Development of retinal mosaics through dendritic competition. The diagram represents a hypothetical array of 10 ganglion cells. Nine of these cells are distributed regularly (black profiles) and the remaining cell (mottled profile) is grossly displaced. The circles delimit a standard dendritic tree attributed to each cell at a given moment of development. This is shown for two profiles only. The dendrites of the mottled profile (shaded circle) are severely overlapped by the dendrites of neighbouring cells. The probability of this neurone contacting underlying afferents from a limited supply should therefore be smaller than that of the nine black cells. As an alternative model, if the abutment of dendrites from different cells should prevent further growth, then a misaligned cell like the mottled profile would again have a reduced chance of receiving afferent connections. In either case, the principle of dendritic competition would predict that the chances of a cell's surviving the period of developmental neuronal death is increased among regularly spaced neurones.

The assumption of analogous time courses for the dependence of developing ganglion cells on targets and on afferents would allow a simple hypothesis to relate the experimental findings reviewed above to the gradients of cell density recognized in the retina. The hypothetical course of events would be as follows (Fig. 9): In the early period of cell death, the predominant loss of late-developing peripheral cells creates a gradient of cell density with a peak in the area centralis. Later, when cells become sensitive to the supporting role of their afferents, dendritic competition begins to operate. The crowding in the central retina leads to more cells dying in central than in peripheral retina because of dendritic competition, while terminal-axonic competition continues to operate with predominant effects in the periphery. The bias of each form of competition towards different cell types leads to further adjustments of the relative proportions of the various cell types, or at least of the large alpha-like cells. At the same time, the regular spacing of the retinal mosaics is tuned by dendritic competition (Fig. 9).

Conclusions

The data reviewed in this paper show that natural neuronal death is a major event in the development of populations of retinal ganglion cells in mammals. The experiments in the retina of rodents support the hypothesis that cell death is controlled by concurrent terminal-axonic and dendritic competitive interactions. The naso-temporal division is not primarily sculptured by selective regression, but the experimental evidence is consistent with other possible roles for the degeneration of large numbers of ganglion cells. These are related to differences between the organization of central and peripheral retina, and to coverage of the visual field representation with dendritic trees of different cell types, presumably endowed with distinct functional attributes. Furthermore, any pattern-generating roles of cell death would be superimposed on a more general effect of adjusting the numbers of neurones in interconnecting populations. The assumptions were kept to a minimum but, while necessary to the formulation of our theories, many of them remain to be verified. Time is probably ripe for exploration of these principles of regulation of neuronal death in retinae like that of the cat or monkey, in which the centro-peripheral gradient of organization, the sharpness of the naso-temporal division and the separation of ganglion cell classes are more marked than in the poorly differentiated rodent retina.

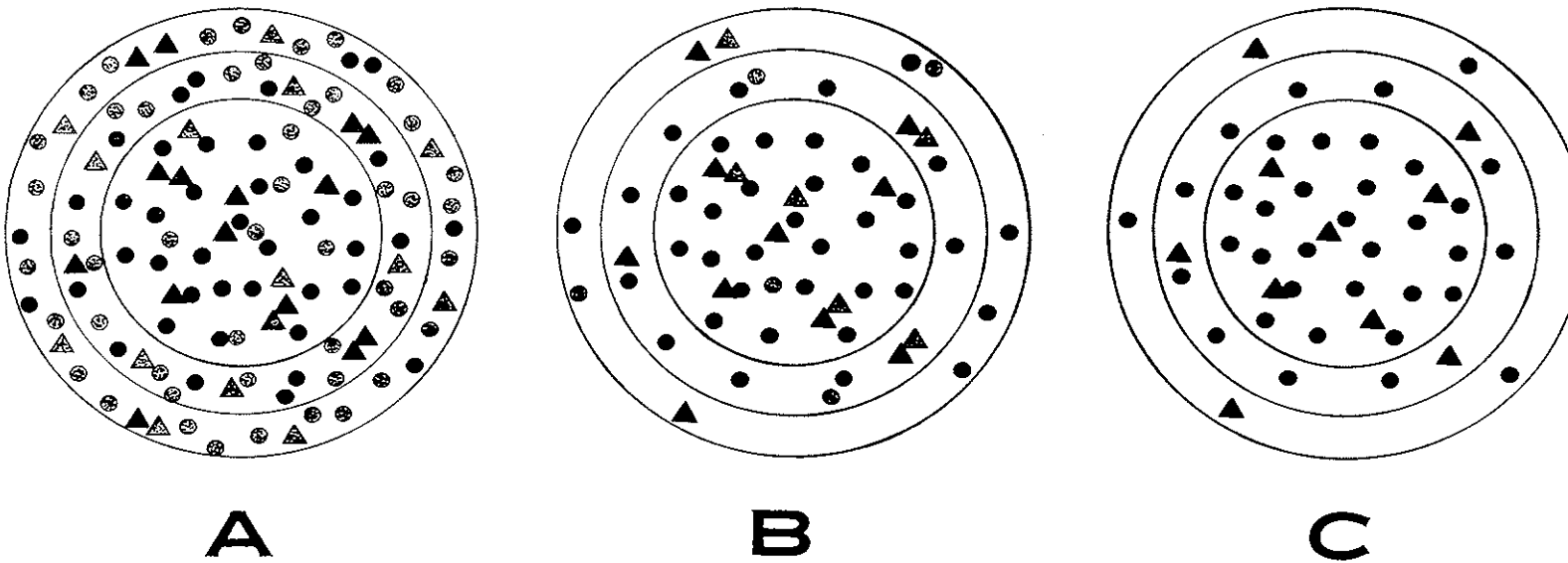

FIG. 9. A simple model of pattern-generating cell death in the retina. Three successive stages of development are represented in A-C. Central, intermediate and peripheral sectors of the retina are suggested for each stage by the equal-area concentric regions. Triangles represent alpha cells, small circles depict other types of ganglion cells. Growth of the retina was ignored for the sake of simplicity. (A) Early stage of development. Cell density (black plus mottled profiles) is homogeneous across the retina; the percentage of alphas is also constant (25%). Cell death is more intense in the periphery (mottled profiles) due to terminal-axonic competition, and particularly damaging to small cells. This leads to a centro-peripheral gradient of cell density. (B) Intermediate stage of development. Dendritic competition operates simultaneously with terminal-axonic competition. Cell death (mottled symbols) still occurs across the whole retina, but now alpha cells of central retina are particularly sensitive to dendritic competition, while terminal-axonic competition takes predominantly small peripheral cells as in A. Dendritic competition, furthermore, allows the survival of regularly spaced cells. (C) Late stages of development. The topographic and type-dependent schedules of cell death generate a retina with a centro-peripheral gradient of overall cell density (approximately 6:1), with both alphas and non-alphas most concentrated in the central retina. The percentage of alphas in central retina is, however, lower than in peripheral retina (18% and 40% respectively). The cell numbers and the percentages of each type are merely illustrative, and not intended to represent a real retina.

On the other hand, the principles of terminal and dendritic competition are hut useful tools to understand in very broad terms the regressive events of neuronal populations. The use of other techniques, hoth *in vivo* and *in vitro*, to explore the mammalian retina is needed in order to extend our knowledge to the actual mechanisms involved in the regulation of natural neuronal death, given their significance to retinal development.

ACKNOWLEDGEMENTS

The author's research reported in this review was supported by grants from CNPq, FINEP, CEPG-UFRJ. The critical comments of my colleagues C.E. Rocha-Miranda, L.A. Cavalcante and C.A. Serfaty are gratefully acknowledged.

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TROPHIC INTERACTIONS AT SYNAPSES AND THE SURVIVAL OF CENTRAL NEURONES

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INTRODUCTION

This review attempts to identify trophic phenomena at synapses which contribute to the survival of central neurones during development. In order to isolate trophic effects which operate at sites of interaction between a neurone and its target cell it is necessary to consider the trophic contributions made by other cells in the environment; these are also detailed. The two central neurones chosen for study are retinal ganglion cells and motoneurones, as they are uniquely accessible for experimental analysis. It is shown that interesting parallels exist in the role and source of trophic factors for the development of these neurones.

RETINAL GANGLION CELLS

The first section below is concerned with describing experiments on the developing visual system which define trophic interactions that may occur between ganglion cells and cells in their immediate environment. This forms a necessary prerequisite for the design of "in vitro" experiments to test for the existence and isolation of trophic factors in ganglion cell development; these experiments are described in the second section.

GANGLION CELL SYNAPSES AND CELL SURVIVAL

Time course of ganglion cell death during development

The death of retinal ganglion cells during the normal development of vertebrates was first indicated by the observations of Rager and Rager [241] they showed that the number of axons in the optic nerve of chicks decreases by 40% between embryonic day 10 (E10) and embryonic day 18 (E18) of incubation. This loss of axons is accompanied by a greatly increased number of pycnotic cells in the ganglion cell layer of the retina between E11 and E15 [125, 240]. Although this large loss of nerve fibres might indicate a similar loss of ganglion cells, other explanations are possible. For example, McLoon and Lund [191] have shown that ganglion cells send large numbers of nerve fibres into tbe contralateral optic nerve in chick embryos between six and fifteen days of incubation; the disappearance of these erroneous fibres may account for the loss of axons from the optic nerve. Similarly, transient projections into contralateral optic nerve were observed by Bunt and Lund [50] during postnatal development of the rat visual system. Furthermore, Langford and Coggeshall [155] have shown that sensory neurones of the rat dorsal root ganglia send on average two nerve fibers into the spinal cord; it is possible that early developing ganglion cells also send more than one process into the optic nerve, and only one remains to mature. It has also been suggested that developing amacrine cells originally send processes into the optic nerve which are then withdrawn during later development.

The loss of nerve fibers in the developing optic nerve of mammals (Fig. 1a) [143, 200, 228, 248] is accompanied by the appearance of pycnotic cells in the ganglion cell layer [72, 271, 272] and the loss of cresyl violet-stained cells in the ganglion cell layer. A quantitative comparison has been made of the numbers of cells which send processes out the optic nerve to the retino-recipient centres of the brain with the number of nerve fibres in the optic nerve [143, 228]. This has enabled a determination of whether a large number of ganglion cells is lost during development. There are over twice as many axons in the optic nerve of the rat at birth as at six days postnatal, when the mature value is reached [143, 228]. Enucleation of one eye at birth, in order to remove the transient retinotectal projection, only reduces the excess of axons by about one-third. It follows that the excess of optic axons in the

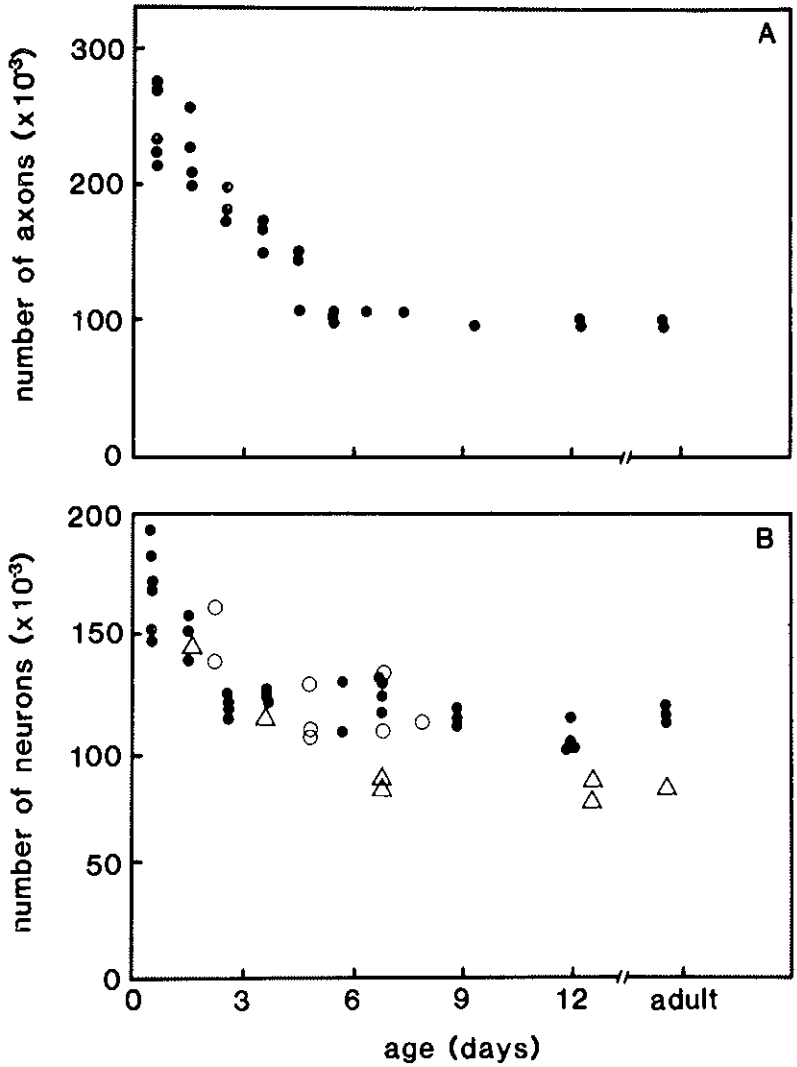

FIG. 1. Death of retinal ganglion cells during development.

A. graph of the number of fibers in the optic nerve (ordinate) of rats of different ages (abscissa). Each symbol represents the value obtained from a single animal (from [143]).

B. graph of the number of HRP-labelled cells (ordinate) in the retinae of rats of different ages (abscissa). The ages indicated on the abscissa refer to the ages of the animals at the time of HRP injection. Filled circles, normal animals; open triangles, animals with a contralateral tectal lesion at birth; open circles, animals injected with HRP at birth but perfused at the times indicated in the abscissa. (From [77, 235]).

neonate is not simply due to the presence of retino-retinal axons. Furthermore, this excess of axons is not due to ganglion cells sending more than one axon out the optic nerve: injection of horseradish peroxidase (HRP) into the visual centres of the brain of neonates labels the same number of retinal neurones as there are axons in the optic nerves of animals with one eye enucleated at birth [235] (Fig. 1B). The excess of axons is also not due to developing amacrine cells transiently sending neurites out the optic nerve: neurones with processes in the optic nerve at birth may be labelled with HRP, and the number of HRP-labelled neurones present in the retina on the sixth day determined; this is the same as the number of neurones labelled witb HRP on the sixth day and counted on the sixth day. If developing amacrine cells were to transiently send axons to the brain, then the number of HRP-labelled cells present on the sixth day but labelled on the first day should be elevated compared with the number present on the sixth day that are labelled on the sixth day; this does not occur [235]. Thus a substantial loss of ganglion cells occurs during normal development of the mammalian retina [77, 228]. No such loss has been reported during normal development of amphibian retina [318], although it occurs if the axons are severed.

The time course of cell death in the retino-recipient nuclei

Neurones also degenerate in the retino-recipient nuclei during normal development. This occurs in the chick tectum between the tenth and eleventh day of incubation, when the rate of loss of retinal ganglion cells is a maximum [55, 241]. Neurones degenerate in both the developing lateral geniculate nucleus [71, 113] and the superior colliculus [7, 72, 92, 95] (Fig. 2) of rodents and primates. In both rat and hamster, cellular degeneration in the superficial layers of the superior colliculus has the same time course as the degeneration of the retinal ganglion cells which provide principal input to the superficial collicular layers [92, 235, 272]. Furthermore, the degeneration of cells in the retina and superior colliculus follows similar spatial gradients related to the topographical relationship between these two structures: there is a greater rate of degeneration of cells in the nasal part of the ganglion cell layer and the caudal part of the superior colliculus than in the temporal part of the ganglion cell layer and the rostral colliculus [72, 95].

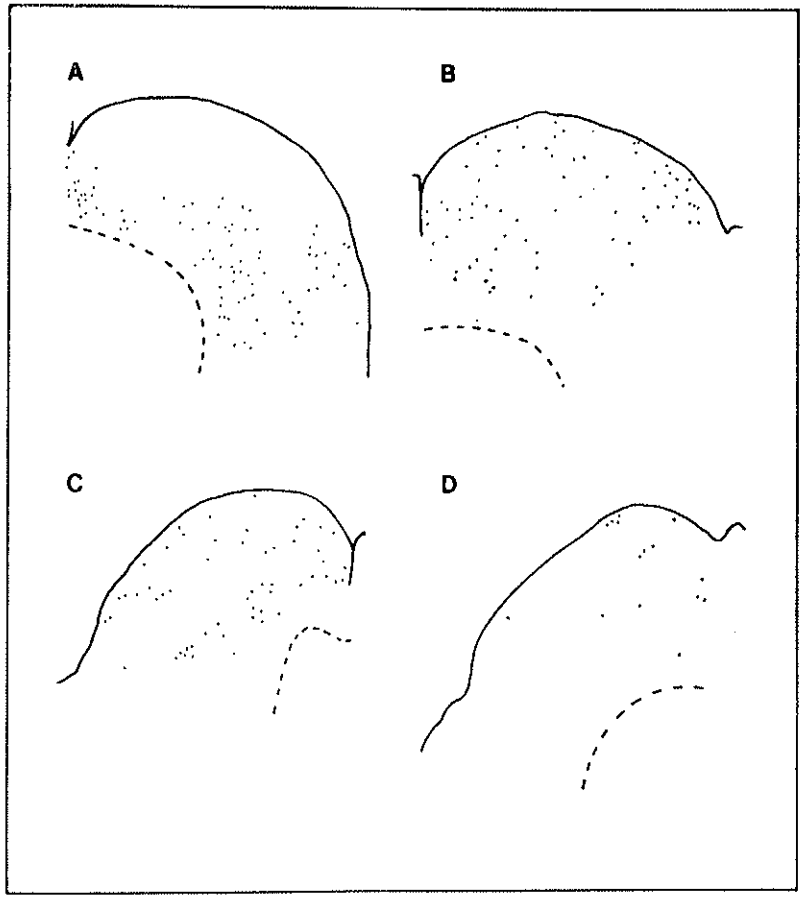

FIG. 2. Death of neurones in the superior colliculus during development. Tracing of micrographs at various levels of the tectum of rats of various ages. A, birth; B, 4 days; C, 7 days; D, 11 days. Each dot represents one degenerating neurone. (From [7]).

Evidence that ganglion cell survival is dependent on the formation of stable synapses

Cell survival that is independent of synapse formation

Explants of fetal mouse and rat retinae taken at 14 d gestation grow in culture with only minimal media for up to two weeks [194, 284]. Both cell division and neuronal differentiation proceed normally: the explants send out neurites for two weeks after which time they disintegrate [284]. During this two-week period the retinal explants retain

their full potentiality for forming appropriate synaptic connections in the brain. If the explants, after 14 d in culture, are transplanted adjacent to the superior colliculus in newborn rats, only cells in the ganglion cell layer form connections with the host's superior colliculus [194]. The survival of fetal retinal ganglion cells in culture may be related to the sustaining properties of the intrinsic glial cells of the retina (Müller cells).

Cell survival that is dependent on synapse formation

Evidence that ganglion cell survival depends on the retino-recipient nuclei. Retinal ganglion cells survive for months in mature rats if the retina is isolated from the retino-recipient nuclei by either removing the nuclei [227] or by crushing the optic nerve [169, 197]. Eventually, the ganglion cells degenerate, so that between the third and seventh post-operative month over 60% of the neurones in the ganglion cell layer are lost. In contrast, as detailed below, isolation of the retina from the retino-recipient nuclei in embryos leads to loss of ganglion cells within a few days.

Destruction of the primordial optic tectum in chicks at E4 results in degeneration of about 72% of the ganglion cells, compared with the 20% loss which normally occurs [124, 125]. The enhanced death of the ganglion cells proceeds in a temporal to nasal progression, just as it does in the presence of normal tectum (but see Rager [240]). As the axons of retinal ganglion cells have not reached the optic tectum by E4, the subsequent loss of cells cannot be attributed to a chromatolytic response, consequent on cutting axons. In rat, removal of the superior colliculus at 1 d postnatal produces a significant increase in the number of degenerating ganglion cells [227, 235]. In carnivores and primates, lesions to the striate cortex at birth lead to a loss of geniculate cells and subsequently of ganglion cells [226, 313]. It is clear that ganglion cells depend on the retino-recipient nuclei for their survival during development in both birds and mammals.

A comparison of the time schedules of neurogenesis for the superficial layers in the mammalian superior colliculus and of the development of optic fibres suggests that migration of neurones to their eventual destination is completed at or after the arrival of afferents [58]. The subsequent survival of many of these neurones is dependent on their receiving innervation from the optic axons. Enucleation of one eye during the early developmental period enhances the naturally occurring

rate of cell death in the retino-recipient nuclei. In chick, retinal ablation at 11 d incubation increases degeneration in the optic tectum [223]. Enucleation of an eye in newborn kittens leads to an increased loss of neurones in the lateral geniculate nucleus [99]. However, enucleation of an eye in primate fetuses at two to three months before birth, although severely disrupting the formation of cellular layers in the lateral geniculate nucleus, does not significantly diminish the number of surviving neurones [246] (Fig. 3C). Survival of the lateral geniculate neurones might be due to the fact that enucleation at such early stages results in the innervation of all lateral geniculate neurones by the fibers from the remaining eye. These observations indicate that the development of the retino-recipient nuclei is to some extent dependent on their receiving an afferent innervation from the retina. This is supported by the observation that cell death in the superior colliculus is reduced if it is innervated by an excess of retinal axons [71].

Evidence that ganglion cell survival depends on the formation of appropriate topographical connections in retino-recipient nuclei. A topographical projection of each retina onto the contralateral superior colliculus exists for vertebrates: temporal retina projects onto the anterior pole of the colliculus whereas nasal retina projects onto the posterior pole. In addition, a small uncrossed projection originating from the ipsilateral lower temporal retina to the anterior colliculus exists in birds and rodents. There is a significantly larger ipsilateral projection during development of the chick retina than that found in the post-hatched chick [216, 297], nearly all these ipsilateral projections are eliminated during the ganglion cell death period in the chick between E10 and E17 of incubation [216]. In the neonatal rat, the ipsilateral projection is not confined to the antero-medial pole of the superior colliculus, as it is in the adult (Fig. 3A): neurones located in the lower temporal crescent send processes throughout the entire extent of the superficial layers of the superior colliculus [177, 216]. The ipsilateral projection to the posterior pole of the colliculus disappears during the first few postnatal days; this is due to cell death of the misprojecting neurones [216]. These neurones do not form synaptic connections before they degenerate [132].

Axons from the contralateral eye of early postnatal hamsters are distributed throughout the entire lateral geniculate nucleus (Fig. 3B; [287]). This contralateral projection is eventually eliminated from the area receiving ipsilateral input. During early gestation of the macaque, retinal projections from the two eyes overlap in the dorsal lateral geniculate

nucleus before sorting out into six alternating layers during the second half of gestation [247]. It is not known if the overlapping distribution of ipsilateral and contralateral projections appears as a consequence of the death of misprojecting neurones, the withdrawal of collateral sprouts or both. The situation is complicated in these primates: the six horse-shoe-shaped layers of cells which comprise the mature lateral geniculate nucleus do not emerge until about the time at which the appropriate projections from each eye are established [247].

Evidence that ganglion cell survival involves competition in the establishment of topographical connections. The question arises as to

FIG. 3. Competition between retinal ganglion cells for efferent connections. Inappropriate projections from retina to retino-recipient nuclei and the effects of an eye enucleation. A. Postnatal development of retinal projections to the rat superior colliculus. Transverse sections through superior colliculi ipsilateral to intraocular HRP injections. Sections were stained with TMB and H_2O_2 to demonstrate the presence of anterogradely transported HRP. The TMB reaction product appears as small dark granules against a light background. The dorsal surface of the colliculus is at the top of each photomicrograph. The midline and a small portion of the contralateral colliculus are to the left. Large dark granules on the surface of the colliculi and at arrows are red blood cells containing endogenous peroxidase. (A) One-day-old normal animal. An ipsilateral retinotectal projection can be seen throughout the colliculus, extending to the pial surface especially in the medial half. (B) Seven-day-old normal animal. While an ipsilateral projection can still be detected across the mediolateral extent of this section through the rostral third of the colliculus, very little reaction product is present nearer the surface. (C) Seven-day-old animal from which one eye was removed at birth. In this litter mate of the animal shown in (B), an ipsilateral retinotectal projection is prominent throughout the superficial portion of the colliculus, and may be slightly denser than the ipsilateral projection at birth. (D) Ten-day-old normal animal. The ipsilateral retinotectal projection at 10 days has become restricted to a deep position in the medial portion of the colliculus, with only occasional foci (arrowheads) evident more laterally. Bar signifies 200 μm (from [148]).

B. Postnatal development of retinal projections to the hamster lateral geniculate nucleus. At 4 d postnatal, the contralateral projection spreads all over the geniculate as indicated by the density of autoradiographic grains following injection of radioactive tracer into the eye. The ipsilateral nucleus only receives a projection over its dorsal part. At 8 d postnatal, the contralateral projection shows a bilaminar pattern as indicated by the grain sparse zone like that in the adult. The ipsilateral nucleus now receives a mature projection (from [287]).

C. Development of retinal projections to the primate lateral geniculate nucleus. (A) Nissl-stained coronal section of the lateral geniculate nucleus (LGd) in a normal adult monkey, showing six cellular layers (1 to 6) and five interlaminar bands. (B) Autoradiograph of the LGd of a normal adult monkey showing labelling of layers 1, 4, and 6 after injection of the contralateral eye with radioactive tracer. (C) Nissl-stained LGd in a monkey of the same age from which one eye was removed at the second fetal month showing the presence of the magnocellular (*m*) and parvocellular (*p*) moiety and the absence of the normal six-layered pattern. (From [246]).

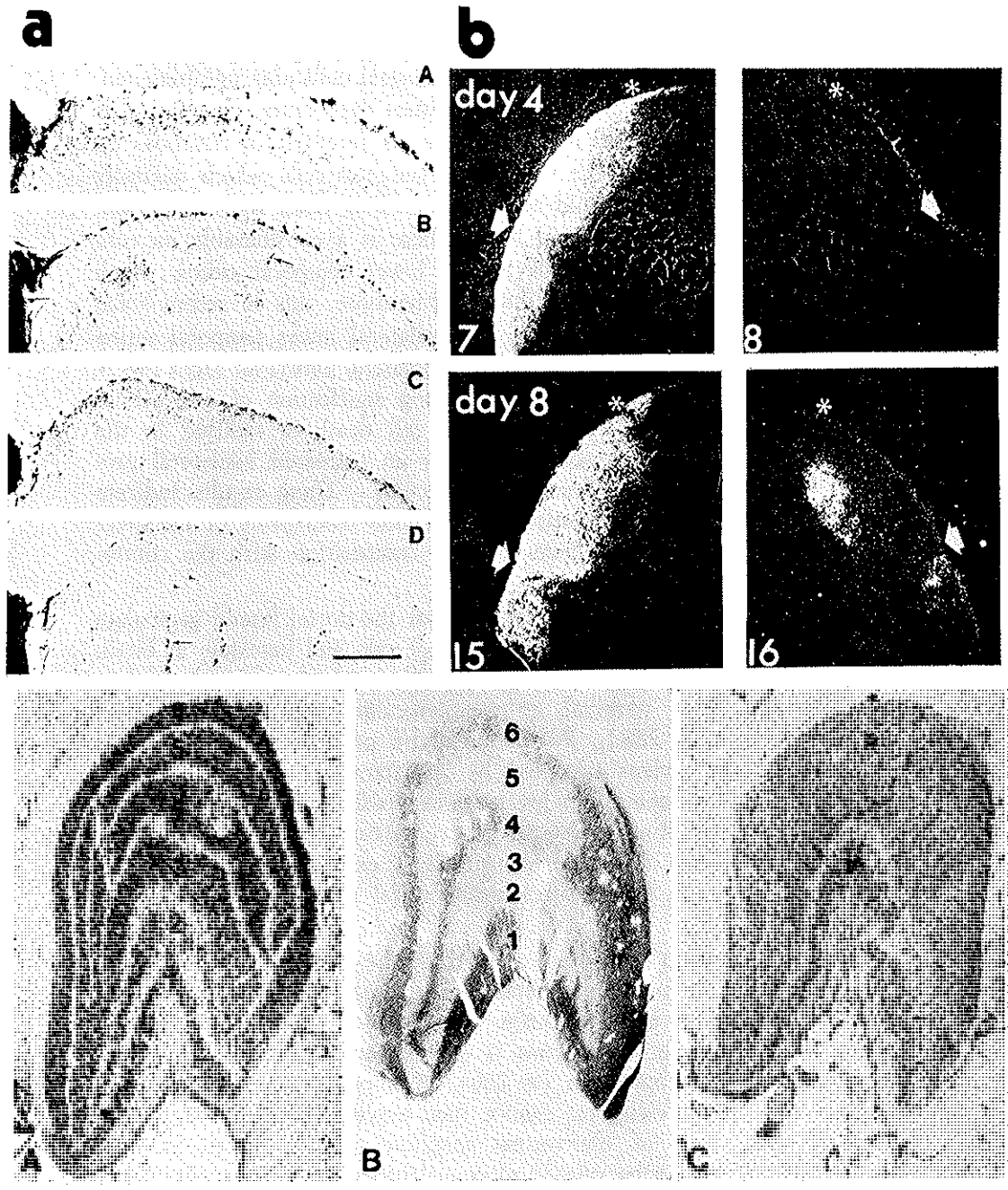

FIG. 3.

whether ganglion cells compete with each other for space in their retino-recipient nuclei. Enucleation of an eye at birth leaves the great majority of neurones in the optic layer of the contralateral colliculus uninnervated. Although some of these deafferented neurones degenerate, ganglion cells of the ipsilateral retina still have much greater numbers of neurones on which to synapse so that the number of ganglion cells which normally die up to 5 d postnatal should be reduced. This takes place after unilateral eye enucleation of rats at birth: there is a considerable increase in the number of ganglion cells in the lower temporal retina which project ipsilaterally [134, 135, 288]; furthermore, this is accompanied by a spread of the projection from the ipsilateral lower temporal retina across all of the tectum, except for its posterolateral part (Fig. 3A; [146]). Similar results have been obtained following enucleation of one eye at birth in hamsters [129, 271]: ganglion cell death is reduced in the remaining eye, and this is accompanied by an increased ipsilateral projection from this eye to the superior colliculus. These results indicate that the withdrawal of the ipsilateral projection from parts of the colliculus during normal development involves competition with the contralateral projection.

Similar observations have been made for the dorsal lateral geniculate nucleus. Following unilateral enucleation of rats and hamsters at birth, the ipsilateral projection from the remaining eye is larger and displays a greater density of terminals in the geniculate than in age-matched controls [131, 170, 288]. Furthermore the proportion of the dorsal lateral geniculate nucleus occupied by ipsilaterally projecting ganglion cells is the same in neonates of a given age as in adults that were enucleated at that age [131]. Enucleation of an eye in cats and monkeys, before the cell death period, also leads to a markedly expanded projection from the remaining eye in the mature dorsal lateral geniculate nucleus [59, 246, 315].

After enucleation of one eye at birth in the rat there is an approximate doubling of the numbers of neurones which project ipsilaterally [133]. However, as mentioned previously, the number of ipsilaterally projecting neurones in the normal rat is only a very small fraction of the total ganglion cell population ($\cong 2\%$). Competition between retinal ganglion cells in the two eyes cannot then explain most of the normal death of over 40% of the retinal ganglion cell population in each eye [235]. This may be related to competition between ganglion cells in a single eye for synaptic sites in the retino-recipient nuclei during the

establishment of a topographical projection from the eye. Enucleation of an eye before the ganglion cell death period in cats and primates [60, 248, 317] ensures that the optic nerve of the remaining eye is larger and contains significantly more retinal axons than in age-matched controls. This is probably due to the much greater number of uncrossed axons in cats and primates, which are available for competition with the crossed axons.

Effect of electrical activity on ganglion cell survival. It is known that blocking electrical activity in muscle with neuromuscular-blocking drugs prevents naturally occurring cell death [233]. Similarly, blocking electrical activity in an eye with monocular tetrodotoxin injections [90, 91] markedly decreases the rate of ganglion cell degeneration in that eye [239]. This may be related to the decrease in electrical activity of principal cells in the retino-recipient nuclei, consequent on the decrease in drive they receive from the blocked optic nerve. The growth factor provided by muscle for motoneurones increases with a decrease in electrical activity (see below). If this is the same for principal cells, it may provide an explanation for the decreased ganglion cell death which occurs following application of tetrodotoxin.

Cell survival that is dependent on synapse formation on ganglion cells

Although in both hooded and albino rats the great majority of retinal ganglion cells which project ipsilaterally are found in the lower temporal crescent, some ipsilaterally projecting cells are located outside the temporal crescent [78]. Lesion of an optic tract at birth also leads to an enlargement of the uncrossed retinotectal projection from the eye contralateral to the lesioned optic tract [135]. Such lesions lead to substantial death of ganglion cells in the contralateral eye: presumably those cells die which are deprived of synaptic connections by the lesion. Perry and Linden [229] have shown that following a large unilateral tectopretectal lesion in rats at birth there is an enhanced survival of neurones in the nasal retina of the contralateral eye which have uncrossed projections. The question then arises as to why there should be an enhanced survival of neurones which have uncrossed projections, in experimental circumstances where there is presumably no increase in the numbers of neurones (or synaptic sites) available to them in the visual centres of the brain. The expansion in the population of aberrantly-located retinal gang-

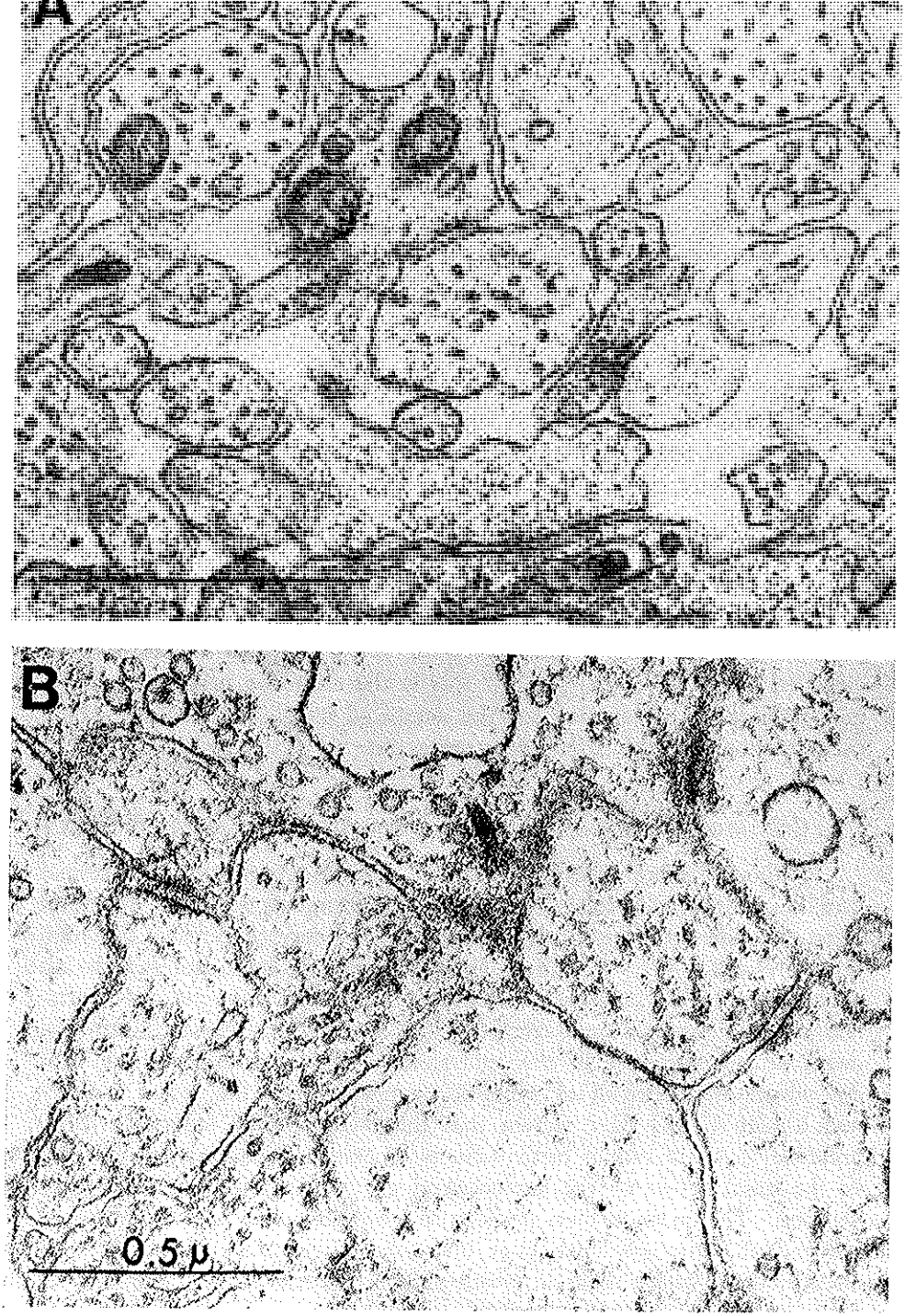

FIG. 4. Development of afferent connections to retinal ganglion cells in the rat inner plexiform layer. A. Inner plexiform layer of the retina at birth. Numerous neural cell processes are seen but no synapses are yet formed. The extracellular space is characteristic of the immature inner plexiform layer. Those spaces will be filled later with the processes of Müller cells. Line indicates 1 μm. B. Inner plexiform layer of the retina on the twelfth

lion cells occurs within regions largely depleted of surrounding neurones. Perry and Linden [161, 229] have therefore suggested that the enhanced survival is brought about by diminished competition within the retina from neighbouring ganglion cells. Indeed, large alpha-like ganglion cells have their dendrites pointing towards the severely depleted nasal areas far more frequently than in normal rats [161, 162, 182]. It is unlikely that the ganglion cells are competing for afferent innervation. In the rat, synapses do not form in the inner plexiform layer, and hence on ganglion cell dendrites, until after the cell death period (Fig. 4; [235, 311]. Nevertheless, the experiments of Linden and Perry [161] do point to some form of competition between the dendrites of adjacent ganglion cells.

Trophic factors released at synapses that mediate ganglion cell survival

Identification of ganglion cells in culture and their degeneration

Trypsin dissociation of retinae into culture allows for the study of factors which determine the survival and maturation of different classes of retinal cells [268]. Retinal ganglion cells can be unambiguously identified in culture of dissociated retina: following HRP injection into the visual centres of the brain, ganglion cells in the contralateral retina can be successfully dissociated into culture and identified at any time by appropriate histochemical staining [206] (Fig. 5); they can also be identified if fluorescent dyes which undergo retrograde transport are injected into the optic chiasm [259]. Ganglion cell cultures have been prepared from dissociated retina of different aged animals in this way, and estimates made of the number of ganglion cells per retina at each age. Such observations in both chick and rat embryos indicate a loss of ganglion cells during the developmental period which parallels that observed in vivo [33, 179]. Counts of HRP-labelled cells from progressively older animals confirm that the peak number of generated ganglion cells occurs on embryonic d 10 in chick and on embryonic d 21 in rat.

The glycoprotein Thy-1 has been localized in the retina by indirect immunofluorescence: it is found in the optic axon layer, the ganglion cell layer and the inner plexiform layer of both adult and neonatal rat retina [16, 19]. Similar results have been obtained for chick retina [62], following the isolation of a chick glycoprotein having the characteristics

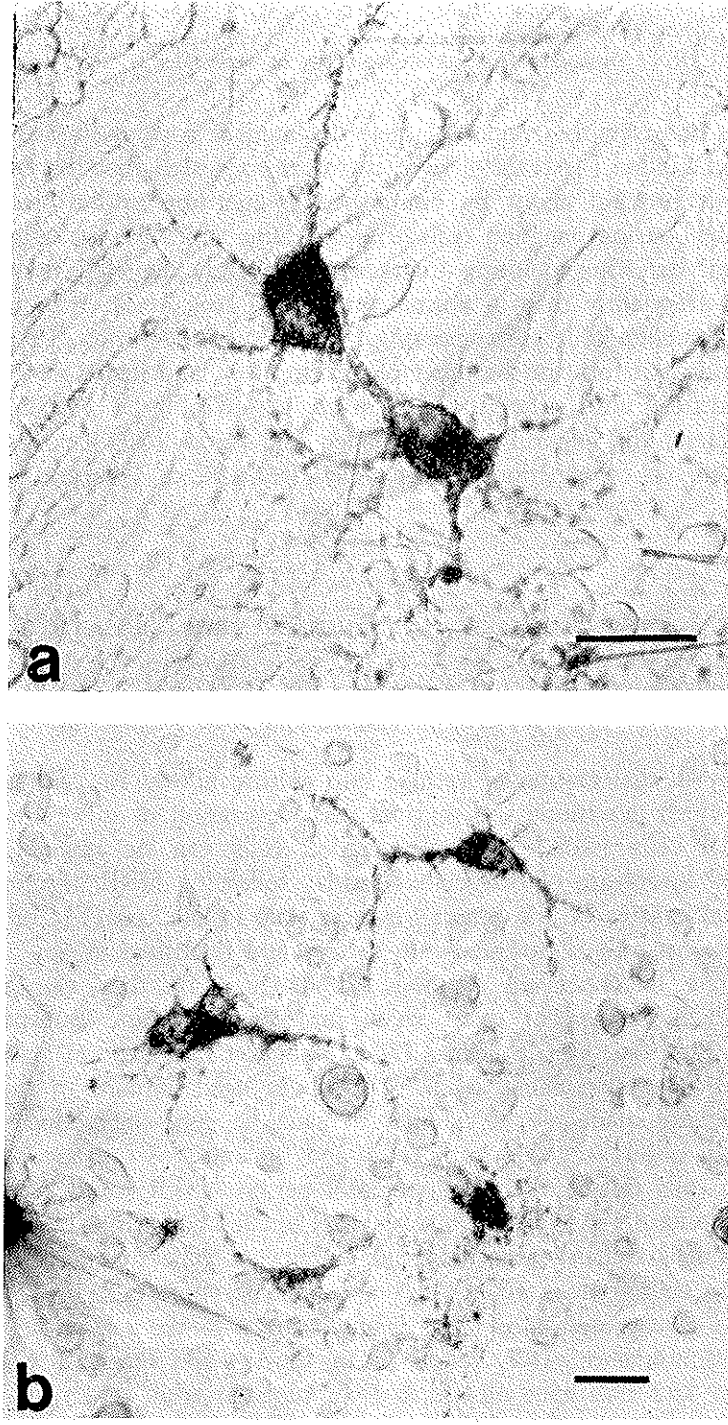

FIG. 5. HRP labelled rat retinal ganglion cells growing on monolayer cultures of Müller glia. These two micrographs illustrate the physical contact between ganglion cells. a, contact formed by the neurite (axon) of one ganglion cell with the soma of the other. b, interaction between somata (arrows) of two ganglion cells. Both photographs were taken with transmitted light. Calibration bars represent 20 μm. (From [243]).

of Thy-1 [157]. Antibodies to Thy-1 also uniquely label ganglion cells in culture [259]; this has enabled confirmation of the times at which peak numbers of ganglion cells are generated.

Retinal ganglion cells degenerate over a few days if cultured in a minimal media at the time when the peak number of ganglion cells is generated, and under conditions in which few glial cells form. The rate of degeneration of chick ganglion cells [206] (50% in 60 h) is much slower than that of rat ganglion cells [179] (50% in 24 h).

Embryonic ganglion cells are maintained by a soluble factor from Müller cells

Dissociated embryonic rat retinal ganglion cells die within about 14 h when cultured in a minimal media, whereas the cells survive much longer in explant culture [191, 193, 194]. These observations suggest that a cell intrinsic to the retina allows for the survival of ganglion cells at this time. It is likely that Müller glial cells fulfill this role, as synapses do not form on embryonic ganglion cells and less than 5% of the non-neuronal cells in the retina are astrocytes [17]. Both the cell bodies of the ganglion cells and the proximal portions of their axons are in intimate contact with Müller cells [43, 45, 250, 252], and these can be identified with antibodies to RAN2 [17] and C1 [290]. Furthermore, Müller cells are the first amongst retinal cells to differentiate and are present at the time when ganglion cells first differentiate [53, 309].

Dissociated embryonic retinal cells are sustained if cultured over a continuous layer of Müller cells [69]. Furthermore, Müller cells exert this survival enhancing effect on identified embryonic ganglion cells in culture. The effect is mediated by soluble factor(s) acting directly on the ganglion cells (Fig. 6) [244]. However, the survival enhancing effects of Müller cells are developmentally regulated, as conditioned media from these cells does not significantly enhance the survival of ganglion cells from 6 d rat retina, when the cell death period ends (Fig. 6 [244]).

Embryonic ganglion cells cultured with Müller glia express neurites

Optic axons growing within the retina and towards the choroid fissure are in close apposition with the endfeet of Müller cells [97, 279, 292]. The question arises as to the effect, if any, which Müller cells have on the expression of neurites by ganglion cells.

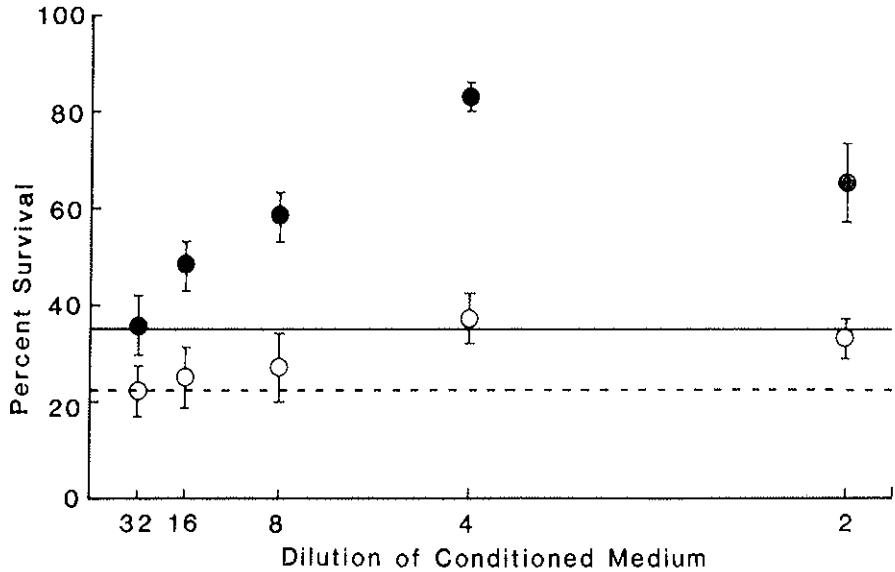

FIG. 6. Dose-response curves for survival of 1 d rat retinal ganglion cells (filled circles) and 6 d ganglion cells (open circles) in Müller conditioned media. The number of surviving ganglion cells is expressed as a percentage of the number originally present in 2 hr culture. Solid and broken lines represent survival at 16 hr of 1 d and 6 d ganglion cells respectively in Dulbecco's minimal medium with fetal calf serum. (From [243]).

Dissociation of chick retina into culture at the time when ganglion cells are first forming (≅E6), leads to the formation of a continuous sheet of Müller cells on the culture plate [301]. Neuroblasts migrate into small clumps on this substratum and extend neurites after about one week [301]. The degree of neurite extension depends on the age of the neurones at culture: neurones plated at E6 express more neurites with a greater average length than those plated from older embryos [301]. Similar results have been obtained for identifiable ganglion cells [243] when neonatal rat ganglion cells are plated onto Müller cells they develop a complex neuritic morphology (Fig. 7); however, such neuritic expression is not present when the ganglion cells are plated onto a polylysine substratum in the presence of colliculus conditioned media [179]. Although ganglion cells express neurites when plated on immature "flat" astroglia, they are not nearly as extensive as those induced by Müller cells. It appears that Müller cells induce a unique proliferation of neurites from ganglion cells.

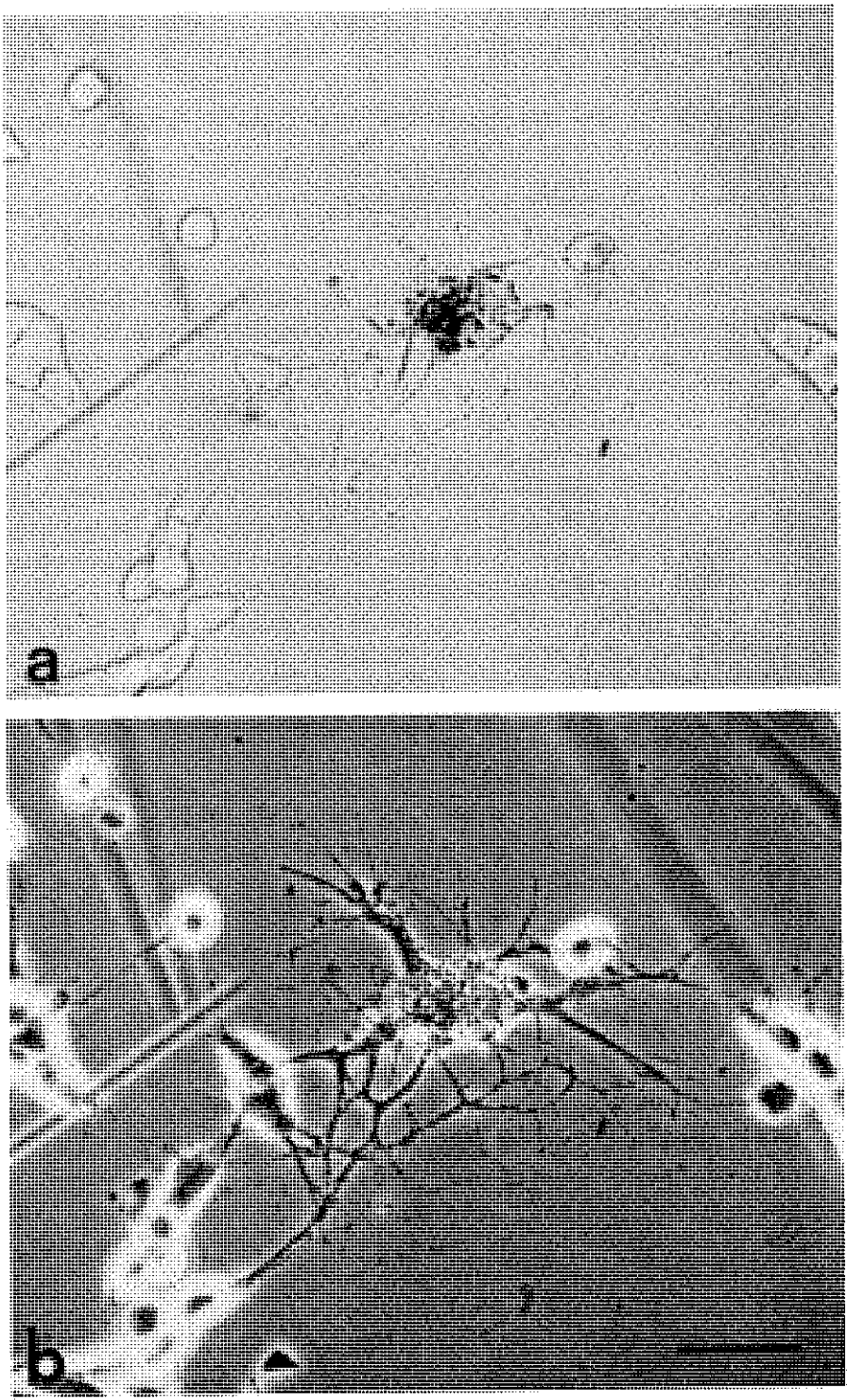

FIG. 7. Transmitted light (a) and phase contrast (b) micrographs of HRP labelled 1 d rat retinal ganglion cells co-cultured with Müller glia for 16 hr. Note the extensive arborization of neuritic processes. Calibration bar is equivalent to 20 μm. (From [243]).

Neonatal ganglion cells are maintained by a soluble factor from the retino-recipient nuclei

Evidence from transplant experiments for trophic factors from retino-recipient nuclei

Lund and his colleagues have made extensive studies of the connections formed between retinal or tectal transplants from rat fetuses to the surface of the tectum of newborn rat hosts. Tritiated thymidine studies have shown that neurones survive and differentiate in the grafts; termination of neurogenesis occurs at successively earlier times in transplants taken from correspondingly older embryos [130]. If retinae are taken from fetal rats and transplanted adjacent to the superior colliculus of neonatal rats, they develop connections with the host's brain [188] (Fig. 8). This occurs even if the fetal retina are first dissociated into single cells and then reaggregated prior to transplantation. The neurones which give rise to these connections are in the ganglion cell layer of the retina [188] and they only project to nuclei in the brain which are normally retino-recipient (superior colliculus, accessory optic tract nuclei, and the dorsal lateral geniculate nucleus) [189]. Furthermore, retinal transplants positioned on the cerebellum send axons forward to enter the superior colliculus [189]. It is not known if a transient axon projection into the cerebellum occurs before these axons reach their final destination in the colliculus; however it is known that regenerating optic fibers in goldfish selectively avoid the cerebellum and grow into the optic tectum [320]. These experiments indicate that retinal axons grow to their appropriate tectal tissue as a consequence of factors released from the retino-recipient nuclei. In contrast to these observations, retinae taken from fetal rats and transplanted adjacent to the superior colliculus of mature rats do not produce stable connections with tbe colliculus: all the ganglion cells degenerate in the transplant [191, 192]. It appears tbat more mature retino-recipient nuclei no longer produce factors for the growth and survival of ganglion cells.

The connections formed by these retinal transplants may be compared with those which form when tectal transplants are made from fetal rats to areas adjacent to the superior colliculus in newborn rats [109, 167]. By six weeks the transplants are situated over the host inferior colliculus and the rostral part of the cerebellum; however they only make connections with those regions of the host's brain which normally project to the superior colliculus, such as the visual cortex. These observations

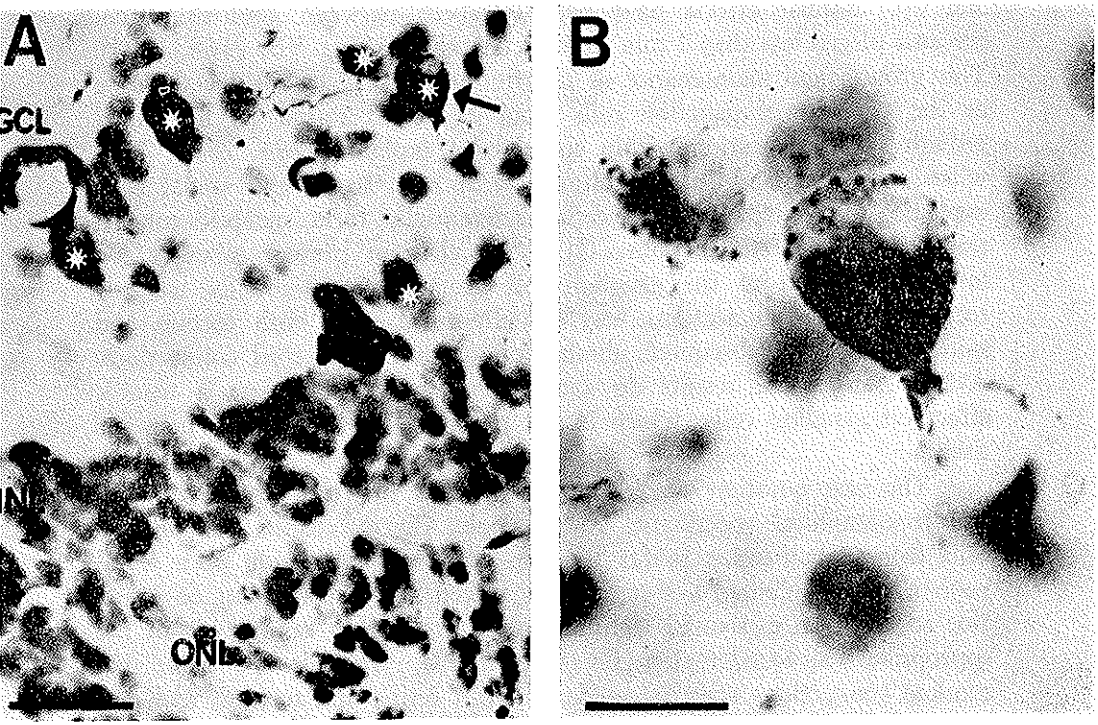

FIG. 8. Projection of ganglion cells from retinal transplants placed on the brains of neonatal rats. Nissl stained section of a transplant from an animal which received an injection of HRP in the superior colliculus. A: numerous HRP-labelled cells (*) are present in the "ganglion cell" layer (GCL) interspersed with bundles of labelled axons. A single labelled cell is also visible along the inner border of the inner nuclear layer (INL). No labelled cells were observed in the outer nuclear layer (ONL). Bar signifies 25 μm. A higher power micrograph of the cell marked by the arrow in A is present in B. Note the dendrites filled with HRP reaction product extending into the plexiform layer. Bar signifies 10 μm. (From [188]).

emphasize the possibility raised by the retinal transplants: neurones may show an affinity for their appropriate target cells [75].

Evidence from explants of retina and tectum in culture for trophic factors

If explants of embryonic rodent superior colliculus are placed within 1 mm of retinal explants in culture, neurites grow from the retina into the colliculus and are maintained for at least 5 weeks [283] (Fig. 9A

and C). Similar effects have been observed between goldfish retinal explants placed less than 1 mm from tectal explants (Fig. 9B; [168, 198]). This growth and maintenance of neurites is peculiar to the tectal tissue as the neurites of ganglion cells from explanted retina do not show growth and arborization within spinal cord explants [283, 285]. Thus when retinal explants are cocultured with both tectal and spinal cord explants, the retinal ganglion cells preferentially form synaptic connections with the tectal explant. It is not clear at present whether the initial outgrowth of the ganglion cell axons is preferentially towards tectal material: connections may initially form with both tectum and spinal cord with subsequent elimination of the spinal cord connections. At any rate, these results do show that the tectum exerts a trophic effect on ganglion cells, which is not provided by some other neuronal tissues.

The next question which arises is whether this trophic effect shows any topographical specificity: for example, do the axons of ganglion cells from a half-retina prefer the "appropriate" half-region of a tectal explant? If explanted half-retinae are placed near the anterior or posterior edges of an explanted tectum, retinal fibers only ramify within the appropriate half-tectal region (Fig. 9C; [285]). Again it is possible that random connections are first made between retina and tectum, with the

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FIG. 9. Trophic interactions between explants of retina and tectum in culture.

A. Explants of embryonic retina maintain their integrity in culture. Thick Epon section (1 μm) of an embryonic day 14 rat retina placed in culture for two weeks; ipl, inner plexiform layer; gcl, ganglion cell layer; ofl, optic fiber layer. Bar is for 50 μm.

B. Neurite expression by retinal explants (R) in the presence of tectal explants (T) less than 1 mm distant. A goldfish retinal explant was placed on a poly-L-lysine coated dish. Bar is for 100 μm.

C. Neurites from a half retina arborize within the appropriate part of the tectum. Cocultures in which a mouse retinal explant was placed near the anterior or posterior tectal edge. A: showing apparent preference for the appropriate half of the tectum. Nasal ratinal fibers enter the cocultured tectum posteromedially, ramify, and form arborizations; in contrast, one large-diameter fiber loops sharply out of this region and continues near the explant edge toward the far side (38.1). B: showing a lack of ramifications upon entering an inappropriate half-region; instead, parallel sheets of ingrowing fibers form. Nasal fibers enter the tectum anteromedially in a thick fascicle which splits into two broad sheets of fibers running in parallel through the tissue (avoiding the central necrotic zone) toward the far side. Some fibers near the point of entry run in a wavy pattern as they fan into one sheet or the other, but the predominant pattern is a lack of ramified crisscrossing of fibers despite the presence of some boutons and terminal arbors (58-4).
(A is from [194]; B is from [198]; C is from [282]).

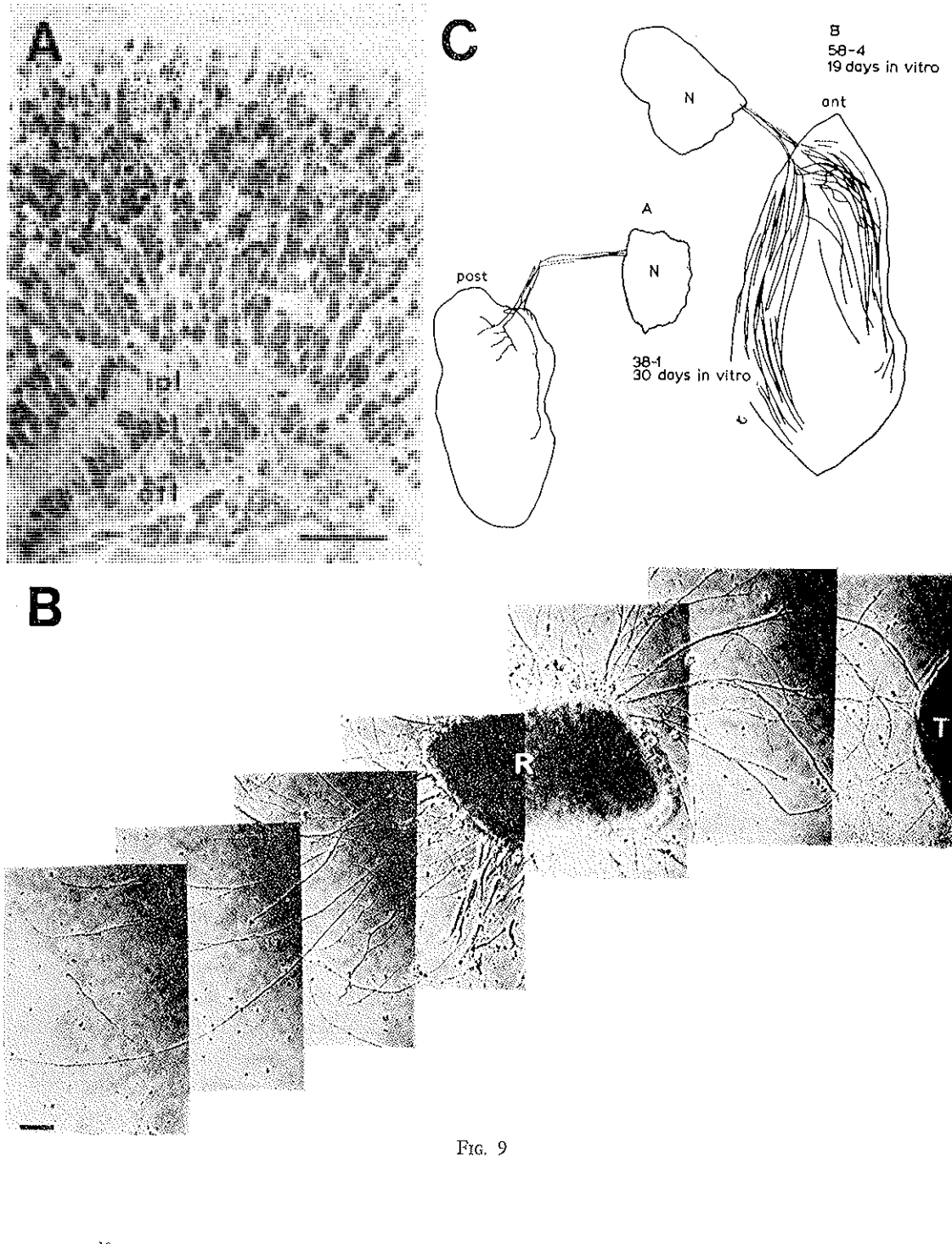
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FIG. 9

subsequent elimination of inappropriate connections: alternatively, the initial outgrowth of axons from the half-retina may be towards the appropriate half-tectum. If this latter case is true, then at least two distinct trophic molecules mediate the formation of retinotectal connections.

Neonatal ganglion cells in culture are maintained by soluble factors from retino-recipient nuclei

Neonatal rat ganglion cells and 10 d embryonic chick ganglion cells survive the cell death period if cocultured with dissociated cells from the retino-recipient nuclei, such as the superior colliculus or optic tectum (Fig. 10 [179, 206]). This survival is not contingent on the ganglion cells forming synaptic connections with neurones in the retino-recipient nuclei: media first conditioned over tectal cells can save ganglion cells from degeneration (Fig. 11; [180, 206, 259]). All the ganglion cells survive over the period in which many of them are destined to die "in vivo". These experiments indicate that ganglion cells are not preprogrammed to die, as all these cells can be saved in culture.

If ganglion cells are transplanted to a site next to the cerebellum, they fail to innervate this structure, and grow instead into the superior colliculus. Coculturing of ganglion cells with cerebellum does not allow for their survival at levels comparable to that when they are cocultured with the retino-recipient nuclei (Fig. 10A & B; [179, 206, 259]). The specificity of the retino-recipient factor for the survival of ganglion cells has been further illustrated by the failure of skeletal muscle myotube conditioned media to maintain the cells (personal observations).

It was noted above that Müller cells fail to support ganglion cells from 6 d postnatal rats, when the retino-recipient nuclei exert a maximum supporting effect [179, 206]. Such a change in the requirement of differentiating neurones for survival factors has been described for dorsal root ganglion neurones in culture by Barde *et al.* [13]. This could explain why more than 60% of ganglion cells degenerate when an optic nerve crush is performed in adult animals [197]: as the ganglion cells are deprived of their targets neither Müller cells nor the mature astrocytes of the optic nerve [180] can maintain the ganglion cells.

Long term cultures of dissociated chick retina, in which glial cells are eliminated, have been developed [1]. In these conditions neurones with a single long process appear in the presence of optic lobe extracts

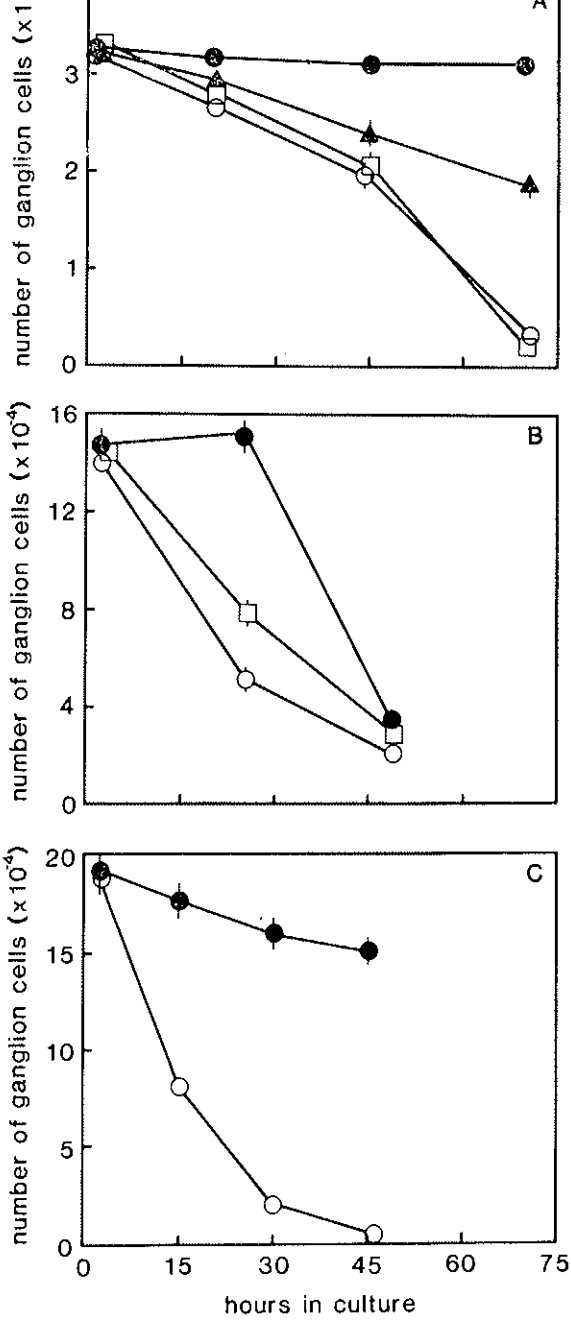

FIG. 10. Trophic effects exerted by tectal tissue on dissociated retinal ganglion cells. A: the effect of tectal tissue on the survival of ganglion cells dissociated from 10 day embryonic chick retina; open circles, cultured alone; filled circles cocultured with optic tectum fragments; filled diamonds, cultured in medium conditioned by previous contact with tectum; open squares, cocultured with cerebellum fragments. B: The effect of superior colliculus on the survival of ganglion cells dissociated from newborn rat retina; open circles, cultured alone; filled circles, cocultured with fragments of superior colliculus; open squares, cocultured with cerebellum fragments. C: The effect of repeated injections of superior colliculus (at 15 hrs and 30 hrs) into cultures of dissociated newborn rat retina; open circles, cultured alone; filled circles, cocultured with the superior colliculus. Note that in A, B & C the embryonic retinal ganglion cells survive much better in the presence of tectum than in controls or in the presence of cerebellum. (A from [206]); B & C from [179])

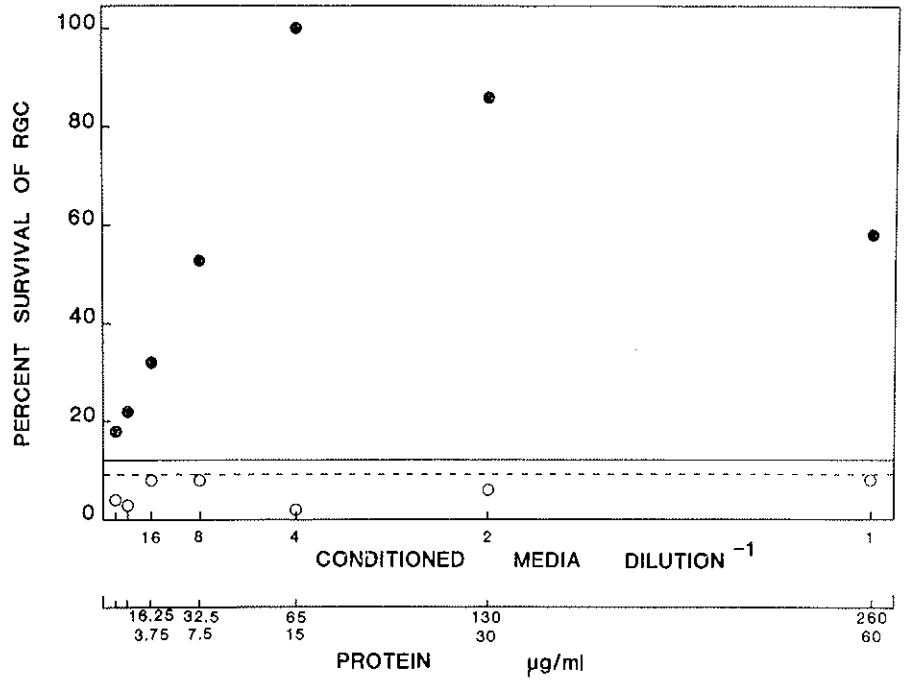

FIG. 11. An example of dose-response curves for the survival of rat retinal ganglion cells over 16 hrs in vitro in colliculus-conditioned medium (filled circles) and glial conditioned medium (open circles). The number of surviving ganglion cells is expressed as a percentage of the number originally present in 2 hr control cultures. Cells were plated in medium plus serum for the first 2 hr, at which time this medium was removed and the appropriately diluted conditioned medium added. Solid and broken lines represent percentage survival in controls (no serum) for colliculus- and glial-conditioned medium experiments, respectively. (From [181]).

[127]. If these neurones are ganglion cells this would provide further evidence for the existence of soluble survival factors in the retino-recipient nuclei.

Failure of soluble factors from astrocytes to maintain ganglion cells. Studies have been made to determine whether neurones or astrocytes in the retino-recipient nuclei provide the survival factor for ganglion cells. It is known that cortical astrocytes release soluble factors which support several types of central neurones [11, 82, 199, 268, 273] and peripheral neurones [13, 15, 163, 164] in culture. However, soluble factors from astrocytes derived from either the superior colliculus or

the cortex do not support neonatal rat ganglion cells (Fig. 11 [180]). This suggests that it is the neurones within the retino-recipient nuclei which supply the survival factor.

Astrocytes express different morphologies in culture [171, 245]. Cultures consisting predominantly of flat, immature astrocytes are capable of maintaining ganglion cells if they come into membrane contact (Fig. 12; [180, 181]). When high proportions of more mature process-bearing astrocytes are present these survival effects are not observed.

Ganglion cells begin sending their axons into the optic stalk at about fetal d 15 in the rat. Skoff, Price and Stocks [280] have suggested that this invasion of axons induces the transformation of ventricular cells into astrocytes. Astrocytes present at late fetal and early postnatal stages are of the immature kind and they begin to differentiate into process-bearing cells from the second postnatal week onward. It is therefore tempting to speculate that these flat astrocytes provide support by contacting developing ganglion cell neurites at the time of their invasion into the optic stalk and progression toward the retino-recipient nuclei. However, it appears that the presence of immature astrocytes alone is not sufficient to prevent ganglion cell death which proceeds from fetal d 20 to postnatal d 5 [143, 235]. Formation of appropriate projections within the retino-recipient nuclei is probably crucial for ganglion cell survival during the cell-death period.

Isolation of principal relay cells from the retino-recipient. In order to directly test the claim that neurones in the retino-recipient nuclei allow for the survival of ganglion cells, it is necessary to isolate the principal relay neurones in these nuclei. Principal relay neurones of neonatal lateral geniculate nucleus have been isolated by back-labelling them from the visual cortex with rhodamine-labelled latex beads. Media conditioned by contact with the neonatal visual cortex maintains the principal neurones in culture, but has substantially less effect on ganglion cells [9]. Whether principal neurones have a direct survival enhancing effect on ganglion cells is now being determined.

Ganglion cells are maintained by depolarizing agents

The extent of the afferent innervation of developing sympathetic neurones contributes to cell maturation, in addition to the supply of sympathetic nerve growth factor (NGF) made available by the target cells [44]. The afferent innervation probably exerts this maturation

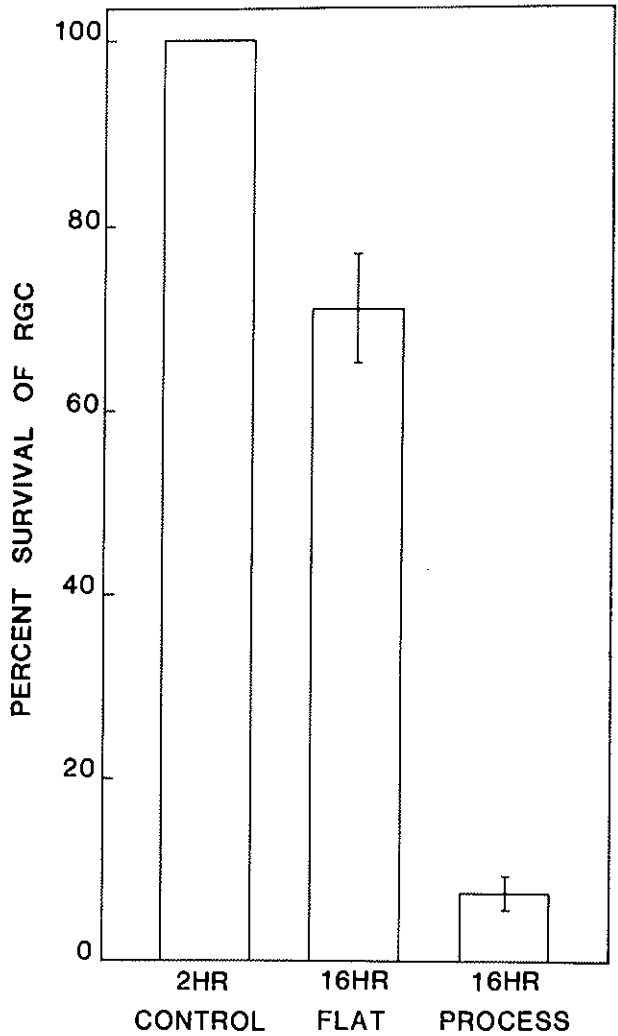

FIG. 12. Quantification of the survival of rat retinal ganglion cells on monolayers of flat and process-bearing astrocytes after 16 hr in vitro. The number of retinal ganglion cells either flat or process-bearing astrocytes at 2 hr was the same and this was taken as 100%. Survival of retinal ganglion cells on flat astrocytes was 71 ± 6% ($\bar{X}$ ± SEM, n = 12) and on process-bearing astrocytes was 7.5 ± 2% (n = 6). (From [180]).

effect through depolarization of the sympathetic neurones by the afferent synapses, rather than by the release of trophic material [231]. It is known that both sympathetic and parasympathetic neurones can survive in culture if their membranes are sufficiently depolarized by increasing the potassium ion concentration [36, 203, 205, 231]. It is not yet known if NGF and potassium depolarization work through a final common mechanism, such as increasing intracellular ionized calcium levels [298].

It was noted above that lesions of an optic tract in a neonatal rat lead to an enhanced survival of ganglion cells which are present in the contralateral eye and have ipsilateral projections. A possible explanation for this enhanced survival is that the ipsilateral neurones receive an increased afferent innervation and that this exerts a survival effect on the ganglion cells through the mechanism of membrane depolarization. If this is so then other means of depolarizing the cells should enhance their survival. Depolarization of cultured ganglion cells by increasing the potassium concentration in the media greatly enhances their rate of survival [179].

Identification of trophic factors for ganglion cells

Growth factors

NGF which has been purified to molecular homogeneity [98], acts to promote the survival of both sympathetic neurones and a class of dorsal root ganglion cells [298]. It is found in the target tissues of sympathetic neurones [84, 276]. The trophic effects mediated by the retino-recipient nuclei on retinal ganglion cells may be mediated by NGF.

NGF is present in the tectal ependymal zone surrounding the optic ventricle of goldfish [37] and optic tectum explants have a growth promoting effect on the neurites emerging from cocultured retinal explants [198, 269]. The retinae of goldfish, treated with NGF in vivo and then explanted into culture, show a much greater neurite outgrowth than controls [137, 306]. Antisera to NGF depress retinal ganglion cell neurite outgrowth in goldfish explant cultures [307, 308] and this inhibition can be partially eliminated by the addition of excess NGF. Similar effects of NGF and its antiserum on the regeneration of optic axons in vivo have been observed in newts [96, 305]. It is therefore possible that NGF in the retino-recipient nuclei of lower vertebrates

does provide a trophic effect on ganglion cells. However, it is not clear if normal ganglion cell death occurs in the retinae of lower vertebrates during development.

Bioassay, antibody neutralization and complement fixation have been used to determine the localization of NGF in the embryos of higher vertebrates. According to these criteria NGF is absent from the brains of developing chick and mice [37, 319]. However, messenger RNA encoding NGF has recently been found in mouse brain; it occurs at levels higher than can be accounted for by the sparse sympathetic innervation of the cerebral vasculature [276]. NGF does not enhance the survival of neonatal rat retinal ganglion cells in culture [179]. It follows that NGF is probably not the trophic molecule from optic tectum which promotes the survival of embryonic rat retinal ganglion cells. Furthermore, NGF is not able to stimulate fiber outgrowth from explants of fetal rat retina in culture, and antibodies to NGF do not block the spontaneously occurring fibre outgrowth [304].

A factor from the avian optic lobe enhances neurite outgrowth from small explants of E6 chick retina [57]; it has a molecular weight in excess of 100,000. Another factor, this time from the mammalian brain, stimulates neurite outgrowth from fetal rat retinal explants [299, 304]; it has been purified to molecular homogeneity [14] and has a molecular weight of 12,300 and an isoelectric point of about 10.2. Neither the avian optic lobe factor nor the brain derived factor are NGF and they do not support the survival of sympathetic neurones. The brain factor may be derived from glial cells [15, 163, 164]. In this case it will be interesting to see what relationship it has, if any, with the soluble factor produced by Müller glial cells that maintains retinal ganglion cells.

Neurite expression factors

The inner limiting membrane of the retina contains the glycoprotein laminin [136, 186], which is known to have a powerful effect on the expression of neurites and their growth [85, 253, 284]. Laminin, when coated onto a polylysine substratum, provides for the extension of ganglion cell neurites of up to 500 μm over 24 hr. The inner limiting membrane is composed of the end feet of Müller cells and a basal lamina, both of which are associated with laminin [186]. Müller cells, like other glial cells, produce laminin in culture [244]. It may be then that Müller cells induce considerable neuritic growth from ganglion cells by secreting laminin.

There are other sources of neurite promoting factors in the retina than Müller cells. Embryonic neural cells from the chick retina release glycoprotein complexes, called adherons, into the culture media [266]. These complexes contain several different proteins and glycosaminoglycans; when adsorbed onto the surface of petri dishes they greatly enhance the adhesion of retinal neurones. Such enhanced adhesion induces neurones to express neurites and is responsible for the guidance of growth cones [157]. Two polypeptides have been isolated from these adherons [63]: one of these is a 170,000 dalton protein which mediates the binding of retinal cells to the substratum. It is not known if adherons exist which are specific for retinal ganglion cells.

Speculations on the role of growth factors in establishing topographical projections between retina and tectum

Observations on ganglion cell synapses and cell survival

The discussion above has been concerned with the role of trophic factors in maintaining ganglion cells through the cell death period. Various forms of surgery on the embryonic visual system have shown that survival of early differentiating ganglion cells in the rat retina is independent of their forming synapses for a week or more. After that time, the ganglion cells must make contact with the retino-recipient nuclei within a few days, and with no other nuclei, such as the cerebellum, if they are to survive. Mature ganglion cells also depend on contact with the retino-recipient nuclei for survival; however, isolation of the retina from nuclei in adults leads to degeneration of the ganglion cells over months rather than days. Ganglion cells are only critically dependent on trophic support from the retino-recipient nuclei at a particular period of development.

Why do some ganglion cells die after projecting onto the retino-recipient nuclei? This problem has been studied in terms of competition between the two retinas for synaptic connections in animals such as rodents that have a substantial cell death in the postnatal period. Two points have been established: firstly, competition for space in the retino-recipient nuclei exists between ganglion cells in a retina which have ipsilateral projections and ganglion cells in the other retina which have contralateral projections; secondly, the elimination of misprojecting ganglion cells by this competition involves death of the cells. This death

only accounts for a very small proportion of the loss of ganglion cells. It may be that ganglion cells within a retina compete with each other for space in the retino-recipient nuclei as the retinae estahlish their topographical projections.

It has not been established that the competition for appropriate space in the retino-recipient nuclei involves synaptic sites in these nuclei. A recent report suggests that misprojecting axons fail to form synapses. It is important to determine if these misprojecting axons form synapses following removal of the appropriate projections: if this should be the case, then misprojecting axons are probably excluded from synaptic sites by appropriate axons; misprojecting axons may then degenerate as a consequence of failing to form stable synapses. This degeneration may then be due to a failure of trophic support arising from a lack of contact between the membranes of ganglion cells and the synaptic site membranes, or from a soluble growth factor which is made available at uninnervated synaptic sites. This latter concept is favoured by the observation that transplanted ganglion cells grow preferentially into the retino-recipient nuclei, ignoring nearby nuclei such as the cerehellum.

The afferent innervation of rodent ganglion cells occurs after the cell death period; ganglion cell death cannot be due to competition between ganglion cell dendrites for afferents. However, there is evidence that a component of cell death does involve some form of competition between the dendrites of adjacent ganglion cells.

Observations on the survival of cultured ganglion cells

The introduction of techniques for the identification of retinal ganglion cells in culture has enabled an analysis to be made of the trophic effects exerted by neurones and glial cells on ganglion cells. Müller cells in culture release a soluble factor which provides for the survival of embryonic but not late postnatal ganglion cells. Immature astrocytes, which are found in the optic stalk, will maintain the viability of cultured ganglion cells if the membranes of the two classes of cells come into contact; mature astrocytes do not give trophic support. It follows that both Müller cells and immature astrocytes could be responsible for the survival of ganglion cells as they project to the retino-recipient nuclei. After that time ganglion cells must receive support from the retino-recipient nuclei. The survival factor released by Müller cells has not been identified. However, it may be related to the recently purified

12,000 dalton factor from brain, which is believed to be of glial cell origin; this factor enhances the growth of neurites from retinal explants.

The growth of axons within the retina may be assisted by the Müller cells, which produce laminin. This glycoprotein has a dramatic effect on the growth of ganglion cell neurites in culture.

Ganglion cells survive through the cell death period if provided with a factor from the retino-recipient nuclei. This factor is not provided by other nuclei, such as the cerebellum. It may be that the factor is responsible for the growth of axons from transplanted retinae into the retino-recipient nuclei rather than into more favourably placed nuclei such as the cerebellum. This is supported by the observation that retinal explants in culture send neurites into explants of the tectum rather than into explants of spinal cord. In both transplant and explant experiments, retinal axons are maintained once they grow into tectal tissue.

It has been argued above that ganglion cell axons compete for synaptic sites on neurones in their retino-recipient nuclei. The ganglion cells that fail in this competition are not able to obtain sufficient amounts of a factor necessary for their survival. Cultured ganglion cells are not maintained by soluble factors from astrocytes in retino-recipient nuclei; however they remain viable when cultured with media conditioned by contact with neonatal relay neurones isolated from the lateral geniculate nucleus. These observations support the concept that it is the target neurones for retinal ganglion cells which supply the trophic factor necessary for their survival during the cell death period.

Motoneurones

Motoneurone synapses and cell survival

Time course of motoneurone death during development

A loss of motor axons from ventral roots at the level of the limbs has been observed during normal development of all species studied (Fig. 13; [13, 61, 93, 107, 122, 123, 238]). The degenerating axons possess numerous vesiculated structures, membrane-bounded autophagic vacuoles, membranous lammelar figures and electron dense bodies. The degeneration process is due to progressive autolysis followed by phagocytosis of the axonal remnants by the surrounding Schwann cells and

mononuclear leukocytes [61]. This loss of motor axons has two phases. In chick there is a 57% reduction in the ventral root axons of the lumbosacral cord between E6 and E10 with a further 14% over the next few days. In rat there is a 60% reduction in the ventral root axons of the thoracic cord between 15 d and 17 d in utero, with a further 14% over the next few days. A postnatal loss of ventral root axons in the brachial spinal cord of rat has been reported (Fig. 13A; [31, 93]).

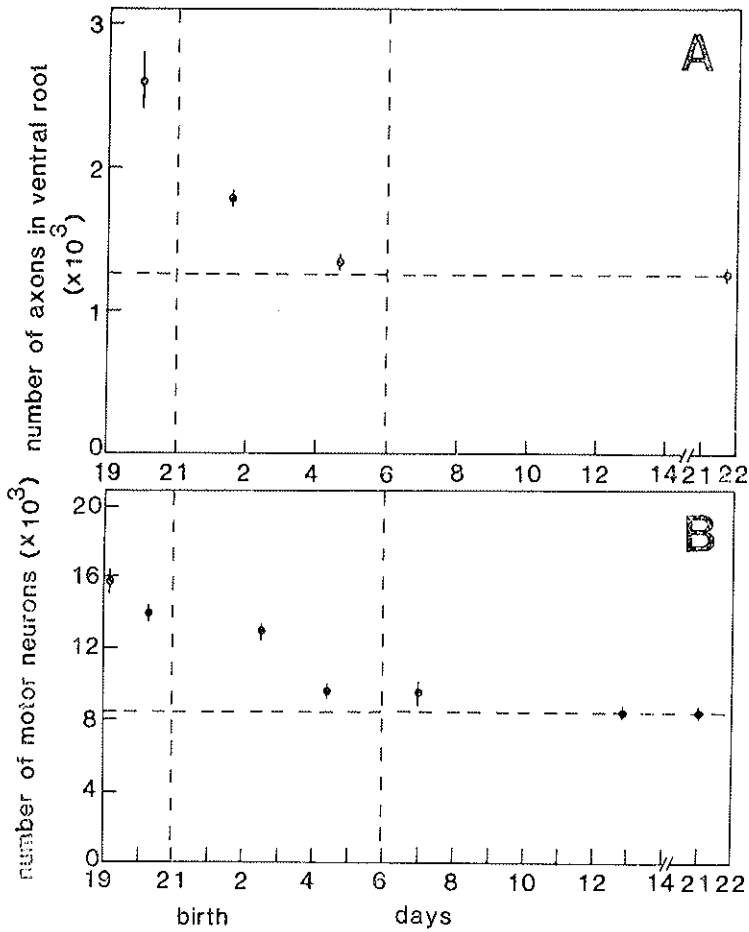

FIG. 13. Comparison between the loss of ventral root axons (A) and motoneurones (B) during development of rat forelimbs. Loss of ventral root axons and motoneurones begins in fetal life and continues until a few days after birth. (Data in A from [31], data in B from [210]).

Motoneurone degeneration accompanies the loss of ventral root axons and has been observed in mammals, chicks and amphibia (Fig. 13B; [18, 61, 103, 108, 121, 123, 142, 144, 146, 210, 238, 255]). There appear to be two types of degeneration: type I degeneration involves the detachment of ribosomes from rough endoplasmic reticulum, vacuolization of mitochondria and the formation of pyknotic nuclei with condensed chromatin; type II degeneration involves dilation of the endoplasmic reticulum, nuclear envelope and Golgi apparatus as well as the formation of rosette-like polyribosomes, the degeneration of mitochondria and the rounding up of nuclei. Degeneration in both types leads to cell death and complete breakdown: the nuclear envelope breaks down and the nuclear contents become mixed with lytic cytoplasm. The degenerating cells then condense into a single large globule or into several smaller fragmented globules. At this stage the cell debris is phagosytozed by radial ependymal processes and mononuclear leukocytes. In the chick lumbosacral spinal cord, motoneurone death begins about E5.5 and is almost complete by E10, by which time about 40% of the motoneurones have been lost [103]. In the brachial cord, an initial loss of about 40% of the motoneurones up to E10 is followed by a slower rate of degeneration which continues up to E18, during which time a further 20% of motoneurones are lost [142]. In the mouse lumbosacral spinal cord, motoneurone death is greatest between 13 d and 15 d in utero when 50% of the motoneurones are lost; this is followed by a slower rate of decline between 15 d and 18 d, during which time a further 15% of the motoneurones degenerate [146]. However, using HRP applied to the mouse sciatic nerve Baulac and Meininger [18] showed that a decrease in the number of motoneurones occurs in the postnatal period. Similar results have been obtained for mouse facial motoneurones, except that because these develop rather late, the greatest loss of motoneurones occurs between 17 d and 19 d in utero, with the slower phase continuing up to 5 d postnatal [10]. In the rat brachial and lumbosacral spinal cord, motoneurone death is greatest between 15 d and 17 d in utero when 60% of these neurones degenerate [28]; this is followed by the slower loss of a further 20% of motoneurones which extends into the first few days postnatal [28, 31, 256].

Motoneurones are therefore lost to the same extent as motor axons [61, 123, 238], so that a close one-to-one relationship exists between the two for the lumbosacral spinal cord. The correspondence between axon and neurone counts suggests that all motoneurones, even

those destined to die, normally innervate limbs [62, 236]. This has been confirmed by injecting hindlimbs with HRP before the onset of cell death and showing that all the motoneurones are labelled in the lateral motor column [61].

Evidence that motoneurone survival is dependent on the formation of stable synapses

Cell survival that is dependent on synapse formation

Evidence that motoneurone survival depends on muscle. During development, the normal loss of motoneurones from the lateral motor columns is greatly affected by alterations to the normal peripheral field of innervation. If limb buds are removed before motor axons grow out of the ventral roots, then the lateral motor column eventually degenerates [103, 121, 219, 236, 237]. For example, if chick limb buds are removed in the early embryo, the lumbosacral column continues to differentiate so that about 90% of the lateral motor neuroblasts are assembled. These deprived motoneurones develop dendritic processes on which synapses form; the cholinergic enzymes acetylcholinesterase and choline acetyltransferase develop normally up to the onset of degeneration; the motor roots are also normal at this stage. It follows that the proliferation of motor neuroblasts, together with their initial differentiation and axon outgrowth are not impaired by the removal of the peripheral field. Eventually, motor hypoplasia is observed which is due to the loss of motor neuroblasts; it is not due to impaired proliferation or enhanced migration away from the lateral motor columns [219]. The lateral motor column then degenerates.

Each motoneurone passes through three phases during development, which are defined by the reaction of the neurone to limb amputation [237]: amputation may have no effect on the neurone (phase I; small neurones with a thin rim of cytoplasm); or it causes it to degenerate within a few days (phase II; small bipolar neurones with little basophilic cytoplasm); or it may cause the neurone to chromatolyse first and only degenerate weeks or months later (phase III; neurones with large, well developed basophilic cytoplasm). The period for which cells in phase III can survive after amputation increases with maturity. Prestige [237] has argued that neurones in phase II die after amputation because

they are no longer obtaining from the leg an essential factor which is normally transported by the motor axons; phase III neurones are less dependent on the factor than phase II neurones.

Increasing the size of the field of innervation should increase motoneurone survival if a factor provided by the peripheral field of innervation is necessary for motoneurone survival. Supernumerary limbs provide such an increased field of innervation. In both amphibians and chicks motoneurone numbers are enhanced on the side of the spinal cord provided with a supernumerary limb; this amounts to an increase of ≅20% over that found on the contralateral control side [119, 145]. Although this percentage is less than may be expected (given the 100% increase in limb mass), factors such as the access of motor axons to the supernumerary and displaced limb and the access of appropriate motor axons to their particular limb segments may be limiting. In early limb bud removal experiments the deprived axons at first end in a tight mesh or tangle of nerve endings (neuroma) some distance from the body wall at the approximate site of the lumbar plexus [221]. Taken together, these observations suggest that the limb releases trophic influences which act at a distance and allow for the survival of motoneurones during development. However, direct evidence that it is the muscle of the limb which is responsible for these trophic effects is not available.

Muscle cells are dependent on a nerve supply. Both the amount of muscle that develops [46, 234] and the maintenance of mature muscle [102] are under neural control. Muscle cell death occurs if neuromuscular transmission is blocked during early development [87, 106, 289], or if muscles are denervated [39, 176]. This degeneration of muscle cells occurs because muscle activity is blocked: paralysis with tetrodotoxin also leads to muscle cell death [106]. Some muscle cell clusters [218] are still generated for a short period in the absence of a functional innervation, but these clusters do not contain secondary myotubes. It follows that the generation of primary myotubes is autonomous, whereas that of secondary myotubes depends on functional innervation [106]. Two classes of myoblasts exist during development: those destined to form primary myotubes and those that form secondary myotubes [46]. Thus a muscle mass is still laid down in the absence of a nerve supply during very early development [214, 321], even though the amount of muscle it contains is less than normal owing to the loss of myoblasts that give rise to secondary myotubes. The maintenance of these already differentiated primary muscle cells depends on the pres-

ence of a nerve supply at about stage 36 in chick: in the absence of nerves these muscle cells degenerate [214, 321].

Evidence that motoneurone survival depends on the formation of appropriate topographical connections in a muscle. The rostro-caudal position of a motoneurone in the lateral motor column of the spinal cord is related to the site of projection of its terminals in the limb [30, 49, 51, 152, 277, 293], so that motoneurones project onto the limb in a topographical manner [23]. Whether a motoneurone pool projects onto its own muscle in a topographical manner depends on the position of the muscle in the limb. For example, the rat soleus muscle occupies a position of complete overlap of the projection from two adjacent spinal cord segments (L_4 and L_5) to the limb: no topographical projection from the soleus motoneurone pool to its muscle then occurs. On the other hand, the rat semimembranosus, gastrocnemius and gluteus muscles occupy a position in which the projections from two adjacent spinal cord segment (L_4 and L_5) to the limb do not completely overlap: a topographical projection from the motoneurone pools of these muscles to their respective muscles does occur (Fig. 14B; [26, 27, 48]). Similar topographical projections occur in the rat for cervical segments C_5 and C_6 to the biceps brachii and pectoralis muscle [26]. Each intercostal muscle in the rat is innervated only by the adjacent segmental nerve [76]. Such topographical projections also occur in amphibia: in the frog, segmental nerves 8 and 9 form a topographical map on the glutaeus muscle (Fig. 14A); in the axolotl, segmental nerves 16 and 17 form a topographical map on the pubioschiotibialis muscle [183].

The topographical projections to these muscles during development have been analyzed with electrophysiological and contraction techniques. In the rat, topographical projections are not established until about one week postnatal in the glutaeus muscle [48], in the biceps brachii and pectoral muscles [26] or in the gastrocnemius muscle (Fig. 14B; [26]) In amphibia, the topographical projection to the frog glutaeus muscle does not emerge until stage 59 (Fig. 14A; [25, 28] and not until the premuscle mass splits in the pubioschiotibialis muscle.

A different method for ascertaining whether motoneurones project to appropriate muscle cells is provided by using the glycogen depletion method [139] to determine the distribution of fibers in single motor units. Each mature motor unit consists of fibers of the same type [139]. However, in the early postnatal period in rats, motor units are hetero-

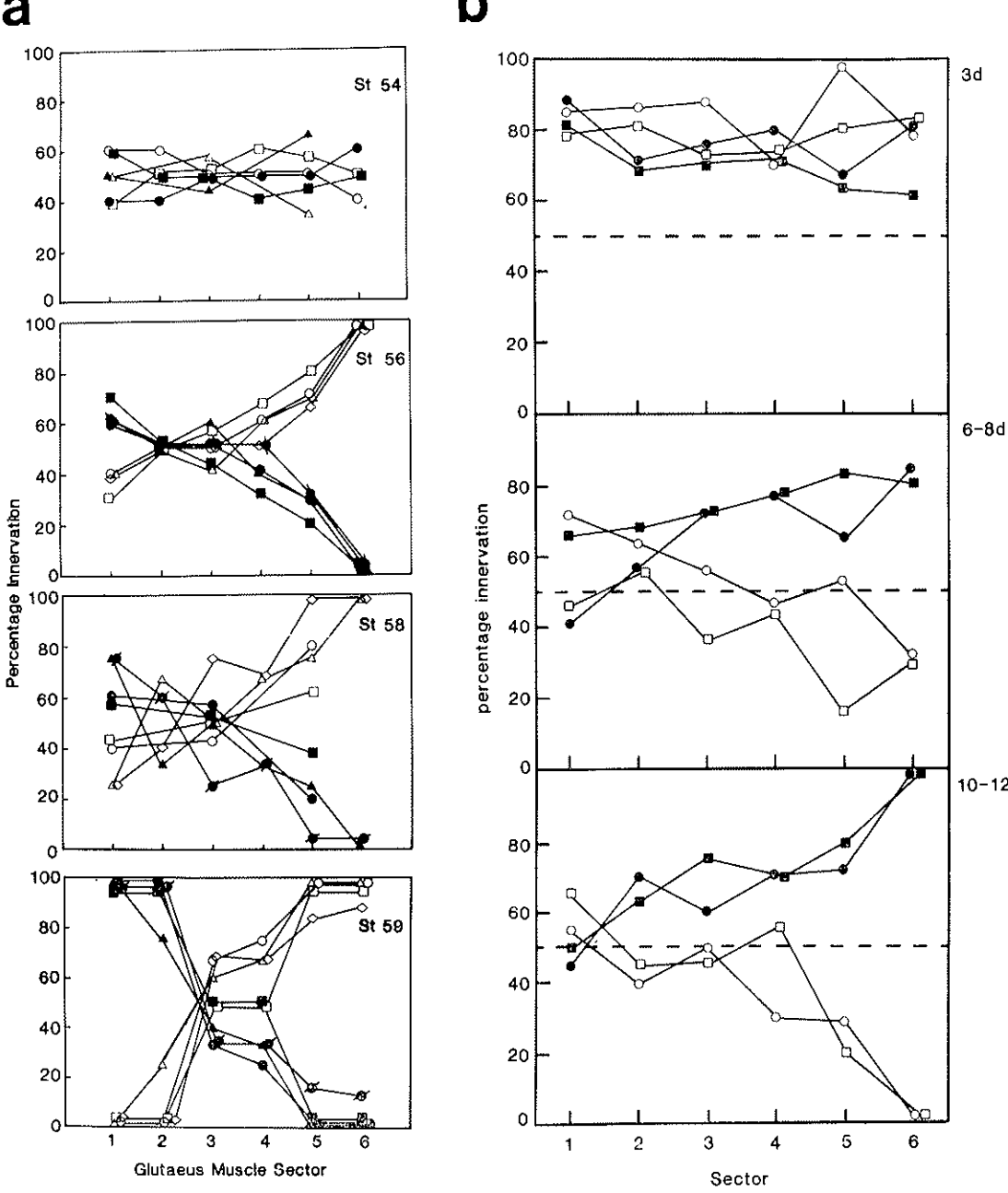

FIG. 14. Development of the topographical projection of motoneurones onto a muscle. *a*: the percentage of muscle cells in different sectors of the frog glutaeus muscle innervated by nerve 8 (open symbols) or nerve 9 (filled symbols) at the stage of development indicated. *b*: the percentage of muscle cells in different sectors of the rat lateral gastrocnemius muscle innervated by L4 (filled symbols) or L5 (open symbols) at the days postnatal indicated. Each symbol at a particular time of development gives the results for one muscle. At stage 54 in the frog and 3 days postnatal in the rat there is an extensive polyneuronal innervation in most sectors due to innervation of muscle cells by both segmental nerves; by stage 59 in the frog and 12 days postnatal in the rat, most of the polyneuronal innervation is eliminated; at this time, sector 6 of the frog glutaeus is only innervated by nerve 8 and sector 6 of the rat lateral gastrocnemius is only innervated by nerve L4. At this time the mature topographical distribution is established in both frog and rat muscles. (*a* is from [253]; *b* is from [261])

geneous although primarily of one type [302]. As the typing of primary myotubes in a muscle occurs independently of the nerve supply [52, 230], these observations suggest that motoneurones have made inappropriate synapses at this early stage of development.

In each of these cases, an approximate topographical map may he established by motoneurone death although no complete study has yet been performed to test this idea. The final sharpening of these maps is probably accomplished by the loss of polyneuronal innervation of synaptic sites [34, 47, 251].

Evidence that motoneurone survival involves competition in the establishment of topographical connections. Competition occurs hetween neurones in a motoneurone pool for connections with appropriate myotubes in a muscle during the period in which topographical projections are established. For example, the motoneurone pool to the rat lumbrical muscle gives rise to axons that enter the muscle via the lateral plantar and sural nerves. If the lateral plantar nerve is cut at birth, the sural nerve continues to innervate a much larger number of muscle cells than it would if the lateral plantar axons were present. Similar results have been obtained for the development of the innervation of the rat lateral gastrocnemius muscle hy L4 and L5 [118]: in this case, if L4 is cut at birth, then L5 axons innervate a much larger number of muscle fihers than they would if L4 had not been cut. Such competition does not seem to exist for muscles which do not receive a topographical projection, such as the soleus [47, 300].

Removal of part of the motor supply to a developing muscle not only ensures that the remaining motor nerves innervate a much larger portion of the muscle than normal, but also that motoneurone death is much less in the remaining motor supply. If, before the cell death period in avian embryos, a spinal cord segment is removed that contains part of a motoneurone pool, then there is an enhanced survival among the remaining neurones in the pool [147]. Partial deletion of two of the four segments containing the motoneurone pool to the chick iliofibularis muscle leads to a twofold enhancement of the number of motoneurones in the remaining two segments after the cell-death period is over. Unfortunately, estimates of the extent to which these deletions allowed the remaining nerves to innervate a greater than normal extent of muscle have not been determined. Evidence that motoneurones die as

a consequence of failure in competition with other motoneurones from the same pool is at present incomplete.

Effect of electrical activity on motoneurone survival. If a muscle is stimulated during the cell-death period, an increased number of motoneurones die [222]. In contrast, inactivation of muscles in chick embryos with either α-cobrotoxin, α-bungarotoxin, curare or botulinus toxin between E4 and E10 prevents the motoneurone death that usually occurs during this period; similar treatment begun after stage 38 has no effect on neurone numbers [233]. Even if a partial immobilization is continued after E10 (in embryos totally immobilized earlier), most of the excess neurones are maintained. If, however, administration of the immobilizing agents is stopped (allowing muscle activation to return to control levels), the excess neurones undergo a delayed cell death, and total cell numbers fall to between control levels by E18; resumption of neuromuscular activity after hatching does not result in a delayed cell death [220]. Thus the maintenance of motoneurones is determined by the activity of muscle and this is regulated differently before and after hatching. These observations on the survival of motoneurones can be explained if muscle cells release a growth factor in inverse proportion to muscle activity. Stimulated and therefore abnormally active muscle would release only a small amount of factor for competing motoneurones whereas inactive muscle would release large amounts, allowing for the survival of most competing motoneurones. Direct evidence for this idea has been obtained (see below).

Cell survival that is dependent on synapse formation on motoneurones

The extent of motoneurone death in the avian lumbar lateral motor column, following elimination of supraspinal-propriospinal inputs or the primary sensory inputs from the dorsal root ganglion, is normal at the end of the cell death period (E10) [215]. However, 25% of the motoneurones are lost in the following six days compared with controls (Fig. 15). An additional loss of motoneurones at the end of the cell death period also occurs in the amphibian spinal cord following removal of the dorsal root ganglion [74]. Removal of both the supraspinal-propriospinal and the primary sensory inputs in avian embryos gives an

additional loss of 37% motoneurones by E10, with no further loss after E10 (Fig. 15; [215]). Chronic treatment of deafferented embryos with curare from E6 to E9 or E10 to E14 prevents the normal loss of motoneurones during these stages, but does not affect the increased loss due to deafferentation [215].

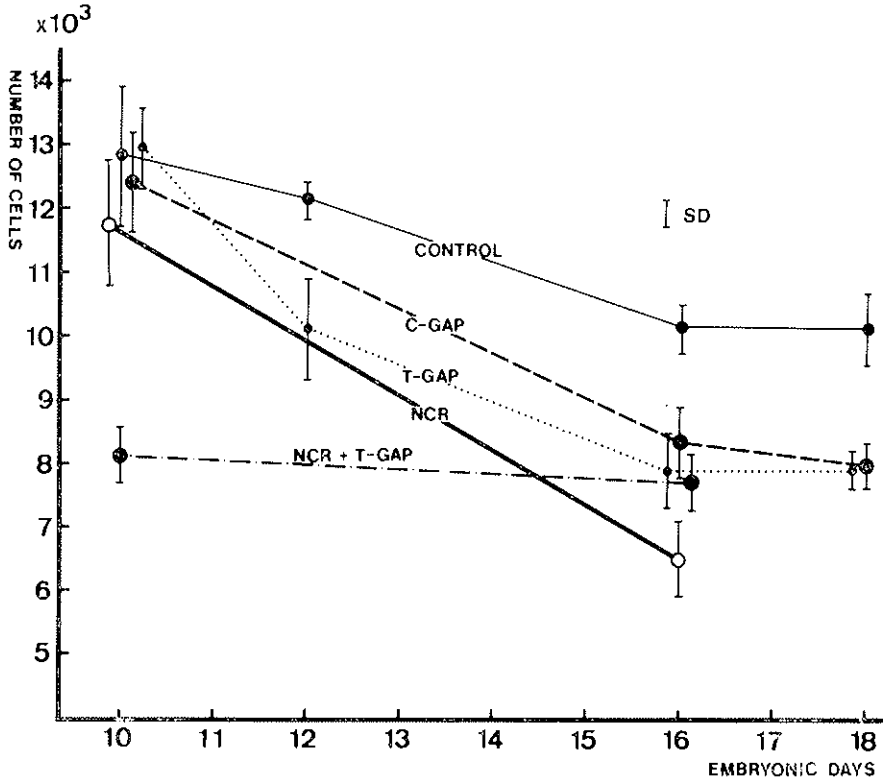

FIG. 15. Evidence that the death of avian motoneurones depends on their afferent innervation. The number of motoneurones (mean ± S.D.) is shown for the lumbar lateral motor column (L_1 to L_8) at different ages after the following embryonic microsurgery. The spinal cord was severed at the cervical (C-gap) or at the thoracic (T-gap) level on embryonic day 2 (E2) so as to eliminate supraspinal cord and/or propriospinal inputs to the lumbar cord; this results in an additional 25% loss of motoneurones between E10 and E16; the entire lumbar neural crest was removed (NCR) in order to eliminate primary sensory inputs arising from the dorsal root ganglion; this also results in an additional 25% loss of motoneurones between E10 and E16. Both T-gap and NCR operations were performed (NCR + T-gap) so as to remove both descending and sensory afferents; this results in an additional loss of motoneurones by E10. (From [215]).

Trophic factors released at synapses that mediate motoneurone survival

Identification of motoneurones in culture and their degeneration

Motoneurones of the lateral motor column can be identified on cultures of dissociated spinal cord if horseradish peroxidase (HRP) is injected into limb muscles prior to dissociation of the cord: motoneurones can then be recognized by appropriate histochemical staining (Fig. 16; [24]). Motoneurones have also been identified by fluorescence using a conjugate of HRP and lucifer yellow; this has allowed for their isolation from other spinal cord cells with fluorescent-activated cell sorters [8, 195].

A monoclonal antibody (NRC2G10) which only labels large Golgi-type I neurones in the avian spinal cord, such as motoneurones, has been generated [9, 56] which is mostly localized to the lateral and medial motor columns of stage 26 embryos. Identification of motoneurones with HRP-lucifer yellow has confirmed that NRC2G10 labels these cells in culture. Motoneurones labelled with either NRC2G10 or HRP, degenerate when cultured in a minimal media; only 50% of the cells remain at 24 hrs in either homogeneous cultures of motoneurones or in heterogeneous cultures which include other spinal cord cells [8, 24].

Embryonic motoneurones are maintained by a soluble factor from astrocytes

It is known that certain classes of neurones can he maintained alive in tissue culture if they are cocultured with glial cells. Astrocytes isolated from the corpus callosum of adult rats support the survival of sensory and sympathetic ganglion from chicks and rats [164]. Furthermore, as noted above, astrocytes from the superior colliculus or cortex can support retinal ganglion cells in culture [180]. Identified motoneurones from 6 d lumbo-sacral spinal cord are maintained alive in culture if they are grown on a monolayer of flat and immature astrocytes from cortex or spinal cord (Fig. 16; [82]). Furthermore, media which has been previously conditioned by contact with immature astrocytes will also allow for the survival of embryonic motoneurones [82]. Mature and process-bearing astrocytes fail to support neurones through contact or the release of a soluble factor. As motoneurones mature they are no longer maintained by astrocytes: motoneurones from 10 d lumbo-

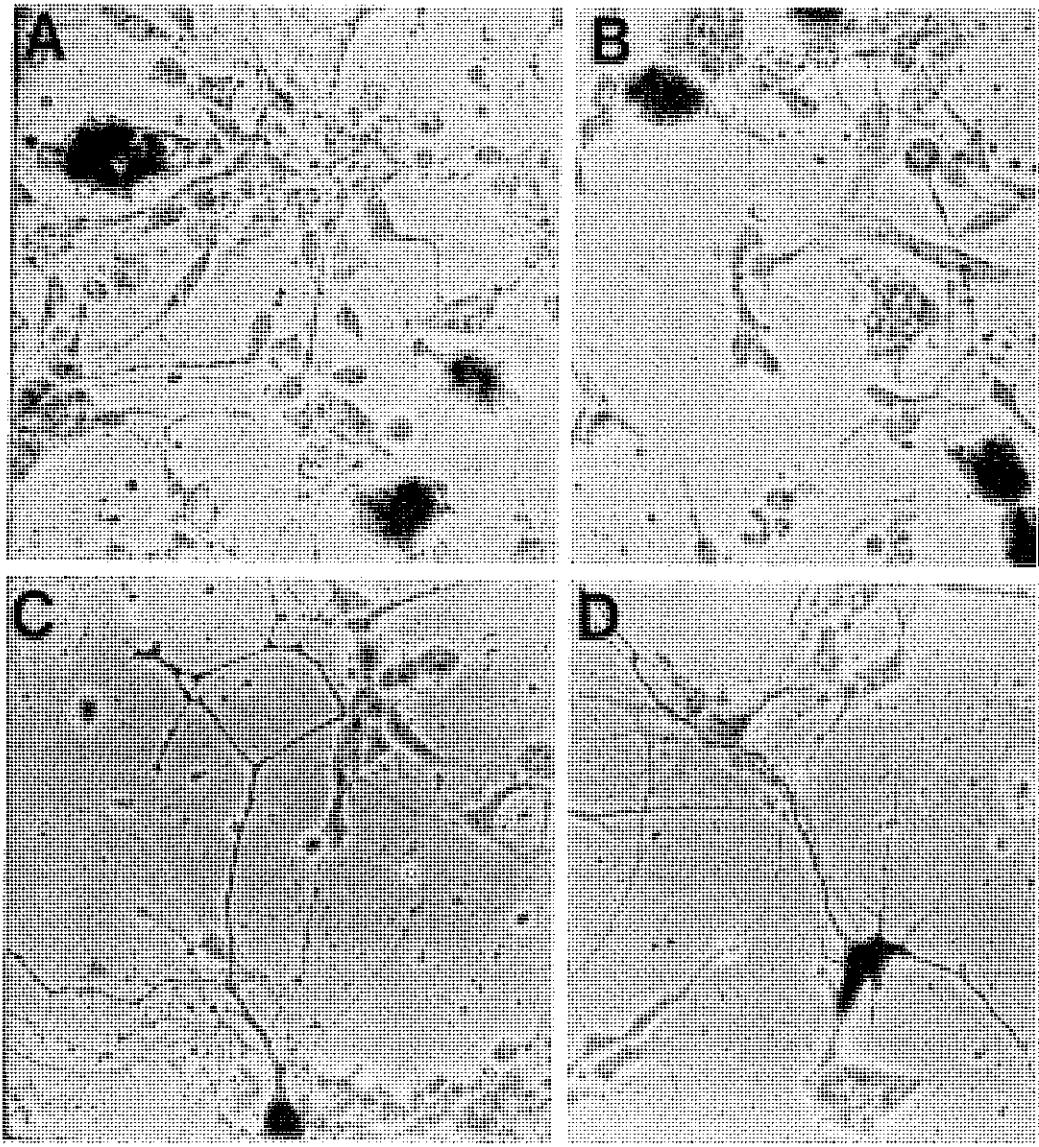

FIG. 16. Identified E6 avian motoneurones in culture. Motoneurones previously labelled "in vivo" with h radish peroxidase are shown after culture for 24 hrs in medium conditioned over predominantly flat imm astrocytes from the spinal cord. Note the large cell bodies of the labelled neurones in comparison with background cells. Some motoneurones have either short or no neurites (A and B), whereas others have neu several times the cell diameter in length (C and D). All photomicrographs were taken with phase con optics. (From [82]).

sacral cord degenerate over 24 hrs in culture in the presence of immature or mature astrocytes (Fig. 17; [83]).

Embryonic motoneurones are maintained by a soluble factor from Schwann cells

Cell cultures which consist of a relatively high proportion of motoneurones have been developed: these consist of dissociated spinal cord cells from E4 chick; at this time a relatively large number of cells in the spinal cord are motoneurones. Media conditioned over mouse Schwann

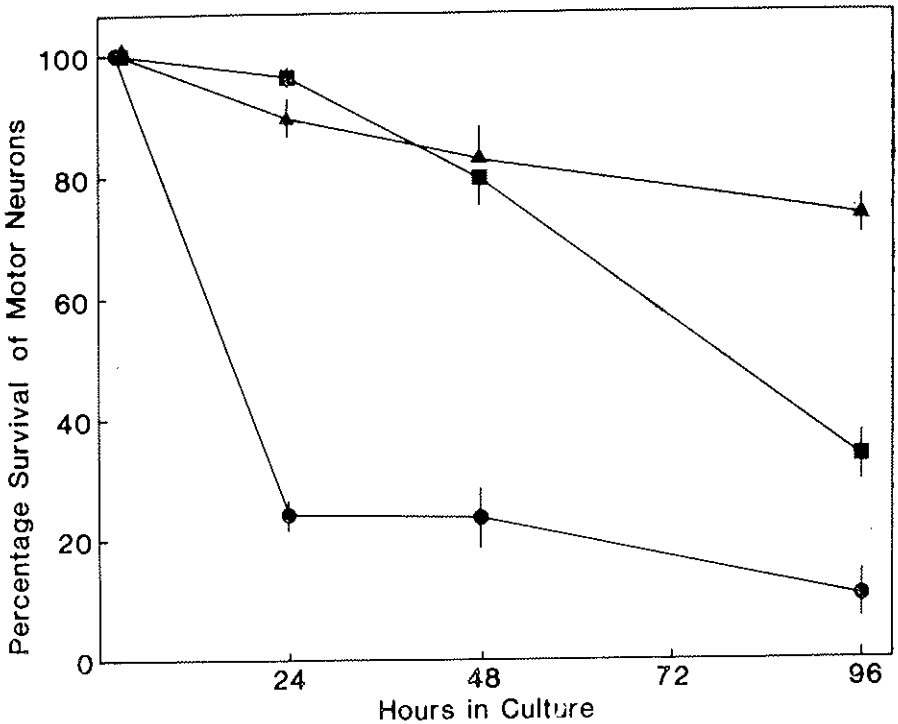

FIG. 17. The survival of E6 avian motoneurones in different conditioned media for different periods of time. Squares give the results for motoneurones in a minimal control medium; triangles for a muscle conditioned medium; circles for an astrocyte conditioned medium. The number of motoneurones present at 2 hrs was taken as 100%; each point represents the mean (± S.E.M.). Note that the astrocyte conditioned media cannot sustain the motoneurones as they age over several days in culture whereas the muscle conditioned can maintain survival over this time. A supply of fresh astrocyte conditioned media during the period the motoneurones are in culture does not assist in their survival. (From [82]).

cells and rat RN22 Schwannoma cells are able to sustain these neurones in culture; in contrast, neither NGF nor a neurotrophic factor from the heart can maintain the cells [166]. These effects are dependent on the density of spinal cord cells plated [174]: in low density cultures, survival for even 1 d is dependent on added RN22 Schwannoma conditioned medium; this medium cannot sustain these cultures for longer periods. As no neurones survive in high density cultures with Schwannoma conditioned medium over 5 d, this medium must be toxic for the neurones [174]. RN22 Schwanoma medium has a particularly powerful effect on preventing the degeneration of identified motoneurones in low density cultures over 1 d [114]; it is not known to what extent this effect is dependent on the age of the motoneurones.

Embryonic motoneurones cultured with astrocytes or Schwann cells express neurites

Peripheral neurones show a dramatic increase in neurite initiation if they are plated onto a polyornithine substratum which has been previously exposed to a medium conditioned from a wide variety of tissues [65]. This substratum-bound neurite promotion factor is distinct from neurone survival factor which is not bound to polyornithine and remains soluble [65]. Peripheral neurones from such sources as E8 chick ciliary ganglia and dorsal root ganglia, E11 chick sympathetic ganglia and neonatal mouse dorsal root ganglia respond in this way; the only central neurones which respond are large spinal cord cells, which are probably motoneurones [2, 119]. Media conditioned over chick astrocytes, mouse Schwann cells and rat Schwannoma cells give rise to this effect; other cell types with activity are chick beart and bovine corneal endothelial cells, as well as chick skeletal muscle (see helow) [2, 150, 207]. When a sharp border is created between a region of polyornithine substratum coated with this factor and a region coated witb unconditioned medium, neurites fail to cross this border; rather, they change their direction of outgrowth so as to remain on the conditioned substratum[65]. The preference of neurites to grow on a suitable substratum is illustrated by observations of Adler and Varon [4]: in the absence of exogeneous neurite promotion factor, explanted ciliary ganglia send out neurites for only a short radial distance before they assume a circular or tangential growth pattern. These authors have shown, by preloading the ganglia with ^{14}C leucine, that the explanted ciliary ganglia are able

to produce material endowed with neurite-promoting properties; this material coats the polyornithine substratum in the immediate vicinity of the ganglia.

Neonatal motoneurones are maintained by a soluble factor from muscle

Evidence from explants of spinal cord and muscle in culture for trophic factors

Neurite outgrowth from rat or chick embryo spinal cord explants is also potentiated by embryonic skeletal muscle conditioned media (Fig. 18; [79, 213, 296]). The outgrowth from 15 d fetal rat spinal cord is not enhanced by NGF or fibronectin [79]. Media conditioned by rat, mouse or chick muscles or rat lung fibroblasts are effective in producing neurite outgrowth from the 15 d fetal rat and embryonic chick spinal cord, although rat and mouse conditioned media are more effective [80].

Preincubation of chick skeletal muscle conditioned media over poly-l-lysine removes the ability of the media to induce neurite expression from embryonic chick spinal cord explants; separable factors exist in muscle-conditioned media for the survival and expression of neurites from these explants [207]. There is an increase in the amount of this poly-l-lysine bound neurite promotion factor produced by chick limb muscle between E5 and E11 when the production of factor declines [208].

Extracts of embryonic avian muscles, immobilized by curare, are more effective than extracts of normal muscle in accelerating the number of neurites, neurones and glial cells that migrate out of an explant of spinal cord [120]. Whether this reflects an enhanced survival effect is not clear.

Neonatal motoneurones in culture are maintained by soluble factors from muscle

Skeletal muscle conditioned media (from E10 chicks) contain neurotrophic activity which supports the survival of identified avian motoneurones (from E9 chicks) in culture (Fig. 19; [24]). Similar results have been obtained for cholinergic enriched fractions of cells isolated from 12 d rat embryos on iso-osmotic metrizamide density gradients [260]: these cells, which presumably include a large proportion of motoneurones, only survive and develop in the presence of muscle cells or in muscle

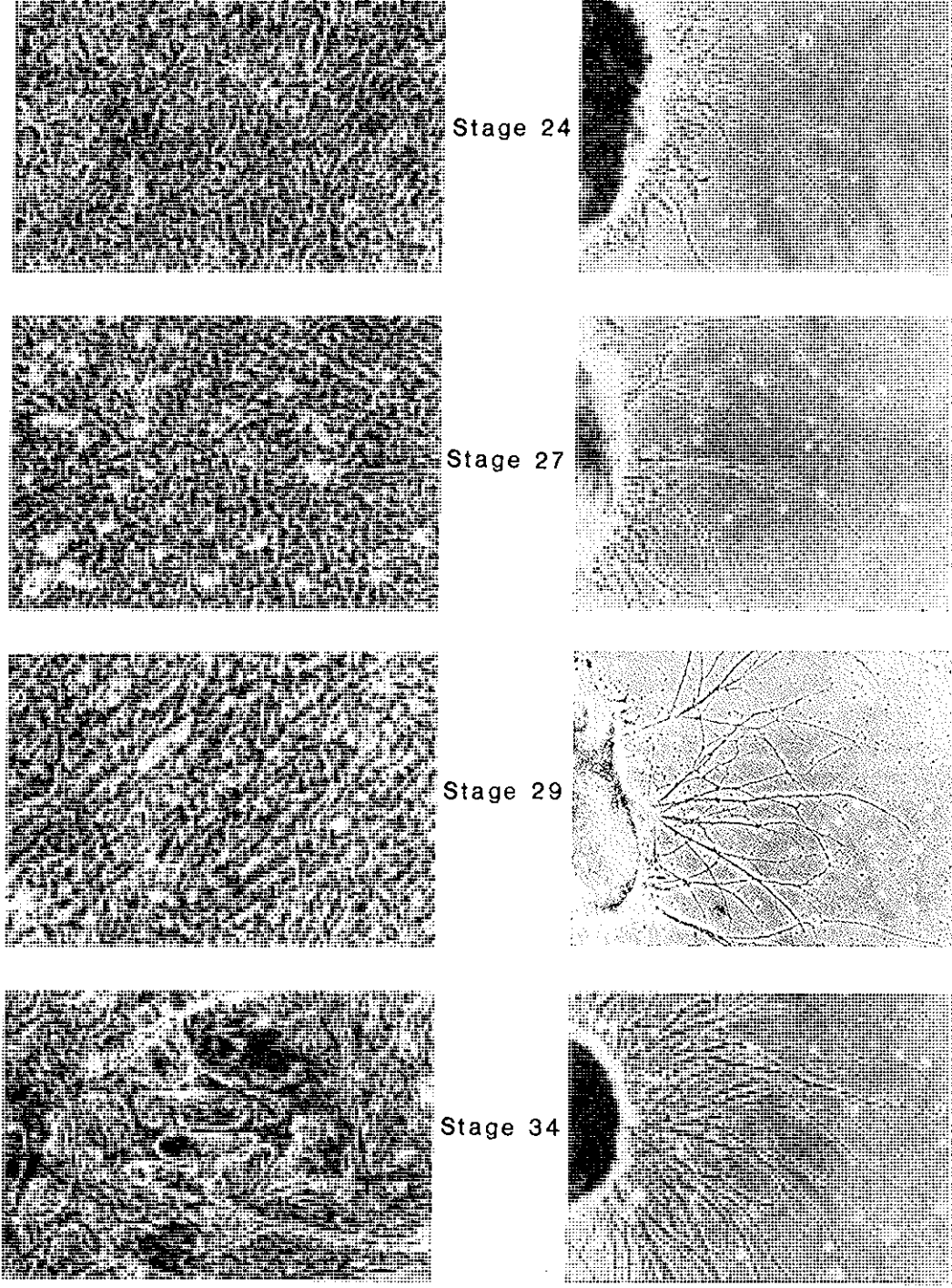

FIG. 18. The effect of conditioned media from different aged premuscle cell masses in the avian limb the outgrowth of neurites from spinal cord explants. Photomicrographs in the left-hand column show fluent layers of premuscle cells from which batches of conditioned media were obtained. Photomicrograph the right-hand column show the spinal cord explant response; this was dependent on the maturation of premuscles and not on that of the spinal cord explant. This response reached a maximum for stage 35 muscles and thereafter declined. Note that myotubes have begun to form by stage 27. Calibration

conditioned media. Tanaka [295] has recently shown that the survival of chick-embryo spinal neurones capable of forming synapses on skeletal muscle cells "in vitro" is enhanced by serum-free skeletal-muscle conditioned media. Presumably a high proportion of these neurones are motoneurones, although preganglionic neurones (which are known to form synapses on muscle cells "in vivo"; [32]) also form synapses. Dissociated spinal cord cultures from E4 chicks, which contain a relatively large number of motoneurones (see above), are sustained in culture by media conditioned over chick embryo skeletal muscle [166]. Neither NGF nor a factor that maintains ciliary ganglion neurones can sustain these cells.

Dissociated spinal cord cells from E4 chicks show a 3 to 4 fold increase in the amount of high affinity choline uptake between 3 and 15 days in culture [12, 38]. Both the magnitude of the uptake and the number of ^{3}H-choline labelled neurones are the same for spinal cord

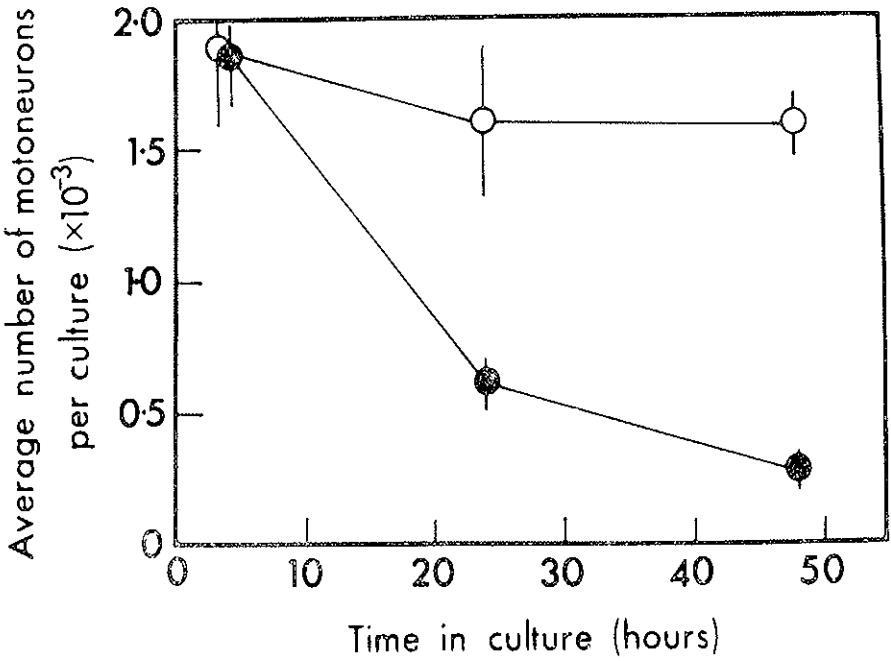

FIG. 19. Average decline in the number of horseradish peroxidase labelled motoneurones in culture from avian embryonic lateral motor column. Filled circles, minimal control media; open circles, skeletal muscle conditioned media. Each point gives the mean ± S.E.M. (From [24]).

cells grown with and without skeletal myotubes. The choline acetyltransferase activity of neurones in these dissociated cultures is enhanced by their co-culture with muscle cells [196], with media conditioned over muscle-cell cultures [94] or extracts of skeletal muscle [286].

Maintenance of motoneurones by soluble factors from myotubes. Myotubes provide the most effective source of motoneurone survival factor in embryonic muscle; the activity of myotubes is greater than that of myoblasts or fibroblasts (Fig. 20; [211, 212]). Furthermore, myotubes have more survival enhancing activity than spinal cord cells (see below; [54]).

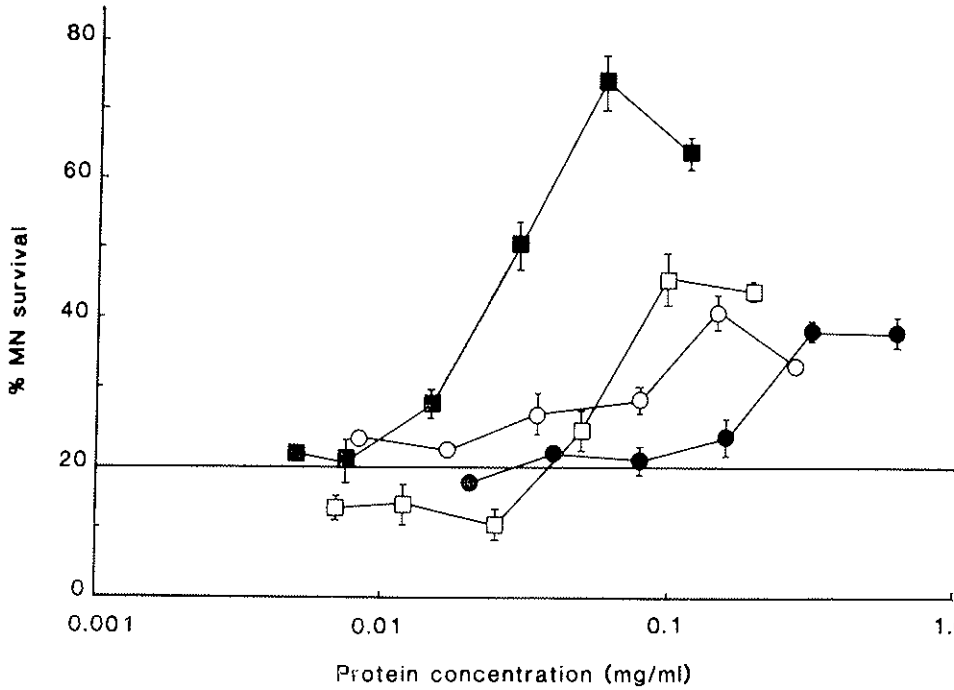

FIG. 20. The dose-responses for the survival of labelled motoneurones in serially diluted conditioned medium (CM). The rates of survival are depicted for myotube CM (filled squares), myoblast CM (open squares), fibroblast CM (open circles) and mesenchyme CM (filled circles). Each set of symbols represents a mean determination from at least 4 preparations (± S.E.M.). The S.E.M. for all points in the lower graph is less than 3%. The horizontal lines in both graphs denote the averages obtained in control media. (From [212]).

Enhanced survival of motoneurones by factors from denervated muscle cells: Extracts from adult rat muscles denervated for a period of about 4 d have optimum survival effects on identified avian embryo (stage 36 or 10 d) motoneurones [211]; extracts from innervated muscle do not have any survival effects above that of the control. This result is specific for motoneurones, as the interneurones do not survive for long periods (48 hrs) in the experimental assay, and only slightly elevated numbers are present compared with controls at 24 hrs. The origins of the factor in muscle responsible for this enhanced survival have been investigated: media supplemented with soluble extract from endplate containing regions of denervated muscle enhance the survival of motoneurones over that of extracts from non-endplate regions of innervated muscle [281].

The effects of denervation in enhancing survival activity are probably due to the cessation of electrical activity in the muscle cells: direct stimulation of denervated muscle cells blocks their enhanced production of survival activity [115].

Neurite expression initiated in motoneurones by factors from myotubes. The expression of neurites by identified motoneurones from E6 chicks as well as of dissociated spinal cord cells from E4.5 chicks and 14.5 d embryonic rats can be greatly enhanced with extracts or conditioned media from embryonic skeletal muscles [2, 110, 149, 211, 212, 213, 286]. The factor in this material is ineffective on neurones if they have been in contact with their target cells for some time [66], but can be restored if the neurones are subsequently maintained in culture for 4 d.

Maximum activity of the factor is found in myotubes rather than myoblasts or fibroblasts [211, 212]. The factor contains heparin sulfate proteoglycan (see below). If messenger RNA from cultured myotubes is injected into oocytes that post-translationally modify and export proteins, then heparin sulfate proteoglycans appear in the media conditioned by the oocytes [138]. This provides direct evidence for the production of this proteoglycan by myotubes.

The activity of the neurite promotion factor from embryonic muscle is maximum with skeletal muscle from 18 d fetal rat and E9 to E12 chicks; it then declines by 80% in the subsequent 3 weeks [208, 286]. At this time a physically different neurite promotion factor is active, which unlike the embryonic factor has a high affinity for tissue culture plastic [112].

The neurite promoting activity for spinal cord neurones, and motoneurones found in extracts of embryonic chick skeletal muscle is greatly elevated after 3 d of denervation [111, 115].

Neonatal motoneurones are maintained by a soluble factor from the spinal cord

Low density cultures of dissociated E4 chick lumbar cord cells degenerate in 1 d [174]. When conditioned medium from high density cultures of dissociated E4 chick lumbar spinal cord is supplied to low density unsupplemented cultures of spinal cord, the neurones survive for five days [174]. Dissociated spinal cord cultures from E6 to E7 chicks are maintained by extracts of spinal cord [294]. It is not clear if the source of this survival-enhancing factor is from neurones or astrocytes within the spinal cord. If it is from neurones, then it may be the factor supplied by the afferent innervation of motoneurones which contributes to their survival.

Identification of trophic factors for motoneurones

Growth factors

Column chromatography shows that skeletal muscle extracts of embryonic (21 d) rat limbs, which provide the survival of identified motoneurones, elute at a molecular weight of about 40,000 daltons, with an additional peak at 140,000 daltons [116]. Similar results have been obtained for the survival of certain classes of dissociated spinal cord cells. Using the outgrowth of neurites from 15 d fetal rat spinal cord as a measure of activity in rat skeletal muscle media, Dribin and Barrett [79] showed that a glycoprotein had maximum activity that is negatively charged at neutral pH and elutes after column chromatography at a molecular weight of about 50,000 daltons. These observations support the existence of a survival factor at about 40,000 to 50,000 daltons, if neurite outgrowth from spinal cord explants gives a measure of cell survival. Recently, Gurney [101] has produced a rabbit antiserum against a protein of molecular weight about 50,000 daltons which is secreted by denervated rat muscle. This antiserum suppresses botulinum toxin-induced terminal sprouting in the mouse glutaeus muscle. It may be that this factor is the same as the motoneurone survival factor.

An alternative approach to the isolation of motoneurone survival activity has used a complementary DNA for NGF (cDNA-NGF) to probe in muscle for a messenger RNA (mRNA) with homologous sequences to that of NGF [126]. Dot hybridization on nitrocellulose has shown that an mRNA homologous to nerve growth factor mRNA is detectable in embryonic but not in adult muscle. Furthermore, this mRNA appears in greatly increased amounts in adult muscles after they have been denervated for 4 d, but not before that time. As motoneurone survival activity is maximum in muscle at these times it may be that the mRNA for motoneurone survival factor is detected by the cDNA-NGF.

The survival enhancing effects of muscle cell extracts and conditioned media may be mediated by their increasing the cyclic GMP content of the motoneurones. Such an increased cyclic GMP is known to enhance the survival of motoneurones through the cell death period [312].

Neurite expression factors

The factor from astrocytes, Schwann cells, myotubes and various peripheral tissues which initiates neurites in isolated peripheral neurones, large spinal cord neurones and motoneurones consists of a complex of several proteins, heparan sulfate proteoglycan and the glycoprotein laminin; it has a molecular weight of several million (Fig. 21; [110, 149, 151, 166]). The neurite promoting effects of this complex are mediated by laminin (Fig. 22). This glycoprotein consists of a long arm (≅77 nm) and three morphologically similar short arms (≅36 nm) joined by disulfide bonds. These rod-like arms terminate in and are interrupted by seven or more globular domains of larger diameter which possess specific binding properties [88]: the terminal globular domain of the long axis binds the heparan sulfate whereas the globular domains of the shorter axons are involved in binding the basal lamina collagen type IV [303]. Thus laminin functions as a mediator between collagen and heparan sulfate in basement membranes and their adherent cells. This probably occurs for two reasons: firstly because an integrated unit is formed by the binding together of type IV collagen, laminin and heparan sulfate proteoglycans; secondly, because laminin in its most extended form exceeds the width of basement membranes so that some of its domains are probably well away from the basement membrane itself. The neurite promotion action of laminin is mediated by the heparan sulfate-binding globular domain at the end of the long arm of the laminin molecule [85]:

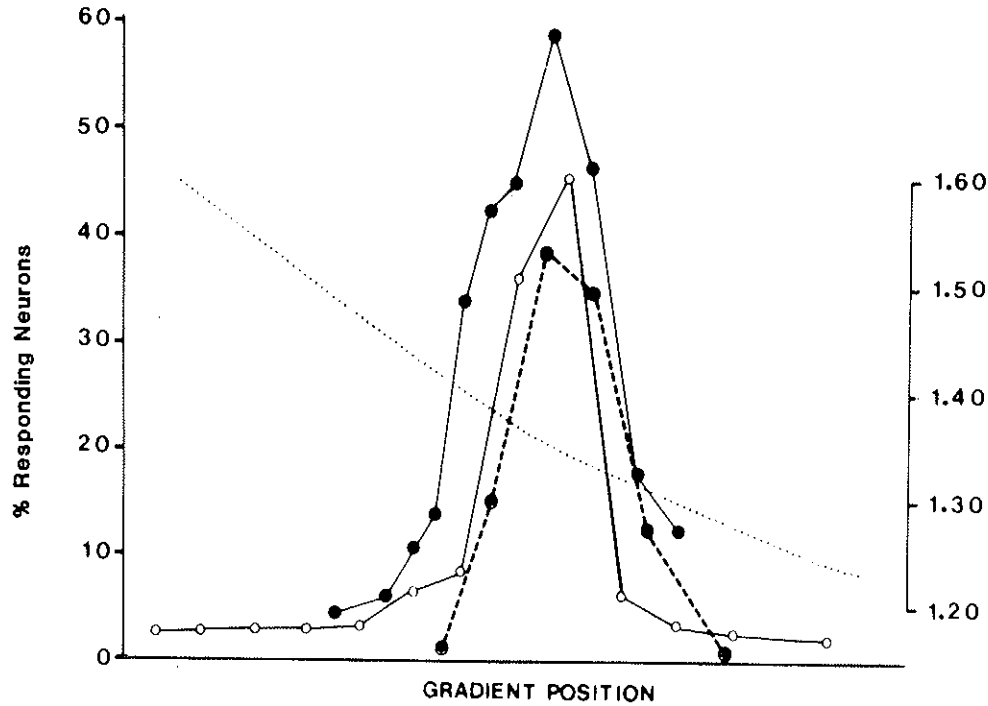

FIG. 21. Isopycnic sedimentation in associative CsCl gradients of the motoneurone neurite promotion factor released by skeletal muscle myotubes. Conditioned medium from different cell types was centrifuged to equilibrium in CsCl containing 0.4 M GuHCl. Fractions were collected and the density of each was measured (...). After dialysis fractions were assayed for neurite outgrowth-promoting activity. Neurite outgrowth from motoneurones was assayed from embryonic chick myotubes (●—●); in addition, neurite outgrowth from sympathetic neurones was assayed from bovine corneal endothelial cells (● - - - ●) and P cells (o—o), and unknown primary cell line whose antigenic properties and morphology suggest they are derived from pericytes. Note that the gradient position for these different sources of neurite promotion material is the same. (From [151]).

antibodies directed against this domain inhibit the neurite promoting effects of laminin.

Laminin occurs in all basement membranes [156, 254]. Within the central nervous system anti-laminin antibodies indicate that the glycoprotein is confined to the external basal lamina and blood vessels [42, 89, 159, 173, 253].

Following the localized injection of neurotoxin into mature nervous tissue, reactive astrocytes which resemble immature astrocytes synthesize laminin [160]. Indeed, cytoplasmic laminin is detectable in cultured

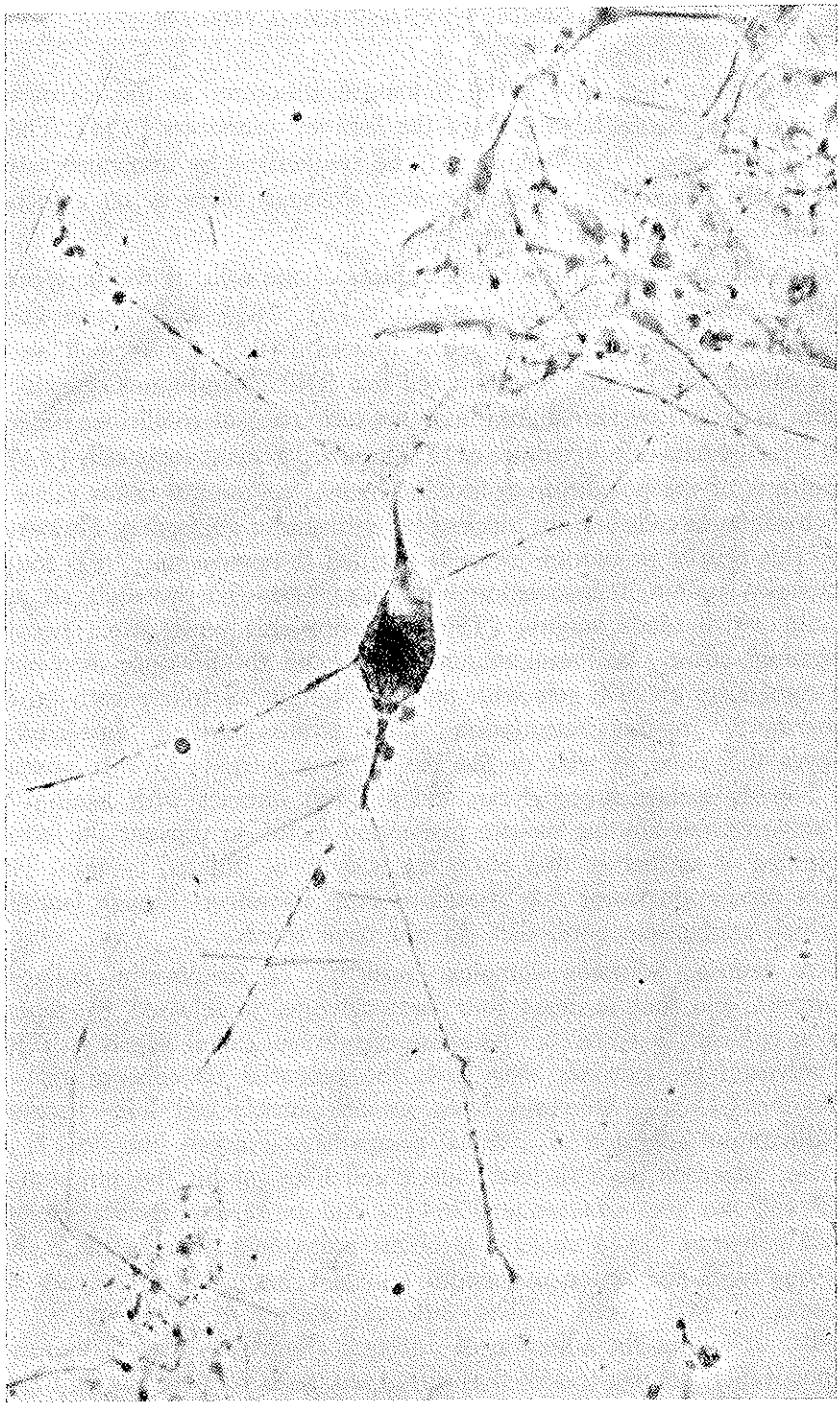

FIG. 22. Motoneurones, identified by horseradish peroxidase labelling, express neurites when cultured on a substratum of the glycoprotein laminin. (From [316]).

astrocytes when they have a flat immature structure; as the astrocytes mature to the process bearing stage the laminin disappears [158]. These observations suggest that laminin may be synthesized by immature astrocytes in the developing nervous system, although there is no evidence for this using antilaminin antibodies.

Laminin is found in the basal laminae of the cells of the peripheral nervous system, including Schwann cells [67, 183] and muscle cells. Schwann cells produce laminin when injured [41, 165] as well as in tissue culture [67]. The Schwann cell line RN22 Schwannoma produces laminin as shown by antilaminin immunoprecipitates of conditioned media from Schwannoma cells in culture or of solubilized Schwannoma cells [224]. The effects of the polyornithine-binding neurite promotion factor released by Schwannoma cells are not blocked by antilaminin antibodies. However, when this neurite promotion factor is purified to homogeneity, it is found to contain laminin in association with heparan sulfate-proteoglycan and entactin which binds laminin [150]. During this purification, neurite promoting activity, laminin-immunoreactivity and the presence of a band that comigrates on SDS gels with purified laminin all follow each other; furthermore, immunoprecipitation with affinity-purified anti-laminin removes this band as well as the biological activity [73]. It appears that heparin sulfate proteoglycan and entactin interact with laminin in the polyornithine-binding neurite promotion factor to prevent the access or binding of antibodies that block the activity of isolated laminin. This neurite promotion factor may therefore exert its effect through laminin, to which antilaminin antibodies do not have access.

A neurone adhesion complex consisting of fibronectin, collagen, hyaluronic acid and at least four proteoglycans is released by muscle cells in culture [261, 267]. As these complexes, called adherons, cause the adhesion of neurones they have the potential to enhance neurite expression [157]. Adherons from smooth muscle cell lines promote cell-substratum adhesion of a clonal sympathetic-like cell line, PC12; in contrast, adherons from skeletal muscle cell lines inhibit the adhesion of PC12 cells to the substratum [264]. An antiserum against skeletal muscle adherons blocks skeletal myoblast adhesion to a substratum but does not block the action of smooth muscle adherons in promoting the adhesion of PC12 cells. Thus different classes of adherons act to promote or inhibit the adhesion of different classes of cells. This raises the possibility, not yet tested, that different classes of adherons will have the capacity to induce neurites in different classes of neurones.

SPECULATIONS ON THE ROLE OF GROWTH FACTORS IN ESTABLISHING TOPOGRAPHICAL PROJECTIONS BETWEEN MOTONEURONES AND MUSCLE

Observations on motoneurone synapses and cell survival

The initial differentiation of motoneurones in the lateral motor columns and the growth of motor axons are not dependent on their gaining access to limb-material. However at some time which has not yet been determined, motoneurones do become dependent on making contact with limbs. Mature motoneurones depend for survival on maintaining contact with muscles in limbs. Isolation of motoneurones from the limbs leads to their degeneration and takes longer the more mature the motoneurone. Trophic support from the peripheral field of motoneurones is therefore required for their survival during development and in maturity.

Most mature motoneurone pools form a topographical map over their respective muscles. Evidence has been presented that this topographical map is not established until the elimination of multiple innervation of synaptic sites is complete. It has not yet been determined if the elimination of misprojecting motor axons within a muscle is accompanied by motoneurone death. Nevertheless, it is clear for many muscles that motoneurones within a pool compete for synaptic sites in their muscle. Elimination of one of the segmental nerves subserving an embryonic motor pool leaves its muscle entirely innervated by the segmental nerves subserving the remaining motoneurones; inappropriate projections are not then eliminated. Following operations of this kind, there is a decrease in the normal cell death occurring in the remaining pool. These observations suggest that competition between motoneurones within a pool for appropriate space within a muscle is involved in motoneurone death. Unfortunately, no careful analysis of both cell death and misprojecting axons following removal of a segmental nerve has been carried out for a single muscle. The most likely scenario is that grossly inappropriate topographical projections are eliminated early by motoneurone death and that more precise projections are established during the later phase of the loss of multiple innervation of synaptic sites.

Synapses are formed on myotubes by the earliest projecting motor axons, probably before the cell death period even begins. It seems likely that embryonic motoneurones are competing for space within their muscles, and that this space is located at the synaptic site on myotubes. However, direct evidence is still lacking, for even a single muscle, that

motoneurones which are eliminated from inappropriate synaptic sites subsequently degenerate.

An afferent innervation of avian motoneurones is present during the cell death period. It is clear that removal of this innervation can increase the loss of motoneurones. This indicates that motoneurones must obtain adequate afferent and efferent synaptic connections to survive embryogenesis.

Observations on the survival of cultured motoneurones

The identification of motoneurones in culture has enabled determination of the effects of glial cells and myotubes on motoneurone development. Immature astrocytes from the spinal cord release a soluble factor which allows for the survival of E6 avian motoneurones; this factor is not produced by mature astrocytes. As the motoneurones mature (by E10) they are no longer maintained by this factor. However, Schwann cells release a soluble factor which maintains E6 motoneurones the age dependency of this support has not yet been determined. It follows that either immature astrocytes or Schwann cells could be responsible for supporting motoneurones as they project to their muscles; after that time motoneurones must receive support from cells in the limbs, probably located in muscle. There is support for the possibility that neurones which provide an afferent innervation to motoneurones do this by means of soluble trophic factors rather than by depolarization during synaptic transmission.

All the cells in the immediate environment of motoneurones synthesize the glycoprotein laminin or a complex of proteins that contain heparan sulfate proteoglycans and laminin; these cells include immature astrocytes, Schwann cells and myotubes. The growth of motor axons to their targets may be assisted by this glycoprotein. Laminin, coated on the substratum of a culture plate, has powerful neurite promoting effects on dissociated motoneurones. Complexes of fibronectin, collagen hyaluronic acid and proteoglycans are released from myotubes: these complexes induce neurites in motoneurones and may help guide them to myotubes.

Motoneurones survive through the cell death period if provided with a factor from embryonic muscle. Myotubes are the main source of this factor, although survival enhancing material is also obtained from myoblasts and fibroblasts. Inactivation of muscle cells by denervation or

blocking synaptic transmission greatly enhances their production of survival factor. A glycoprotein that has trophic effects on motoneurones has been partially characterized: it is negatively charged at neutral pH and has a molecular weight in the range 40,000 to 50,000 daltons.

The main hypothesis in this study is that motoneurones compete for synaptic sites on muscle cells and thar motoneurones which fail in this competition are not able to obtain sufficient amounts of a factor necessary for their survival. Unfortunately, there is only indirect evidence for this proposition: no experiments have been done on a single embryonic muscle which show that following removal of part of its motoneurone pool, before the cell death period, there is an enhanced survival of motoneurones in the rest of the pool and that these innervate all of the muscle. Furthermore, although cultured motoneurones are maintained by soluble factors from myotubes, their survival is also enhanced to a lesser degree by myoblasts, fibroblasts and even undifferentiated mesenchymal cells within the developing limb bud. There is no evidence for a qualitatively different effect of myotubes on motoneurone survival than that exerted by other muscle cells. Although the most likely hypothesis is that motoneurone survival is contingent on their forming stable synapses and therefore obtaining access to a growth factor, convincing evidence in support of this is still required.

Summary: Trophic interactions at developing synapses and cell survival

The following points are offered as generalizations, based on the study of embryonic retinal ganglion cells and motoneurones.

1. Embryonic central neurones are maintained viable during the period of axon growth to targets by immature glial cells in their immediate vicinity (Müller cells for retinal ganglia and immature astrocytes for motoneurones).

2. Axon initiation is promoted by complexes of glycoproteins including laminin. These are synthesized by immature glial cells (Müller cells for retinal ganglia and both reactive astroglia and Schwann cells for motoneurones).

3. As central neurones mature they become dependent on their peripheral field of innervation for survival. At this time, astrocytes have

matured to a stage at which they can no longer support neurones. Also as neurones mature they are no longer capable of being supported by even immature glial cells. (Müller cells cannot support mature ganglion cells and immature astrocytes cannot support mature motoneurones).

4. The target cells on which central neurones synapse are the principal if not unique source of survival factor in the peripheral field of innervation. (Only retino-recipient neurones release a survival factor for ganglion cells; myotubes are the principal source of survival factor for motoneurones).

5. The amount of survival factor provided by these target cells declines with maturation, as the dependence of central neurones on their target cells declines. However, the amount of survival factor provided by maturing target cells is inversely proportional to their electrical activity: cessation of activity leads to enhanced survival factor synthesis. (Ganglion cell survival is enhanced following probable reduction of target cell activity after injection of tetrodotoxin into an eye; motoneurone survival is increased if muscle is made inactive with neuromuscular blocking drugs).

6. There is increasing evidence that central neurones with grossly inappropriate topographical projections are eliminated by cell death. (Direct evidence for the ipsilateral misprojections from retina to colliculus and geniculate; indirect evidence from different experiments supporting this notion during elimination of grossly inappropriate topographical projections from motoneurone pool to muscle).

7. The most likely hypothesis for the cause of cell death is that neurones with grossly inappropriate topographical projections either completely fail to form synapses or are eliminated from synaptic sites by more appropriately projecting neurones; as a consequence of failing to form stable synapses they do not obtain a survival factor made available at synaptic sites. (Ganglion cells which make grossly inappropriate ipsilateral projections do not make synapses at all, and subsequently degenerate. It is not known if motoneurones with grossly inappropriate topographical projections form synapses; it is known that motoneurones with moderate topographical misprojections form synapses).

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Landmarks in "the NGF uncharted route"

Discovery and definition of the Nerve Growth Factor

The term Nerve Growth Factor was introduced to define the effect of a macromolecular agent endowed with the property of inducing, in an *in vitro* test, rapid and exuberant fiber outgrowth from sensory and sympathetic embryonic neurons (Fig. 1). This growth response, which occurs in a 10-24 hour period, offered a fast, most reliable test to assess the presence of NGF and to uncover its putative biological sources.

The NGF's mysterious effect was first noticed when a mouse tumor, known as mouse sarcoma 180, which had been implanted into the body wall of 3-day chick embryo was innervated by nerve fibers outgrowing from the adjacent spinal root ganglia of the host. The original interpretation, that this effect was due to the rapid growth and biochemical properties of the neoplastic cells [8], was later revised and replaced by the hypothesis that the implanted tumor released a humoral factor whicb selectively promoted fiber outgrowth from sensory and sympathetic ganglia [32]. This hypothesis was tested and confirmed by the *in vitro* bioassay mentioned above.

NGF effects on its target cells

The first NGF target cells to be identified were (1) the large majority of sympathetic long adrenergic neurons, (2) the small late differentiated sensory neurons which represent 50-60% of the root dorsal spinal ganglia. A decade ago a third neural crest derivative cell, normally destined to become chromaffin, was found to undergo, upon exposure to NGF during early developmental phases, phenotypic differentiation toward the neuronal sympathetic rather than the chromaffin cell type [2, 36].

The target nerve cells listed above respond to NGF action according to a pattern which is similar for all cells, namely, maximal during early ontogenetic stages and gradually decreasing — but still persisting — in adult life. A paradigmatic and most investigated case is that of sympathetic cells, which offer a most favorable model system to explore the NGF's effects. Three experimentally distinguishable NGF effects during early ontogenetic phases have been described for this cell type: *trophic*, *differentiative* and *neurotropic*.

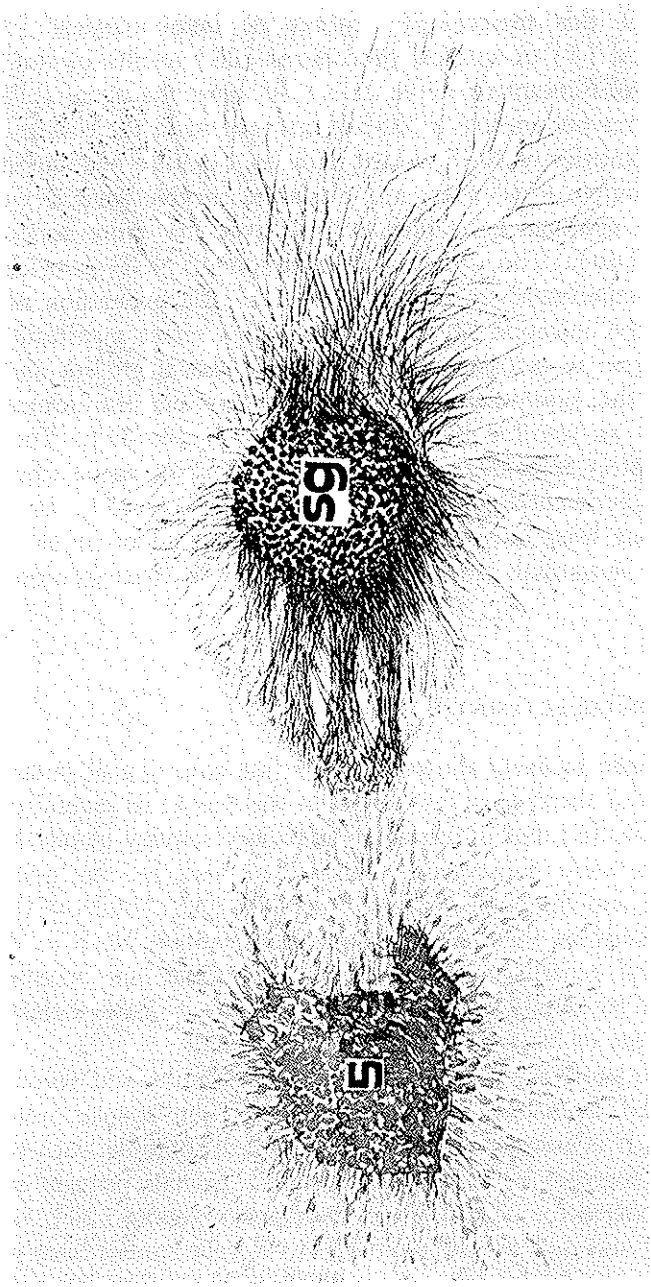

FIG. 1. Sensory ganglion (SG) of an 8-day-old chick embryo cultured for 36 hours *in vitro* with a fragment of mouse sarcoma 180 (S) (Indian ink drawing sent by one of us (R. L-M) to Dr. Hamburger from Rio de Janeiro, November 1952.

The essential trophic role played by NGF in early ontogenetic stages is evidenced by *in vivo* and *in vitro* studies. In the developing organism, deprivation of this molecule by means of immunological [33, 41], pharmacological [5] or surgical procedures [26] results in the death of sympathetic NGF-receptive nerve cells. *In vitro* the same cells neither produce neurites nor survive if NGF is not present in the culture medium. Nanogram quantities of NGF, added daily to a culture medium consisting of essential amino acids, salts and vitamins, survive several weeks and build a dense neuronal net which extends over the entire surface of the culture dish [40]. The differentiative action of NGF is evidenced by the greater commitment to, or acceleration of, the expression of the cell phenotype upon injection of the protein. This effect manifests itself as cell enlargement, in cytoskeletal organization, axonal growth and branching, and in the synthesis of neurotransmitters via the stimulation of enzymes such as tyrosine hydroxylase [11, 12, 38, 53]. The *in vivo* neurotropic effect is evidenced by the channeling of nerve fiber growth toward an experimentally produced NGF pool [35, 45]. *In vitro* the same effect has been unequivocably demonstrated to occur as the directional neurite outgrowth toward a calibrated NGF concentration gradient [22, 31].

The NGF molecule and its gene

The *in vitro* bioassay allowed for the fast screening of putative NGF sources. Two of these were discovered in the venom of several poisonous snakes [13, 14, 16] and in mouse submaxillary salivary glands [15, 43]. Large sources were subsequently uncovered also in the guinea pig's prostate [23] and the bull's seminal fluid [24, 25]. While all the above-mentioned glands or their secretory products contain NGF in the order of μg/mg total protein, a large number of normal and neoplastic cells from different vertebrate species synthetize minute — nevertheless measurable — amounts of NGF [18, 42, 47, 48, 59, 61].

NGF is a protein whose primary structure (aminoacid sequence) was first elucidated from the molecule purified from mouse submaxillary glands [28]. It is a dimer made of two identical subunits, each of 13,250 daltons, held together by non-covalent bonds. Similar primary structures have been reported for NGF isolated from snake venom [29], guinea pig prostate [51] and bull seminal fluid [24]. Recent studies have identified the mRNA for the mouse salivary gland NGF [52]. The latter codes for

a much larger precursor of 322 aminoacids (the biologically active NGF contains 118 aminoacids) which demonstrates that the NGF protein is part of a much larger precursor, as is the case for several hormones and specific growth factors. Processing of this larger precursor is mediated by specific proteases which attack the NGF precursor at specific sites [52, 54]. In the mouse salivary glands, it has been hypothesized that the processor enzyme remains connected to NGF to form a large, multimolecular complex also known as 7S NGF [55, 56, 60]. This complex consists of 3 different molecular species: the alfa subunit of unknown function, the gamma subunit, endowed with protease activity, and the beta subunit, which is the biologically active NGF. Although this complex is detectable both in salivary gland homogenates and in mouse saliva [58] its presence in other large or small NGF sources has not yet been established. Upon removal of 8 aminoacids at the C-terminal and of an N-terminal arginine, β-NGF is converted into a slightly smaller, biologically active molecule, known as 2.5S NGF because of its sedimentation coefficient.

The isolation of NGF mRNA in the mouse was followed by the cloning and isolation of its gene not only in rodents [52] but also in the human species [54]. Preliminary studies demonstrate that the NGF gene as well as mRNA is present in chick [19]. Its structure in mammals, from rodents to primates, has been highly conserved — as one can infer from sequence studies of the protein product [52, 54]. This is also true in the non-mammalian taxa [19, 52, 54].

The NGF's mechanism of action

The action of NGF on its target cells is mediated by specific, high affinity receptors present on the external surface of their plasma membrane. These receptors have a Kd for NGF which varies, in different target cells, between 10^{-11} and 10^{-9} M. The optimal NGF concentration for the *in vitro* bioassay employed for NGF screenings in biological sources is 1 — 10 ng/ml., i.e., $2.5-5.0\times10^{-10}$ M, a value of the same magnitude as the Kd of NGF receptors in these nerve cells. NGF receptors are present in the plasma membrane of undifferentiated target cells and in the nerve endings of axons and dendrites of the same fully differentiated cells. Binding of NGF to its receptors is followed by the internalization of their complexes and intracellular distribution into different cellular compartments, including lysosomes which eventually destroy the factor. NGF internalization from nerve endings is followed by retrograde transport along nerve fibers up

to the cell perikarion [27]. Evidence that this transport is crucial at least for the vital trophic effect of NGF, is provided by different immunological, pharmacological, surgical manipulations, which lead to death of target cells, an effect which is counteracted by an exogenous supply of NGF [3, 30, 44]. It is not yet clear whether there are one or more distinct types of receptors possibly mediating different NGF effects, nor whether these receptors operate as transducers of NGF action via as yet unidentified second messengers (cyclic AMP, Ca^{++} etc.). An alternative mechanism to the amplification of the NGF message through second messengers might be a direct action of NGF or of NGF-receptor complexes on certain intracellular structures or components (e.g., the cell's genome, the cytoskeleton, etc.) which, in turn, due to their vital function or strategic position, control several other properties of the cells [10].

Morphological, ultrastructural and chemical changes of target cells are already detectable minutes after NGF interaction with its receptors, and become more pronounced and of a broader range within hours or days [20]. They involve increased intake of aminoacids and other nutrients across the plasma membrane, stimulation of the synthesis of specific classes of mRNAs and of their corresponding protein products, and organization of cytoskeletal elements which provide the force-generating structure for axonal growth and elongation [10]. These anabolic changes are accompanied by an increased intake of glucose and its consumption through oxidative pathway [14, 46]. This pleiotypic response of target cells to NGF is particularly evident in certain cells such as those of a clonal cell line known as PC12 cells [21]. In the presence of NGF, their phenotype changes from that of a neoplastic, undifferentiated and proliferating cell to that of a mitotically-arrested, neurite-bearing cell population, morphologically and biochemically undistinguishable from normal sympathetic adrenergic nerve cells.

Some recent developments

Of the numerous lines of investigation pursued in the last 5 years, we shall consider here only three which have been the object of studies on the part of our group, that is of the two authors of this review, of Dr. Enrico Alleva of the Istituto Superiore di Sanità in Rome, and of Dr. Ariela Böhm, who recently joined our team. Our studies were directed at examining the effect of mouse NGF (M-NGF) in lower vertebrates, a topic barely touched upon in all these past years, at

continuing and extending the investigation, begun 9 years ago in our laboratory, on the effect of M-NGF on rodents' mast cells, and at testing a hypothesis on the functional significance of the synthesis and storage of such a large amount of the NGF peptide in the submandibular glands of adult male mice. We report here the main results of these investigations.

1) *The NGF spectrum of action in lower vertebrates*

The object of the studies which were begun three years ago in our laboratory was *Xenopus laevis* tadpoles between one day (after hatching) old, and the pre-metamorphic stage 52 according to the table of Nieuwkoop and Faber [50]. The possibility of rearing these amphibians in the laboratory all year around and their remarkably high survival rate after all sorts of surgical or pharmacological intervention make them an ideal object of experimental analysis. Here we shall briefly describe the results of these previously reported studies [37].

M-NGF was injected in tadpoles which had been anaesthetized and immobilized by immersion for a few minutes in ice water. NGF or vehicle solution was injected by means of a calibrated micropipette with a tip diameter of about 10-15 μm. The larvae were injected for periods of 3-7 days with 2 μg (stages 40-44), 4 μg (stages 45-48), and 10 μg (stages 49-52) of NGF in 1-2 μl of vehicle solution. Mortality in the NGF- and vehicle-injected tadpoles was about 20%, and slightly higher in the former than in the latter. NGF- and vehicle-injected tadpoles, sacrificed after shorter or longer treatments as indicated above, were the object of a) histological, b) histofluorescence, c) immunofluorescence and d) radioautographic studies that are described in detail in the PNAS article [37]. Here we shall report only the main findings of studies a), b) and c).

Histological studies. The tadpoles were sectioned serially and stained in hematoxylin/eosin, or toluidine blue dyes. Some specimens were also stained with a modified silver impregnation technique (unpublished method) which gave excellent results, and permitted the visualization — in the minutest detail — of nerve cells and their axons in the central nervous system and in their ramification in peripheral non-neuronal tissue. Morphometric studies performed using the technique described in previous articles [37] gave evidence of the extraordinarily large increase in volume of sensory ganglia in 48-stage tadpoles that had been treated for 5 days with M-NGF. The 8-fold volume increase was the result of increases

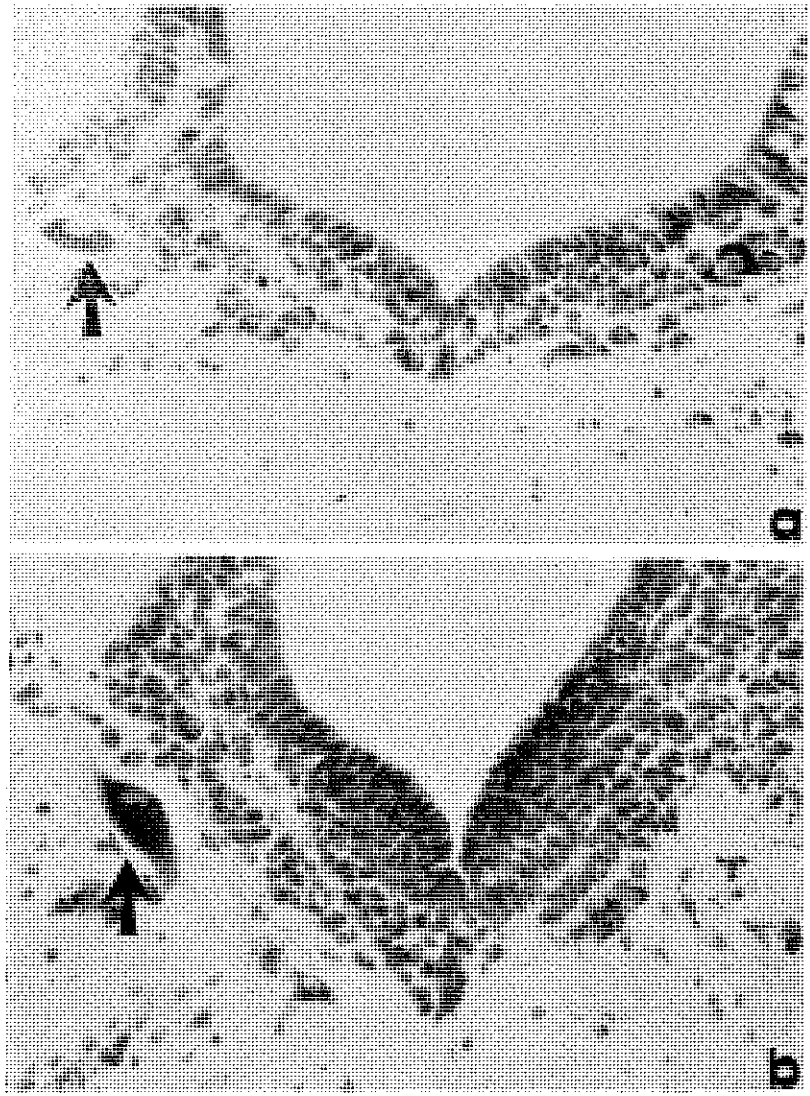

FIG. 3. Comparative histological section of the pontomesencephalic area of *Xenopus laevis* tadpoles at stage 48 injected for 6 consecutive days with saline (a) or NGF (b). Arrows point to Mauthner cells. Toluidine stain. X 120.

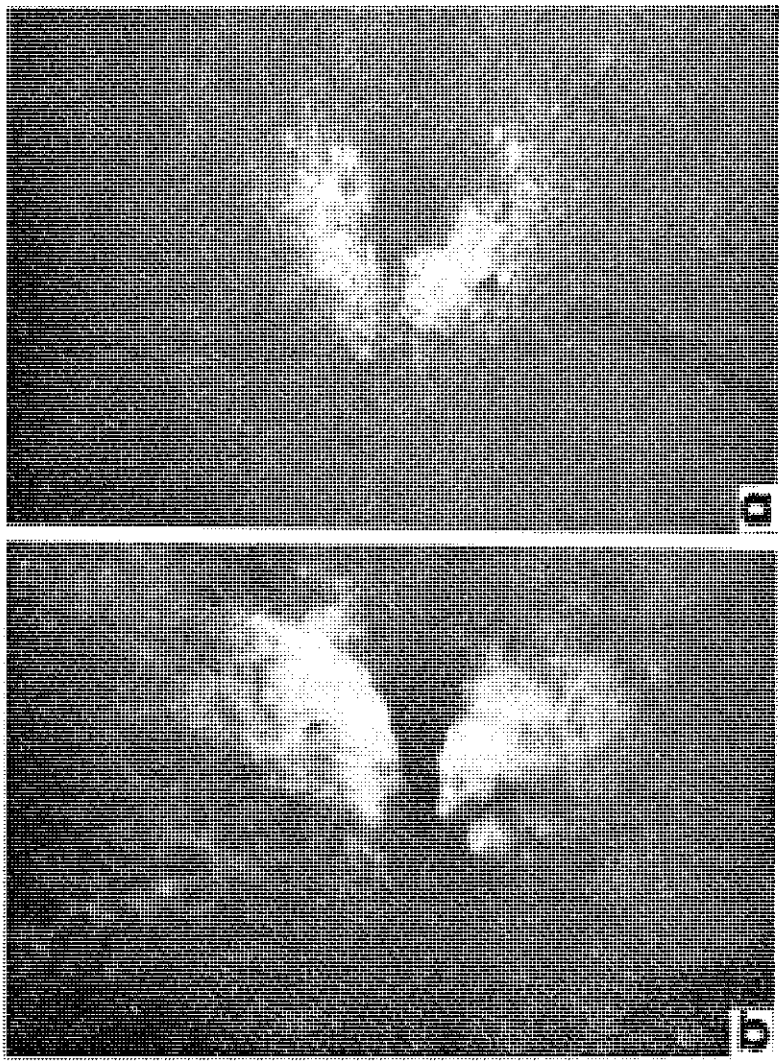

FIG. 4. Formaldehyde induced fluorescence in the diencephalic section of tadpole at stage 49 injected for 8 consecutive days with saline (a) or NGF (b). Adrenergic neurons in the paraventricular nucleus of the NGF-treated tadpoles are larger and more intensely fluorescent than in control nucleus. X 100.

P (S.P.), somatostatin (Som), and enkephalin (Enk), a generous gift of Dr. Claudio Cuello, were employed in these immunofluorescence studies. Immunoreactivity to Som of diencephalic nerve cells, their dendrites and axons in NGF-46-52 stages injected tadpoles, was more pronounced than in controls. A more vivid fluorescence reaction to S.P. monoclonal antibodies was well apparent in neurons in the ponto-mesencephalic centers of experimental compared to control tadpoles. Immunoreactivity to Enk, still very weak at these stages in experimental and control nerve cells, became in subsequent pre- and post-metamorphic stages more marked in experimental tadpoles. Figs. 5, 6 show the differences in immunofluorescence of diencephalic and ponto-mesencephalic nerve cells processed with monoclonal antibodies to Som and to S.P. respectively.

In vitro *studies*. The brains of 50 stage 46 to 48 tadpoles injected for a 5-day period with NGF and the same number of brains of tadpoles injected for the same amount of time with vehicle solution, were removed from soft surrounding tissue, carefully washed, dissociated through a 30 minute suspension in a 1% trypsin and 0.2% collagenase containing buffer solution, washed in culture medium, dissociated and plated in a culture medium consisting of BEM/RPM inactivated horse and fetal calf sera, with NGF or vehicle solution, and incubated for a 20-day period. Each day the cultures were examined under an inverted Leitz microscope. In NGF-enriched media, nerve cells not only survived but produced axonal processes extending at a distance, whereas in control cultures all nerve cells died within the first two days of incubation *in vitro*. Studies in progress are directed to ascertain whether only a percentage of the nerve cells of the CNS survive and are receptive to M-NGF and, if this is the case, an attempt will be made to identify these cells.

Preliminary autoradiographic studies with ^{125}I-labelled NGF showed that a rather large percentage of cultured nerve cells of the Xenopus tadpoles's central nervous system selectively bind to the labelled NGF, thus providing additional evidence of their capacity to respond to this protein molecule. These findings, which unequivocably prove that nerve cells in the peripheral and central nervous system of specimens of this amphibian species are highly receptive to M-NGF, beg that a search for its source of origin in this species be made. Experiments by other investigators (Drs. Salvatori and Cattaneo) that are in progress in our laboratory have provided suggestive evidence for the existence of a Xenopus DNA sequence which hybridizes *in vitro* with segments of a denaturated probe in the NGF region of the human gene.

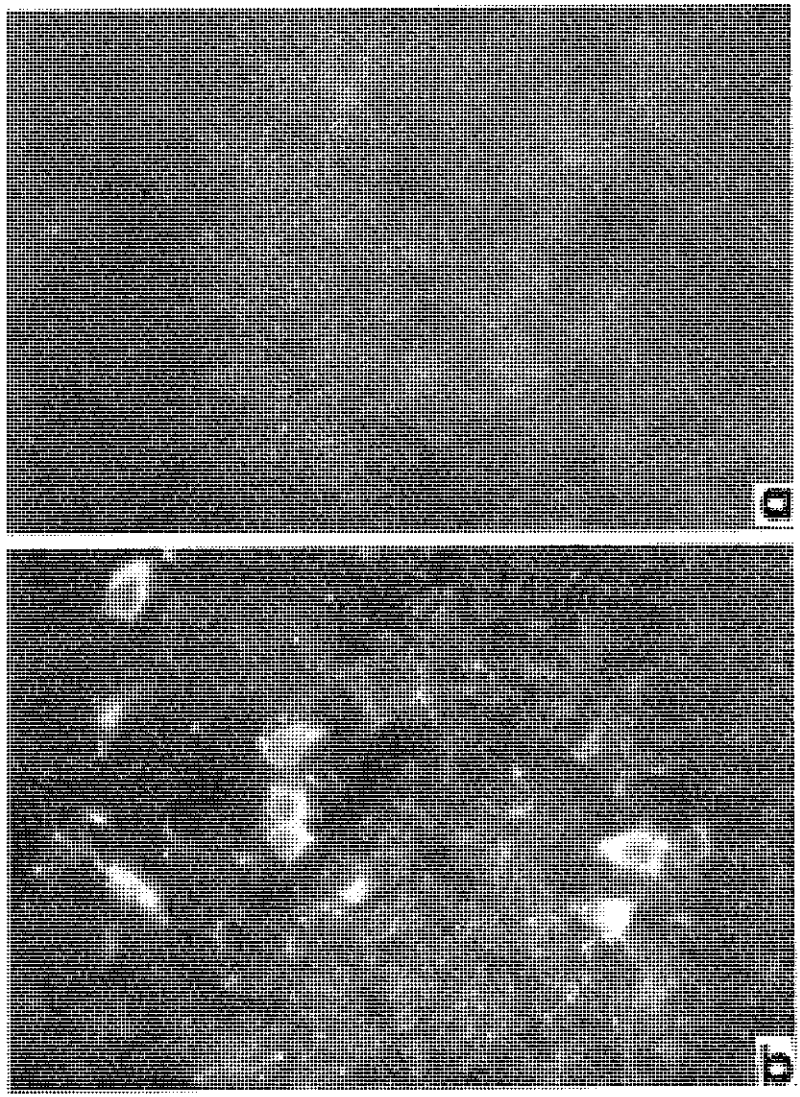

FIG. 5. Somatostatin immunoreactive cells of the tracts in the diencephalic area of tadpole brains injected for 6 consecutive days with saline (a) or NGF (b). X 370.

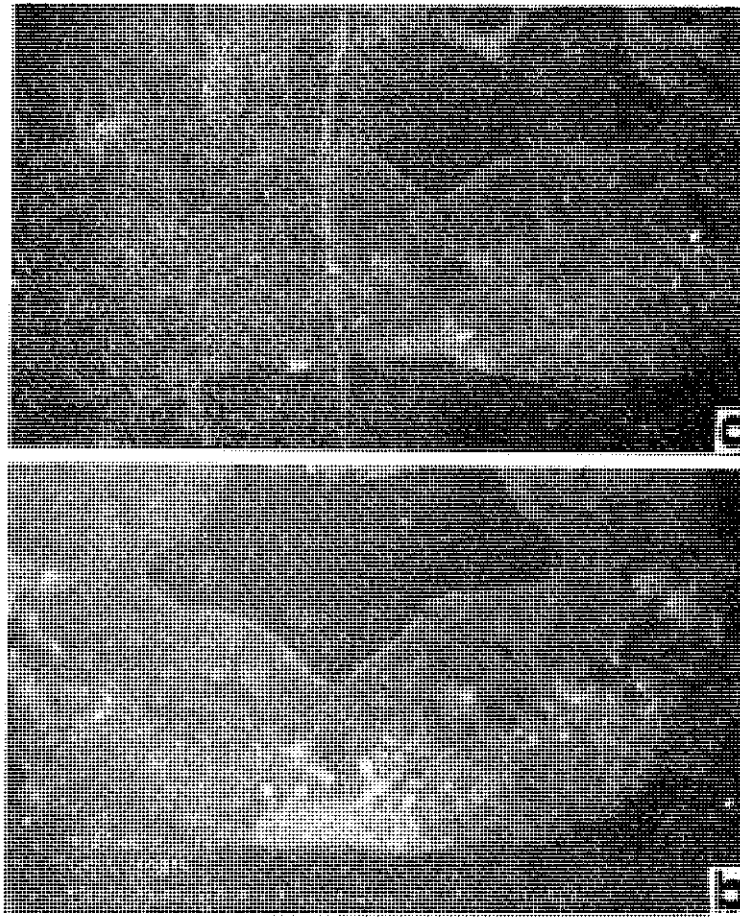

FIG. 6. Substance P immunoreactive cells and tracts in the pontomesencephalic area of tadpole brains at stage 48 injected for 6 days with saline (a) or NGF (b). X 100.

2) In vivo *and* in vitro *studies on rodent mast cells as NGF target cells.*

In an article published in 1977 [1] we reported data of experimental studies performed on neonatal rats which gave unequivocable evidence for the marked numerical and size increase of mast cells in neonatal rats injected with NGF. It was possible to obtain decisive evidence against the hypothesis that such an effect could result from the strong antigenic properties of NGF and to demonstrate that instead it can be compared

in all respects to the effects of NGF on its neuronal cell targets. In this past year the study of the effect of NGF in neonatal and adult rats was carried further by one of us (L. Aloe), and it was proven that in adult female rats autoimmunized against their own NGF, mast cells decrease in number; the peritoneal mast cells are depleted of granules and show more or less pronounced degenerative marks (unpublished results). In parallel with the studies *in vivo*, L. Aloe and A. Böhm examined the effect of M-NGF on mast cells *in vitro*. Here we shall summarize the main findings of this investigation which is still in progress in our laboratory. Spleen cells of newborn Sprague Dawley rats, once dissociated from the organ, were cultured in semisolid medium (1×10^6 cells/ml) in 24 wells tissue culture plates in Dulbecco modified Eagle medium supplemented with 30% FCS, 10^{-4} M mercaptoethanol, 2 mM glutamine, antibiotics and metilcellulose at a final concentration of 1%. NGF in the amount of 10 μg/ml, was added to half the cultures from the beginning of the incubation period, and at 4 day intervals. The cultures were incuhated in a high humidity atmosphere with 5% CO_2 at 37°C. The results demonstrate that dissociated spleen cells of neonatal rats when cultured for 2-3 weeks in presence of physiological amounts of NGF, acquire characteristics similar to those of fully differentiated mast cells. These include: (a) presence of metachromatic granules in toluidine blue staining preparations and prominent dark cytoplasmatic granules after May-Grünwald staining, (b) presence of serotonine revealed by immunohistochemical techniques, (c) demonstration of the presence of IgE receptors on the cell membrane, (d) electron-dense granules of varying size and density throughout the whole cytoplasm. The role of NGF in allowing survival and differentiation in mast cells of dissociated splenocytes *in vitro* is illustrated in Fig. 7. The results are reported in detail in an article now in press [7]. These findings illustrate an entirely new property of NGF, namely that of enhancing differentiation in a type of cell belonging to the immune system, and they suggest the search be extended to other putative NGF target cells belonging to this system.

3) *Functional role of NGF in the mouse submaxillary salivary glands.*

The third line of investigation pursued in these past two years, was directed at elucidating one of the most intriguing and still unsolved problems that confronted us ever since the 1960 discovery, that is, that the submaxillary salivary glands of adult male mice synthetize a large

THE OUTLOOK: THIRTY-THREE YEARS LATER

Thirty-three years have elapsed since the bright morning of November 2nd, 1952, when the still unnamed NGF revealed itself in Rio in such a glamorous way as to leave all of us who witnessed the event breathless as if we had been in front of a sort of miraculous apparition.

None of us, in fact, could have anticipated the formation, in the space of few hours, of the magnificent halo of nerve fibers around the ganglia facing a neoplastic source of this factor.

Many no less dramatic events and discoveries have taken place in these last decades, and certainly many more are in store for future years. It may

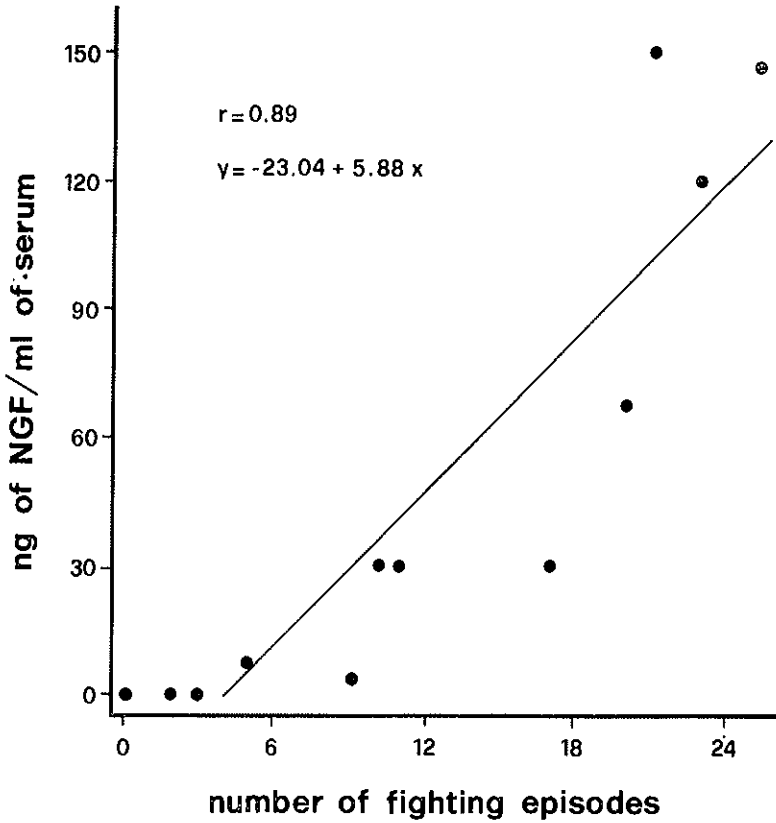

FIG. 8. NGF level in the blood serum of fighting mice as a function of aggressive behaviour scored during a 6 minute session. NGF values refer to mean levels of two fighting animals.

therefore be premature to attempt to predict where the "NGF uncharted route" will bring us, but among past accomplishments, one perhaps may be remembered as having been the most important: that of having revealed the existence of an outstanding role played by polypeptides endowed with specific growth and differentiation properties on different cell lines. As far as the nervous system is concerned, NGF has uncovered some most important properties of the NGF target cells and the large investments now being made in many laboratories all over the world for the study of this peptide, of its mechanism(s) and its spectrum of action allow one to predict that new avenues will be opened in the coming decades. Among these, we believe that the NGF effects on one cell type belonging to the immune system may shed light on one of the most fascinating problems now under investigation: the interaction between the nervous and the immune systems and its possible important clinical implications.

Acknowledgements

This work was supported by grants from Medicina Preventiva e Riabilitativa and Progetto Strategico Consiglio Nazionale delle Ricerche. We thank Ms. A. Sebastiano for typing the manuscript.

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THE CHANGING ASTROCYTE: ITS ROLE IN CNS AXON TRACT DEVELOPMENT, IN REGENERATIVE FAILURE, AND DURING INDUCED REGENERATION UPON TRANSPLANTATION

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INTRODUCTION

The consequences of damage within the central nervous system (CNS) of mammalian embryos or neonates are often less severe than those of an equivalent lesion in adults, and as a result usually far less functionally detrimental [7, 13, 14, 18]. The functional recovery of embryos or neonates with CNS injuries has been suggested to be produced by: (1) the ability of non-lesioned neurons to shift their positions into the lesion site [17, 33], (2) the growth of axons from later maturing neurons through or around the lesion site [7, 26, 30, 41, 47] or (3) an actual regeneration or sprouting of severed neurites [4, 10, 18, 20, 23, 24, 31, 32, 46]. In all mammals, functional restoration from CNS injury diminishes significantly and rapidly during early postnatal stages, establishing a "critical period" in which extensive axon elongation through or around the wound will no longer occur [13, 14, 18]. Unlike the embryonic brain which does not tend to scar when injured [4] (also see below), when penetrating types of CNS lesions occur in the "post-critical period" animal, astroglial, mesenchymal and a variety of other non-neural cells infiltrate the wound producing a complex, collagenous, glial-mesenchymal scar which tends to impede most regrowing axons [8, 9, 11, 26, 43]. Despite the scarring, axons proximal to the area of injury, at all ages, are usually capable of a considerable amount of sprouting [32] and have the potential for long

distance growth [2, 12, 44]. However, instead of innervating their appropriate synaptic targets, regenerative sprouting is usually abortive and axons rarely reach appropriate synaptic targets [3, 8, 9].

Unlike most early induced CNS insults, there is one particular embryonic lesion that does have devastating consequences on outgrowing axons. When the presumptive terrain for the developing corpus callosum, the so-called glial "sling", is surgically lesioned, the would-be commissural axons whirl into tortuous neuromas (Probst's bundles [39]) which persist indefinitely in an ectopic location lateral to the cerebral midline [21, 39, 49] (Lent and Schmidt, this volume). In early postnatal acallosal animals, Silver and Ogawa [51] (see figs. 1, 4 of present study) have shown that an untreated, properly shaped nitrocellulose (Millipore) filter placed adjacent to one or both neuromas and spanning the cerebral midline can support the migration of astrocytic glia from the subependymal zone. These astrocytes attach to the filter surface, artificially reproducing the sling-like embryonic callosal substratum [50, 51] (Silver and Levitt, in prep.) (see fig. 1). In turn, the de novo synthesized glial bridge provides a terrain suitable for ectopic "callosal" axons in Probst's bundles to traverse the midline, form a proper commissure, and enter appropriate regions of the opposite hemisphere (fig. 1).

In the present study we have determined that a "critical period" exists for this form of induced callosal axon growth that is similar in length to that established for the lateral olfactory tract, an early spontaneously regenerating CNS fiber pathway in rodents [18]. In addition, we have used the nitrocellulose implant in acallosal animals as a paradigm to analyse the subpopulation of cells that interacts with both the prosthesis and newly growing axons. By observing animals implanted at successively older ages, we have gained insight into the factors that determine why axon growth refractory states develop within the mammalian brain. Finally, we have documented a variety of intriguing environmental changes at

FIG. 1. In this previously acallosal animal, a Millipore implant (I) was placed into the brain on postnatal day 5. When it was sacrificed 5 weeks later, a new callosum (CC) had already formed above the implant (b and c). Uniquely, the animal had both longitudinal neuromas (LB) and a callosum in the same plane of section. Horseradish peroxidase injected into the cortex of one hemisphere (*) labels neurons on the opposite side of the brain (bracketed area of c; higher mag, d, e). The reformed callosum has grown to its appropriate region of synaptic termination and perhaps has made functional synapses. (a) X90; (b) X90; (c) X90; (d) X220; (e) X350.

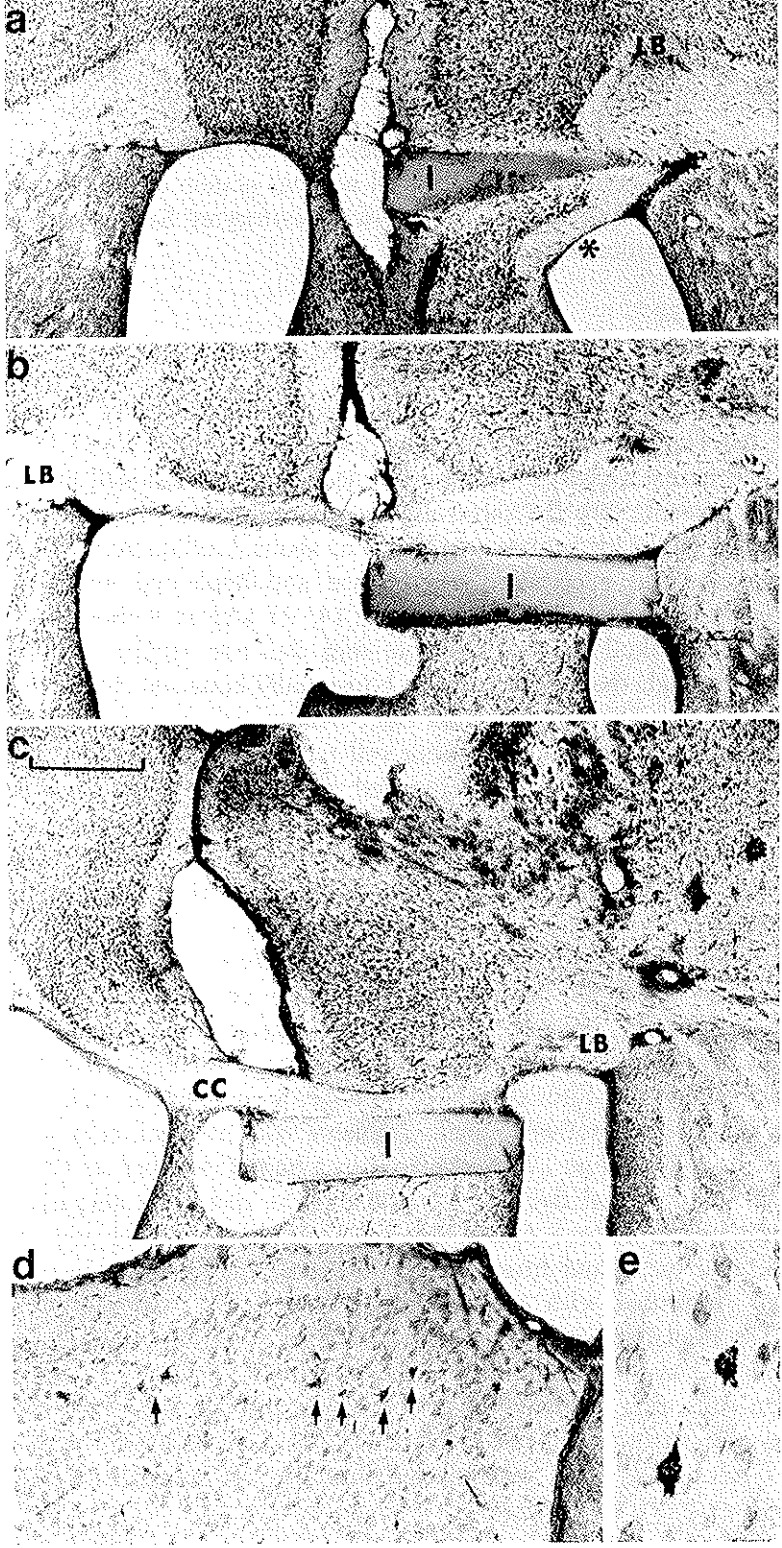


FIG. 1

the host/implant interface and have observed an induced axon "regeneration" in the post-critical period central nervous system of acallosal animals that were given implants pre-coated with astrocytes harvested from the forebrains of critical stage donors.

Materials and Methods

Timed pregnant C57BL/6J mice were obtained from the Jackson Labs, Bar Harbor, Maine. The glial sling of day 16 embryos (E16) or the sling and immature corpus callosum of neonates were lesioned by insertion of a microneedle into the calvarium approximately 1 millimeter rostral to the cranial landmark lambda to a depth of about 2 millimeters [49]. Such induced acallosal mice were anesthetized and implanted with Millipore bridges on postnatal days (P) 2, 5, 8, 14, 21 and at 8 months. In the neonates the skull was still pliable and did not require drilling. An incision through the skin was made horizontally between the eyes and the skin retracted. The surface of the skull was scraped free of tissue in order to minimize contamination of other cell types onto the implant as it was inserted. The prosthesis to be inserted was a specially designed piece of nitrocellulose filter (Millipore) similar in shape and size to that described by Silver and Ogawa [51]. This was then inserted 2 to 5 millimeters into the stab wound, depending on the age at which the animal was implanted.

In animals receiving transplants, filters were removed from decapitated acallosal mice, 48 hrs. after implantation on postnatal day 2. The tissue around the implant was carefully dissected and the glial coated implant was removed with a specially designed forceps which prevents the glia along the filter's surface from being crushed or stripped away when placed into another animal. Implants with attached cells were then dipped in N-2 medium [7] and placed in a humid chamber at 37°C. Host animals (at postnatal day 17, 34 or 8 months that were surgically made acallosal in the embryo or on the day of birth) were prepared, and the donor, glial pre-coated filters were transferred in the same manner as in animals implanted with naked filters.

The implanted mice were then sacrificed at various time periods and analyzed. Anesthetized animals were killed 0.5, 1, 2, 3, 5, and 7 days or 2 months after implantation by perfusion through the heart. The perfusion was performed in two steps; first, 2 to 5 ml of a 0.15 M phosphate buffer solution at 37°C was injected into the left ventricle of the heart,

followed by fixative (a combined 0.5% glutaraldehyde/2.0% formaldehyde solution in the same buffer with 0.5% DMSO). The brains were quickly dissected from the cranium and placed in the same fix overnight at 4°C. The brains were dissected and the filter and surrounding tissue were subsequently embedded in Spurr's plastic using standard procedures. Serial 1 micron sections were taken through the implant and stained with toluidine blue. Certain regions were sectioned ultrathin, stained with uranyl acetate and lead citrate, and viewed with a Zeiss 109 electron microscope. For specimens to be examined by SEM, the tissue above the implant was gently dissected away and the specimens osmicated and dehydrated through a graded series of alcohols. The samples were critical point dried in a Balzers CPD 020 and sputter coated with gold using an Edwards E306 device, mounted on aluminum stubs and viewed with an Etech scanning electron microscope.

Immunohistology. Postnates were anesthetized and perfused through the heart with 2 to 5 ml of 4.0% formaldehyde in phosphate buffered saline (PBS, pH 7.5). The brains were dissected from their calvaria and immersed in the same solution for 2 hours. The brains were then cryoprotected using a graded series of a sucrose PBS solution (10% sucrose PBS solution for 30 minutes, 15% for 30 min., and 20% for 2 hours to overnight). 10 micron sections were taken on a Slee HR Mark II cryostat microtome.

Polyclonal antibodies against purified laminin and fibronectin were received from Dr. G. Martin (NIH) and antibodies against N-CAM from Dr. U. Rutishauser (CWRU). The sera were used at dilutions of 1:50 for laminin, 1:100 for N-CAM, 1:1000 for GFAP and applied for 1 hour at room temperature. The sections were then rinsed in PBS (3-15 min washes) and incubated with peroxidase conjugated goat anti-rabbit IgG (Cooper Biomedical, Malvern, PA.) at a dilution of 1:100 for 30 minutes at room temperature or goat anti-rabbit FITC at a dilution of 1:50 for 1 hour. Sections were rinsed again in PBS and peroxidase conjugates were incubated in a solution containing 15 mg 3,3 Diaminobenzidine tetrahydrochloride, DAB (Eastman Kodak Co., Rochester, N.Y.) per 100 ml Tris buffer (pH 7.5) for 30 to 45 minutes at room temperature and in the dark.

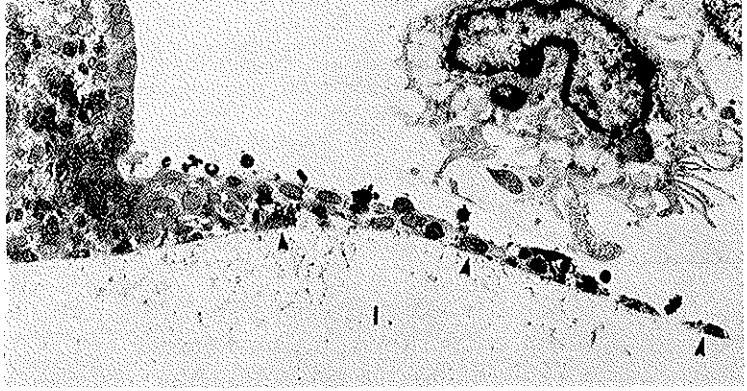
HRP injections. In order to determine the location of cell bodies that contribute axons to implant induced callosi, postnatally (P5) implanted acallosal animals after 5 weeks survival were injected with a single, small

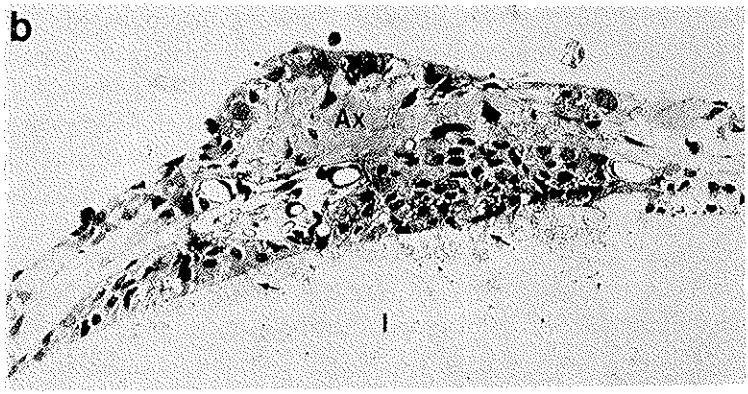
wedge of crystalline HRP (Sigma VI), which was inserted (with a spinal needle) very superficially into the cortex in a region immediately lateral to the implant. On the following day the animals were killed, perfused with 0.5% glutaraldehyde and 2% formaldehyde in 0.15 M PBS, and the brains were removed and placed in the same fix for 4 hours. Sections were cut on a vibratome at 65 μm. They were then incubated in 50 mg of DAB or TMB in 100 ml Tris buffer (pH 7.5) for 20 minutes, a solution of 0.6% hydrogen peroxide was added and incubated 15 to 20 minutes longer. The sections were counterstained with neutral red and mounted on slides.

Results

Host glial reaction to implantation of untreated nitrocellulose bridges at various postnatal ages. At early stages (P 2 and P 8) the glial cells that invaded the implant were capable of rapid tangential migration along the filter surface, as well as rapid extension of cytoplasmic processes deep into the filter's system of 0.45 micrometer pores (figs. 2a, b). Such immature cells expressed vimentin but not glial fibrillary acidic protein (GFAP) as they migrated along and became entrenched within the surface of the filter (not shown). Once the vimentin positive cells sent processes into the implant (approximately 24 hrs after implantation) they began to synthesize GFAP in higher amounts. The GFAP staining also revealed extensive branching of glial processes both into the prosthesis and encompassing tissue (fig. 10). Thus, as quickly as 12 to 24 hours after implantation in P 2 animals, primitive astrocytes have the ability to migrate out of the hemisphere and establish a foothold within the filter. The astrocytes rapidly incorporate the implant within the brain by interdigitating with each other, the implant, and the surrounding neuropil. In P 2

Fig. 2. Coronal sections through filters implanted into postnatal day 2 acallosal mice and examined 24 (a), 48 (b), and 72 (c) hours later. (a) 24 hours after implantation numerous glia (arrowheads) have migrated out of the hemisphere and along the implant. As they attached to the implant (I) they extended cytoplasmic processes into the pores; note that the leading glial cell (far right) has extended few processes. (b) Within 48 hours, glia coat the majority of the filter's surface providing a substrate on which axons (Ax) and blood vessels have extended. (c) In some specimens 72 hours after implantation the axons fasciculate over the glia above the filter, a configuration similar to that of the normal developing corpus callosum (CC) and « sling » (SL; d). (a) X450; (b) X360; (c) X450; (d) X540.

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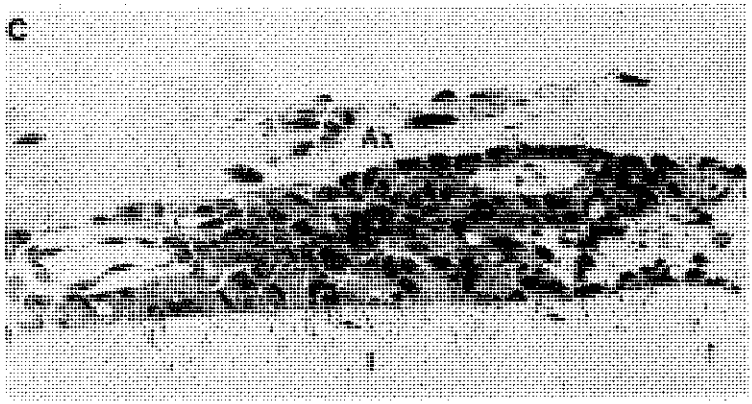
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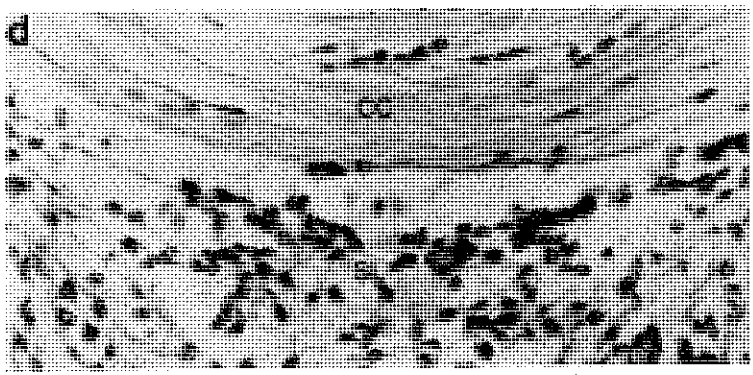

d

Another significant variation of the host glial reaction in older animals was the inability of the flattened, mature form of reactive cell to insert processes into the implant. Filters introduced intracerebrally at later stages (P 21 and 8 months) and examined after 7 days had limited penetration of glial processes into their pores (figs. 4e, 10). Rather than inserting, the flattened glia appeared to meld with the mesenchyme in the longitudinal fissure, together, encapsulating the prosthesis by forming sheets several cell layers thick around the circumference of the implant.

The anti-GFAP staining pattern of P 21 individuals also showed sheets of flattened astrocytes with very short or no processes penetrating into the implant (fig. 10b). These flattened astrocytes were often surrounded by non-staining fibroblast-like elements that seemed to comprise a much larger proportion of the cells encompassing the implant than at earlier stages.

Extracellular matrices associated with the gliotic response at different ages. The gliotic reaction that appeared 48 hrs after implantation in P 2 neonates did not stimulate the production of ectopic collagen or basal lamina. Only basal laminae which normally occur around capillaries and at the longitudinal fissure could be found (figs. 3, 9a). In some P 8 individuals examined after 2 days, very small amounts of ectopic collagen and basal lamina appeared in isolated patches among the cells surrounding the implant (fig. 5).

When animals implanted at P 2 and P 8 were examined for laminin (a major protein component of basal lamina), an unusual staining pattern was revealed. Thus, as expected, laminin appeared to be concentrated in the basal laminae of the blood vessels and the pia mater throughout tbe brain, but, surprisingly, was also found within the pores of the filter in regions containing inserted glial processes that were not associated with

FIG. 4. Coronal sections through the nitrocellulose bridge (I) and associated tissue in the area of the presumptive callosal pathway of acallosal mice. Panels a, b and c represent animals implanted at « critical » stages; postnatal day 2 (a) and 8 (b, c), both examined 48 hours after implantation. Compare a, b and c to animals implanted at « postcritical » stages, 14 (d) and 21 (e) days after birth, both examined 7 days postoperatively. In a, b and c the glia are more stellate, sending many cytoplasmic processes into the pores of the filter (I), whereas the cells near the implant in c and d appear flat, lacking extensive infiltration of processes. Directly above the infiltrated stellate glia of « critical » period implants are numerous axons (asterisks; a,b,c), but such axons were not apparent in « postcritical » stage implants (d,e). (a) X360; (b) X110; (c) X360; (d) X360; (e) X360.

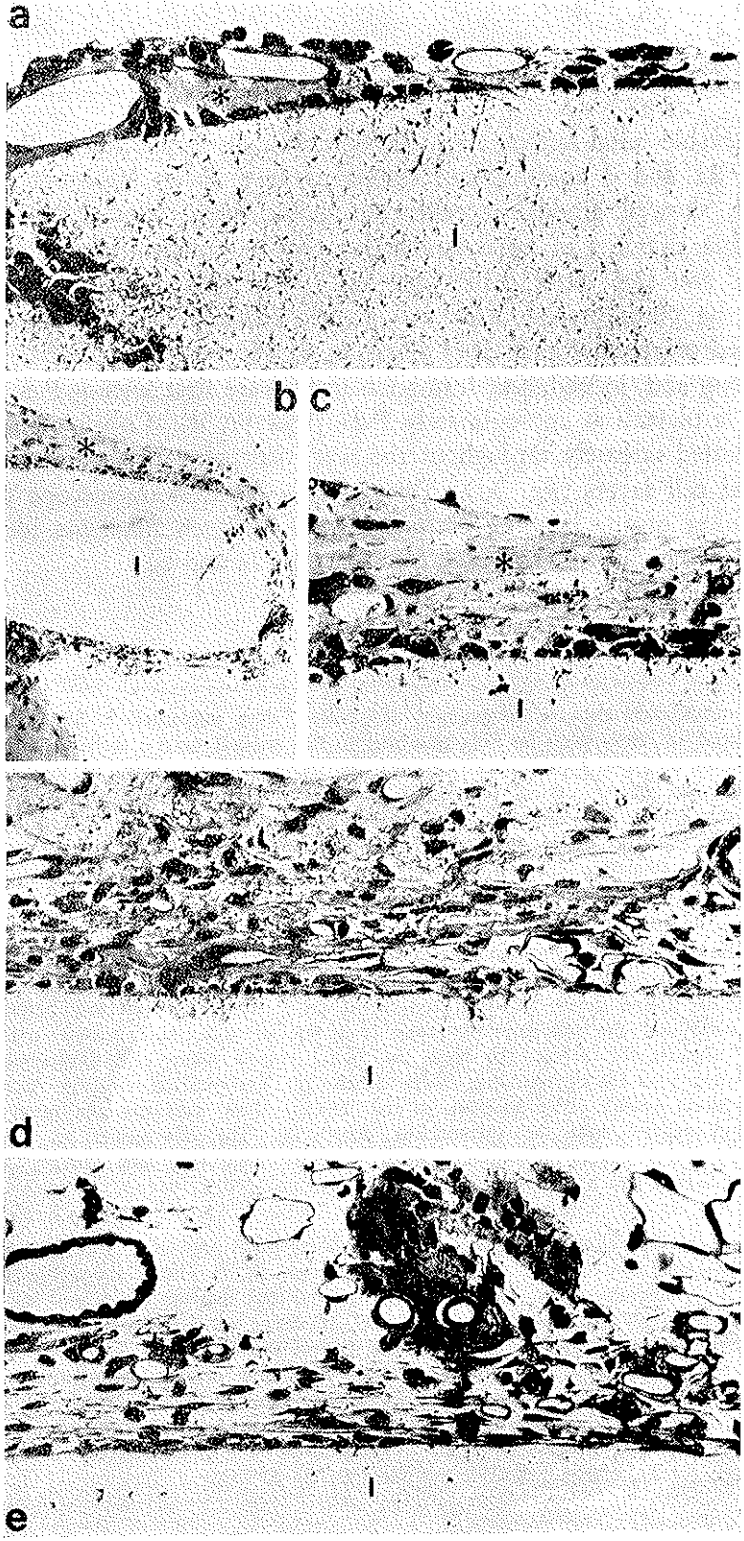


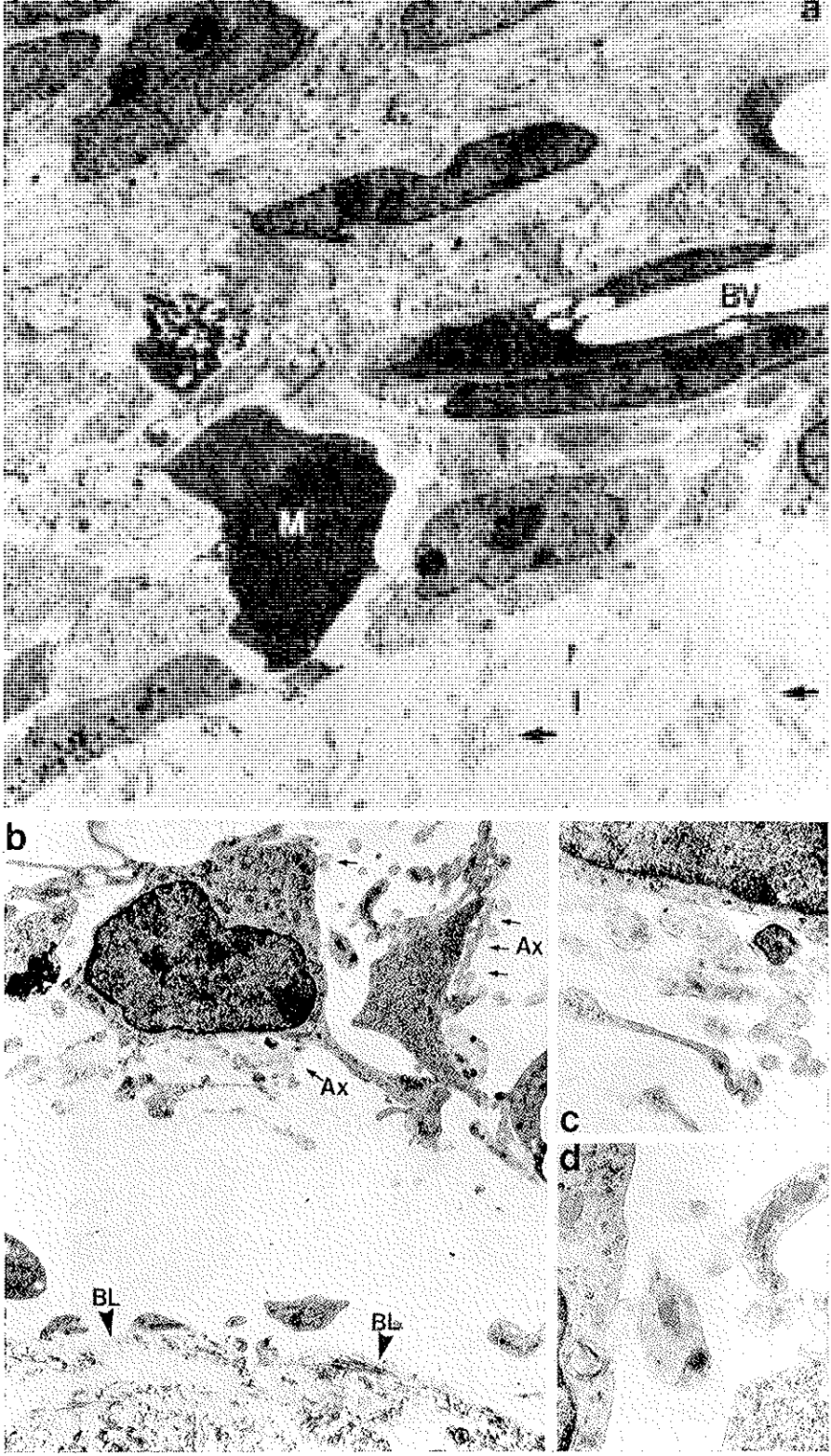
FIG. 4.

an observable basal lamina (figs. 9a, b). Animals implanted at P 2, P 8, P 14, P 21 and 8 months, and compared as a group after one week's survival, showed gradual increases at consecutively later stages in the observable amounts of basal lamina, as well as collagen associated with the glial scar (i.e., after P 14; figs. 5a, 7, 9). The anti-laminin staining within the pores of the filter was greatly reduced or absent in brains implanted on or later than P 14 (figs. 9c, d). Scar-associated basal lamina production and, indeed, scar formation itself were first seen at P 8-10 and reached a plateau at approximately P 21 (figs. 9c, d). Collagen filaments were located diffusely throughout the scar, occupying the spaces between cells and cell layers (fig. 7d). Transmission electron microscope (TEM) examination of the banding pattern for the collagen filaments identifies them as being composed of type I [22] (figs. 7a and b).

FIG. 5. Transmission electron micrographs of acallosal mice implanted 8 days after birth and sacrificed 48 hours later. (a) Glia attaching to the implant have a stellate morphology; microglia (M) are also apparent. Above the glia infiltrating the pores of the filter are axons (Ax) and Blood vessels (BV). (b) The axons (arrows) that extend into areas where basal lamina (BL arrowheads) appears, are positioned immediately adjacent to the glia, but not the basal lamina. Higher magnification in (c) shows axons associated with astrocyte processes containing intermediate filaments and glycogen granules (d). (a) X4000; (b) X4450; (c) X10,650; (d) X10,650.

FIG. 6. Coronal sections through the filters and associated tissue of acallosal mice implanted on postnatal day 14 and examined 48 hours later. (a) Note that the filter is surrounded by tissue debris (N) and red blood cells. Along the interface between the necrotic tissue and the subventricular zone is a line of glia (large arrows) presumably migrating towards the debris. (b) Near the blood vessels many cells (arrowheads) are also migrating into the surrounding tissue debris. (c) Transmission electron micrographs of intact blood vessels (BV) adjacent to the implant show that the glia remove their endfeet and migrate into the surrounding debris (c). The glia nearer to the vessel are connected by puncta adherentia (small arrows), while the glia further removed are not connected. (a) X110; (b) X260; (c) X2850.

FIG. 7. Transmission electron micrographs of acallosal mice implanted with nitrocellulose filters at postnatal day 21 and examined 7 days later. (d, f) The glia and mesenchymal cells that have migrated into the wound site around the implant (I) appear to have a flattened morphology and are arranged in layers. (f) Some of the glia are vacuolated and extend short processes into the filter (I). Interspersed within the cell layers are microglia (M), and collagen (type I; a,b) filaments (cf), establishing a glial-mesenchymal scar. (e) Throughout the scar are an abundance of macrophages, some of which have dense bundles of intermediate filaments (*) and basal lamina (arrow) along their surface. (c) Basal laminae are adjacent to many cell processes and in multilayers. (a) X72,650; (b) X42,750; (c) X25,650; (d) X3050; (e) X10,250; (f) X5470.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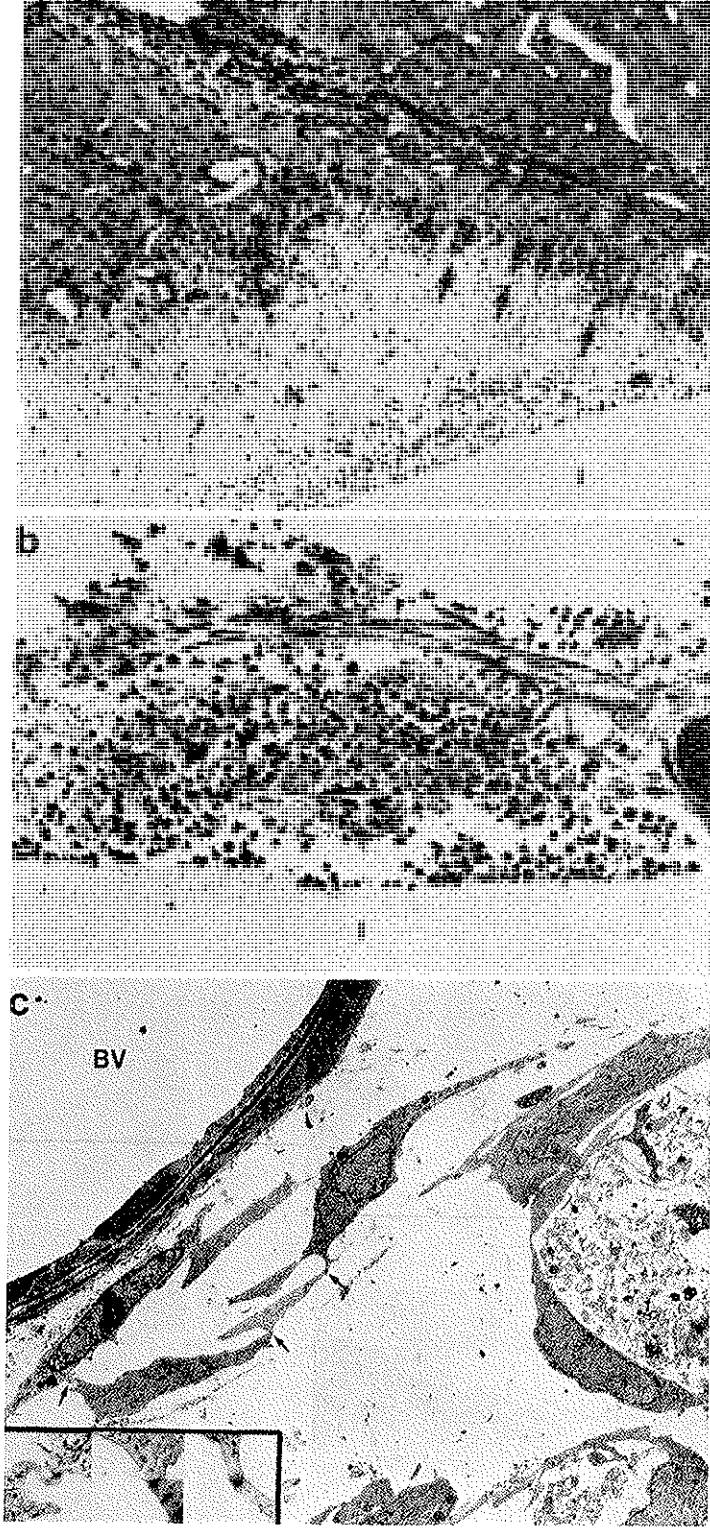


FIG. 6.

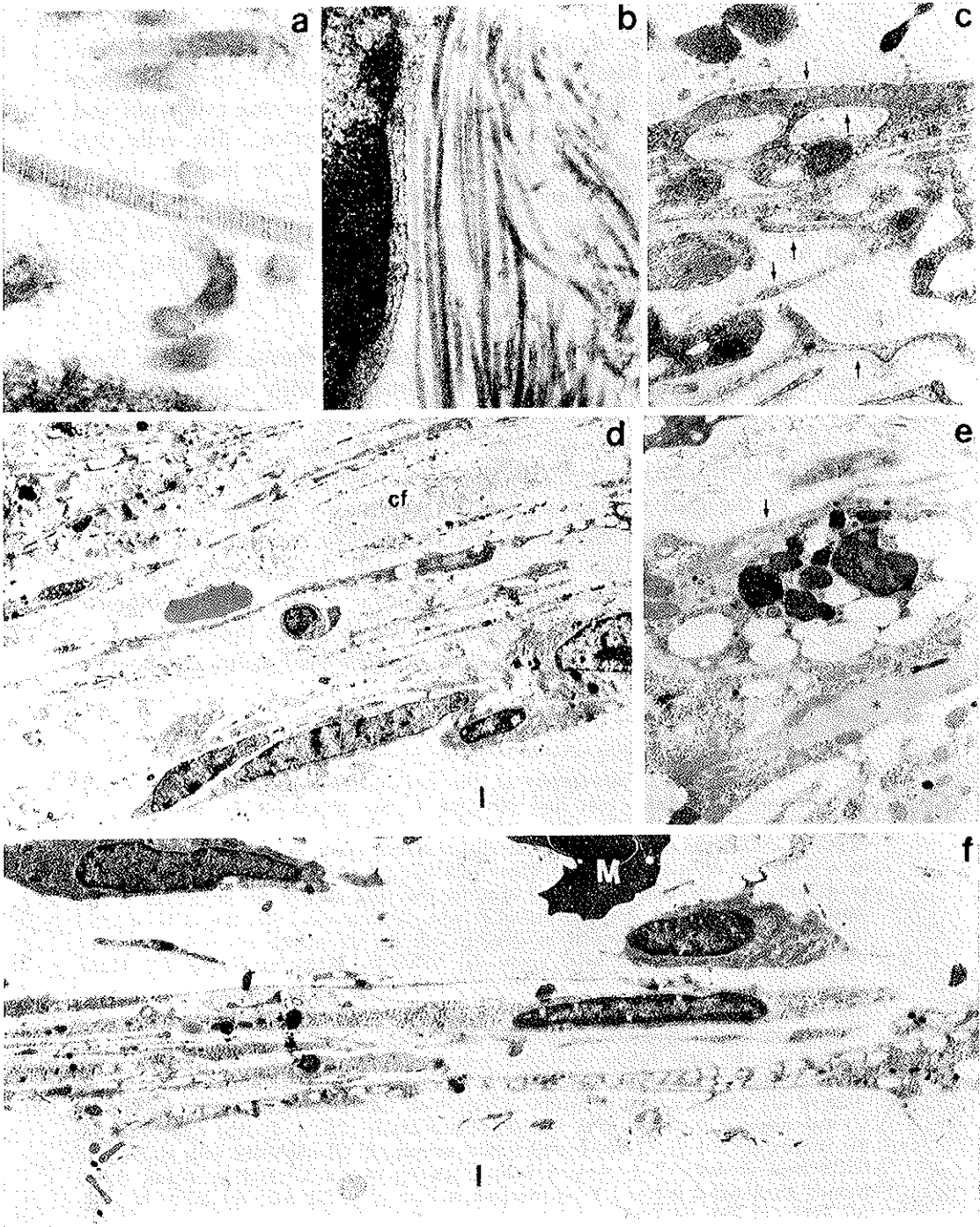


FIG. 7.

SEM examination of the scar in an animal implanted on P 21 demonstrated how the basal lamina blankets an extensive area of tissue above the filter (fig. 8a). There were many unidentified cell types and cytoplasmic processes upon the basal lamina's surface. The cells that had made contact and were beginning to attach to the laminar surface appeared spherical with many ruffles and microextensions (figs. 8a, b). Other cells had a morphology similar to fibrous or type II astrocytes [32] that had spread or were in the process of spreading by extending long, slender, multibranching cytoplasmic processes (fig. 8c).

Axon elongation over implants — defining the critical period. Acallosal animals were implanted in the forebrain with untreated nitrocellulose bridges at various stages (postnatal days 2, 8, 14, 21 and 8 months). This was done not only to evaluate and compare the gliotic response at these time periods (see above) but, also, to evaluate the ability of the glial coating around the implant to provide an adequate substratum for axonal elongation out of Probst's bundles and between the hemispheres (fig. 1). We have found that the gliotic response, 48 hrs after implantation, is capable of producing a terrain readily suitable for axon extension in animals implanted before or on postnatal day 8. This is shown by the presence of many unmyelinated axons interspersed

FIG. 8. Scanning electron micrographs of the surface along the filter implanted into postnatal day 21 acallosal mice and examined 7 days postoperatively. (a) The basal lamina (BL) produced by cells which react to the implant and wound, blanket over an extensive area of the filter's surface. The folded surface of the basal lamina is populated by a multitude of cells (a,b,c). (b) These cells extend a myriad of processes over the entire surface of the basal lamina. (c) Many of the cells attached to the surface of the basal lamina are spread flat and resemble type II astrocytes. (d) The cells attached to the filter (I) also have a flat morphology, but do not radiate extensively branched processes as those along the basal lamina. (a) X350; (b) X1230; (c) X2190; (d) X1230.

FIG. 9. Coronal sections showing the staining pattern produced by antibodies against laminin protein. (a) In critically implanted P 8 animals laminin not only appears to be confined to the basal lamina of blood vessels and the pia, but is also along glial processes (arrow) within the filter (b). When animals were implanted at post-critical stage (P 21) laminin stained in massive whorls, basal lamina extended around the implant (I) and appeared continuous with the longitudinal fissure (LF; c, d). The cells producing the laminin are flat. (e, f) Post-critical period animals (P 17) implanted with glial coated filters from neonates (P 2) show a laminin staining pattern identical to critical period animals given naked implants alone (compare a, b to e, f). (a) X85; (b) X215; (c) X85; (d) X215; (e) X85; (f) X215.

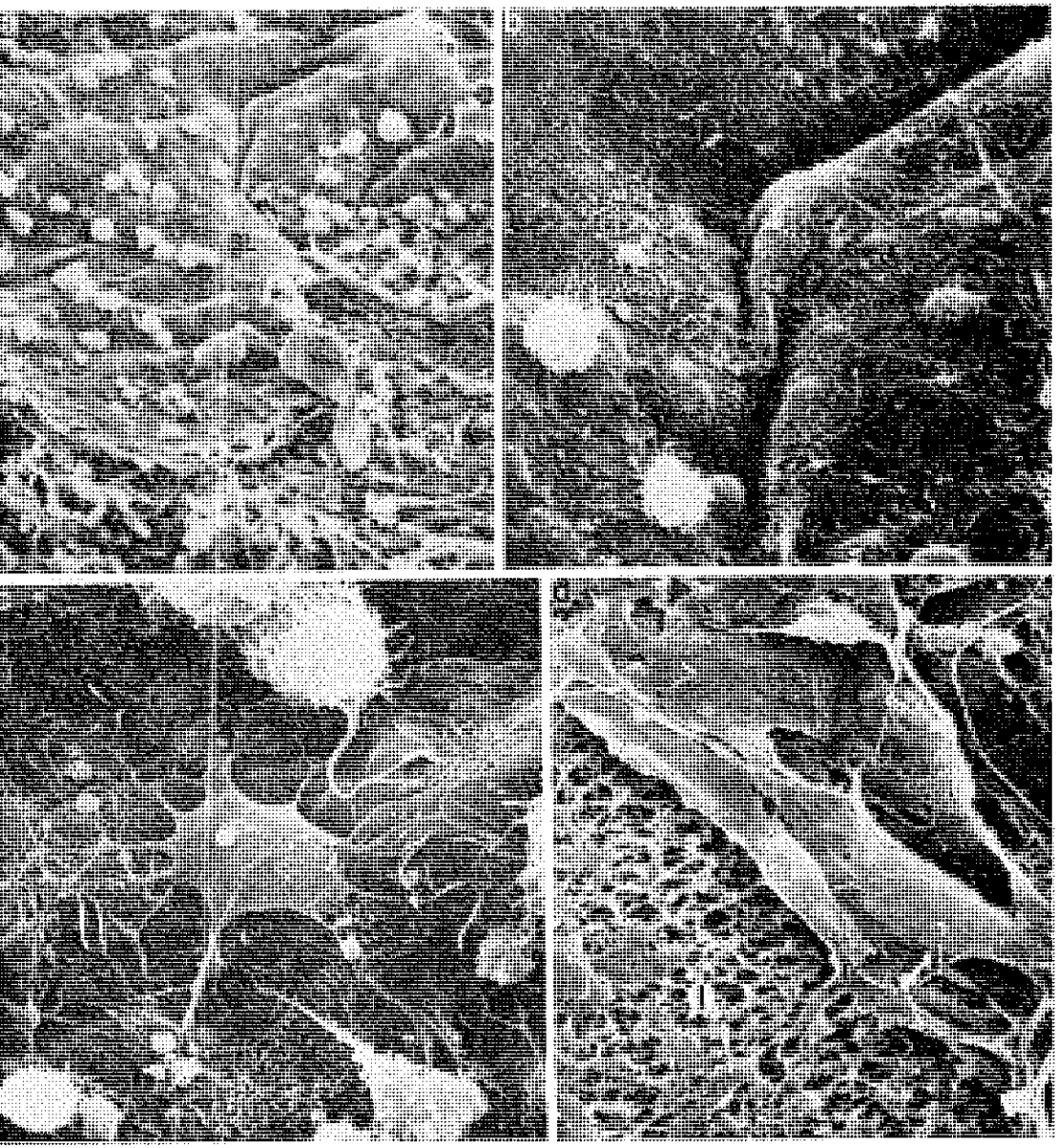


FIG. 8.

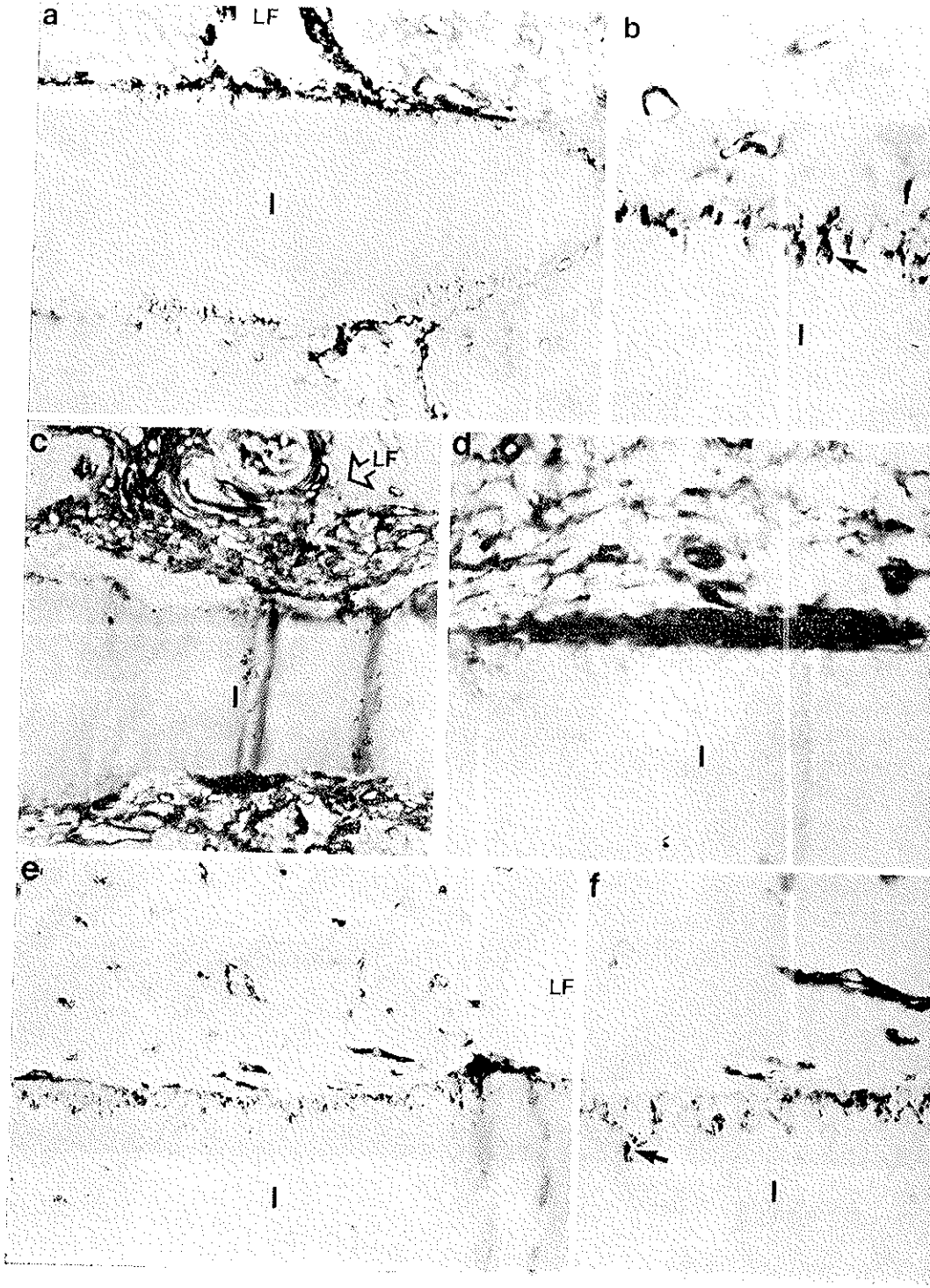


FIG. 9.

among the attached glial cells (figs. 1, 2, 3, 5). In P 2 and P 8 implants the astrocytes, which are above and attached to the filter, appeared to react quickly to the presence of axons by sending out many cytoplasmic processes around the fibers and into the axon bundles, binding them into fascicles (figs. 3, 12, 14b, 16). The affinity of axons for critical period glia was also illustrated in various P 2 implants, in which elongating axons actually grew into the pores of the filter, again intimately associated with inserted glial processes (figs. 2b, 3). In these animals, axons were found directly adjacent to astrocytic processes in the marginal third of the implant. However, in the center of the filter (beyond the depths of the glial processes) a few axons could also be found adjacent to each other in small compact bundles. In some animals implanted on P 8, small patches of basal lamina were located near astrocytes and growing axons. Axons were not observed along the entire length of the ectopic basal lamina. However, axons were observed clustered along the plasma membrane of astrocytes less than 10 μm from the basal lamina (fig. 5b).

Interestingly, towards the caudal aspect of a P 8 implant that only became embedded in one hemisphere with the other end suspended freely in a cyst, axons extended over the glial coated upper surface and continued around the filter's edge to the underside and back into the same hemisphere (fig. 4b). The presence of axons inside filters, as well as the unusual shapes and positions of the de novo formed commissural fiber fascicles associated with Probst's bundles and the implant, clearly indicates that the prosthesis can support the de novo growth of axons (also see 46, 48 for a full discussion of this issue).

In order to determine if newly forming axons arose from cortical neurons and grew to appropriate regions of the opposite cerebral hemisphere, would-be acallosal animals implanted on P 5 and sacrificed after one month, were given a small wedge of HRP crystals superficially into the cortex. In the selected animal shown in figure 1, the representative sections (all taken rostral to the hippocampal commissure) contain retrogradely labelled cortical neurons in a position contralateral and homotopic to that of the injection site (434 cells were labelled in layers II and III predominantly and all but 6 were located homotopically; figs. 1c, d, e). Thus, at least some of the commissural axons in this animal have emerged from one Probst's bundle and have grown across the midline using the implant as a pathway (figs. 1b, c). En route to the

opposite hemisphere, the fibers have even managed to traverse the second neuroma before reaching their appropriate destinations.

The CNS gliotic response generated by implantation of animals later than P-10 did not produce a terrain readily suitable for axon extention. Animals given naked, untreated implants postnatally at 2 and 3 weeks, and 8 months showed little or no growth of axons through or around the glial scar encompassing the implant when examined at one week and as late as 2 months after implantation.

Axon reaction to astrocytes in P 2 neonates induced to flatten by compressing the pores of the implant. Which of the several changes that occur in the host gliotic response to implantation are critical in generating the change from a growth permissive to a growth refractory state within the CNS? Does the change in morphology from stellate to flat alter the astrocyte's functional ability to provide a conducive substratum for axon elongation? In order to answer this question, portions of implants were precrushed midsagittally before insertion into the neonatal brain. We postulated that by crushing filters, the reduction in size of the pores into which glial processes usually extend would force attaching cells into a

FIG. 10. Coronal sections illustrating the staining pattern for antibodies against GFAP. (a) Acallosal neonates (P 2) implanted with filters and examined after 5 days show extensive amounts of astrocytic processes within the implant (I) and the cortex, retaining their stellate morphology. (b) The astrocytes in acallosal animals implanted at post-critical stages (P 21) and examined after 1 week appear flat within the scar above the implant (I). (c) Transplanted neonatal glia on Millipore (I) placed into post-critical period animals (P 34) retain their stellate morphology. (a) X280; (b) X280; (c) X280.

FIG. 11. Scanning electron micrographs of axons extending over the glia attached to a filter implanted into an acallosal postnate (day 2) and examined 48 hours later. The inset shows callosal axons extending out of a neuroma (LB) and across the implant (I). The caudal tip (CT) and borders of the filter are apparent. Higher magnification shows large fascicles of axons traversing the implant towards the opposite hemisphere. However, not all axons retain their orientation and wander in the middle of the filter. X570 (inset) X65.

FIG. 12. Scanning electron micrographs of acallosal mice implanted on postnatal day 2 and examined 24 hours later. (a) View from above, showing a filter which was placed horizontally across the midline extending into each hemisphere. (b) Higher magnification of the surface of the filter showing many attached glia. (c) Axons extend along the glia that have attached to the implant arrowheads. (d) Astrocytes respond to the presence of axons by extending small processes which encircle the axon. (a) X17; (b) X610; (c) X1750; (d) X13,900.

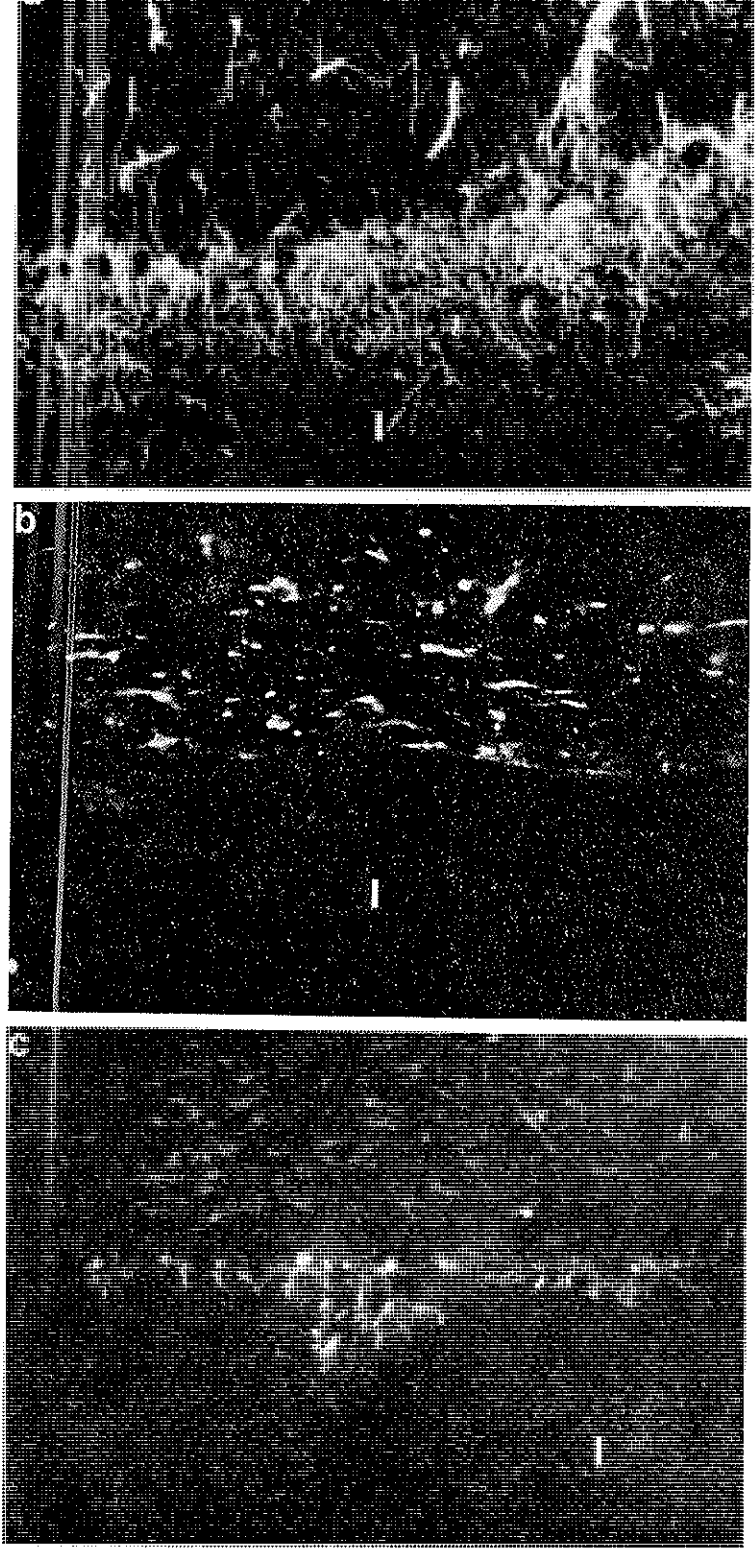
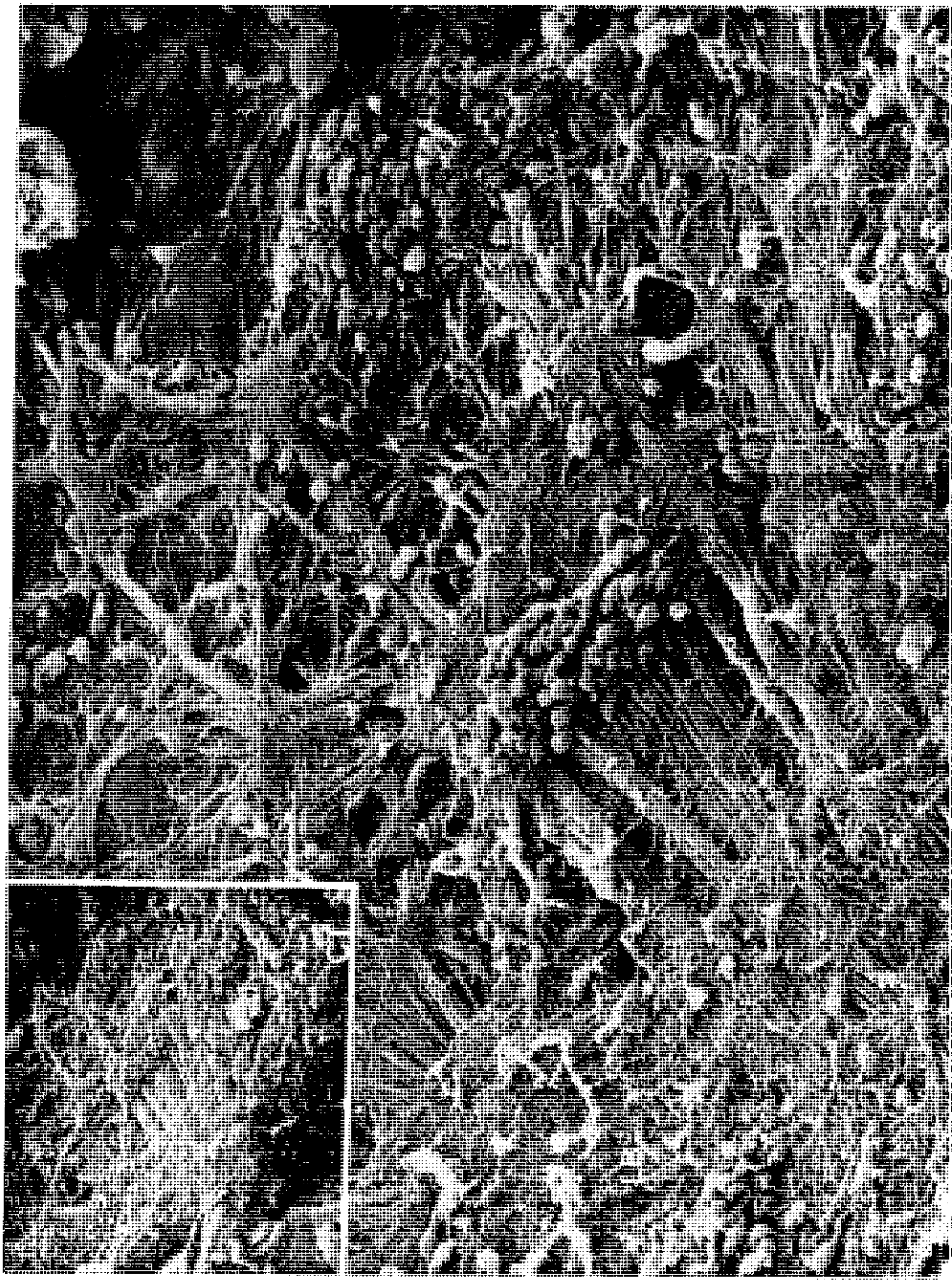


Fig. 10



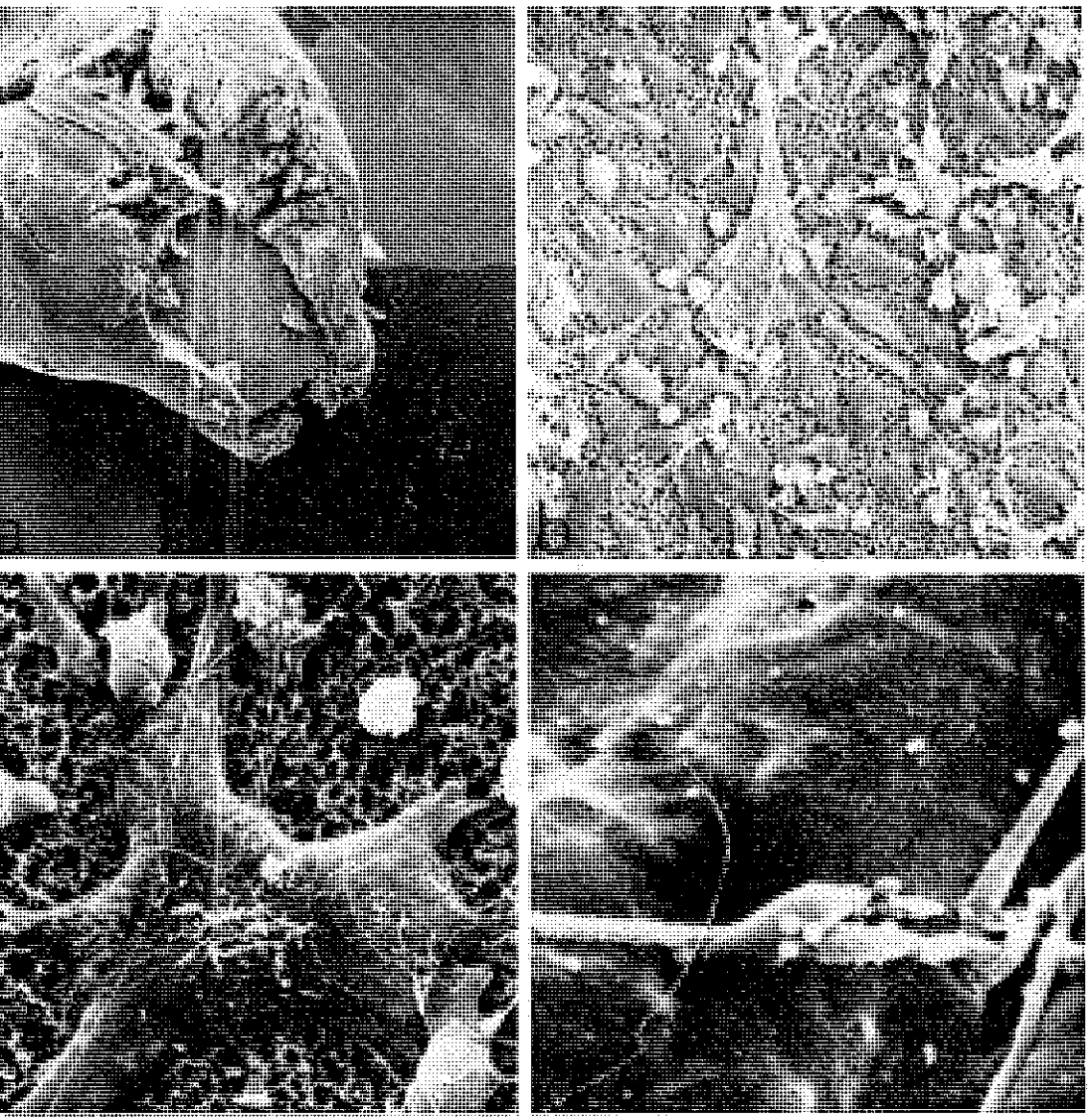


FIG. 12.

flattened configuration (figs. 13a, b; 14). Our observations are compatible with this concept. 48 hrs after insertion on P 2, many GFAP positive (not shown) astrocytes in the crush region spread out flat, some extending to a diameter of 300 μm or more. Some of the flattened glia had vacuolated lamellipodia at their leading edges (fig. 14). SEM of a crushed specimen (but one lacking axons) shows several examples of glia flattened out on the implant surface (fig. 13). The interface between the compacted and noncompacted areas of the filter can be readily demonstrated by SEM, TEM and light microscopy because of the distinct difference in morphology of the attached cells. As usual, the astrocytes that accumulate in layers over the uncrushed aspect of the prosthesis were stellate with many ruffles, blebs and cytoplasmic extensions (fig. 13f). In contrast, the GFAP positive cells over the crushed region had a flattened morphology and often formed a monolayer (figs. 13, 14). In a few instances, however, the upper surface of the flattened astrocyte monolayer became ruffled with a myriad of small processes and blebs (figs. 13c, d, e). In close association with the flat glia undergoing this cytological change was another nearby cohort of cells that encroached upon the territory of those in the crushed region. Such cells ramified profusely along the upper surface of the flattened astrocyte population producing a pile of cells with a flat-celled bottom layer (figs. 13c, e). The glia attached to the flattened astrocytes did not themselves flatten, but instead, assumed a stellate shape like those in the implant's uncrushed sector (figs. 13c, e).

When given a choice, growing axons extended over the glia infiltrating the porous portion of the implant but did not grow among flattened cells on the crushed portion. In a particularly interesting specimen where axons had extended out over the porous part of the filter (fig. 14a), serial sections revealed that those over the lateral aspect of the implant were oriented parallel to the plane of section, whereas those more medially ran perpendicularly. Thus, in this specimen axons extend-

FIG. 13. Scanning electron micrographs of acallosal mice implanted on postnatal day 2 with partially crushed filters and examined 48 hours later. The glia above the crushed portion of the filter (C) are flat, having a smooth surface with few attaching cells (a, b), whereas the glia attached to the non-crushed area (NC) are more stellate in shape (a, c, f). In places, some of the flat glia over the crush rippled and extended many very short processes (d). In areas where this occurred other glia moved on to the flattened cells establishing a cellular pile (e, also fig. 14). (a) X170; (b) X1525; (c) X510; (d) X2950; (e) X1610; (f) X1780.

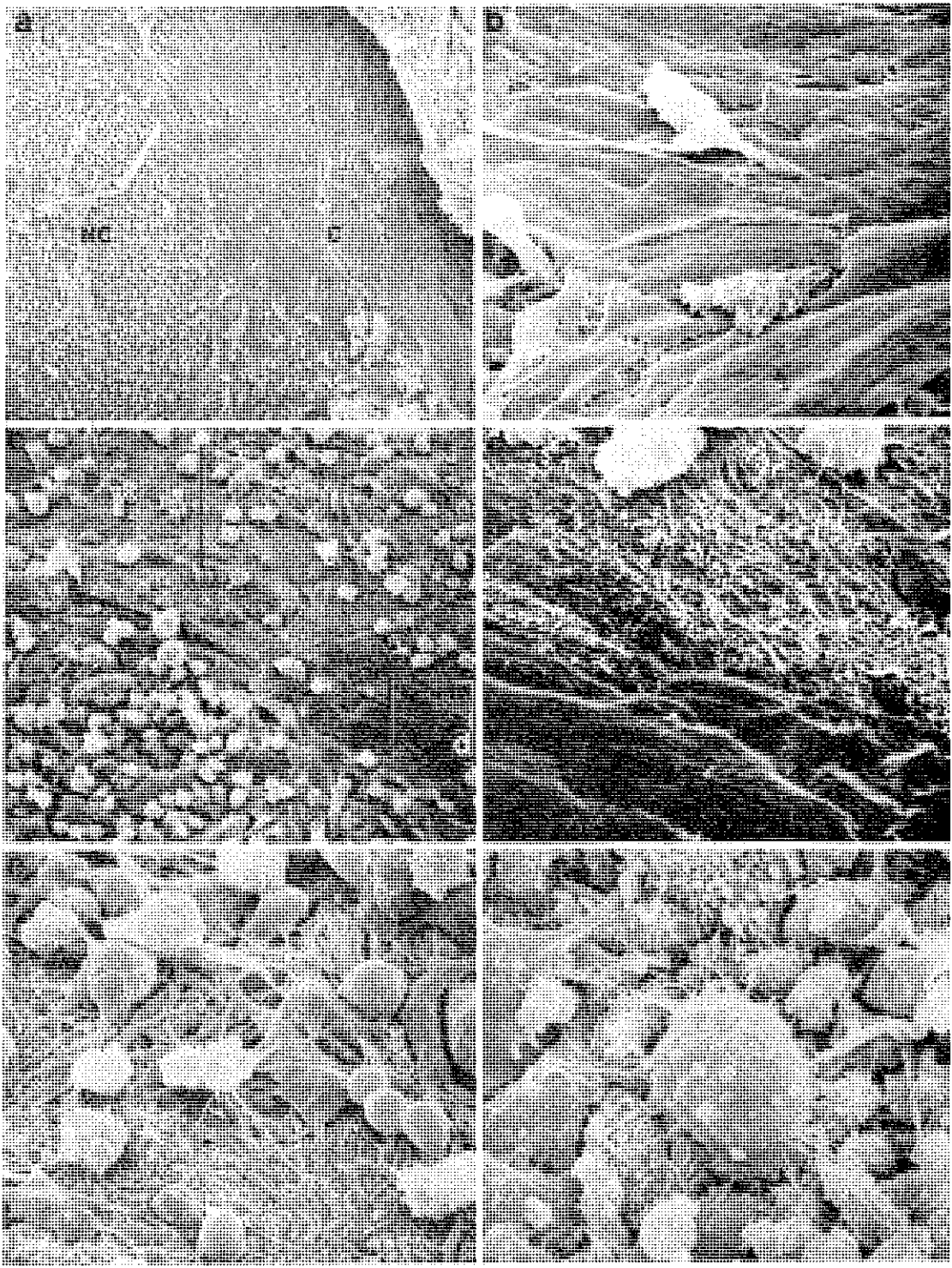


FIG. 13.

ed onto the filter toward the midline but turned abruptly at the crush/non-crush interface, where they collected into a huge fascicle that very much resembled a Probst's bundle. The fascicle extended rostrally above the filter, directly adjacent to the interface (fig. 14a, b). It was well vascularized and surrounded by glia from below as well as above. In places, the tightly bound fascicle was suspended above the compressed portion of the filter, anchored to the porous part by a large group of infiltrated cells. The axon bundle extended rostrally along the interface for approximately 150 μm and finally traversed the compressed area of the implant in a place where a dense mound of cells with a flat cell under layer had formed (fig. 14c). The glial mat that promoted axon crossing over the crushed area most likely developed under similar circumstances as that which was described earlier (i.e. by the subsequent addition of stellate cells to the flat cell monolayer; see figs. 13c, d, e). Once across the midline of the implant, some of the axons extended caudally while others travelled approximately 240 μm rostrally where they passed into the other cortical hemisphere.

Transplantation of glial coated implants from critical to post-critical period animals. Will glia that migrate onto an implant from P 2-4 (critical stage) donors and then transferred to an older (post-critical period) animal, alter the gliotic reaction at the lesion site? Will such precoated implants (i.e., transplants) also promote callosal axon growth across the cerebral midline in post-critical period animals? In order to answer these questions, 2 day old acallosal mice were implanted with nitrocellulose filters. After 48 hours the glial coated implants were harvested and transplanted into 17 or 34 day old (post-critical period) acallosal mice. In most animals (14 out of 20 transplants) the glia survived the trans-

FIG. 14. Coronal sections through a partially crushed filter implanted into a postnatal day 2 acallosal mouse and sacrificed 48 hours later. (a) The axons (Ax) traverse the surface of the implant along the glia that extend processes into the non-crushed portion of the filter (NC), but they whirl and turn abruptly at the crush (C)/non-crush (NC) interface forming a neuroma. The astrocytes attached to the surface of the crushed portion flatten (arrowheads and upper inset), displaying a mound where the nucleus resides and lamellipodia at their periphery (inset). (b) The axon bundle turns perpendicularly and travels along the crush/non-crush interface. (c) Fibers crossed over the crushed portion of the implant above a mat of glia with a flat celled bottom layer (open arrow; c). The fibers then grew rostrally on another inserted group of cells on the other side of the crush (Ax at the right in b). (a) X340; inset-left side X535, right side X2820; (b) X340; (c) X340.

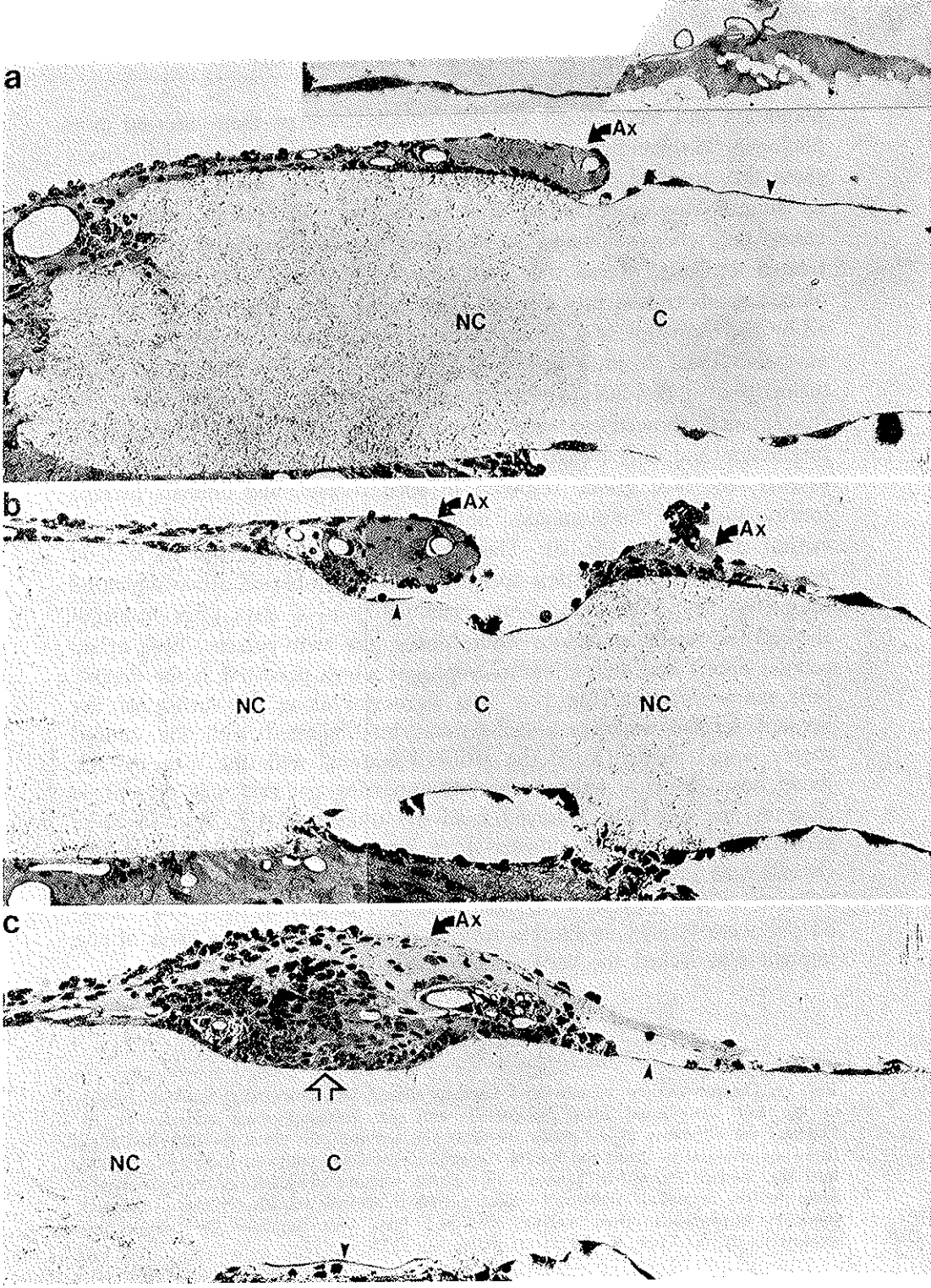


FIG. 14.

plantation and were clearly demonstrable because of their inserted morphology. ^{3}H-thymidine experiments are now in progress to definitively show that attached cells come from the donor. The brains of transplanted animals displayed distinct changes in the glial reaction around the transplant, when compared with brains receiving non-glial coated, untreated implants. Recall that implantation of naked filters into P 14 or older animals consistently resulted in rampant tissue degeneration, followed by the formation of a dense, flat-cell form of glial scar complexed with extensive basal laminae and collagen (figs. 15a, b). In contrast, the majority of individuals given implants pre-coated with immature glia (transplants) showed no such scar formation, with little basal lamina production and only meager amounts of tissue trauma and bleeding. In essence, the host gliotic response in such transplanted animals became indistinguishable from animals implanted with naked implants during critical stages (figs. 15c, d). These transplanted animals also showed an anti-laminin staining pattern identical to critical period filter-implanted mice (fig. 9). Most transplants examined 3 to 6 days after insertion showed (especially in regions where donor glia were present) little or no cellular debris ond only a few macrophages which persisted at the donor/host tissue interface (fig. 17). The astrocytes along the surface of the implant formed multiple branches that interdigitated with the injured cortex, appearing to "knit" the artificial material with the tissue of the living host. In most animals, normal appearing neuropil was present as close as one cell layer removed from the transplant (fig. 17). In these successfully transplanted animals there was minimal invasion of mesenchymal cells into the wound site. However, in a few instances a dense collagenous scar with layers of basal lamina, mesenchymal cells, and flattened glia were located in discrete regions of tissue adjacent to areas of the implant that lacked the penetrating stellate form of glial cell.

FIG. 15. Coronal sections of untreated implants placed into post-critical acallosal animals (a, b), and transplanted filters pre-coated with glia harvested from neonates (c, d). Compare the difference in the gliotic reaction. The reacting cells along the untreated filter (a, b) are arranged in sheets and have a flattened morphology, with few processes extending into the implant. (c, d) In contrast, the gliotic reaction produced in the post-critical brain by the transplant resembles critical period implanted animals. Numerous inserted processes (arrowheads) from stellate cells and little scar formation or necrosis are evident (c, d). (a) X104; (b) X335; (c) X104; (d) X335.

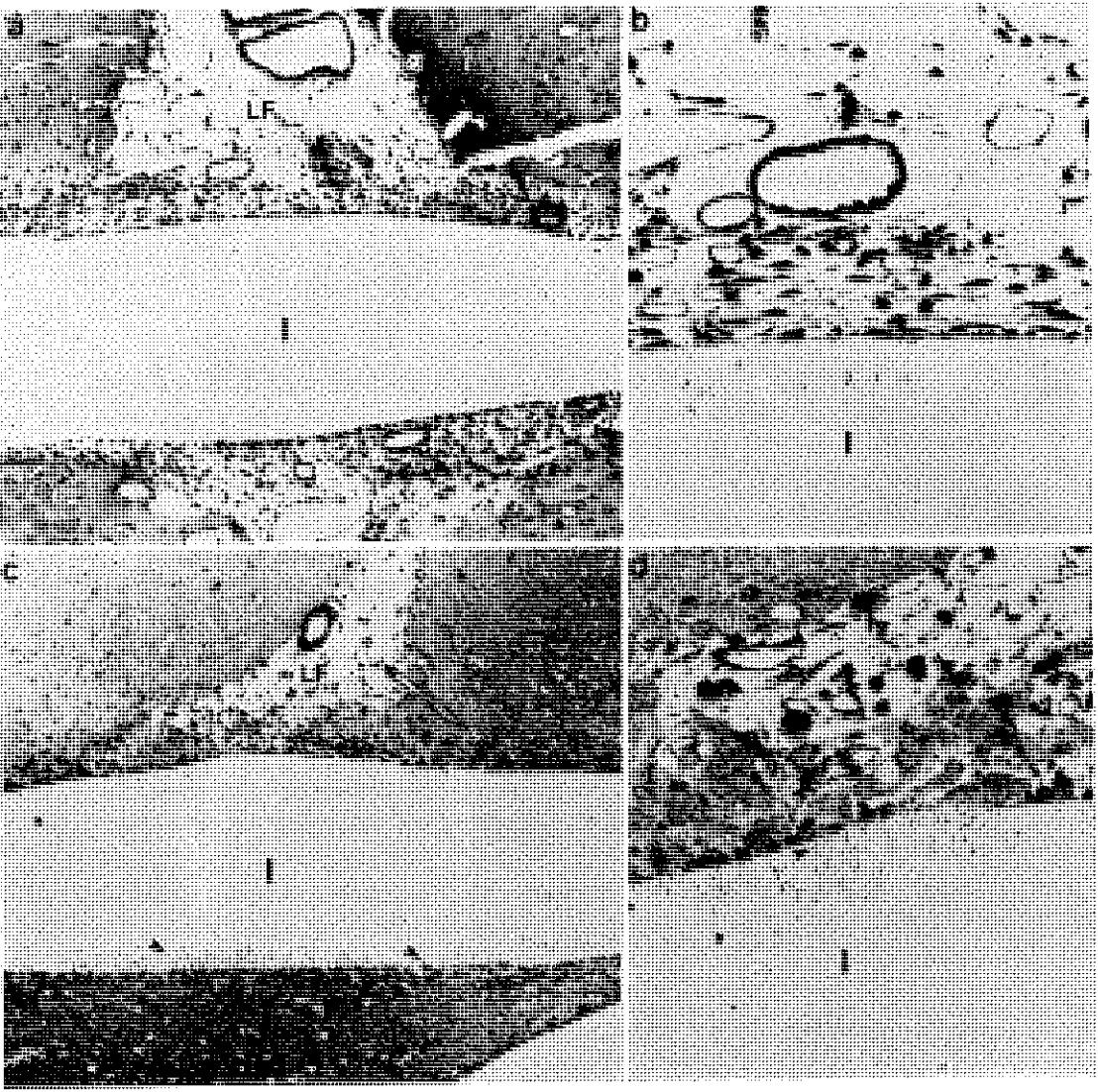


FIG. 15.

Induced axon growth over glial transplants. In 2 of the 20 post-critical period, transplanted animals examined after 4 to 11 days, several hundred "regenerating" or sprouting axons were present at the previously lesioned cerebral midline among the transplanted glia attached to the implant (fig. 16). The axons were all unmyelinated and bundled in small fascicles by glial processes (fig. 16b). Extensive examination of the cells along the surface of the implant indicated that no neurons and only axons were present along the transplant surface itself. As our transplant techniques improve, the cortical source of the neurons that produce the regrowing axons and their eventual destinations can be determined.

Discussion

In a previous study of surgically induced acallosal mice it was shown that, at early postnatal stages, axons entrapped within Probst's neuromas retain the potential to regrow between the cerebral hemispheres when they are presented with a properly aligned, glial-covered scaffold [51]. The present study demonstrates the existence of a critical period for such substrate supported axon re-elongation. The de-novo growth of commissural axons across the cerebral midline was observed in acallosal animals implanted with untreated prostheses prior to postnatal day 10, but not later. Horseradish peroxidase analysis has shown that such fibers originate from cells primarily in layers II and III of the cortex and terminate in appropriate homotopic locations in the opposite hemisphere (fig. 1).

During "critical" stages the migration of astrocytic glia onto the implant and the insertion of their processes are extremely rapid events, occurring within 12 to 24 hours. The cells migrating onto the implant initially express vimentin, while those that have sent processes into the

Fig. 16. Transmission electron micrographs of a postnatal day 17 acallosal mouse that received a pre-glial coated implant from a 2 day neonate donor and then examined 6 days after it had resided in the host. (a) The glia attached to the implant have retained their stellate morphology and infiltrated cytoplasmic processes. Scarring and ectopic basal lamina are lacking. Among the glia are many de novo formed axon bundles (arrowheads). Higher magnification shows loosely fasciculated unmyelinated axons (b) and others adjacent to astrocytic processes (c). Two daughter cells above the implant (a) share a midbody (d), thus, transplanted cells can divide. (a) X8260; (b) X16,530; (c) X16,530; (d) X2730.

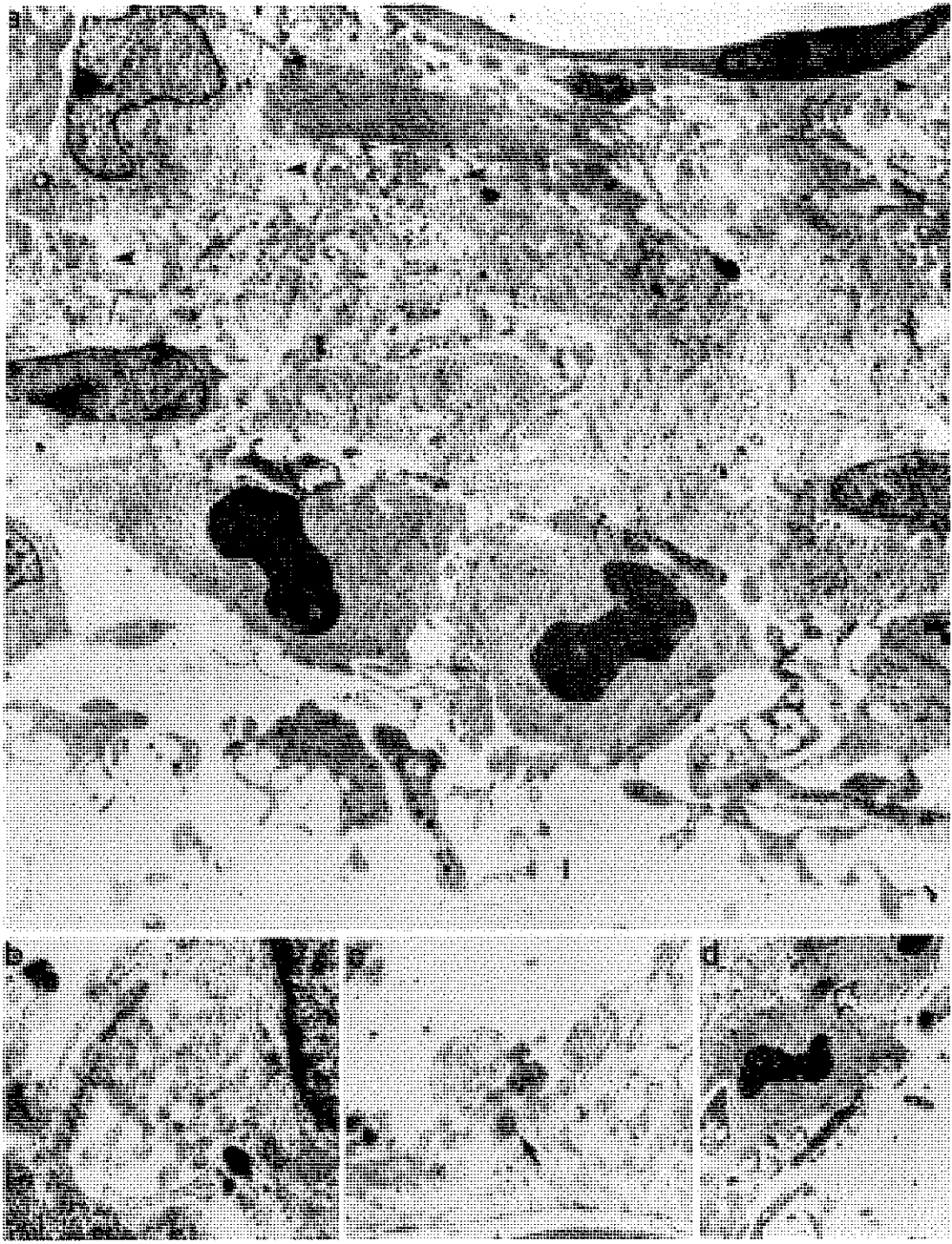


FIG. 16.

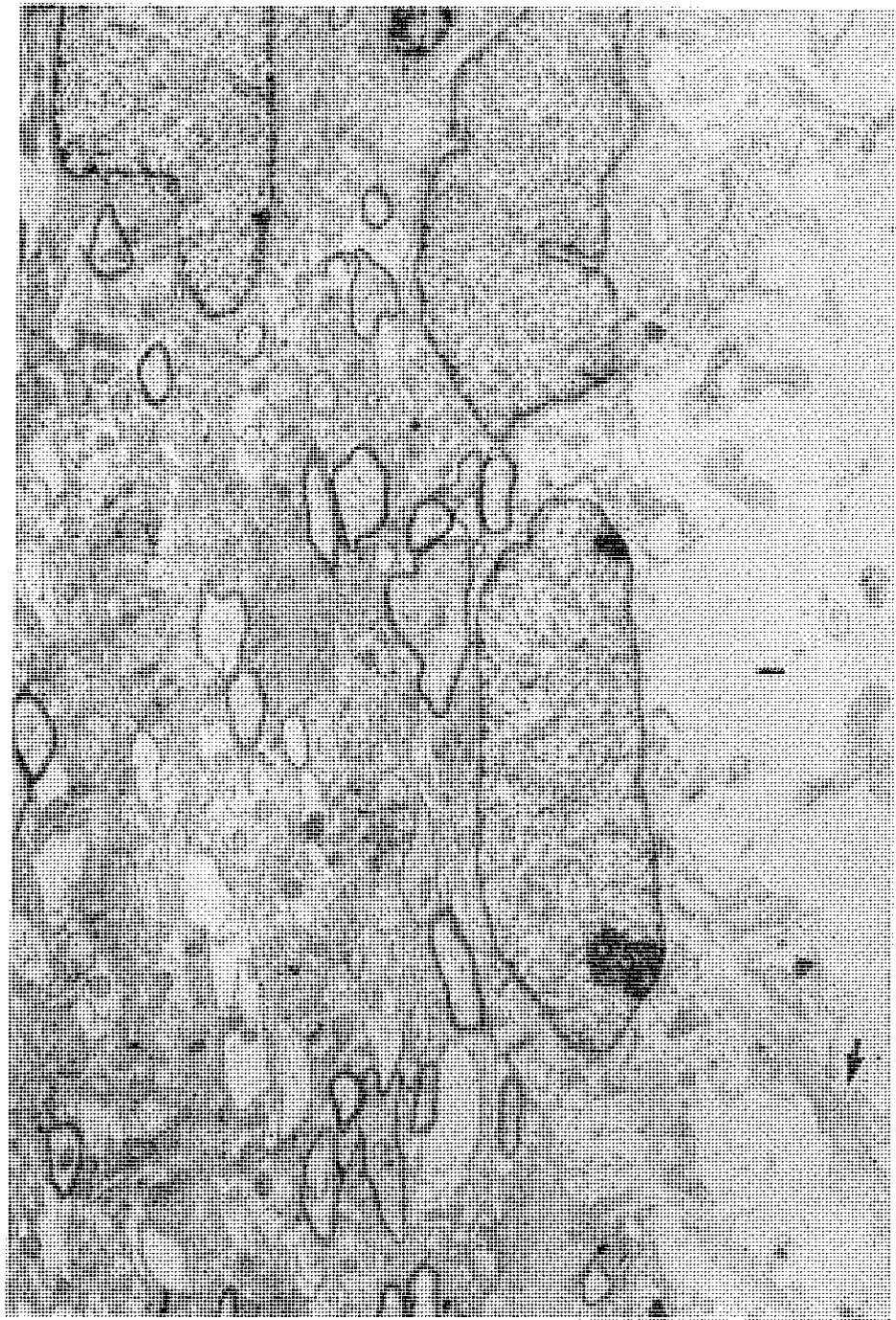


FIG. 17. Transmission electron micrograph of the host/donor interface (lateral to the midline) from a P 17 acallosal animal transplanted with pre-coated glial implants and examined after 11 days. The astrocytes attached to the implant (I) retain their inserted processes, which are rich in intermediate filaments (arrow). The cortex above the attached glia shows no signs of tissue degeneration or scar for-

filter expressed GFAP. During and after the initial glial invasion phase, the same gliotic cell population that moves onto the implant has the capacity to support axons as well as vascular elements to grow between the hemispheres. In turn, astrocytes appear to respond to the presence of growing axons by extending cytoplasmic processes around the fibers. The reactive glial response in animals implanted later than 14 days after birth showed distinct differences from gliosis observed in neonates. The length of time it takes glial cells to reach the surface of the implant, the amount of bleeding from the implant site, the extent of secondary necrosis, and the degree of basal lamina production at and around the filter, all increase with age (see table I). The morphology of the migrating cells surrounding the implant also becomes altered and, most importantly, these reactive cells lose their ability to stimulate axon outgrowth. Thus, in contrast to adult "reactive" gliosis, we suggest that the gliotic response in the neonatal mammalian CNS be an active rather than reactive phenomenon and, thus, when controlled with a prosthesis, be considered a beneficial and constructive process. We suggest the nomenclature "activated gliosis" when referring to the stereotyped, immature form of glial response to injury. Phenomenologically similar, "activated astrocytes" have been described in the developing [52] or regenerating optic nerve of *Xenopus laevis* [5, 42], suggesting that the adult CNS of amphibia may retain the primitive "activated" form of glial cell. Consequently, there may be many features shared by the immature "activated" astrocyte in the mammal and the adult astrocyte of regenerating species of amphibia [1, 59].

During the critical period the de novo forming commissural axons grow mainly among the astrocytic processes which lie a cell layer or more removed from the implant. What is special about this region, early on, that establishes it as such an attractive growth substratum for axons? What are the critical changes that occur here in older animals that lead to a failure in axon outgrowth?

Recently, astrocytes have been shown to produce laminin and fibronectin (major components of the basal lamina) in culture [28]. Laminin immunoreactivity has also appeared on GFAP positive reactive astrocytes produced from neurotoxin induced injury in the CNS of adult rats [29]. It has also been suggested, that laminin may play an adhesive role in attracting axons along their stereotypical pathways during normal development [29, 56]. Acallosal animals implanted with nitrocellulose bridges at precritical stages showed a laminin staining pattern that was

TABLE I - *This table illustrates our qualitative impressions of the changes that occur during and after the gliotic reaction induced when acallosal mice are implanted with nitrocellulose filters at critical and post-critical stages, and when implants are transplanted from neonates to post-critical period animals.*

	P 2-8 Critical *	P 14-Adult Post-critical *	P 2 to P 17,34 Transplant +
NUMBER OF GFAP + CELLS PRESENT	+++	++	++
LAMININ ALONG GLIAL PROCESSES IN FILTER	++	—	++
N-CAM ON GLIA ON IMPLANT	+	—	?
AXON OUTGROWTH OVER FILTER	+++	—	+
NECROSIS	+	+++	+
BLOOD SURROUNDING FILTER	+	+++	+
BASAL LAMINA	—	+++	+
COLLAGEN	—	+++	+
ASTROCYTE SHAPE	STELLATE	FLAT	STELLATE
INSERTED GLIAL PROCESSES	+++	—	++
TIME TO GLIAL CAPSULE FORMATION AROUND FILTER	24 to 48 hrs	5 to 7 days	2 to 3 days

* Untreated implants.
\+ Implants from P2 critical period mice.

confined to the area where glial processes extend into the filter and around blood vessels, but not in the surrounding cortical tissue away from the implant, where the majority of de novo formed commissural axons grow. The ability of the astrocytes' cytoplasmic processes within the filter to express laminin suggests that either these cells can polarize their distribution of laminin protein or that laminin is produced in a uniformly low amount on processes in and above the filter, but is concentrated by the nitrocellulose. Since all areas where laminin staining

was apparent (e.g. the glial limitans of the pia, and blood vessels) contained cells which were foreign to the CNS, as was the implant itself, it may be that astrocytes have the ability to recognize and react to non-brain cells or substances by producing laminin. It is also conceivable that laminin may attract a small number of axons into the filter. However, it is yet premature to conclude that laminin plays a major role in stimulating the bulk of axonal growth that occurs on astrocytes above the implant.

During development, the CNS is isolated from non-neural tissue by a basal lamina that forms at the endfeet of perivascular and pial glia. In the developing optic [48, 53, 54, 55] and olfactory [18] pathways as well as the embryonic spinal cord [38, 55] of most species of vertebrates (except perhaps teleosts [15]), growing axons tend to travel adjacent to the endfeet, but rarely touch the basal lamina. When injuries occur within the CNS, the resulting cellular disruption often stimulates the reconstitution of the glial limiting membrane throughout the wound site [43]. In implanted acallosal neonates where axons were induced to extend across the midline there was no or very little extraneous basal lamina formation and growing axons were usually found directly adjacent to astrocytic processes, as they are in normal development [48, 53, 55]. In animals implanted with prostheses on postnatal day 8 (a late critical stage when small patches of basal laminae on flattened cells sometimes developed within the pathway of stellate astrocytes) axons were rarely observed directly apposed to the basal lamina. Rather, they were usually positioned along membranes of stellate shaped astrocytes even when basal lamina and astrocyte membranes were closely juxtaposed. This observation suggests that when given a choice, callosal fibers prefer to associate directly with the plasma membrane of immature glia, rather than with basal lamina. Implantation of nitrocellulose prostheses during the post-critical period resulted in astrocyte scarring and the estahlishment of multilayers of intracerebral basal laminae. Axons did not regenerate across the midline through such a terrain. During adult reactive gliosis, the dense encapsulation of the scar by limiting membranes has been suggested repeatedly over the past century [9, 25, 40, 57] to produce a barrier to regenerating axons. Our observations provide support for this hypothesis. However, we would also suggest that the basal lamina may not merely form a mechanical impediment to axons. Since the basal lamina that forms within a scar covers the surfaces of many astrocytes (fig. 7c and e), it may reduce the axon/astrocyte interaction, which is essential for the support of axonal guidance across the midline.

Our experiments suggest that the physico-chemical state of the astrocyte itself is crucial for axonal growth. Reactive glia are flat and do not readily promote axonal extension. Activated astrocytes are stellate and support axonal extension. When partially crushed filters were implanted into P 2 animals (a critical period when no basal lamina forms within the wound), the astrocytes encasing the implant assumed a flattened shape over the compacted area and a stellate shape in the uncrushed region. Axons extending onto the prosthesis turned precipitously at the crush/non-crush interface, traveling tangentially to, but rarely onto the surface of the flattened astrocytes. This observation suggests that growing axons have a preference not only for astrocytic membranes, hut specifically for the penetrating, stellate variety.

These growth preference phenomena may be explained, we suggest, by either of two mechanisms: (1) that there are at least two different astrocyte populations that have different affinities for the crushed and non-crushed areas of the implant and, in turn, have different affinities for axons, or (2) that there is a single astrocytic cell type that invades the filter but expresses a different morphology depending on its age or the conformation of the implant's surface, and this morphological alteration is directly involved with the cell's ability to stimulate or sustain neurite outgrowth. At present we are unable to distinguish between these possibilities. However, *in vitro* studies suggest that the induction of a flattened morphology does decrease the adhesivity of the upper surface of fibroblasts and epithelial cells [19, 37]. Therefore, it is conceivable that the flattened form of young or old astrocyte may suffer a reduction in some adhesive axon growth promoting property [27, 55, 58]. In this regard some preliminary studies on the role of N-CAM, the neural cell adhesion molecule [45], may be relevant. We have recently demonstrated with anti N-CAM antibodies, that N-CAM is produced by critical stage astrocytes, but primarily on their processes within as well as outside of the implant. Glial cells within a reactive scar at post-critical stages make much less detectable N-CAM. Furthermore, flat glia in contrast to stellate glia exhibited a reduction of cytoplasmic process formation. Thus, in a more mechanical sense, flattened cells may not provide a large enough surface area for cell-to-cell contact and may have a limited ability to erect a proper scaffolding for a large number of growing axons [16]. Thus, our results suggest that many progressive changes in the CNS glial response to injury appear soon after birth, reducing the effectiveness of potential axonal growth that can occur as the brain matures.

Transplantation of neonatal, glial coated implants into postcritical stage animals. In comparison to post-critical period animals that received naked implants, older animals transplanted with bridges that were precoated with glia from P 2 donor mice showed dramatically reduced amounts of tissue degeneration and glial-mesenchymal scarring. The lack of extensive tissue degeneration and bleeding in transplanted animals suggests that the transplant increases the survivability of cortical tissue near the site of injury. Nieto-Sampedro *et al.* [36] have shown that extracts containing injury-induced neurotrophic factors from lesioned animals increased the survival of transplants, when the tissue was transplanted with the extract. In adults, the production of injury-induced neurotrophic factors has a considerable lag time when compared with the neonatal counterpart [35]. We have shown that transplantation of immature "activated" astrocytes into post-critical period animals buffers the traumatic effect of the wound itself, perhaps by the production of a similar type of neurotrophic substance. In turn, the increase in survivability of the cortical tissue near the lesion site and the reduction of gliosis may further enhance the regeneration of axons onto a transplanted glial substrate that is very attractive to axons.

Taken together, our results [and also see Hankin and Silver, in press and 49, 51] suggest that immature astrocytes in the region of the presumptive callosal pathway of neonates have the capability of attaching to a nitrocellulose sheet and functionally reproduce the "sling-like" structure that stimulates axons to extend between the hemispheres during normal development. The effectiveness of the glia to recapitulate this structure postnatally and provide a properly aligned substratum for axon extension is transitory, and diminishes quickly with age. When untreated filters are introduced into acallosal animals later than postnatal day 10, the resident astrocytes react to the wound and implant by producing a dense glial-mesenchymal scar that lacks the ability to promote axon extension. Finally, when axon growth promoting glia of the critical stage are removed from a neonate (retaining structural integrity) and transferred to a more mature or adult acallosal animal, the negative effects of reactive gliosis and scarring in the host are repressed, potentially reestablishing an environment conducive for axon regeneration. Our major goal for the future will be to attempt to understand the cellular and molecular events that lead to the inhibition of scarring, secondary necrosis and induction of axonal outgrowth by such glial transplants.

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classes of cells [1, 5, 6, 37]. This approach has not however had the same success in the study of microglia and the picture appears to be confused. Apart from our own studies to be considered below only two reports have shown that microglia share antigens with macrophages [11, 26]. There are a number of other reports that antisera directed against macrophages only stain macrophages in the neonatal or injured brain but not microglia [28, 31, 41, 43]. Possible reasons for these differences will he considered later.

In the course of a series of experiments on cell death in the developing retina of the rat we [35] noted that there were large numbers of macrophages on the vitread surface of the retina of newhorn animals, but few in adult animals. To further study these cells and to follow their fate during development we have used the monoclonal antibody F4/80.

The rat monoclonal antibody F4/80 defines a 160K plasma glycoprotein expressed by mouse macrophages [2]. The antigen is sufficiently stable to allow aldehyde fixation and wax embedding prior to immunohistochemical localization, thus giving good preservation and morphology. At the time of writing F4/80 has been used to identify most cells of the mononuclear phagocyte system, as judged hy other criteria, and there is no example of an F4/80 positive (F4/80+) cell which clearly does not belong to this type [19].

The retina

Retinae from mice ranging in age from embryonic day 16 through to adulthood were prepared for the localization of F4/80 using the avidin-biotin-peroxidase method [20]. In the retina at embryonic-day-16 the vast majority of F4/80+ cells are found lying on the vitread surface among the developing blood vessels and have the appearance of round monocytic cells. At progressively later embryonic stages and in the first postnatal week F4/80+ cells migrate into the retina following an inner to outer sequence. Very few cells are found within the most outer third of the developing neuroblastic layer or the outer nuclear layer. As the F4/80+ cells move into the retina they lose their monocytic appearance and fine processes emerge from the cell body which in older animals become more crenelated and ramified.

The period over which we observed the migration of F4/80+ cells into the retina was coincident with the onset of cell death within the nuclear layers. We noted that the maximum numher of pyknotic cells in

the ganglion cell layer occurred on the day of birth while the maximal number in the inner nuclear layer occurred on the fifth day after birth. Few dying cells were found at any age within the outer nuclear layer. Thus, cell death in the mouse retina progresses in an inner to outer sequence [20, 49]. Approximately one-half of the pyknotic cells were found to have F4/80+ processes around them. Between ten and twenty days of age the numbers of dying cells declines dramatically and the F4/80+ cells adopt a form and distribution similar to that seen in the adult. The F4/80+ cells are largely confined to the two plexiform layers and their shape is best seen in horizontal sections or wholemounts. These adult F4/80+ cells are similar in morphology to cells described as retinal microglia seen in other species [9]. A notable feature of these cells is the regular pattern they form with each cell having its own territory (Fig. 1).

We concluded from this study that macrophages migrate from the vascular supply into the retina, phagocytose the dying cells and differentiate to become microglia. It could be argued that the F4/80+ cells in the retina are derived from the ventricular cells adjacent to the retinal epithelium and dividing cells do not express F4/80; but mitotic macrophages in culture are F4/80+ (Gordon, unpublished), as are mitotic macrophages in tissue sections (see below) and such cells have not been observed at the ventricular surface. The suggestion that dying cells are the stimulus for the invasion of macrophages into the retina is supported by the finding that cell death is relatively uncommon in the outer nuclear layer [20, 49] and F4/80+ cells are absent from this layer. Our results provide strong evidence for the monocytic origin of microglia.

The brain

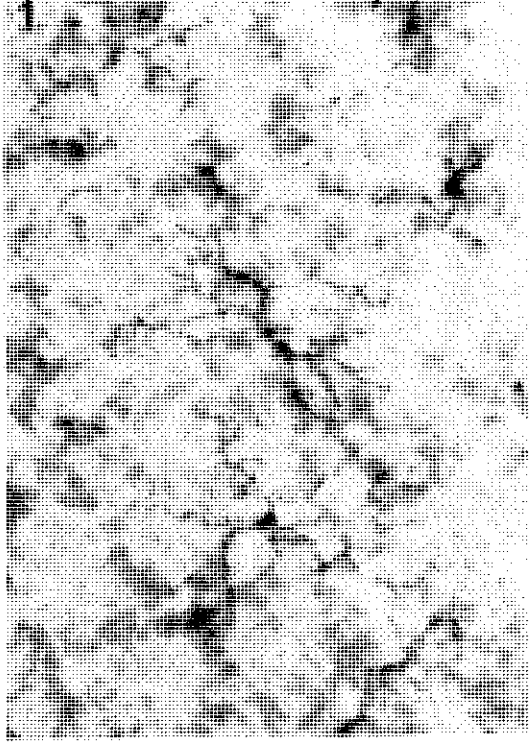
The purpose of our studies on the developing and adult brain was severalfold. We were interested to learn whether microglia could be detected in the central nervous system using F4/80 and other antibodies to surface antigens known to be present on macrophages. We were also interested in examining the distribution of microglia to determine whether their final distribution could be clearly related to the amount of cell death known to occur in different brain regions. The detection of the F4/80 antigen was done in the same way as described previously [20]. However, this method was not compatible with the localization of the type 3 complement receptor, CR3, as revealed by the monoclonal antibody Mac-1 [4, 40] or the IgG1/2b Fc receptor as revealed by the monoclonal anti-

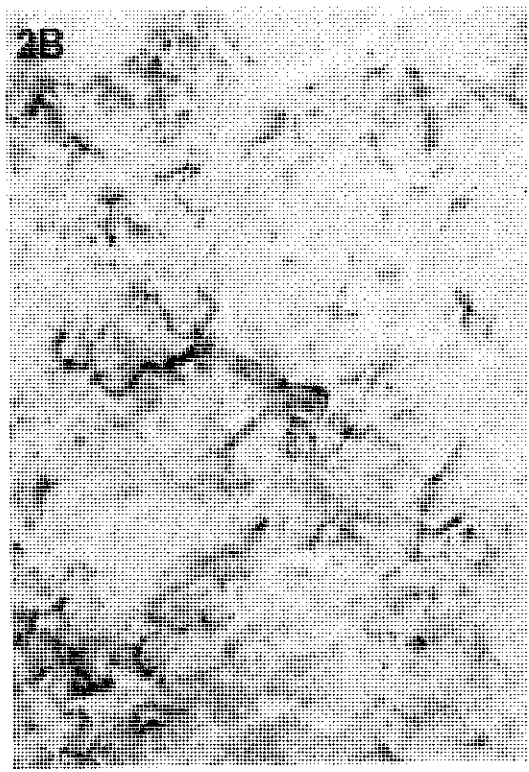
body 2.4G2 [42]. Both of these receptors are known to be present on macrophages. To localize these antigens the animals were fixed with periodate-lysine-paraformaldehyde [27] and cryostat sections were cut and processed [36].

In the brain of the adult mouse all three antibodies labelled microglia; the morphology, distrihution and number were not found to differ with each of the antibodies. These cells at the light microscopic level are similar in all respects to cells previously described as microglia by other authors (Fig. 2). The cells have a small nucleus which appears heterochromatic when stained with basic dyes. The fine processes leaving the cell body are crenelated and may branch several times. The longest processes seen in a single section are about 40 μm in length. Although difficult to appreciate in a single section it appears that each cell has its own territory and we have rarely observed two cells lying immediately adjacent to each other. At the electron microscopic level we see that these cells have many of the characteristics previously described for microglia (Fig. 3) and some of these cells in the normal adult mouse brain are found to be phagocytosing debris, a feature of macrophages.

In the regions of the brain that we examined microglia were ubiquitous, there were no areas from which they were consistently absent. The numbers of microglia were not apparently related to the amount of cell death known to occur in different structures in the rodent brain. For example, the number of dying cells or cells lost from the different layers of the cortex varies, there being greater loss from the superficial layers than the deep layers [14, 16] and yet the numbers of microglia remain uniform across the layers. Furthermore, it is clear that the density of microglia in white matter is much less than in grey matter despite the fact that many fibre tracts contain many degenerating axons during development.

Brains from embryonic and postnatal mice show a similar picture to that found in the developing retina. The first F4/80+ cells found at embryonic day 16 are monocytic in morphology and are frequently close to large blood vessels particularly in the region of the developing cortical white matter (Fig. 4). At this stage there are no cells with the appearance of microglia. At later embryonic stages and the first postnatal week the number of F4/80+ cells increases as does the number of pyknotic cells. Many of these pyknotic cells are surrounded by F4/80+ processes. The F4/80+ cells can be followed through a series of





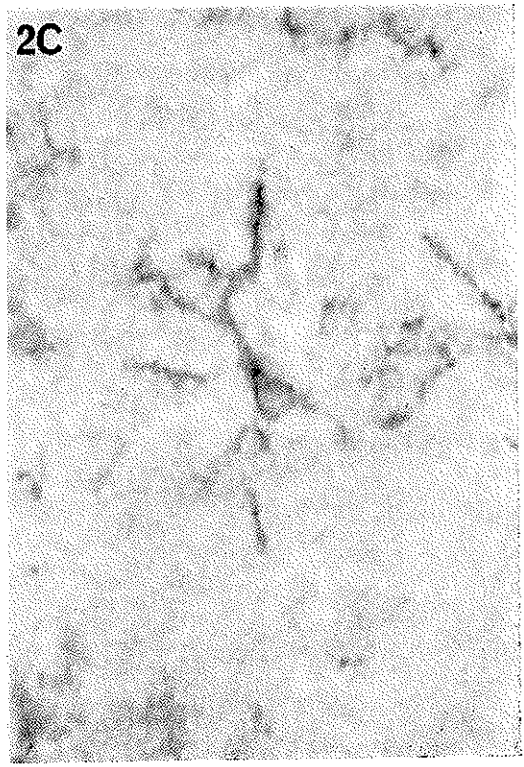


FIG. 1. F4/80+ microglia in the inner plexiform layer. Wholemount retina preparation. x410.
FIG. 2. (A) F4/80+, (B) Mac-1+, (C) 2.4G2+ microglia in the adult muose brain. (A) x260 (B & C)

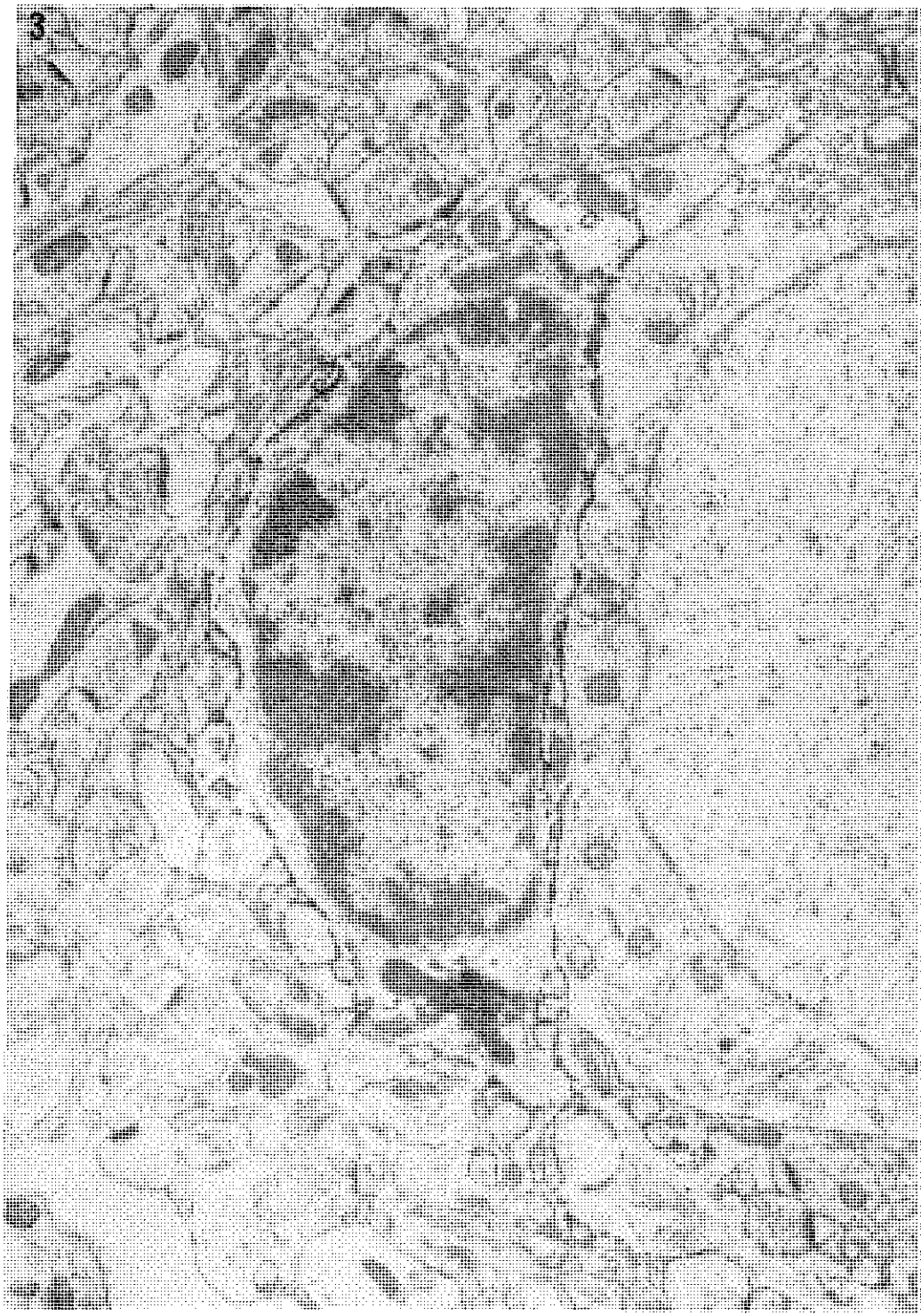


FIG. 3. Electron micrograph of an F4/80+ microglial cell in the adult mouse brain. x 16000.

transitional forms as they differentiate from their monocytic form to become microglia (Fig. 5).

The results from this study provide further strong evidence for the monocytic origin of microglia and again suggest that the initial stimulus for the invasion of the nervous system by macrophages is naturally occurring cell death. While it is possible that microglia are unrelated to the macrophages and yet share a common antigen, F4/80, the presence of the CR3 and Fc receptors on the microglia leaves little room for doubt as to their monocytic origin. The ontogeny of the F4/80+ cells within the brain provides further support for the contention that the F4/80+ cells are delivered to the nervous system via the vascular supply and are not neuroectodermal in origin. We have not observed dividing F4/80+ cells at the ventricular surface at any age.

The question now arises as to why we have succeeded in demonstrating that microglia have surface antigens found on macrophages while others have not (see Introduction) and in particular we have shown that microglia possess at least two receptors previously thought to be absent from microglia [32, 34, 48]. At least part of the answer lies in the sensitivity of the detection system that we have used, the avidin-biotin-peroxidase method [21]. In addition our results clearly demonstrate the importance of testing different fixatives for different antigens before concluding that a particular antigen is absent. It will be interesting to examine the surface properties of microglia using other antibodies directed against known macrophage antigens to see which are common to both.

The stellate morphology of various tissue macrophages is well documented [19] and yet there are clear differences between these cells and microglia. The apparently unique shape of microglia which develops as macrophages migrate into the parenchyma cannot simply be attributed to the geometry of the surrounding neuropil since microglia in the white matter are often indistinguishable from those in the grey matter. On the other hand it is interesting to note that F4/80+ cells associated with the peripheral nervous system are more like other tissue macrophages (Fig. 6). Thus, it is not simply the contact with neurones that induces the microglial morphology and this is also true of *in vitro* studies [37]. It is not hard to imagine that the change in form from monocytic to microglial is associated with other changes in the phenotype of these cells, but what the nature of these changes is remains to be explored.

Another question raised by our observations is whether the macrophages play any role in the development of the nervous system other than

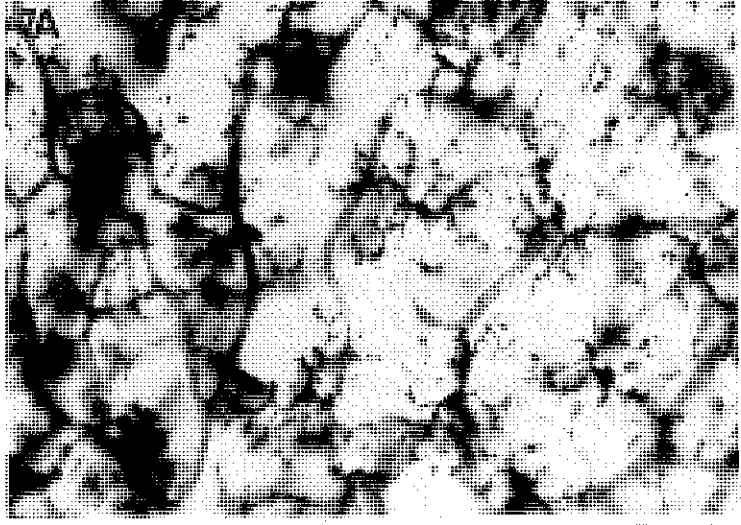
for the rd gene the photoreceptors develop normally until about seven days after birth but then start to degenerate such that by twenty days after birth there are very few remaining [10]. It has been demonstrated in such mice that the loss of the photoreceptors has no detectable effect on the number of cells in the inner nuclear layer at least up to 41 days of age [8] but in older animals there is some evidence for a small cell loss from the inner retinal layers [15]. By examining the distribution of microglia in the retinae of rd mice it is possible to see whether there is a relationship between the amount of cell death and the numbers of microglia and whether the spacing of the microglia is related to a particular class of neurone. We have found during the phase of photoreceptor cell loss that the number of F4/80+ cells in the retina is greatly increased when compared to normal, showing that the macrophages do indeed respond to the enhanced cell death [39]. In adult rd retina the number of microglia in the outer plexiform layer (which now lies adjacent to the retinal epithelium) is decreased by about 25% whereas the number in the inner plexiform layer is increased by about 30% (Fig. 7).

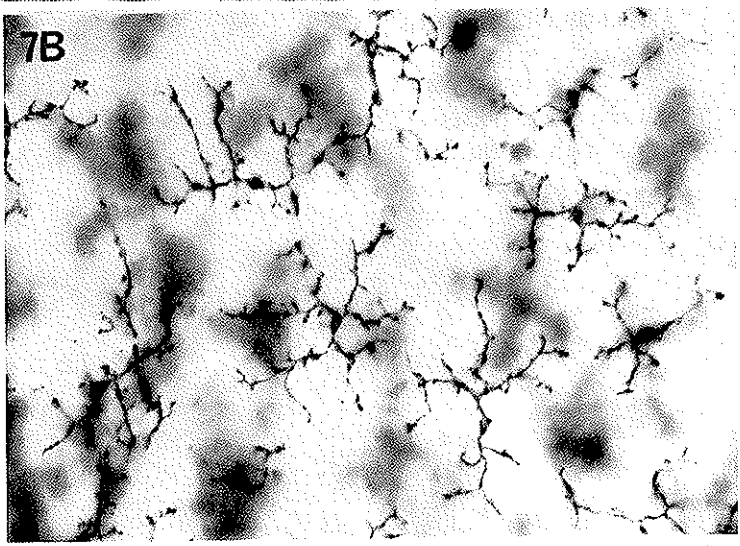
Our results suggest that the final distribution of microglia is not simply related to the amount of cell death, as we have already noted in our study on the brain, and furthermore the final distribution does not seem to depend on a particular class of neurone. We also found that the size of the territory of the microglia changed with their density such that the area covered by their processes was larger when the density was lower. Thus it seems that one factor limiting the density is the amount of overlap that the cells will tolerate; to what extent some component of the tissue environment determines the numbers remains to be shown. It should be noted that these changes can not be related to a change in the vascular supply in any simple way. In the retinae stained for adenosine diphosphatase the distribution of the blood vessels can also be seen and in the rd mouse their number is greatly reduced (Fig. 7B) [25].

Conclusion

Our studies have shown that during the period of naturally occurring cell death macrophages migrate into the nervous system, phagocytose dying cells and then transform to microglia. The microglia not only retain F4/80 on their surface but also complement and Fc receptors. It is clear that the final distribution of microglia is not simply related to the amount of cell death in a particular structure but must respond to some other local

Fig 7. The microglia in the outer plexiform layer of (A) normal (B) rd/rd mouse. The microglia have been stained for adenosine diphosphatase [30]. In the normal mouse the microglia are denser and smaller than in the rd/rd mouse but there is a regular array in both. Note the conspicuous capillary bed in (A) which is absent from (B). x260.





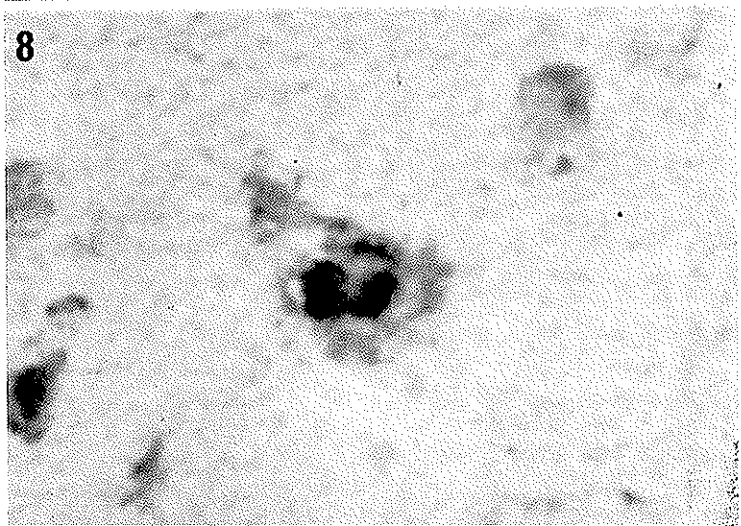


Fig. 8. An F4/80+ mitotic cell in the cortex of mouse three days after a small cortical lesion. It is not known whether this cell is a dividing microglial cell or has migrated from the blood to this location in response to the lesion. x1800.

cues. Our experiments have not addressed the thorny problem of whether any macrophage or only a particular subclass differentiate to become microglia, neither have we examined whether microglia can divide in either the normal adult brain or following local damage. Our studies do show, however, that with the appropriate markers we can help to resolve many of these issues (Fig. 8). The possible role of macrophages and microglia in the developing and adult brain in functions other than the clearance of degenerating cells is unknown, but the use of immunohistochemical markers to different biosynthetic products will certainly aid in this task.

ACKNOWLEDGEMENTS

This work was supported by the MRC. V.H.P. is a Locke Fellow of the Royal Society. The experiments reported here were done in collaboration with S. Gordon, D.A. Hume, B. Baker and L. Hayes.

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age, and region of the nervous system. Loss of nerve cells, the interruption of axons or dendrites, alterations in neuronal membrane properties, changes in excitatory and inhibitory inputs and also in transmitter or peptide contents can lead to transient, permanent or progressive cell disfunction and disconnection [15]. Furthermore, the non-neuronal cells that surround the neuronal somata and their cellular processes also undergo changes in number, shape and molecular constituents [16, 17] whose effects on neurons are still poorly understood.

The functional recovery from neural injury in adult mammals is limited largely because few neurons replicate most of the developmental events described above. For instance, lost neurons other than those which originate from the olfactory sensory epithelium [18] are not replaced by cell division. While an extensive axonal regrowth follows the cutting of peripheral nerves in all vertebrates and some central nervous system (CNS) neurons in fish and amphibia regain function as their axons extend and synapse with appropriate targets in the brain [19], the regenerative responses of most axotomized central nerve cells in adult mammals are restricted to short-range changes in neuronal connectivity and synapse organization [20]. As a result, some of the functional deficits that follow injuries to the mammalian brain may become permanent because renewed neuronal interactions and neuron-target interdependencies are impossible between the more widely separate nerve cells. Paradoxically, this well-documented failure of axonal extension in the adult CNS appears to coexist with a preserved intrinsic ability of the mature central neurons to initiate and sustain a lengthy regrowth of their interrupted axons [21] and to receive inputs from neighbouring indigenous neurons [20] or from transplanted immature nerve cells [22, 23].

The precise mechanisms that curtail axonal extension within the mature mammalian CNS are unknown, but such effects could be mediated by local inhibitory influences and a lack of certain cellular or extracellular growth-promoting components that are perhaps only expressed transiently during development [21]. Interestingly, the results of several *in vivo* and *in vitro* experiments suggest that specific molecules related to non-neuronal elements of the peripheral nervous system (PNS) and the immature CNS may permit or promote the extension of axons in a manner that resembles that observed during the development of the PNS and CNS and in regenerating peripheral nerves [24-30].

As an experimental method to provide CNS axons of adult mammals with an environment that is normally restricted to the PNS, we have

transplanted long autologous segments of peripheral nerve and used them as "bridges" between selected regions of the CNS to investigate the relative role of intrinsic and extrinsic influences on axonal regrowth (neuron-substrate interactions) and to explore the possibility of establishing renewed contacts between widely separate nerve cells (neuron-neuron interactions). In these studies of the regenerative capacities of injured neurons in mature rats with PNS grafts in the brain or spinal cord, the following relevant observations have been made: a) A broad spectrum of central neurons of different types and locations are indeed capable of extensive axonal regrowth into these grafts [21]; b) Some of the axotomized neurons regenerating long axons retain or regain normal functional properties [31-33]; c) Certain regenerating central axons can establish new synaptic contacts with the CNS neurons which they reach across these conduits [34, 35].

Although these conclusions were drawn from several studies of the responses of injured neurons in different regions of the CNS, we focus the present review on observations made in the visual system of adult rats [34-38]. In the experiments to be described here, the neural paths that normally link the eye and the midbrain were interrupted and replaced by peripheral nerve grafts joining the retina and the superior colliculus (SC). The retino-tectal projection, which originates in the ganglion cells of the retina and courses through the optic nerve (ON) and tract to synapse with neurons in the SC, was chosen for these studies because it provides special advantages for the study of several anatomical, functional and molecular aspects of the responses of injured central neurons.

The minimal requirements for the reconnection of retinal and tectal neurons in our experiments are: 1) The survival of retinal ganglion cells (RGCs) that have been axotomized near their somata; 2) The capacity of such retinal neurons to produce, extend, and maintain axons long enough to reach the dorsal midbrain, a distance of approximately 2 cm in adult rats [39]; 3) The guidance of these regenerating axons into the SC; and, 4) The ability of the RGC axons to synapse with tectal neurons. It is anticipated that many other circumstances — including the number of RGCs that survive axotomy, the state of the intrinsic retinal circuitry, the conduction properties of the new axons and the number, functional characteristics, appropriateness and persistence of the re-formed synapses in the SC, as well as the responsiveness of tectal neurons to new inputs — will need to be investigated before it can even be suggested that the

anatomical restoration of some retino-tectal connections may have any beneficial effects on the visual behaviour of these animals.

We have previously demonstrated that axotomized RGCs share with other CNS neurons a capacity to regenerate axons into PNS grafts inserted directly into the retina of adult rats [40] and that some of these neurons regain or retain normal electrophysiological responses to ocular stimulation by light [33]. We review here our recent attempts to explore anatomically; a) the complex issue of the capacity of axotomized ganglion cells from the entire retina to regrow nerve fibers long enough to reach the tectum along an extracranial route; and, the tracing of the RGC axon terminals that enter the superior colliculus, which is the natural target of most retinal projections in rats.

Methods

In female Sprague-Dawley rats weighing 200-300 gm, a 2-4 cm segment of the common peroneal nerve was removed and one end was sutured to the ocular stump of the intra-orbitally transected optic nerve [34-36]. The remaining portion of the graft was extended along a boney groove drilled in the skull and its free end placed subcutaneously over the occipital bone. The PNS-grafted rats were then divided into two main groups. In Group I animals, the retinae were studied after intervals up to 9 months to determine: the capacity of RGCs to elongate axons that could reach the midbrain (Group Ia); the incidence of axotomized RGCs that survive injury (Group Ib); and, the proportion of these cells that regenerate axons along the PNS grafts (Group Ic). In group II animals, PNS grafts 3-4 cm in length were used to "bridge" the eye and the tectum, to demonstrate the course and termination of the regenerated RGCs that reach the superior colliculus.

The specific assessment of the responses of ganglion cells is made difficult by the high incidence of amacrine cells in the ganglion cell layer [41-43]. In an attempt to circumvent this problem, we estimated the population of RGCs by: a) determining the numbers of retinal neurons with somata areas greater than 80 square microns [44]; b) applying retrogradely transported labels to the axons of RGCs; and, c) using a monoclonal antibody (RT-97) that reacts with phosphorylated 200 kD neurofilaments [45], to delineate RGC processes.

Group Ia: The origin of axons regenerating along PNS grafts was investigated in 19 animals by exposing the unattached caudal end of each

graft in its extracranial position over the occipital bone 8 to 10 weeks after the rostral end of the PNS segment had been grafted to the ON. HRP (40%, Sigma) was applied near the end of the nerve graft approximately 2-3 cm from the posterior pole of the eye. Forty-eight hours later, the animals were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer and the retinae reacted for HRP histochemistry [46]; some retinae were also incubated with RT-97 to visualize 200 kD neurofilament-immunoreactivity by fluorescence microscopy. Control and grafted retinae were examined by light microscopy to determine the number and distribution of the retrogradely-labelled retinal neurons that had regenerated axons along the PN grafts and to compare these numbers with the total population of nerve cells in the ganglion cell layer of these animals, estimated by others [44].

Group Ib: The survival of axotomized retinal ganglion cells in PNS-grafted and non-grafted retinae was compared in 43 animals. In 20 rats, the autologous sciatic nerve was grafted intraorbitally to the transected left optic nerve; in 19 animals, the left optic nerve was transected at the same level near the optic disc but no graft was implanted. Groups of these experimental animals were studied after intervals of 15 days, 1, 3, 6, and 9 months. Four animals with intact optic nerves served as controls. After fixation, the retinae of these animals were prepared as flattened whole-mounts, dried on gelatin-coated slides, stained with 0.02% methylene blue [47], and mounted. Twelve photomicrographs were printed at 700x for each retina by photograpbing the retinal quadrants at distances of 1, 2, and 3 mm from the optic disc. The areas of all neurons in these photographs were determined with the aid of an IBAS-I analyzer and size-frequency histograms prepared.

Group Ic: The survival of ganglion cells in grafted and non-grafted retinae as well as the proportion of these cells that had regenerated along the PNS grafts was also examined in eight experimental and three control (non transected) animals following the application of retrogradely-transported tracers. In eight rats, the left ON was transected and an autologous graft implanted; in 7 of these animals the right ON was also transected but was not grafted. At the time of ON transection, small crystals of the fluorescent carbocyanine marker, diI-C18 [48-50] (D282), were applied to the ocular stump of the optic nerve to label RGCs by retrograde axonal transport. After intervals of 15 days (n=2) and 1 month (n=6), a second fluorescent marker, Fast blue, was applied to the distal end of

the grafts to identify the retinal neurons that had elongated axons to the end of the grafts. After forty-eight hours, the retinae were mounted on coated slides [51] and observed by fluorescence microscopy with different filters to visualize neurons labelled with Fast hlue and/or D282. As controls, D282 was applied to the transected ON in 7 animals and Fast Blue injected into both SC of 4 animals 48 hours prior to perfusion-fixation. Photographs of these retinae were also prepared at 700x and the densities of both types of labelled cells expressed per mm^2 for each retina.

Group II: The course and termination of regenerated retinal ganglion cell axons that reach the superior colliculus was determined for 26 animals by inserting the caudal free end of the graft into the superficial SC two to three months after the initial grafting to the optic nerve stump. These animals were allowed to survive an additional 6-8 weeks to determine if, within such time periods, the RGC axons coursing along the entire graft would penetrate the superficial layers of the SC and form synapses with tectal neurons.

Horseradish peroxidase (HRP) was the main tracer used to visualize by light and electron microscopy the precise termination of the regenerated fibers that leave the graft and enter the SC. Additional orthogradely-transported tracers, including rhodamine B isothyocyanate [35, 52], have also been used for these investigations; the present descriptions will be confined to the HRP studies of axonal terminals in the SC. The intravitreal injection of HRP identifies the regenerating retinal axons within the graft and tectum and excludes axons arising from injured peripheral nerves in the orbit, nearby muscles, scalp and meninges, all of which are a frequent additional source of innervation of these and other grafts [21].

Six to eight weeks after the insertion of the distal end of the PN grafts into the superior colliculus of this group of animals, 3 µl of 30% HRP (Boehringer-Mannheim) were injected into the vitreous body of the eyes. Two days after intraocular injection of HRP, the rats were perfused with fixative solution (2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer). To visualize the HRP reaction product and to stabilize it for electron microscopy (EM), serial sections were cut at 50 microns with a vibrating microtome and reacted first with tetramethylbenzidine and then diaminobenzidine-cobalt [53]. Wet-mounted sections were surveyed by light microscope and selected for EM examination after osmication and flat-embedding in epoxy resin.

Several precautions were taken to assure that labelled terminals were

due to orthograde transport of HRP along regenerated RGC axons rather than the result of spurious labelling of SC axons and synapses arising from tectal neurons. Extraneous neuronal labelling near the site of graft insertion into the SC could have resulted from the orthograde transneuronal transport of label from the eye [54]. To reduce this possibility, HRP rather than HRP-labelled lectins was used for the present experiments [55]. Furthermore, the soma and local branches of tectal neurons, also capable of regenerating axons along PN grafts [56] could have been labelled retrogradely if their axons had reached the eye by the time of the intraocular injection of the tracers. To minimize this risk, the insertion of the distal ends of the PN grafts into the SC was delayed until 2-3 months after PN grafting to the eye. To further lessen the chances of a spurious labelling of axon terminals by incorporation of the tracer substance into neuronal cell bodies in the tectum, labelled terminals in the SC were excluded from this study in the infrequent instances in which there were also labelled somata in the vicinity.

Experimental Results

1. *Growth and Survival of Axotomized Retinal Ganglion Cells*

In the retinae of group I rats, many neurons whose shape, size, position within the retina, and intraretinal axonal patterns as revealed by RT-97 immunoreactivity suggested that they were ganglion cells, were retrogradely labelled after the application of HRP to the graft approximately 3 cm from the posterior pole of the eye. In the 19 rats of Group Ia, the mean number of such labelled neurons per retina was 3500 (range: 949 - 12,385). The labelled cells were distributed throughout the PNS-grafted retinas (Figure 1). Such total numbers of retrogradely-labelled cells prove that up to 10% of the normal population of RGCs approximately 110,000 [44]) had regenerated their axons to the site of application of the tracer. Because a large proportion of the RGCs die soon after axotomy (see below) and also because some sborter regenerating axons may not have reached the site of application of the tracer, the incidence of regeneration among surviving RGCs is estimated to be considerably higher.

By 2 montbs after grafting, the perykarial area of 680 HRP-labelled retinal neurons ranged from 60 to 614 square microns (control range: 52-483; n = 653). This range of RGC somata measurements is similar to

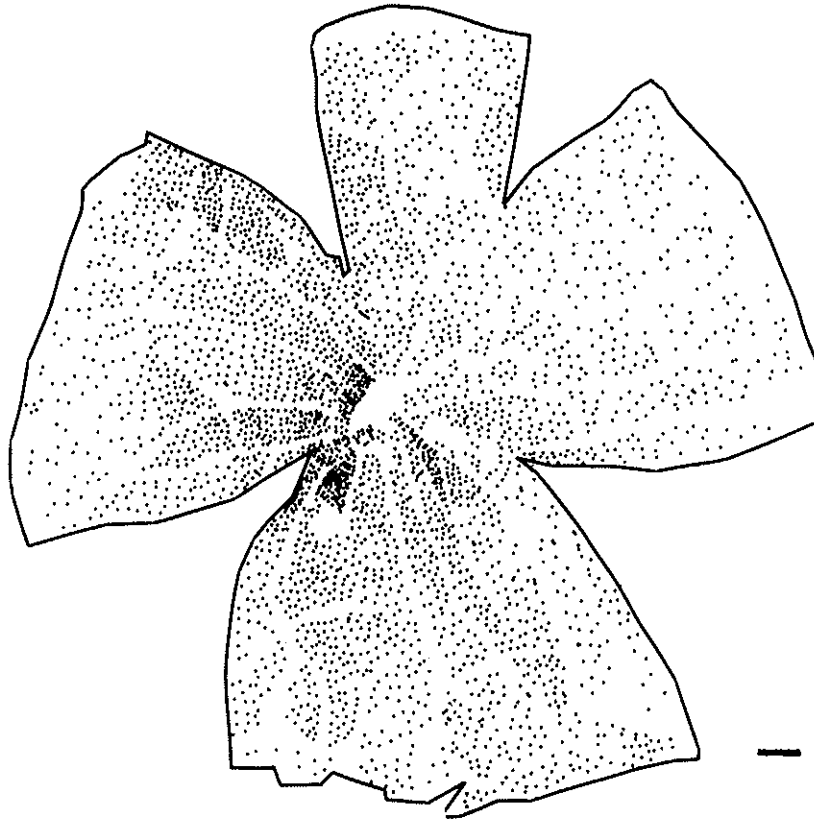

FIG. 1. Diagram, drawn from a camera lucida observation of a flattened retinal whole-mount, indicating the location of 3,323 ganglion cells (dots) retrogradely labelled with HRP applied near the distal end of the PNS graft. (Bar = 500 microns) (Modified from Aguayo *et al.*, [82]).

those reported previously from experiments in which PNS grafts were inserted directly into the retinae of adult rats [40] and may relate to the electrophysiological observations that retinal ganglion cells of different types regenerate axons into the grafts [33].

Survival of Axotomized Retinal Neurons: In other experiments in which the fate of retinal neurons was investigated anatomically after crush or transection of the optic nerve [43, 57-59], approximately one-half of all neurons in the ganglion cell layer degenerated within the first month after injury. Similar results were obtained for the non-grafted animals of Group Ib in which one optic nerve was transected near the optic disc and

the densities of all neurons in the ganglion cell layer of the retina were counted by light microscopy after 15 days or one month (Table I).

Because there are large numbers of amacrine cells in the ganglion cell layer of the retina [41-44], the effect of axotomy on the numbers of RGCs was estimated by comparing the numbers of large retinal neurons (cell somata areas greater than 80 square microns) that are more likely to

TABLE I - *The effects of axotomy with or without PNS grafts on the survival of neurons in the ganglion cell layer of the adult rat retina.*

	Number of Neurons per mm²			
Animals	Total		Large (soma > 80 μm²)	
CONTROL				
R 25	5,218		1,656	
R 28	4,777		2,044	
R 29	6,048		3,520	
R 30	6,362		2,608	
Mean ± SEM	5,601 ± 365		2,547 ± 404	
15 DAYS AFTER AXOTOMY				
Animal	Right	Left	Right	Left
N 208	3,970	3,851	519	622
N 284	2,636	2,906	449	563
N 209	3,358	—	384	—
N 283	2,643	—	446	—
N 204	—	3,444	—	405
N 207	—	3,262	—	531
Mean ± SEM	3,151 ± 321	3,365 ± 196	450 ± 27	530 ± 45
% Survival	56.3%	60.1%	17.7%	20.8%
30 DAYS AFTER AXOTOMY				
N 192	3,693	3,658	560	856
N 199	3,274	3,547	267	499
N 212	2,574	2,496	286	414
N 196	3,162	—	540	—
N 199	—	2,274	—	786
Mean ± SEM	3,176 ± 230	3,111 ± 289	413 ± 79	639 ± 107
% Survival	56.7%	55.6%	16.2%	25.1%

Right = axotomized; Left = axotomized + PNS graft.

% Survival was calculated by dividing the mean number of cells surviving at each time period by the mean number obtained for the controls.

be RGCs than amacrine cells [44]. In the non-grafted animals of Group Ih, less than 20% of such large neurons survived axotomy (Table I). On the basis of previous studies in the peripheral nervous system, it has also been reported that axotomy near the cell soma can cause the retrograde degeneration of 25% to 70% of the cells of origin [60-62].

Effects of Nerve Grafts: In the four Group Ib animals with PNS-grafted optic nerves, the density of large neurons (areas of somata greater than 80 microns^2) in the ganglion cell layer 30 days after axotomy was 26% compared to a value of 17% in the four non-grafted animals (Table I). This trend suggests that the PNS graft itself, and/or the axonal regrowth that it permits, has had a protective effect in preventing the loss of axotomized neurons. This possibility was explored further in the Group Ic animals in which one fluorescent label (D282) was applied to the optic nerve at the time of transection to mark retrogradely the ganglion cells whose axons were severed and a second dye was applied to the end of the graft after the RGC axons had extended along the PNS segment. In all seven animals in which the effects of such PNS grafts were compared, the density of D282-labelled neurons in the grafted retinae was greater than the density of D282-labelled cells in the ungrafted retinae (Table II). Furthermore, because the proportions of surviving neurons in the grafted retinae (labelled with D282 at the time of ON transection) that were also labelled with Fast blue (indicating that their axons had regenerated along the grafts) ranged from 10.6 to 19.8% (Mean 15.7%) (Table II), the data from these experiments also suggests that the incidence of neurons that have grown along the grafts may approximate 15.7% of the surviving RGCs rather than the 3.1% estimated for the Group Ia experiments (see above).

The fact that greater numbers of RGCs survive in the PNS-grafted retinae than in the non-grafted eyes implies that interactions between the RGCs and the PNS grafts may mitigate the retrograde effects of axotomy on these neurons. Similar protective influences on neuronal survival have been observed following the implantation of fetal grafts into the CNS of adult [63] and developing [64] rodents and also after the introduction of specific molecules into the stump of autonomic nerves [65]. If, as suggested previously [21], axonal regrowth into the grafts and neuronal survival are eventually proven to be related, it would indicate that conditions created by the release of soluble factors in the graft or by surface interactions with graft components can modify intrinsic cellular mechanisms

TABLE II - *The effects of PNS grafts on the survival and axonal regeneration of axotomized retinal ganglion cells in adult rats.*

	Number of Neurons per mm²				
Animal	A	B	C	B/A	D
15 DAYS					
T 54	105.6	342.9	118.2	3.2	12.5%
T 55	149.2	202.8	90.1	1.3	16.0%
30 DAYS					
T 44	72.3	282.3	151.1	3.9	19.4%
T 45	96.4	128.0	70.0	1.3	19.8%
T 46	51.6	158.4	76.9	3.0	17.6%
T 48	13.7	231.8	67.7	16.9	10.6%
T 49	22.9	229.5	81.4	10.0	12.8%
A 26	—	138.0	64.0	—	16.8%

A: Right retina - density of ganglion cells retrogradely labelled with D282 at the time of axotomy.

B: Left retina - density of ganglion cells retrogradely labelled with D282 at the time of axotomy + PNS grafting.

C: Left retina - density of ganglion cells doubly labelled with D282 (at the time of axotomy + PNS grafting) and Fast Blue (applied to the end of the grafts, 15 or 30 days later).

D: Estimated incidence of axonal regeneration among surviving axotomized RGCs, calculated according to the formula $\frac{C/X}{B/Y} \times 100$, where X is the average density (2,116 cells per square mm) of Fast Blue labelled RGCs in four control animals, and Y is the average density (767 cells per square mm) of D282 labelled cells in seven control animals.

that regulate neuronal survival as well as growth, as observed elsewhere [21, 66].

Based on data from Group Ib animals studied at 3, 6 and 9 months after axotomy/PNS grafting [37, 38], it is possible that such graft-dependent effects on the integrity of nerve cells may only be temporary. The progressive structural abnormalities that become apparent within the retina of some of the PNS-grafted rats 6-11 months after transplantation

(unpublished observations) may be related to this phenomenon. One of many possible explanations for these findings is that the RGCs that initially extend their axons along the blind-ended PNS grafts are protected from cell death and stimulated to elongate but that certain other critical conditions are no longer expressed after graft reinnervation [29]. Under such circumstances, the persistent integrity of axons and perikarya may be conditioned to the eventual establishment of terminal connections with target tissues [67]. A protracted loss of neurons is well known for axotomized cells that are prevented from making terminal connections with the periphery [62, 68, 69]. It has not yet been determined if the establishment of synaptic connections between RGC axons and tectal cells further influences the survival of RGCs in the experimental animals we have studied.

2. *The Extension and Termination of the Retinal Axons that Re-enter the Superior Colliculus*

Using the orthograde transport of lahels injected into the PNS-grafted eyes of the group II animals, we traced axonal profiles that had extended along the entire lengths of the graft and into the SC (Fig. 2). Because the nerve grafts that joined the eye and the superior colliculus across a mainly extracranial course measured 3-4 cm, this observation confirmed that many of the retinal axons that grew the full length of the PN grafts had elongated distances that are greater than the normal retino-tectal projections in the intact adult rat (approximately 2 cm [39]).

In contrast to the extensive growth of the regenerating retinal axons along the PNS grafts, these axons only lengthened into the CNS for distances of up to 500 microns beyond the tips of the grafts inserted into the SC. Thus, at least some of the regenerated RGC axons, which had grown along the substrate of the PNS grafts for distances that are even greater than those acquired during normal development, were able to re-enter the SC through the PNS-CNS interface but were thwarted in their elongation within the superior colliculus itself. In other words, the successfully growing axons entered the injured CNS substance that surrounded the graft tip but failed to extend substantial distances beyond this region. We interpret these differences in fiber extension as further evidence of the decisive role played by substrate conditions in the PNS and CNS. It remains to be determined if the formation of synapses [70] between

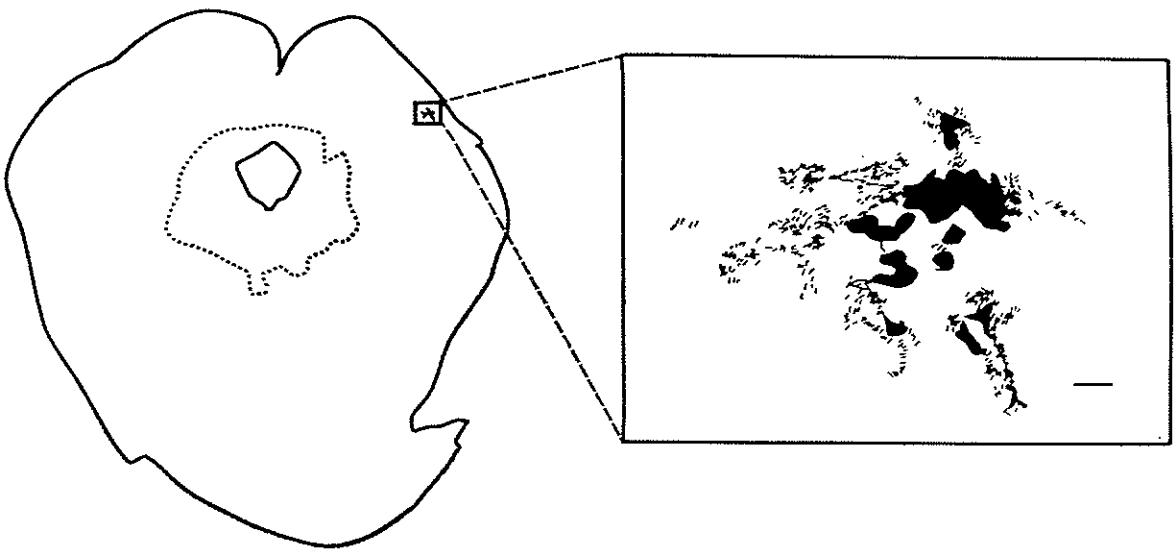

FIG. 2. Drawing of an HRP-labelled arborization at the end of a PNS graft connecting the retina and the contralateral SC in a Group II rat. On the left, the diagram of the epoxy resin-embedded cross-section of the midbrain illustrates the location of the arborization (box) in the superficial layers of the lateral aspect of the SC. The diagram on the right is a camera lucida reconstruction of the arborization; the interrupted lines represent the discontinuous HRP reaction product seen in serial sections by light microscopy. (Bar = 20 microns).

RGC axons and cells in the tectum is one of the factors that contribute to the curtailing of the further extension of these axons in the SC.

Within the SC, some of the HRP labelled structures consisted of single fibers with little branching. More commonly, the labelling pattern outlined multi-branched arborizations up to 300 microns in length. Along their short course within the SC, the labelled axonal profiles were often close to collections of macrophages or blood vessels near the tips of the grafts; they were not accompanied by peripheral nerve components such as fibroblasts, Schwann cells or their basal lamina. Because axonal branching was uncommon along these and other PNS grafts used in similar experiments [71, 72] but increased as axons entered the CNS, it is conceivable that putative different substrate properties of the PNS and CNS have an inverse influence on the extension of axons and on the formation of arbors. The existence of regional glial conditions that favour axonal arborization has been suggested [73].

By electron microscopy, we observed small HRP-labelled axonal profiles within the territory of both patterns of labelling seen by light microscopy. Many of these profiles contained synaptic vesicles and contacted unlabelled neural elements, usually dendrites. Some of these contacts showed pre- and post-synaptic specializations (Fig. 3). Since the tracer-injected eyes and the SC were only linked by nerve fibers that extended through the PNS "bridges", the labelled profiles found in the SC are presumed to be the terminals of RGC axons that have re-entered the SC. Because it has been reported that severed axons in mature rats may undergo a presynaptic differentiation in the absence of a post-synaptic neuronal contact either in neuroma-like formations at sites of optic nerve transection [74] or in regenerating spinal roots that abut against glia [75], the present finding in the SC of both pre- and post-synaptic specializations at the sites of contact between labelled axons and post-synaptic processes suggests that the synapses we observed in these PNS-grafted rats are the result of a more advanced interaction between RGCs and tectal neurons. However, we do not know if the synapses we have observed are numerous, appropriate or sustained. Furthermore, although we have demonstrated electrophysiologically that some of the axotomized-regenerating RGCs that extend their axons along PNS grafts inserted into the retina [33] or attached to the optic nerve stump (unpublished observations) retain or regain their normal electrophysiological responses to light, it remains to be proven that this activity can be relayed

transynaptically to the tectal neurons on which synaptic contacts have been demonstrated.

General Comments

Previous anatomical studies of the responses of injured retinal ganglion cells in adult mammals reported a local growth of axons within the injured retina [76-78] and into peripheral nerve grafts attached to the optic nerve [79-81]. It has also been established that the cutting of

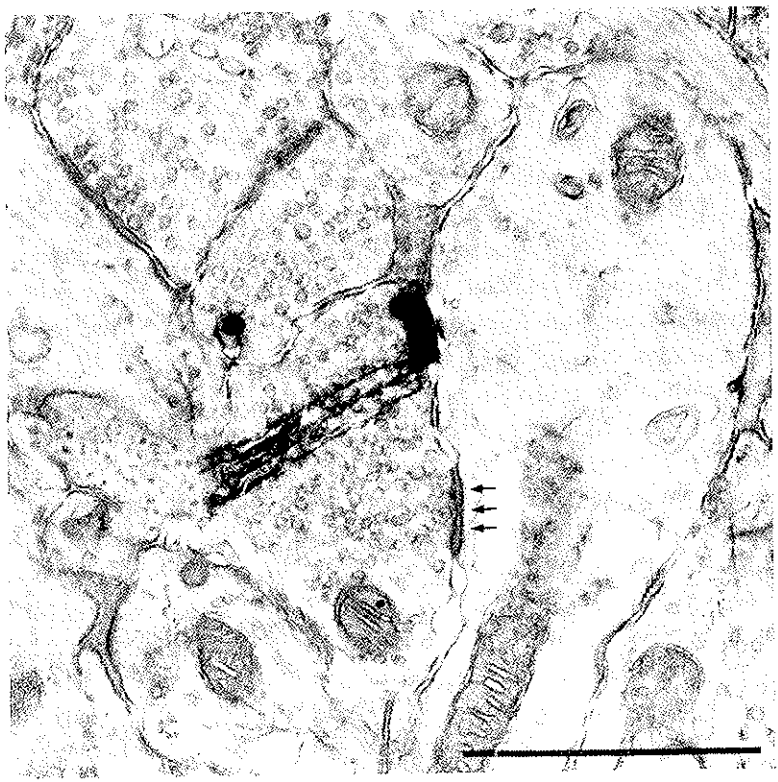

Fig. 3. Electron micrograph of a presynaptic profile in the superficial superior colliculus (strata zonale and griseum superficiale) that contains the characterictic crystalline HRP reaction product [53] indicating that they are terminals of axons originating from the retina. The presynaptic terminal contains vesicles that are predominantly round or spheroidal. The thickenings of the pre- and post-synaptic membranes at the synaptic contacts are indicated by arrows. (Bar = 1 micron) (Reproduced from Vidal-Sanz *et al.*, Journal of Neuroscience, 1987, Courtesy of Oxford University Press [35]).

the optic nerve is followed by an extensive loss of retinal ganglion cells [43, 57, 74]. The recent availability of improved microsurgical techniques, dependable neuroanatomical tracers, and specific cell markers has made possible a more accurate assessment of the intrinsic capabilities of these cells of the central nervous system to survive injury, sprout, elongate, and form synapses with target neurons in the brain.

The present investigation of RGC axonal regrowth and connectivity in mature rats in which one optic nerve and tract were substituted by a PNS bridge indicates that some axotomized RGCs survive and are capable of the additional steps needed for the reconstitution of the retino-tectal projection. Indeed, such damaged neurons prove able to: i) extend their axons well beyond their natural length; ii) penetrate the CNS substance for the short distance needed to reach their target neurons in the superficial layers of the SC; and, iii) make synaptic contacts in the SC.

It has also been learned that although approximately 80% of the RGCs die soon after axotomy, an average of 15.7% (range 10.6 to 19.8%) of the surviving RGCs regenerate lengthy axons into the PNS grafts. Furthermore, unknown interactions between the injured retina and the PNS grafts appear to protect some RGCs from degeneration following axotomy. In other words, influenced by the conditions created by grafting, some of the injured RGCs prove capable not only of regrowing lengthy axons but also of mounting a metabolic response that allows them to survive axotomy. A better understanding of the mechanisms regulating the cell rescue and axonal growth effects observed in these animals may help increase the number of surviving neurons and perhaps, consequently, the population of new axons that may be guided to selected targets.

These observations, which underline once more the regenerative capacities of neurons in the adult mammalian central nervous system, suggest the existence of similarities between the intrinsic responses of neurons whose somata are located in the PNS or CNS and indicate further the powerful influences exerted by constituents of the environment on the survival and growth of mature nerve cells. Although the mechanisms responsible for these phenomena are unknown, the findings described here suggest that certain stages of both normal development and the successful regeneration of retino-tectal projections in anamniots [19] can be replicated in adult mammals. It is tempting to speculate that similar molecular determinants may regulate these processes.

Acknowledgements

The authors thank Dr. S. Thanos for the introduction to the technique of the carbocyanine label, D282. The technical assistance of M. David, J. Laganière, S. Shin, J. Trecarten, and W. Wilcox is gratefully acknowledged. M. V.-S. was supported by a grant from the Spanish Ministry of Education and Science and the Medical Research Council of Canada. M.P. V.-P. was supported by a grant from the Spanish Ministry of Education and Science. The Medical Research Council, the Multiple Sclerosis Society, and the Muscular Dystrophy Association of Canada provided research grants.

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EARLY DISCONNECTED BRAINS: DEVELOPMENTAL PLASTICITY OF THE CEREBRAL CORTEX IN ABSENCE OF THE CORPUS CALLOSUM

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INTRODUCTION

Congenital absence of the corpus callosum in eutherian mammals has attracted the interest of investigators since the end of the last century. Not only acallosal animals [53-55, 108, 119] have been found, but also humans with defective or absent corpora callosa [5, 83, 85]. However, it was only after the work of Myers, using experimental animals [78-81], and that of Sperry and his collaborators, using adult patients subjected to surgical transection of the commissures [28-30, 105], that the scientific background was ripe to provoke questions about the effects and causes of the early absence or interruption of the callosum. Sperry himself was interested in a group of congenitally acallosal patients, whose only neurological deficit seemed to be the commissural anomaly. He studied these asymptomatic cases of radiologically-detected agenesis of the corpus callosum [103, 104], using the same tests applied to commissurotomized patients. Quite surprisingly, he found that congenital acallosals did not present the interhemispheric disconnection syndrome characteristic of split-brain adults [105], responding to the tests similarly as matched controls. Other behavioral studies confirmed Sperry's results [13, 14, 23, 24, 34, 92, 93], although indicating cognitive [13, 14] and perceptuomotor deficits [34, 47, 49]. It became evident that the differences between congenital acallosals and split-brain patients were due to plastic mechanisms — either compensatory, maladaptive or both — which would take place during development.

Many hypotheses were offered to explain those differences (recently reviewed by Jeeves [48]). A first one postulated a reorganization of asymmetric specialization of the hemispheres, resulting in a bilateral representation of language, which would allow the patient to express verbally the perceptual events of both hemispheres [31, 34, 56, 103]. However, while a case subjected to intracarotid injection of sodium amytal showed bilateral language [34], another revealed unilateral representation of language (B. Milner, cited in Gazzaniga [27]). Another hypothesis considered the possibility of a compensatory use of the other cerebral commissures [24, 103]. In this case, support was provided by the occasional finding of a hypertrophy of the anterior commissure in human acallosals [64]. A third hypothesis predicted an enhancement of ipsilateral pathways and/or mechanisms which would allow the subcortical integration of the sensory hemifields [13, 14]. Gazzaniga [27] raised another possibility: acallosal subjects could learn to perceive cues from the experimenters or even from the patient's opposite hemisphere, and thus be able to solve behavioral tests of interhemispheric integration. Finally, it was recently suggested [96] that certain congenital mental disturbances could be explained by the occurrence of aberrant neural connections which form after early cerebral lesions. In this case, anomalous connections would form between and within the hemispheres, which could either restore the integrative functions of the absent corpus callosum or cause abnormalities in their physiological performance. Whatever the mechanism, it is clear that early disconnected brains are subject to some degree of plastic reorganization during development.

It is our purpose to review briefly the experimental models and paradigms used so far to disclose the plastic effects of early removal or congenital absence of the corpus callosum upon the morphology and function of the brain, as well as the behavioral consequences. Basically, three animal models have been used: (1) acallosal mutants; (2) animals subjected to early surgical transection of the callosum; and (3) animals born acallosal as a consequence of prenatal irradiation.

Congenital Defects of Corpus Callosum in Animals

Although callosal agenesis has occasionally been reported in species other than man (cat [119], monkey [108]), only in mice has it been systematically studied. King and Keeler [55] first recorded the absence of corpus callosum in the mouse, and attributed this phenotype to a single

gene with total penetrance [53]. It soon became clear [54] that a simple Mendelian mechanism was not sufficient to explain the complex inheritance of callosal defects in the mouse.

Total and partial callosal agenesis were later reported in two inbred strains of mice, BALB/c and 129 [111, 112]. Although the inheritance mechanisms are still incompletely understood, it seems certain that these brain defects of mice are completely recessive [113], but not monogenic as previously suggested. In addition, environmental factors during gestation were shown to have considerable influence on the frequency of appearance of defective individuals in these strains [113, 114, 116].

Although a genetic determination of callosal agenesis in humans has also been suggested [71, 82, 99] it has not been unequivocally proven. Therefore, the adequacy of the mouse model for heredity studies of callosal agenesis remains to be demonstrated.

A detailed description of brain morphology of acallosal mice was provided by King [54], and some of its aspects were confirmed by later investigators [101, 111]. At least two types of commissural defects were recognized. One was characterized by the complete absence of the corpus callosum and the second presented an anterior callosal remnant but no genu and absent posterior callosum. A third type was described by King, with a posterior callosal remnant but not the anterior half of the commissure. However, this latter type was not found in BALB/c mice [111].

An aberrant longitudinal bundle of fibers (equivalent to the Probst bundle of acallosal humans) was consistently seen medially in all defective animals underneath the cortical white matter (Fig. 1B). Myelinated fibers therein were described to run tortuously in a gross longitudinal direction. Some of the aberrant fibers were seen to deflect out of the bundle in a ventromedial direction, join the fornix and presumably terminate in the septum. Other architectural anomalies were detected, such as malposition of the indusium griseum, fornix longus and angular bundle, but these were considered to be secondary to the callosal defect and due to mechanical strains [54]. Apart from these anomalies, general brain architecture looked normal.

As the main morphologic characteristics of the brains of acallosal mice — normal architecture of most cortical areas and the presence of the longitudinal bundle — are also found in humans with primary agenesis of the corpus callosum (Fig. 1C), King [54] suggested that those mutants should be considered as convenient experimental models for studies of that neuropathological entity. The suggestion remains tenable even if the

inheritance of callosal defects is shown to be determined by different genetic mechanisms in each species. In fact, even when no genetic factors operate as the cause of callosal absence, as when the callosum is mechanically interrupted perinatally [59-61, 101], these characteristics can also be observed.

The mutant model was recently utilized in studies of brain development by Silver and his collaborators [101]. They noticed that a transient interhemispheric bridge of glial cells (the glial "sling") forms at prenatal day 15 in normal mice just after fusion of the septal midline. In acallosal BALB/cCF mice, however, septal fusion is delayed by a couple of days,

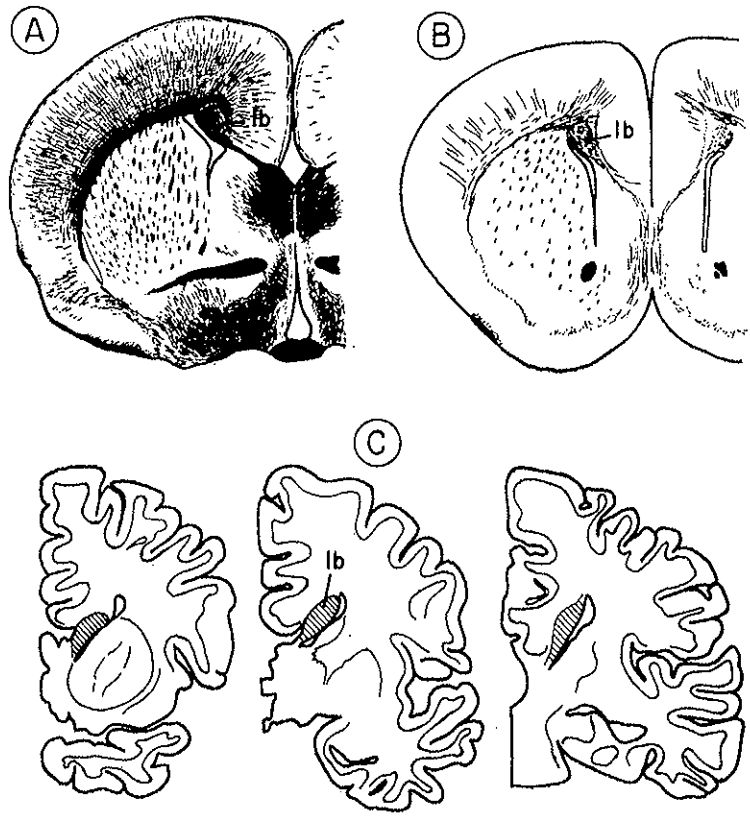

FIG. 1. Similar characteristics of brains of early transected hamsters (A), congenitally acallosal mice (B; modified from King [54]) and acallosal humans (C; modified from Bossy [4]): normal architecture of most cortical areas and the aberrant longitudinal bundle of Probst (Ib).

and the glial bridge does not form. A hypothesis was put forward [52, 101] (but see refs. 109 and 110 for a different explanation) which attributes to the interhemispheric glial cells an essential role in guiding the pioneer callosal fibers across the midline. In defective animals these fibers would grow normally in medial direction, but when arriving at the midline they would not find the guidance cues provided by the glial cells, being diverted longitudinally to form the Probst bundles. Long delays in the fusional process and consequently in the formation of the sling would result in completely acallosal brains, while shorter delays would allow some late arriving callosal fibers to cross the midline and form a subnormal callosum [115].

Apart from these genetic and developmental studies, however, no published account has appeared so far on the occurrence of either morphologic, physiologic or behavioral plasticity in these mutant mice. This is probably due both to the relatively low incidence of defective individuals among normals (10-20%), and to the fact that acallosals are indistinguishable from normal subjects under routine laboratory conditions, both anatomically and behaviorally.

Morphological Effects of Perinatal Transection of the Corpus Callosum

The most common experimental paradigm utilized for the study of cortical plasticity in absence of the corpus callosum has been the surgical transection of this commissure during different stages of its prenatal and postnatal development. From this work, two parallel, somewhat unrelated sets of information have arisen.

On the one hand, investigations have concentrated upon the effects of early callosal transection on the architecture and hodology of the cerebral cortex. In view of the finding of aberrant connectivity in many subcortical and cortical systems consequent to different kinds of environmental and surgical intervention, it has become natural to expect the formation of such abnormal circuits after early callosal transection. A second line of investigation has been the study of the physiological and behavioral consequences of early removal of the callosum.

Despite these efforts, however, in very few instances could the physiological and psychological characteristics of early disconnected subjects be related to the morphological brain abnormalities that they present.

As is well known, plastic phenomena can be viewed as compensatory mechanisms of correction of deranged brain hardware which can either be successful, restoring the normal functions disturbed by the environmental insult, or a failure causing abnormal function and wrong behavioral patterns. Unfortunately, this association bas seldom been established for the mammalian cerebral cortex grown in the absence of its grand commissure.

We have been involved, during the last few years, in a study of the morphological consequences of early postnatal transection of the callosum in rodents [59-62].

Golden hamsters are particularly suited for this type of paradigm. Females have very short (16 days) gestation periods, after which they give birth to 7-14 pups in a relatively immature developmental stage as compared with other eutherian mammals [57]. Neonates were removed from the nest during their first 24 postnatal hours, and cooled for some minutes in a freezer to provide anesthesia, inhibition of motility and slowness of blood flow. Body hypothermia was maintained during surgery either by putting the animals onto an ice cube covered with gauze, or by inserting their trunk into a metal cylinder immersed in broken ice. A slit was made onto the cartilage over the sinusal lambda, exposing the pretectal region, which at that age is uncovered by the cerebral hemispheres. A miniscalpel was then inserted caudorostrally underneath the dorsal sagittal sinus and moved downwards while pulled back, in order to transect the corpus callosum. A piece of gelfoam was inserted through the slit to avoid excessive bleeding, and the animals were sutured, warmed and returned to the nest, where they remained until weaning.

In a first series of experiments, some of these early transected animals were simply anesthetized and perfused with aldehydes. Their brains were removed, cut frozen and stained for the visualization of cyto- [72] and myeloarchitectural [26] details, in order not only to evaluate the extent of the callosal defect but also to compare the general brain architecture of these surgical acallosals with that of genetical acallosals such as BALB/c mice.

A comparison between the brains of an early transected acallosal hamster and a normal, unoperated control is provided in Figure 2. It can be seen that the general architecture of these brains does not differ significantly, apart from the callosal defect and some secondary midline abnormalities. These latter consist basically of the ventral displacement of cingulate and retrosplenial cortical fields, which presumably occupy the

space left vacant by the absent callosum. In most acallosals, despite the complete absence of the callosum, a few fibers succeed in crossing the midline at septal levels, sometimes forming a tiny commissural remnant, or even coursing through septal tissue. These remnant callosal fibers could be seen emerging from a bulky, longitudinal bundle of myelinated fibers (lb in the figure), and heading towards the midline. The aberrant bundle, situated medially underneath the cortical white matter, is a very consistent feature of surgical acallosal brains, its diameter holding a gross inverse relation with the width of the callosal remnant. It is interesting to note that the other forebrain commissures looked normal, except when they had been directly severed by the scalpel.

By comparing the brains of surgical acallosal hamsters with those of genetical acallosal mice (Figs. 1 and 2), it is noticeable that a striking similarity characterizes the architecture of their brains. Whenever they have a pronounced defect of the corpus callosum, both present the aberrant longitudinal bundle. Both animals show fibers leaving the bundle in a medial direction, and present a ventral displacement of medial cortex. Finally, both defective animals display normal architecture of most brain regions, when compared with their normal littermates. These same characteristics were observed in mice subjected to prenatal sagittal transections [101], but not in mice transected postnatally [62], which suggests the existence of a critical period roughly coincident with the time course described by Silver and his collaborators [101] for the events which lead to the process of commissuration of callosal axons. Normal architecture in humans with primary callosal agenesis has been reported by some authors [4, 102, 107], although others have found some hypotrophic anomalies [1, 100]. The Probst longitudinal bundles are consistently found in human acallosals [64, 118], with few exceptions (Fig. 1). In partial acallosals, the bundles are present in those regions where the callosum is absent [64]. The anterior commissure is always present, albeit sometimes enlarged [41, 64], but the hippocampal commissures may sometimes be absent.

The lack of gross architectural anomalies in the surgical acallosal brains, together with the occurrence of the longitudinal bundle, which presumably contains callosal fibers, converges with the hypothesis that the genetic mutation(s) which presumably cause(s) the callosal defect may act on the process of commissuration of callosal axons [54, 88], possibly by interfering with the interhemispheric glial "sling" [52, 101]. Conceivably, the guiding cues for commissuration of the callosal fibers would be absent,

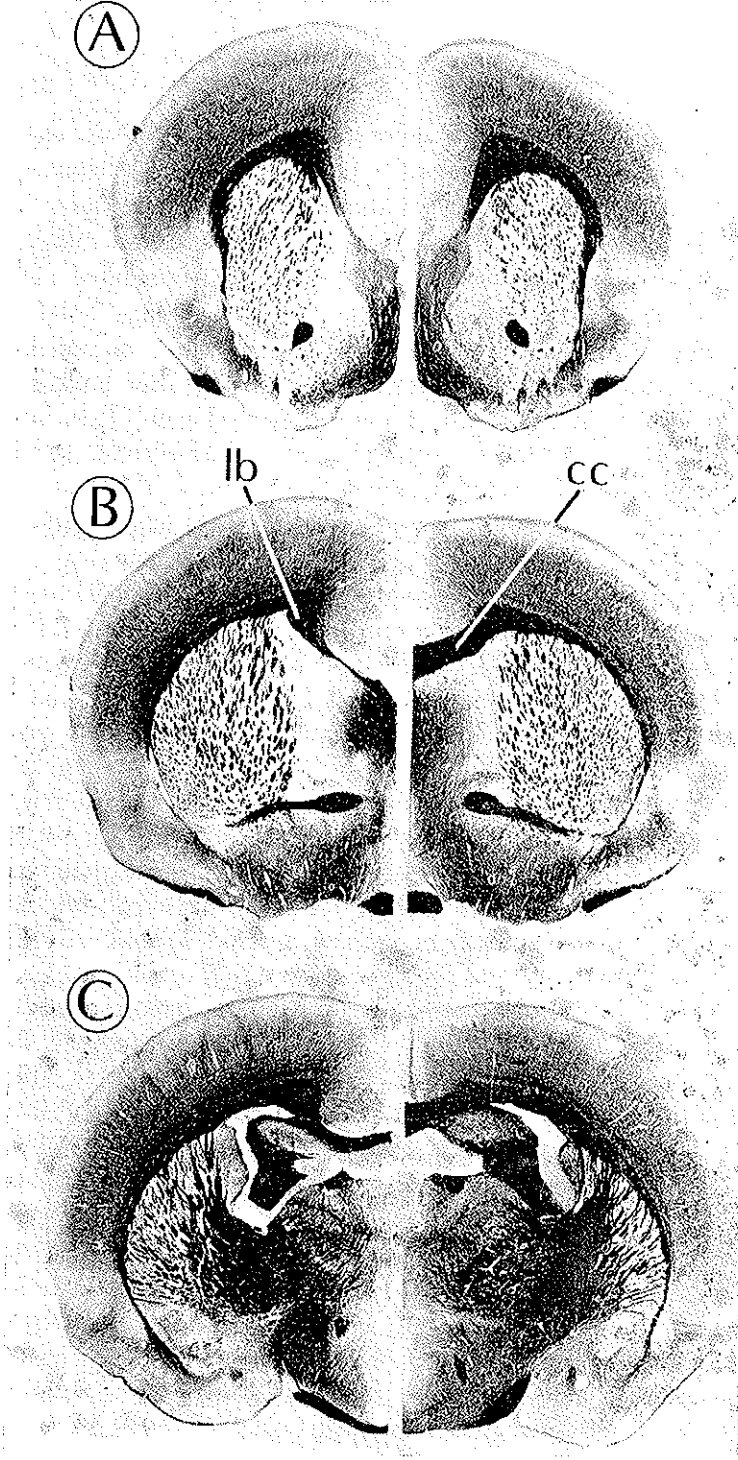

FIG. 2. A comparison between the brain of a hamster subjected to early transection of corpus callosum (left side), and that of a normal, unoperated control (right side). *A* is rostral; *C* is caudal. Gallyas technique [26]. Modified from Lent [61]. Abbreviations: cc, corpus callosum; lb, longitudinal bundle.

and the callosal axons would be misdirected to form the aberrant longitudinal bundle of Probst.

Aberrant Cortico-Cortical Connections in Early Disconnected Brains

In a second series of experiments, we decided to look for aberrant connectivity in acallosal brains. The corpus callosum of different mammals is prone to modification of its cells of origin, pathways and terminal fields after manipulation of the sensory input [2, 11, 22, 44-46, 65-67], of the primary sensory pathways [10, 45, 68, 77, 89, 90], and of the cerebral cortex itself [6, 32]. Therefore, it was reasonable to expect that the surviving callosal neurons (or other cortical cells) would show plastic reshaping after direct transection of callosal fibers.

A group of early transected hamsters had small pieces of polyacrilamide gel containing horseradish peroxidase (HRP) implanted into different cortical loci. After a few days survival, the animals were perfused with aldehydes through the heart, and their brains were removed, cut frozen and processed for HRP histochemistry following Mesulam's [73] protocol.

HRP was transported both retrogradely and anterogradely in most cases, and as a result of the slow delivery characteristics of polyacrilamide gel pellets [38], streams of labeled fibers could be followed from the sites of implant through terminal fields or the projection zones. From sites of gel implant situated in regions of the frontoparietal cortex corresponding to cytoarchitectonic fields 6, 8, 4 and 3 [ref. 7], sets of labeled fibers could be seen entering the cortical white matter and taking the longitudinal bundle, within which they were arranged in a topographical manner similar to that of the callosum [61].

Figure 3 shows schematically the results of one such experiment. Two groups of fibers were seen emerging from the bundle, one taking a lateral direction, the other heading medially. The lateral branch consisted mostly of fibers which either terminated or originated in the subcortical sites normally connected to those cortical regions. Other labeled fibers coursing laterally within the infragranular cortical layers constituted the normal ipsilateral complement of cortico-cortical fibers. While the bulk of these labeled fibers entered the internal capsule, some of them continued ventrad to take the external capsule and finally enter the posterior branch of the anterior commissure (single arrowheads, Fig. 3a). Following their

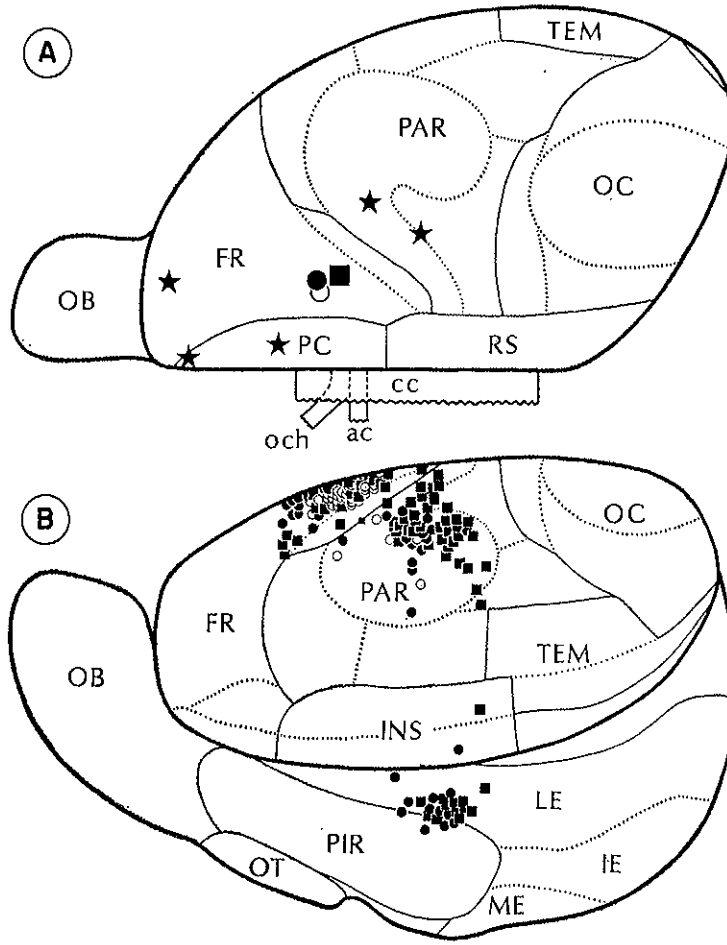

FIG. 4. *A*. Dorsal reconstruction of a hamster's right hemisphere, indicating the positions of HRP implants into the cortex of a normal animal (open circle), and of some early transected cases (black symbols). *B*. Lateral reconstruction of a hamster's left hemisphere, indicating the positions of labeled neurons corresponding to the three cases of implants into the posterior frontal cortex, as depicted in *A*. Abbreviations: ac, anterior commissure; cc, corpus callosum; FR, frontal cortex; IE, intermediate entorhinal area; INS, insular cortex; LE, lateral entorhinal area; ME, medial entorhinal area; OB; olfactory bulb; OC, occipital cortex; och, optic chiasm; OT, olfactory tubercle; PAR, parietal cortex; PC, prefrontal cortex; PIR, piriform cortex; RS, retrosplenial cortex; TEM, temporal cortex.

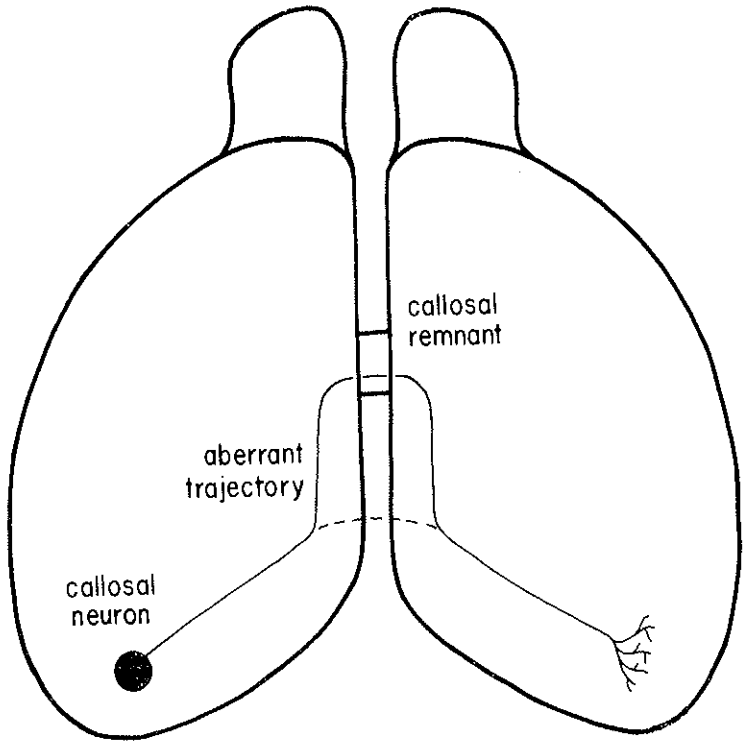

FIG. 5. Schematic representation of the aberrant longitudinal trajectories taken by posterior callosal fibers of early transected hamsters.

cases, a few labeled neurons were identified in layer III of the visual cortex.

From this series of experiments, the most important conclusion is perhaps that interhemispheric connections are capable of some reorganization after early callosal transection. It has long been suggested that the anterior commissure might be used as an alternative pathway for interhemispheric transfer in acallosal subjects [23, 24, 103]. However, although most congenital acallosal patients show signs of interhemispheric communication [8, 74], not all autopsied brains present a hypertrophied anterior commissure. On the other hand, work in cats [80], monkeys [40] and humans [33, 36, 37] shows that only a small complement of callosal fibers is necessary for the transmission of visual information from one to the other hemisphere. This implies that only a few con-

nections through the anterior commissure might be enough for achieving the interhemispheric communication seen in acallosals, a possibility enhanced by the finding that commissurotomized patients with an intact anterior commissure may show transfer of visual, auditory and olfactory information [91] (but see McKeever *et al.* [70]).

The fact is that so far nothing can be affirmed as concerns the role of the aberrant interhemispheric connections of surgical acallosal rodents. It remains to be shown, first of all, whether similar plasticity occurs in other animal species, including humans. Secondly, the functional status of these aberrant connections is not known. It is conceivable that, even if normal synaptic transmission is shown to exist in these interhemispheric pathways, maladaptive function may result from wrong wiring up of the circuits. In fact, maladaptive visuomotor behavior has been observed in hamsters after early lesions [94], due to anomalous retinotectal projections formed during postnatal development of the lesioned animals [95, 97]. If this is shown to be a general phenomenon following early brain lesions, as suggested by Schneider [96], symptoms would occur as a consequence of the aberrant projections, along with, or instead of, recovered functions. Although so far a speculation, it is conceptually tenable (and of great heuristic value) that the perceptuomotor deficits shown to coexist with the lack of a disconnection syndrome in acallosal patients [8, 74] may be due to the activity of aberrant projections. Along the same line, it is conceivable that the existence of interhemispheric communication in acallosals may be due, at least in part, to rewired interhemispheric circuits similar to those observed in surgical acallosal hamsters.

Physiological and Behavioral Effects of Neonatal Callosal Transection

Although many neuropsychological studies of congenital acallosal patients have been made, very few physiological and behavioral studies have been performed in animals. To our knowledge, no published account of work in acallosal rodents has yet appeared, and all available data have been obtained from early split-brain cats, the visual system having been selected for most studies. It is therefore premature to establish relations between the morphological reorganization of cortical connections which occurs in rodents, and the functional and behavioral plasticity described in cats.

A first attempt to evaluate the behavior of experimental acallosal

animals was made by Sechzer and collaborators [98]. Reasoning that myelination should be considered as the terminal stage of maturation of the forebrain commissures, they managed to transect surgically the corpus callosum of kittens during their first 3 postnatal days, well before the beginning of myelination [25, 35]. When tested, the acallosal kittens showed abnormal "home orientation", hyperactivity, learning deficits and a paradoxical response to amphetamine, most of which are similar to those symptoms characteristic of the minimal brain disfunction syndrome of children [106]. Sechzer and collaborators did not offer any explanation as to how callosal transection would lead to these symptoms. Furthermore, there is no indication in the neurological literature that congenital acallosals (see Jeeves [48], for a recent review) and young commissurotomized patients [63] present that syndrome.

A more specific approach was taken by Elberger in a series of experiments with early transected cats. She first detected a persistence of the divergent eye misalignment of young kittens when they had the posterior half of the corpus callosum transected between 13 and 29 days after birth [15]. A loss of about 35 degrees of the contralateral visual field of each eye was also detected in these kittens, as compared with late-sectioned controls. The animals were tested until they were 7 months old, but no signs of recovery were observed. Since both the integrity of the corpus callosum and the matched interplay of information from both eyes were known to be necessary for depth perception [76, 117], a study was undertaken on the development of depth perception [16] and of visual acuity [18-20] in neonatal split-brain cats. Animals were tested in a visual cliff apparatus for depth discrimination and with gratings of different spatial frequencies for acuity determination. Results showed a delay on the gradual acquisition of these capabilities in the operated animals. In the case of visual acuity, the developmental delay was shown to be more severe the more precocious the disconnection surgery. After the fourth postnatal week, callosal transection was no longer effective on visual acuity [19]. These results were interpreted as an indication of the role played by the callosum on the fine tuning of vision during postnatal development [18]. Although this interpretation is undisputable *latu sensu*, the subtractive logic used for lesion experiments in adults — to consider a deficit as the very function of a lesioned brain region — may not be appropriate for the analysis of lesion experiments in immature nervous systems since it underestimates the influence of plasticity. It is possible, in these cases, that a reorganization takes place after early

callosal transection, either causing function restoration or provoking behavioral pathology. The former possibility would mean that the callosum is much more important for visual development than Elberger's interpretation would imply. A recovery might have occurred after callosal interruption, and other interhemispheric or intrahemispheric pathways might have taken up the callosal function. The developmental time course of depth perception and visual acuity would be delayed in a much milder way than if no plastic response occurred. The opposite possibility, on the other hand, would mean that — if one takes an extreme situation — the corpus callosum does not participate at all in these developmental processes. Maladaptive plasticity would be responsible for the developmental delay observed in the experiments, causing the subnormal performance of neonatal split-brain cats. Current evidence is not sufficient to clarify this issue.

Positive evidence of behavioral plasticity has been provided by Ptito and his colleagues [86, 87] in kittens subjected to callosal transection at 20 days after birth. They tested interocular transfer of visual learning in these animals, as compared with cats operated both at 45 days postnatally and in adulthood. The optic chiasm was sagittally cut before testing. These experiments revealed some transfer in the earliest operated group, but not in the other cats.

Neonatal split-brain cats were also utilized in physiological studies of binocularity of visual cortical neurons. It was shown that the proportion of monocular cells increased significantly in the striate cortex [17] but not in the lateral suprasylvian area [21] of cats operated during the second postnatal week. Although there is some controversy concerning the influence of the corpus callosum on the binocularity of cortical neurons in adult cats [3, 75, 84], the data from split-brain neonates seem to indicate that such an influence does exist during development, at least for the striate cortex. However, plastic reorganization of the visual system may have masked the actual role of the corpus callosum during development in the same way as suggested above.

Callosal Agenesis After Prenatal Irradiation

Defects of the corpus callosum in rodents irradiated *in utero*, ranging from total callosal agenesis to variable degrees of hypoplasia, have been detected by different investigators [9, 42, 43, 50, 51]. The severity of the anomaly was shown to depend both on the dose of radiation (Schmidt

and Lent, in preparation) and on the gestation age at irradiation. Moderate doses in the range of 1-4 Gy [43] were shown to be the most effective to produce callosal anomalies in rats when irradiation was performed between the 17th and the 18th gestational day [51].

Differently from callosal agenesis produced by genetic mutations and from that provoked by perinatal surgery, the commissural defects which result from foetal irradiation are considered to be caused by the death of callosal cells [50]. Postmitotic, predifferentiating neurons are known to die when irradiated [12, 43], but the phenomenon probably occurs non-selectively among the whole population of cortical cells irrespective of their architectonic or hodological categories. No direct demonstration of death of callosal neurons has yet been produced, but its occurrence in the rat brain has been inferred from the correlation found to exist between counts of neuron numbers, measures of neocortex radial dimensions and the degree of reduction of the corpus callosum [50].

We were interested in exploring the characteristics of callosal agenesis due to irradiation in order to get some insight as to the mechanisms of plastic reorganization of cortex in early disconnected brains, particularly concerning the formation of the aberrant longitudinal bundle [62].

Swiss mice were mated between 4 PM and 8 AM, and when the females were pregnant, gestation day 1 (E1) was considered to have begun at 0 AM. At E16, two groups of pregnant females were individually exposed to homogeneous fields of 2 and 3 Gy, respectively, from a ^{60}Co gamma source (mean dose rates $\cong$ 0.50 Gy/min). Birth took place at term, and the litters were allowed to develop with their mothers under routine conditions for 60 days. All of the animals were intracardially perfused with aldehydes under anesthesia, and their brains were removed, photographed and cut frozen at 25 μm in the coronal or parasagittal planes. Series of alternating sections were stained with cresyl-violet and a silver technique for myelin [26], for qualitative and quantitative analysis.

The cerebral hemispheres of animals irradiated with 3 Gy were pronouncedly hypotrophic, especially at their caudal half, leaving the superior colliculi exposed. In the 2-Gy group the hemispheres were not so reduced, but the difference between the groups was not very large. Cortical lamination was very anomalous in both groups, but the damage due to irradiation was more severe in those animals irradiated with 3 Gy (Figure 6). All cortical layers of area 6 [ref. 7] were greatly reduced in both groups, relative to the non-irradiated controls, but again the reduc-

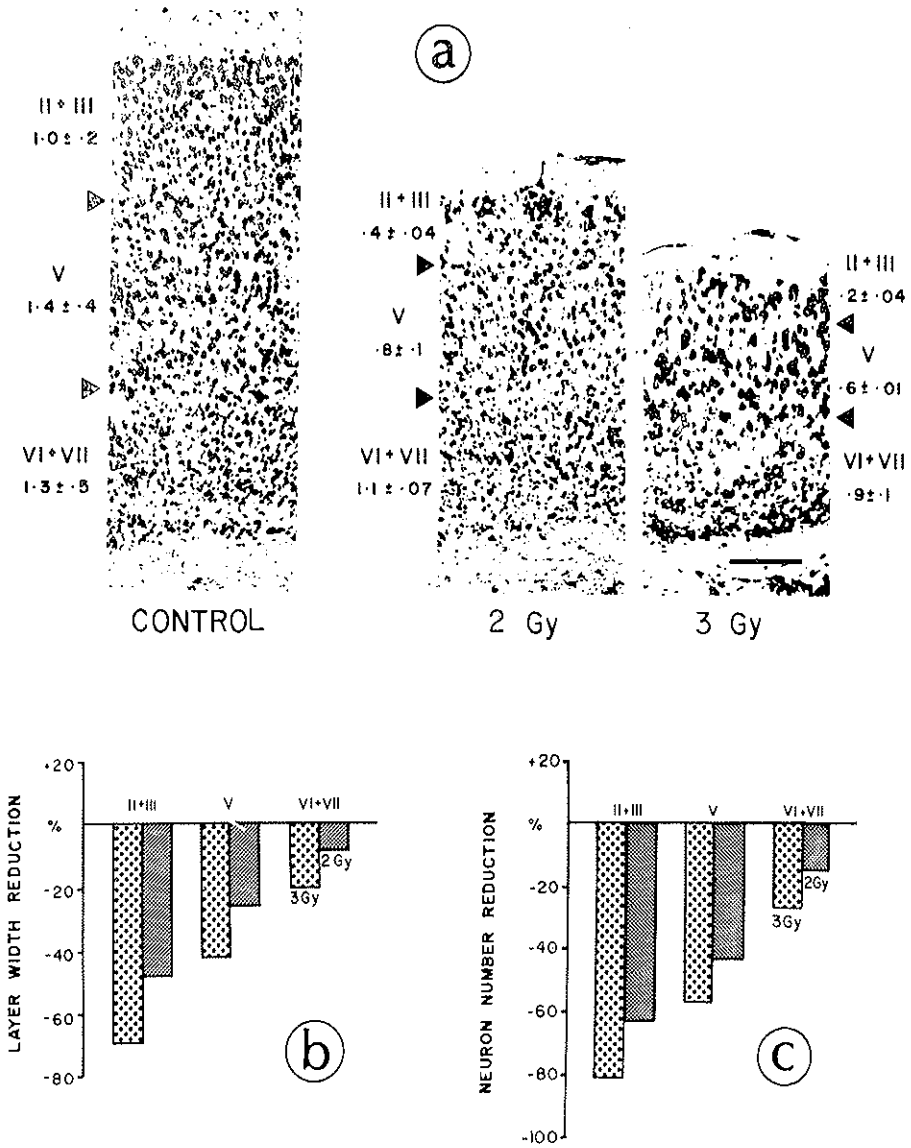

FIG. 6. *a*: Nissl-stained sections through frontal area 6 of a control animal and of mice irradiated with 2 Gy and 3 Gy. The triangles mark the borders of layer V. Mean numbers (X 10^4) of neurons (± 1 SD) calculated as described in text. Calibration bar, 250 μm. *b* and *c*: Percent reduction of layer widths and neuron numbers for the irradiated animals, as compared with the controls. From Lent and Schmidt [62].

tion was greater in the 3-Gy group. As can be seen in Figure 6, the damage was much more severe in the supragranular than in the infragranular layers.

In both groups, the corpus callosum was totally absent, although in a few cases some myelinated fibers were seen crossing the midline over the septum. The hippocampal commissures were hypotrophic in most cases, and absent in a few animals. The anterior commissure did not present any obvious abnormality in all of the irradiated animals.

A striking architectural difference between mice irradiated with 2 Gy and those irradiated with 3 Gy was the occurrence, in the former, of a tiny aberrant longitudinal bundle of fibers which was absent in the latter (Figure 7). As it was suggested that the longitudinal bundle of genetical acallosal subjects could be formed by misdirected axons of cortical neurons [54, 88], we decided to check whether the greater width reduction of cortical layers in the 3-Gy group as compared with 2-Gy animals would imply that more cortical neurons were spared in the latter. We then calculated the number of neurons in an arbitrary volume contained within area 6 of non-irradiated animals, correcting it in the irradiated groups by the reduction factors determined for the three dimensions of cortex. This procedure was chosen in order to circumvent the difficulty of determination of cytoarchitectonic boundaries of area 6 in the irradiated animals. As can be seen in Figure 6, a sizeable proportion of neurons survives in all cortical layers ($\cong$ 15%) of animals irradiated with 2 Gy, as compared with those irradiated with 3 Gy.

The lack of specificity of the effects due to irradiation, however, opens up a wide range of possibilities for the interpretation of these results. First, the formation of the aberrant longitudinal bundle may depend on the survival of a critical number of cortical neurons, some of which may be callosal. Secondly, it is conceivable that the glial sling may have been damaged by irradiation at E16, provoking the misdirection of surviving callosal axons in animals irradiated with 2 Gy, and consequently the formation of the aberrant bundle. Alternatively, the absence of both the callosum and the aberrant bundle in the 3-Gy group may have been due to the damage of appropriate targets, rather than to a disturbance of the interaction between callosal neurons and their guiding glial cells. Further work is needed to clarify these issues.

Summary and Conclusions

Three different experimental paradigms have been used so far for studying the consequences of early removal or congenital absence of the

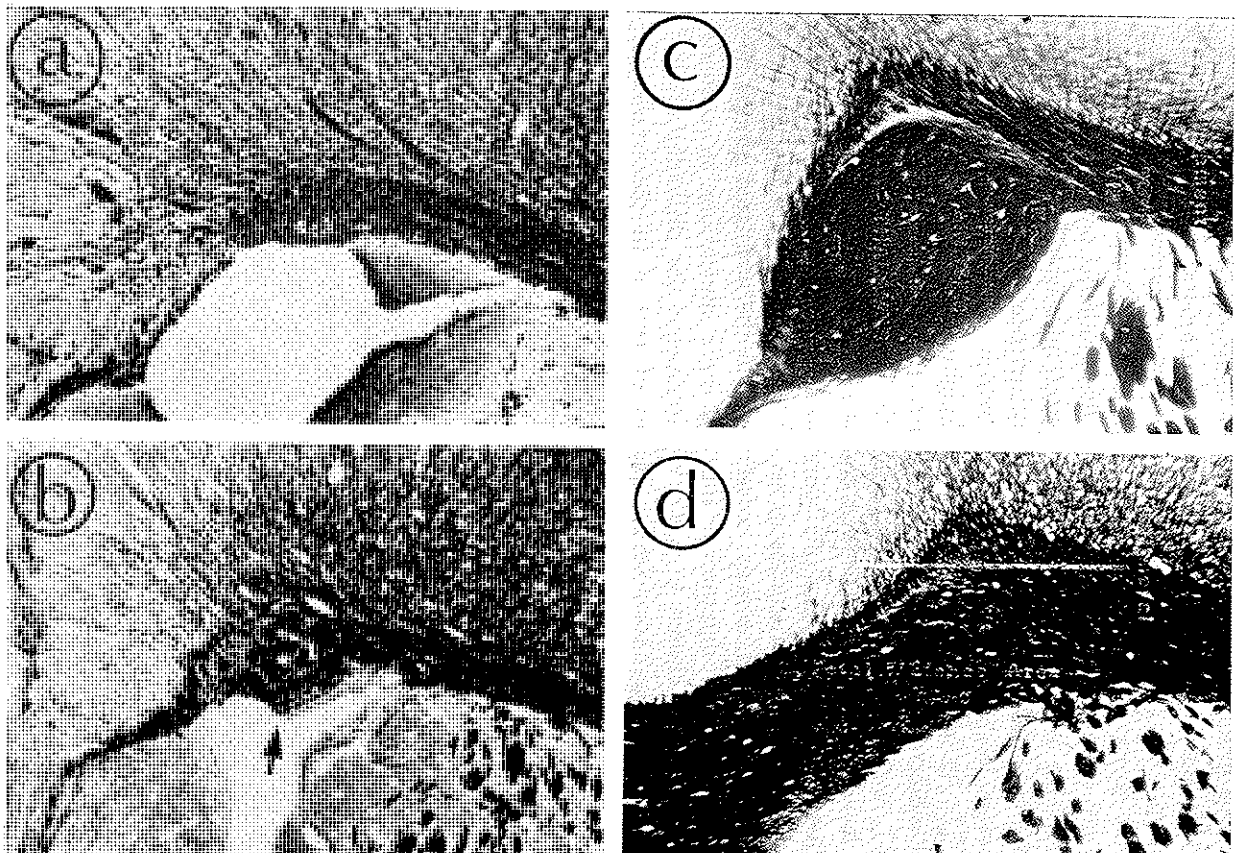

FIG. 7. *a*: The lack of an aberrant longitudinal bundle at the medial sector of the cortical white matter is visible in a 3-Gy mouse brain. *b*: A tiny longitudinal bundle (arrow) in a 2-Gy mouse brain. *c*: The typical longitudinal bundle of an acallosal BALB/cCF mouse. *d*: Control brain. From Lent and Schmidt [62].

corpus callosum: (1) acallosal mutants, (2) animals subjected to early transection of the callosum, and (3) animals born acallosal as a consequence of prenatal irradiation.

Although callosal agenesis has been reported in a few different species, only in certain strains of mice has it been systematically studied. Some knowledge has been acquired about the genetic mechanisms determining the expression of callosal defects in these mice, and the architecture of defective brains has been described.

Rodents subjected to perinatal transection of the corpus callosum have brains which are architecturally similar to those of genetically acallosal mice. In addition, early transected hamsters were shown to present some aberrant interhemispheric connections. Neonatal split-brain cats show some signs of physiological and behavioral deficits, but they also show behavioral plasticity, expressed by the restoration of their ability to transfer learning from one to the other hemisphere.

Rodents irradiated during prenatal development may have variable degrees of callosal defects, depending on the radiation dose and on the age at irradiation. Their brains, however, were shown to be very different both from those of genetical and those of surgical acallosals, in that a considerable disorganization of cortical architecture takes place, along with great reduction of cell numbers within the neocortical layers.

Current evidence for the occurrence of developmental plasticity in the cerebral cortex of early disconnected brains is still not enough, however, to attribute a functional significance — either restorative or maladaptive — to the aberrant circuits which form in these brains. Further experimental work is needed in this direction, until one is able to generate concepts which may be extended to human acallosal patients.

Acknowledgements

Support for the authors' work has been provided by the Fogarty International Center (USA), the Conselho Nacional de Desenvolvimiento Científico e Tecnológico (CNPq, Brazil), Financiadora de Estudos e Projetos (FINEP, Brazil) and the Fidia Research Laboratories (Italy).

We thank E.S. Silva Filho, J.S. Ramoa and J. Nilson Santos for skillful technical assistance, J.F. Tiburcio for animal care and our colleagues who participate in the weekly Callosal Tea, for helpful discussions.

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STRUCTURAL, FUNCTIONAL AND BIOCHEMICAL PLASTICITY IN THE RODENT BRAIN

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INTRODUCTION

Plasticity implies molding. When the term plasticity is applied to the brain it suggests that the brain itself can be molded. The list of factors now known to be of importance in this molding is long and varied. It includes a number of active molecules including hormones and trophic factors [10], selective experience with the environment [12, 31] and changes provoked by deleting or adding inputs to the brain as with lesions [27, 64] and transplantation [65] respectively. The most dramatic changes in the brain can be provoked in developing systems (e.g. ref. 45) but there also is a wealth of information showing that plastic changes occur in the adult brain (e.g. ref. 43).

Why the mammalian brain should be plastic or lacks plasticity is a fundamental question. Factors such as a limited genome, the value of adapting to the environment, the need for adjusting to imperfect and changing body dimensions, and a means for long-term storage have all been implicated (e.g. ref. 11). It is of theoretical and practical interest to better understand these phenomena. On the one hand, insights into how the brain actually conducts its business is a goal; on the other, how it can be altered to compensate for severe and now largely permanent injury is a practical hope. Most of the experimental work in this area is based on induced pathology.

Dramatic changes in brain architecture and function are observed when manipulations are conducted on developing animals. For many systems and features of these systems there is a limited period during which manipulation(s) produce an observed effect. Depending on the system and the phenomenon, the periods have been termed "sensitive" or "critical" since these are times when changes can be provoked and in some cases reversed (e.g. refs. 8, 35, 67). Critical and sensitive periods are clearly linked to the sequences of development which normally occur in the brain. As such their definition provides clues to the mechanisms involved in the formation of the brain and its connectivity (e.g. ref. 48). Other plastic changes — so called because their time course is several orders of magnitude longer than synaptic and modulatory events — have been defined in adults [5]. Most of those documented are apparently reversible [43]. The precise linkage or lack thereof between the two classes of phenomena which are timed by the age of the relevant organism is an outstanding question. Some insights are provided elsewhere in this volume.

I propose here to review studies on a brain pathway that relate to the plasticity of the mammalian brain and its functional meaning. The rodent trigeminal pathway is described briefly with emphasis on its useful features; its development is delineated. Experimentally induced changes in the brain, morphological, functional and biochemical, are outlined.

The Rodent Trigeminal System

With occasional exceptions the world of the myomorph (mouse-like) rodents, mice, rats and their relatives is one of dark small spaces. The sensory and behavioral characteristics of these animals reflect (as do many species, e.g., raccoons, monkeys, bats, songbirds, etc.) the ecological niche they occupy [9]. The animals are highly olfactory (macrosomatic) and have relatively poor vision for detail. The tactile sense is important, especially that derived from the whiskers or vibrissae which are prominent on the animal's face [74]. These are used in active exploratory behaviors [75, 82], but have many analogies to guided and reflexive visual behavior in primates [89; see Fig. 1].

The whiskers are tactile organs arranged in grid-like arrays that are characteristic for a given species and are genetically determined [19, 69]. Each whisker consists of a central hair of constant length which is em-

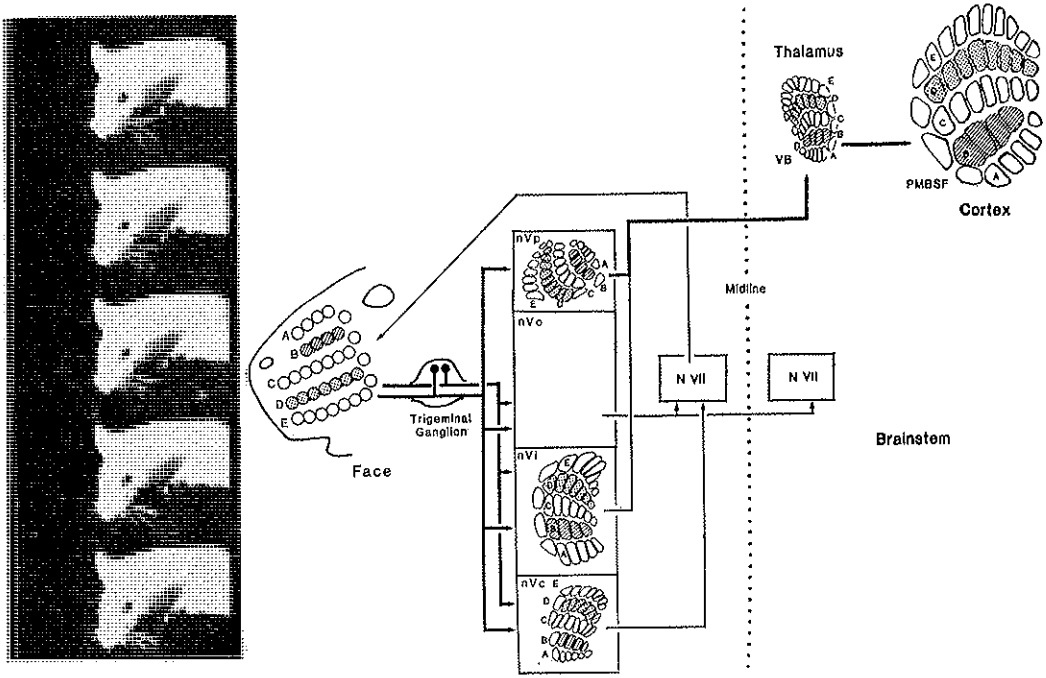

FIG. 1. The trigeminal system of the mouse. *Left.* A mouse exploring the environment in sequential cine frames. The prominent whiskers provide tactile inputs from the face. *Right.* The grid-like array of whiskers or vibrissae is evident from the skin and in sections. Each whisker receives a fascicle of sensory fibers from the infraorbital nerve. Cell bodies of the sensory fibers are located in the trigeminal ganglion where there is topography with respect to the principal divisions of the fifth nerve but no clear whisker-related organization of the cell bodies. The axons of the ganglion cells course along the lateral aspect of the brainstem as a relatively pure fiber bundle to targets in the brainstem trigeminal complex (BTC). Three of these have a segregation of terminals and cell bodies clearly related to the whiskers. The thalamus receives an input from the BTC where fibers and cell bodies have a whisker-like pattern. The cerebral cortex has a two-dimensional map of the body surface which resembles it in a distorted fashion consistent with density of peripheral innervation. Descending connections not shown. Abbreviations: nVp = principal trigeminal nucleus; nVo = oral trigeminal nucleus; nVi = interpolar trigeminal nucleus; nVo = caudal trigeminal nucleus; N VII = facial n nucleus; VB = ventrobasal complex; PMBSF = posteromedial barrel subfield.

bedded in a hair sinus that is densely innervated [32, 39a, 75]. The innervation to each whisker is supplied by nerve bundles that can be recognized on gross dissection [17]. The nerve bundles carry consistent numbers of myelinated fibers of at least three diameter classes which presumably correlate with the several different kinds of endings found in each tactile

organ [3, 91]. The whiskers are under efferent control from both the facial nerve and the autonomic nervous system [75, 82].

The infraorbital nerve, a branch of the maxillary division of the trigeminal nerve, supplies the larger whiskers. This very stout nerve courses from the trigeminal ganglion on the floor of the skull across the floor of the orbit and out the infraorbital foramen [59]. At this point it is easily recognized and can be surgically manipulated. The related cells of the trigeminal ganglion are recognizable in sections as to the division of the fifth nerve with which they are associated but there are no cell groupings which correlate with each of the whiskers [28]. Central axons from the ganglion cells are attached to the pons via the trigeminal root and course along the lateral aspect of the brainstem as a relatively pure tract giving off collaterals to the four distinct nuclei of the brainstem trigeminal complex (Fig. 3; ref. 4). Four of these nuclei are characterized by having whisker-related patterns of primary afferent terminals and three having segregated target cell bodies which contribute to the pattern [6, 42]. It is now known from Golgi studies of these neurons that the dendrites of a majority of the brainstem target neurons are confined to a whisker-related module which extends tube-like throughout the rostrocaudal extent of a particular subnucleus [41].

When the somatosensory thalamus is injected with a retrograde axonal tracer, a majority of the neurons in the principal nucleus is labeled and some neurons in the interpolar nucleus are labeled. Neurons in the other two subdivisions are not labeled [24]. The axons of the cells projecting to the thalamus cross the midline as the trigeminal fillet just medial to the nucleus of origin. There is no clear evidence that the axons in the lemniscus are bundled according to the part of the trigeminal periphery with which they are specifically associated. With modern experimental methods it has been possible to confirm the earlier findings of Ramon y Cajal [50] and the Scheibels [55] on the manner in which the lemniscal fibers terminate in the somatosensory thalamus [7]. They do so as curvilinear tubes which can be recognized as patches of mitochondrial densities and, in the mouse, as clusters of thalamic neurons [21, 68]. The afferent patterns persist into adulthood although their definition becomes less sharp. While the evidence is less clear-cut both Golgi impregnations and retrograde filling of the thalamocortical neurons suggest that their relatively short dendrites are oriented toward the incoming lemniscal terminal clusters [7]. In many respects the arrangement of fibers, terminals, target cells and target cell dendrites is similar to the pattern in the brainstem nuclei [7]. There

are modules in the somatosensory thalamus each associated with a particular whisker.

Axons of the thalamocortical projection leave the thalamus and rotate just lateral to it before traversing the striatum to the subcortical white matter. There is no evidence that these axons traverse the basal ganglia as whisker-related bundles but rather seem to collect as whisker-related groups near their final destination near the target cells [7]. Axons from the somatosensory thalamus as described in the visual cortex have terminal fields in upper layer VI and throughout layer IV spilling over into layer III [39, 40]. As in the subcortical stations the terminal clusters in the somatosensory cortex can be recognized with the mitochondrial stains [36, 38]. The target cells, principally in layer IV but to a lesser extent in layer VI, are segregated into characteristic architectural patterns that resemble the patterns of input [93]. The processes of the layer IV target cells are oriented toward the inputs from the thalamus. Modern studies have confirmed the earlier studies of Lorente de No [40].

Based on the name given to the cellular aggregates in the cortex, the part of the pathway to first be understood in the context of the organization of sensory periphery, the modules along the pathway are all named barrels, barreloids and barrelettes in the cortex [93], the thalamus [68] and the brainstem [42] respectively (see Fig. 1). Each module, regardless of location, has the same fundamental organization — a central core of clustered axon terminals ending on dendrites which are confined to the field of termination of the clusters and a ring of cell bodies around the terminals. The arrangement is shown schematically in Figure 2 and although the arrangement differs in dimensions according to brain location it is fundamentally similar in overall anatomical organization regardless of location. The incoming axons meander prior to reaching their final destinations. This is especially true for single axons entering the hrainstem or cerebral cortex where the axons have been demonstrated by several methods in both the rat and the mouse [7, 41]. These observations are consistent with the two-stage developmental process outlined by Schneider and his colleagues [56]. It has been suggested that fasciculation of projection axons is a major corollary of the observed projection patterns [26] but although the general topography in any part of the axonal trajectory is preserved [13] it is also clear that, as in the optic axons of the primate [59, 83], axons change their nearest neighbor relationship often and that it is the terminals that correlate with the modular arrangement seen in all parts of the pathway.

Functional studies in 1967 first suggested the correlation of the anatomical modules with the whiskers on the face [87]. Subsequent microelectrode and 2-DG studies have verified the relationship at the cellular level [23, 63, 81]. With both means it has been shown unequivocally that a majority of neurons in each module in each part of the pathway respond to one and only one whisker. Because of the anatomical arrangement of the pathway, it is possible to show that the responding neurons are consistently located in the appropriate anatomical location [91]. That is, when a whisker or groups of whiskers are stimulated, the appropriate modules are activated. The neurons in the somatosensory

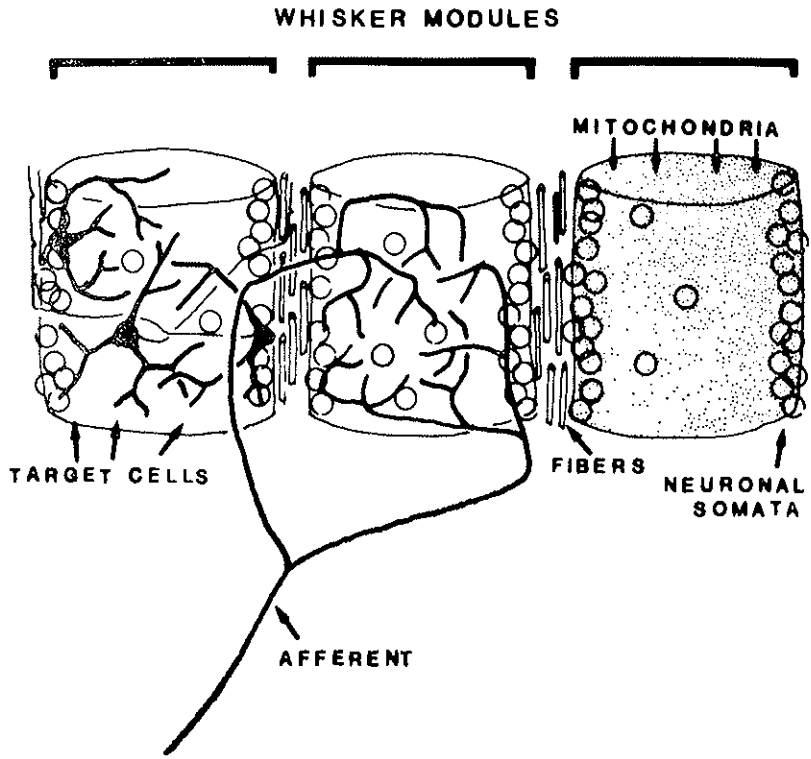

FIG. 2. Schematic drawing of a whisker-related module .Evidence from all parts of the pathway suggests that afferent fibers form terminal clusters on cells with directed processes. It is these terminal clusters that are partially responsible for the patterns seen in histochemical stains for mitochondria. The directed dendrites and axons and the consequent differential packing of the cell bodies make each of the whisker modules detectable in routine histological preparations.

cortex are organized as columns extending throughout the entire cortical depth [20] and the studies of Simons indicate that processing of whisker-related information parallels the sensory processing so well described in the primate visual cortex [61].

From this review of the basic anatomical and functional organization of the rodent trigeminal system it is clear that the system is behaviorally important to the animal, that it has many favorable anatomical features which aid in interpretation of experimental studies and that the functional correlates of the anatomical organization are precise and are arranged in a fashion similar to that reported for other well studied mammalian sensory systems.

Development of the System

Some of the myomorph rodents offer distinct advantages for studies of development. In addition to their small size, which facilitates appraisal of the entire nervous system, the animals breed well in the laboratory, have large litters, have relatively short gestation times (3 weeks) and are immature or altricial at birth. Although all the neurons of the somatosensory pathway have been generated at birth many events such as neuronal migration, axon outgrowth and terminal elaboration, and differentiation occur in the week after birth [92]. Thus it is possible to follow important developmental events *ex utero* (see Fig. 3).

The principal features of the development of the somatosensory system are known. For instance, the timing of the divisions of neurons in each part of the pathway has been determined using the thymidine labeling technique. As summarized in Figure 3 the neurons of the central nervous system follow an ascending order of birth from the brainstem to the cerebral cortex. The gradients of neurogenesis have been mapped in a general way at each location [92]. The neurons of the trigeminal ganglion are born slightly later than their targets in the brainstem. The establishment of a pattern of the receptor organs on the face is clearly related to organizing centers in the dermis [14, 18]. Although neurites have been associated with these centers early in life, a number of experiments have shown conclusively that innervation is not required for the vibrissae to develop properly [1, 71].

The pattern of maturation of the different levels of the somatosensory system seems to follow the same inside-out sequence as described for neurogenesis but over a longer time course, which for the central and

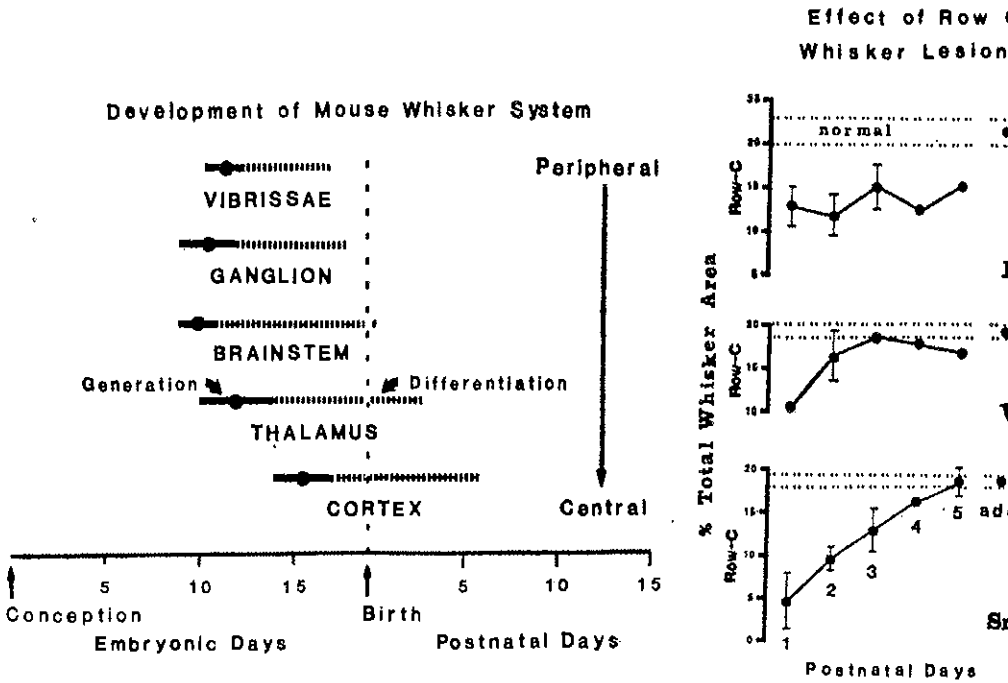

FIG. 3. *Left*: Schematic diagram showing the ascending patterns of maturation of the trigeminal system. Neuronal differentiation continues into the postnatal period. *Right*: Lesions to row C whisker on different postnatal days effect terminal areas in an outside in sequence. PS = brainstem, VB = thalamus; SmI = cortex. (Data from ref. 21).

peripheral nervous systems extends into the postnatal period. In the interval between the birth of the neurons and the maturity of the relevant loci, axons connecting one part to another grow out. The axons establish their approximate trajectories quite early in gestation having an approximately appropriate topographical order. But as described in other animals these fibers have extended latent periods where they "wait" near their targets before invading those targets to establish contacts with the target cells [84]. Thus the system seems to follow the two stage rule laid out by Schneider and his colleagues [56]. It is known that the axons to the vibrissae grow out in the infraorbital nerve in late gestation, establish contact with the vibrissal follicle primordia and appear to have an approximate order in the nerve related to the peripheral targets [26]. It has been suggested that the pattern of fasciculation is important in establish-

ing the patterns of connectivity between the periphery and the brainstem targets but several lines of evidence indicate that fasciculation is not necessary for the process [59].

Direct observation of the developing axons in their preterminal segments indicates that the initial location of these fibers is at best only approximate. Since several fibers can approach a single target (a barrel or barrelette) after originating from different locations in the parent bundle it would seem clear that the initial position of the axons is only approximate and that as described in other parts of the nervous system the axons have a certain freedom to search for and find their proper targets. The fiber trajectories leave a trail indicating these wanderings [7, 41]. In the brainstem the fiber terminals from the trigeminal nerve become progressively more elaborate in the postnatal period [41]. A similar sequence occurring later in the cerebral cortex has been less well characterized.

The segregation of neuronal somata appears at about the time that afferent fibers grow into a target (see Fig. 4). For instance, there are no cytoarchitectonic entities in the cortex before clustered terminals arising from the thalamus can be demonstrated [53]. In the brainstem this relationship has been studied in detail. When the mouse is born neither cell clusters nor segregated histochemical patches are evident in the brainstem. The patches appear some 12 to 24 hours before the cytoarchitecture becomes evident [41]. Later in the animal's development the same sequence occurs in the cerebral cortex. In the brainstem, synapses are found on proximal growing neuritic processes and these synapses mature into the characteristic complex endings that arise from the incoming trigeminal axons of the adult. The findings are consistent with the hypothesis of Vaughn and co-workers [72] which suggests that the formation of synapses on growing neuronal processes directs the growth of dendritic trees [30, 41]. It is the directed growth of the target cell processes and the continuous elaboration of afferent terminals that produce a differential growth of the neuropil at each level of the neuraxis. A result is the apparent concentration of cell bodies in the cytoarchitectonic units associated with each vibrissa.

From a strictly descriptive point of view then, it would seem as if all the parts of the pathway have similar sequences of developmental elaboration. Incoming axons are roughly arranged in a topographic fashion but are not specifically bundled with respect to an individual vibrissa. The terminals of these axons elaborate after growing into the appropriate target tissue. The resultant pattern is consistent with the axons behaving

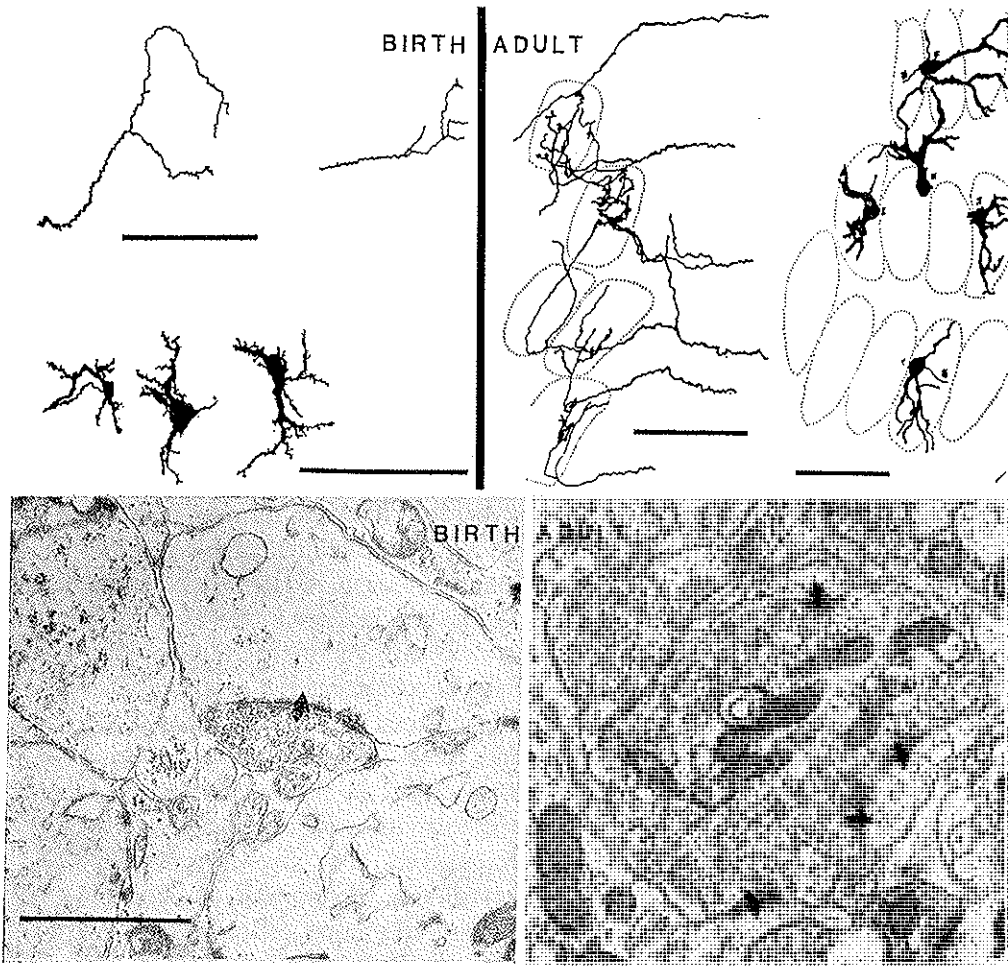

FIG. 4. Afferent arbors, target cell processes and afferent synapses become increasingly elaborate in postnatal life. Examples from the brainstem illustrate the sequence. *Left*: On the day of birth trigeminal axons are simple and target cell dendrites are short. In the electron microscope (EM) the presumed primary afferent terminals are simple (curved arrow). *Right*: In the adult primary afferents are more complex and elaborate and target cell processes are longer. Both afferents and target cells are related to cytoarchitectonic boundaries (dotted lines). Primary afferent terminals are complex and much larger. Drawings from ref. 41; bars = 100 μm. EM's prepared by Jon Christensen; bar = 1 μm. A similar sequence is followed later at higher stations in the pathway.

as if they were a single source as in seeding a crystal [57]. The terminals contact target cells after they grow in and stimulate directed target cell process growth which temporally correlates with the appearance of afferent synapses on the target cells. The principal feature which distinguishes one location from the next is the *time* at which these events occur. This is in an ascending sequence from the brainstem to the cortex. Based on the general and detailed similarities from one station to the next it is parsimonious to think that the manner in which the detailed arrangements are conferred on each station is the same regardless of location in the nervous system [88].

The Morphological Plasticity

Because the whiskers are discrete and grossly recognizable at birth, as are the nerves to them, it is possible to selectively manipulate the sensory periphery to see if this has any effect on the developing central nervous system. Since our demonstration that the morphology of the cortex could be altered by surgical manipulation of the somatic periphery [70] many workers have employed this approach to better understand the principles of the development of the rodent somatosensory pathways (e.g. refs. 29, 73, 77).

Although there are many ways in which the lesion experiments can be done, the two principal methods have been to lesion selected whiskers [21] or to surgically transect the infraorbital nerve [6]. All of these manipulations, except lesioning one whisker alone, will alter the organization of the central whisker pathways (see Fig. 5). Approaches designed to alter activity in the pathway such as chronic whisker clipping [88] or section of the seventh nerve which activates the muscles which move the whiskers back and forth [52] have not produced a morphological change in the system.

The effects of whisker lesions in early life on the system depend on the time the lesion is made and the locus. In contrast to adults, lesions to the distal processes of the trigeminal nerve are followed by prompt degeneration of the parent cell body in the trigeminal ganglion and the central processes of the fibers in the trigeminal tract and target nuclei [54, 76]. In the newborn the degeneration is sufficiently swift to be virtually complete within several days. In mice similar lesions at about 20 days of life do not produce significant cell loss in the ganglion or loss

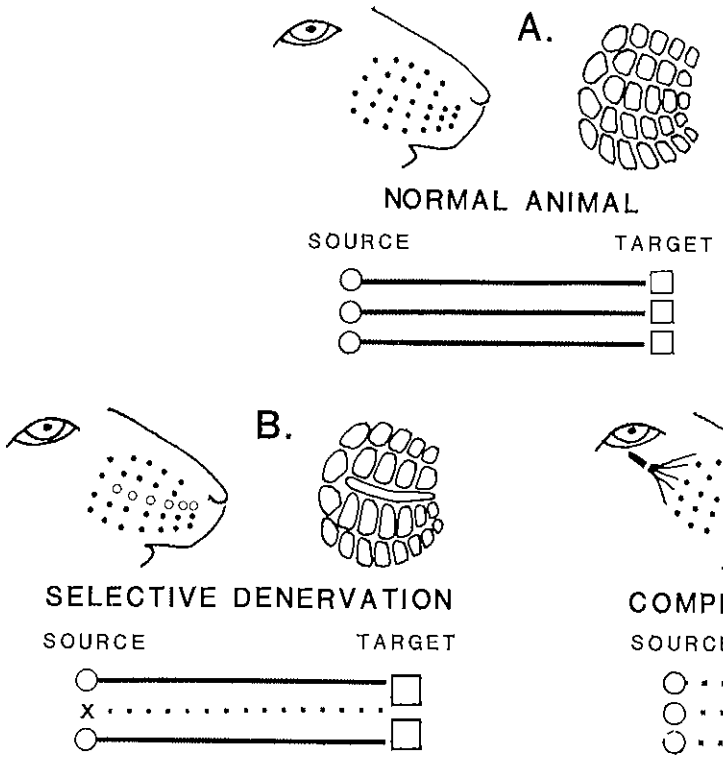

FIG. 5. Schematic diagram showing patterns of projection in the mouse brain in normal animal (A) and after selective (B) and complete (C) denervation. Patterns that result depend on the target, the age of the animal and the pattern of manipulation done. The larger boxes in B under target indicate the expansion of connected terminals at the expense of the disconnected terminals possibly by competitive mechanisms. The figure illustrates the two principal lesion paradigms generally used. Figure prepared by L.M. Sikich.

of their central processes. Lesions within the first two weeks of life produce loss of the ganglion cells and their processes although there is some evidence that the rate of degeneration slows as a function of age [41]. Abnormally large peripheral receptive fields of the trigeminal neurons after such lesions have been reported and are consistent with the notion that the peripheral processes either sprout or fail to retract [51]. Although single row lesions have been made at a time when the pattern of barrelettes is forming, there is no evidence that the projections from the remaining whiskers enlarge their trajectories in the brainstem postnatally [21, 41]. The changes after single row lesions made throughout

the first two weeks of life are largely the same in all relevant nuclei in the BTC and can be largely attributed to the degeneration of the primary afferent fibers. Electron micrographs of the principal nucleus show an absence of primary afferent terminals and though the target neurons are atrophied they do not appear to undergo a true transneuronal degeneration [41]. Retrograde labeling with HRP indicates that these atrophic neurons still support axons to the thalamus [25]. In the brainstem the patterns that result are always consistent with the patterns of peripheral disruption and this anatomical evidence correlates with functional mapping of these nuclei [23, 47, 58].

In contrast to the findings in the brainstem, changes in the thalamus and cerebral cortex are critically time-dependent both for the period over which they can be produced and the patterns that result (see Figs. 3 and 6). In the mouse thalamus, the period during which the patterns of projection can be modified by peripheral lesions terminates on about the third day of life. Interestingly this correlates well with the time at which this nucleus is first seen to begin to differentiate into its segmented modular pattern [90]. Other workers studying rats disagree with this finding but the two species may not be entirely comparable in terms of the timing of early postnatal events [6, 21]. The other interesting difference between the thalamic and brainstem patterns which result from selective lesions to the periphery is that in the thalamus representations adjacent to the lesioned whiskers expand as if to compensate for the attenuated projections related to the lesioned whiskers [21, 90].

Similar results are found in the cortex, where these effects were first noted [94]. The neighboring whisker projections expand so that the total cortical area related to the entire vibrissal representations remains unchanged although the relative size of the representations is radically changed [22]. Always the projections which seem to be related to the intact whiskers encroach on the cortical zones to which the missing whiskers would normally project. The alterations are graded such that the earlier the peripheral lesions the more dramatic the shifts in cortical territories associated with the intact and missing whiskers [94]. The changes are reflected not only in the pattern of inputs from the thalamus, but also the orientation of cortical dendrites and the architectonic patterns of the cells [30, 66]. In comparison to the thalamus, the extent of these alterations in the cortex is always greater and at a given time point and the time over which they can be produced is longer, terminating on the fifth day of life [21]. The evidence is clear that the time course of these

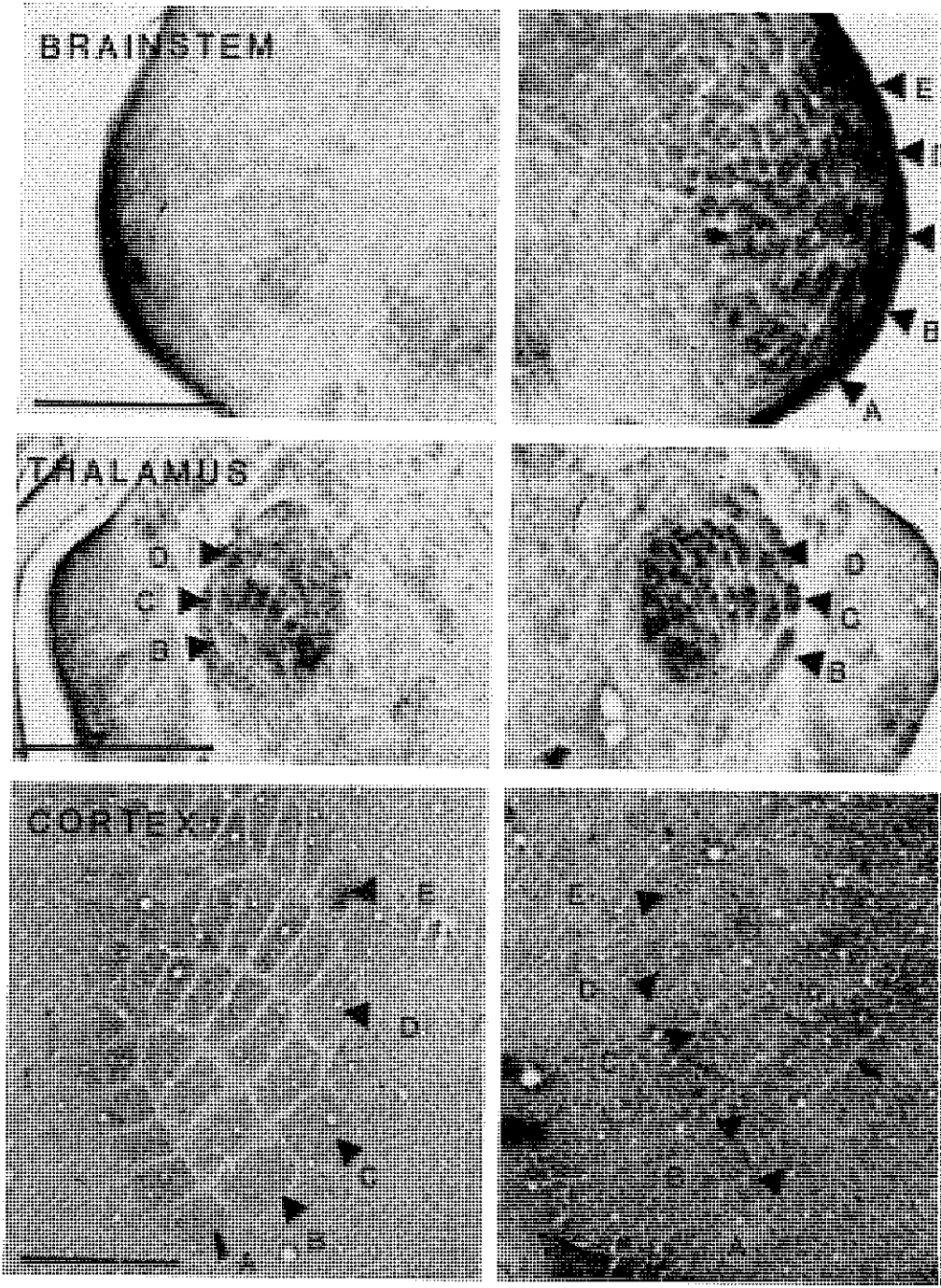

FIG. 6. The trigeminal pathways of the mouse stained for cytochrome oxidase after infraorbital nerve section to the sensory periphery on the third day of life. The animal survived for 3 days. The whisker pattern is absent in the nucleus interpolaris; is is apparently normal in the contralateral VB and severely altered in the contralateral cortex. Sections prepared by Cathy Ifune. Bars = 500 μm.

experimentally produced alterations in cortical anatomy correlates temporally with the differentiation described in the normal animal. The observed findings are consistent with a competitive imbalance produced by tbe lesions such that the parts of the projection pattern still connected to the periphery have a competitive advantage over those which have been disconnected.

The altered patterns in the cortex and presumably the thalamus seem to follow a sequence of pattern arrangement. If the infraorbital nerve is sectioned on different postnatal days the resulting patterns follow a time related order. In mice, ION lesions on the first day of life produce no recognizable pattern in the afferent projections to granule cells when those animals are studied as adults. If examined early enough the histochemical methods for mitochondria show a similar arrangement but the interpretation of these results is complicated by the paling of the reaction product (see below) [86]. On subsequent days a pattern of first rows then segmentation of rows into whisker modules occurs with each later time point being more sharply defined in terms of pattern than the one before it (see Fig. 6). These findings are interpreted to indicate a sequence of organization of the fiber terminals which is stepwise and are consistent with the very different results found when single rows of vibrissae are lesioned as opposed to groups of whiskers across different rows [92].

The behavior of the patterns of cortex and thalamus have been interpreted as indicative of a sequential transfer of pattern information from one trigeminal station to the next which temporally correlates with the observed patterns of postnatal development in the normal rodent brain [92]. The lesions perturb this process and lead in some cases (ION lesions) to an arrest of the developmental process in question [41].

The system behaves as if detailed pattern information is being transferred across the three synapses from the periphery to the cerebral cortex. If this were true then it should be possible to surgically disconnect the cerebral cortex from the underlying white matter and either replace it or transplant it to another host. If such a graft were to survive then the expectation would be that appropriate patterns could form in the transplanted tissue. Recently the experiment was done and in a few cases the pattern which resulted resembled the normal one [2]. This experiment indicates that no mechanical guides are necessary for the axons to reach their targets.

Whether or not any cortex (i.e. visual, motor, etc.) will accept the

ingrowing axons has yet to be determined. In the transplant experiment and in a compression experiment, which leads to the same conclusion, the cortex seems to have certain boundaries which are obeyed by the incoming fibers. If neonatal lesions of the somatosensory cortex are made then a nearly perfect diminutive map is formed so long as the thalamocortical fibers in the white matter are not disturbed [33]. There may well be an overall polarity in the cortical tissue, since rotations of the cortical implants described above do not result in interpretable patterns. As the authors point out, such a negative finding is difficult to interpret [2].

The lesions described above have several disadvantages since they deafferent the periphery entirely or part of the periphery while leaving the rest intact. If in the latter competitive mechanisms are involved then a complete partial denervation of the periphery should not affect the central patterns produced although the size of these patterns could be reduced accordingly. Recently we studied an animal model which leads to a profound and apparently uniform denervation of the periphery. This is the autoimmune model which Johnson and coworkers have developed in the guinea pig [34].

It is known that the guinea pig trigeminal system has similar organized whisker related projections although the animal has a much longer gestation than mice and rats and the nervous system of the newborn animals is fully differentiated. The placenta of the guinea pig is permeable to antibodies which cross it freely at the time the somatosensory pathways are beginning to develop. Female guinea pigs can be immunized with mouse nerve growth factor (NGF) and in some cases the antibodies cross-react with guinea pig NGF. Offspring of such females have substantial neurological deficits which are most clearly related to deficits in the autonomic and pain pathways. On clinical examination there are changes in the response to tactile stimuli as well. Histologically, these animals have less than 20% of the normal trigeminal ganglion cells and only 50% of the normal number of nerve fibers in the infraorbital nerve and to individual vibrissae (see Fig. 7). The central pathways associated with the vibrissae in these animals are indistinguishable from normals. Since the balance between the relative numbers of fibers to the vibrissae is preserved although the absolute numbers are greatly reduced, it would seem that the result favors a competitive mechanism to explain the effects that are observed in the thalamus and

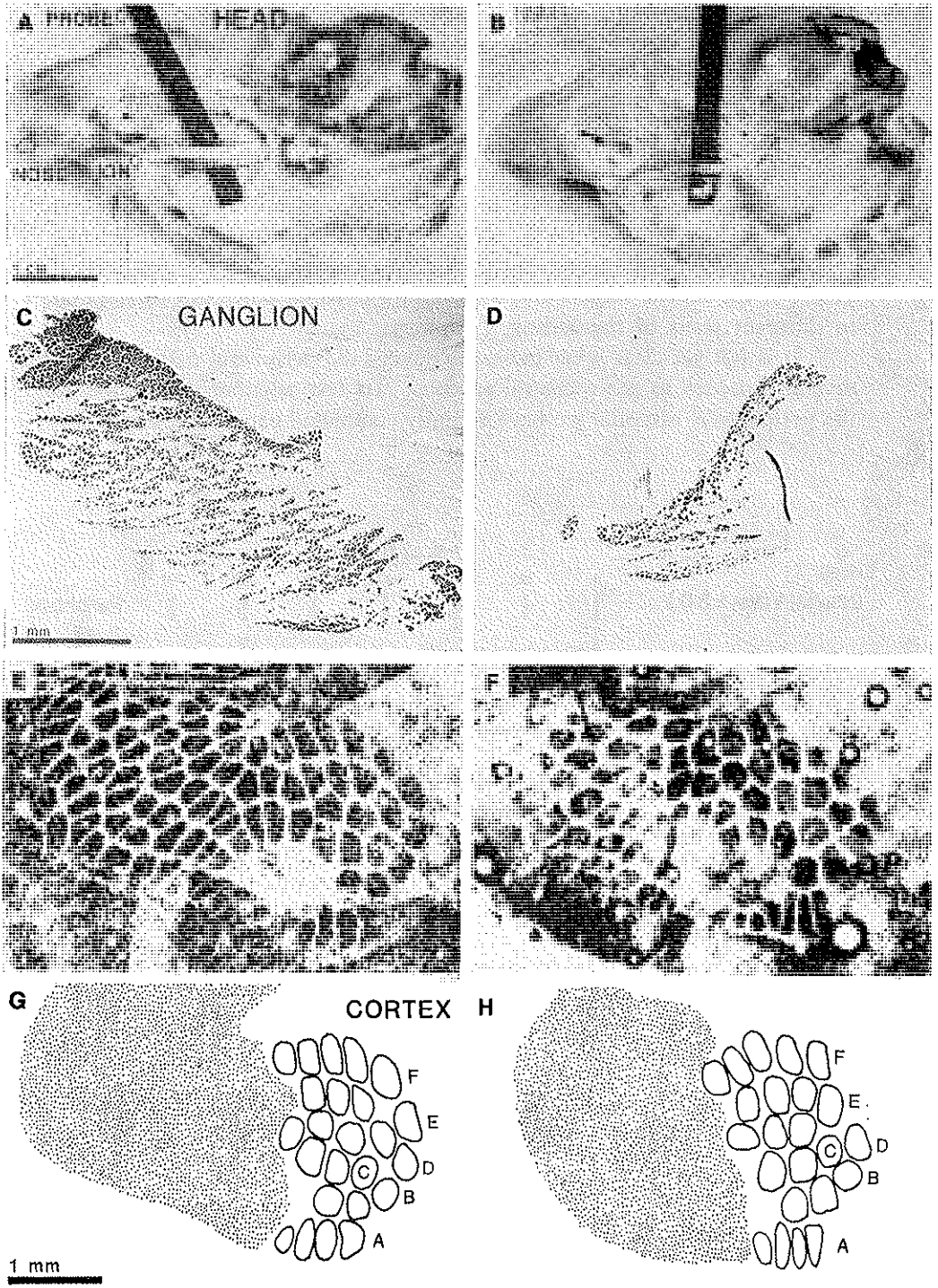

FIG. 7. In guinea pigs, fetal NGF deprivation (Right) results in a universal loss of ganglion cells (C vs. D) and their peripheral axons (ION = infraorbital nerve) (A vs. B) yet the central patterns of representation are preserved (E-H). The latter are not dependent on absolute innervation density but do depend on a competitive balance which is disrupted

cortex of animals in which the periphery was partially denervated [59] (see Fig. 8).

The results also indicate that a small fraction of the final complement of peripheral fibers is sufficient to establish the proper central projection patterns. That these patterns occupy the same volumes as in normal animals points to independent mechanisms for determining the spatial extent of the central targets and the division of those targets to correspond to different parts of the sensory periphery.

It is not yet clear what message conveys the peripheral pattern across several synapses to more central stations. To test whether connection to the periphery is essential we have recently completed experiments in which

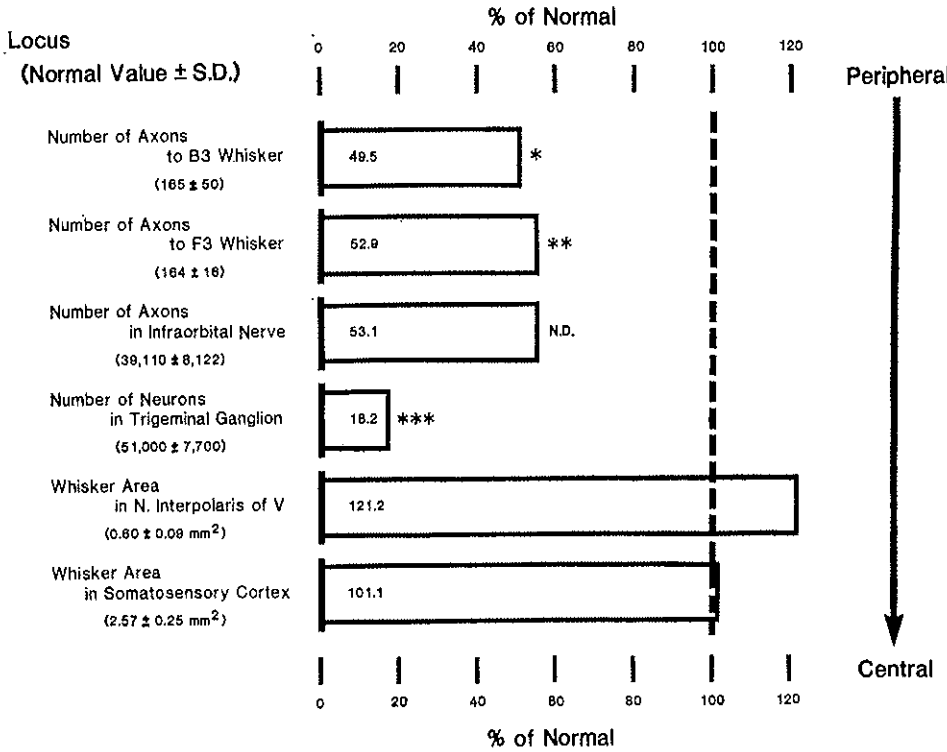

FIG. 8. Figure summarizing quantitative differences between various parts of normal (100%) and NGF deprived guinea pigs. In spite of the reduction in ganglion cells and their axons (asterisks indicate statistical significance) central structures are quantitatively normal. (Modified from ref. 59).

it was possible to preserve the central processes of the trigeminal afferents after the infraorbital nerve was sectioned. Systematically administered NGF prevents the degeneration of about 50% of young ganglion cells after peripheral axotomy [96]. In these cases a central pattern in the BTC and the central ganglion cell axons in the tract of V are preserved as long as exogenous NGF is available. The number of these cells and their central processes is apparently in excess of those necessary to set up the central patterns. In spite of the preservation of the pattern in the brainstem throughout the time during which the more central parts of the pathway establish the whisker-related patterns, the central stations appear just as they would without exogenous NGF [41]. Thus simply maintaining a pattern in the central nervous system is not sufficient to lead to proper whisker pattern formation. A conclusion is that the brain must be directly connected to the sensory periphery throughout morphogenesis. The experiment does not shed any light on what it is that tbe central nervous system gathers from the periphery to instruct its development. Logical candidates are activity and/or trophic factors for wbich there are precedents.

The Functional Plasticity

Against the growing list of morphological changes that can be provoked in tbe central and peripheral somatosensory system, one can ask what functional correlates if any exist for these changes? Here it is important to keep in mind that altbough the anatomical results obtained are in general agreement with each other, there are significant variations as to what manipulation has been done and the means to evaluate the functional consequences from laboratory to laboratory (e.g. refs. 37, 49, 77).

Our approach to this question has focussed on the cerebral cortex but incidental findings in our laboratory and those of others suggest a similar arrangement throughout the pathway. The basic strategy we have employed is to lesion a single row of whiskers in rats and mice on different postnatal days up to the end of the cortical critical period. The animals are studied as adults using two functional assays: single unit recording to evaluate the receptive field characteristics of individual cortical neurons [62] and ^{3}H-2DG to determine the population responses and to improve tbe spatial localization of these neurons [22].

Witb both assays the basic result is the same. The altered cortical anatomy still accurately defines functional boundaries in layer IV. If the

peripheral lesions are done at a time when the territory associated with the intact whiskers can expand, then the receptive fields of the cells in the expanded territories are related to those normal whiskers. With recordings, cells in the part of the cortex related to the missing periphery are abnormally silent and the usual background "hash" heard as the electrodes advance across the cortex is absent (Fig. 9). Cells which can be activated by peripheral stimulation in these quiet regions of cortex almost always can be related to the appropriate adjacent whiskers [62]. In the 2-DG brains we fail to detect increased numbers of labelled neurons in the deafferented layer IV which is consistent with the recording results. There is increased labeling of the neuropil in these cases, the significance of which is not clear [22]. However given that the cortex is electrically silent and that the 2-DG method presently does not allow a distinction between excitatory and inhibitory activity, the results are consistent with increased tonic inhibitory drive to neurons which must for the animal generate neural nonsense (Fig. 10).

From the recording experiments there are clearly a number of abnormal receptive fields. In the normal animal all of the cells in layer IV are activated only by the whiskers and about 80% of these by one whisker only [63]. In animals with whisker lesions some of the neurons adjacent to the deafferented cortex are activated by stimulation of skin in the region of the facial scar (see Fig. 9). When this happens the relevant skin is always adjacent to the whisker which activates the cortical neuron [62] The effect differs in rats and mice (which could either be a question of absolute size and/or developmental stage) and the time at which the lesion was made. The columnar properties of the somatosensory cortex (i.e. activation of cells in the nongranular layers) are coupled to the patterns in layer IV. These cells do not appear to differ significantly from their normal counterparts (see Fig. 9). Although abnormal receptive fields could be generated at any level of the neuraxis, the most parsimonious explanation is that there is either sprouting of the peripheral axons or maintenance of existing collateral peripheral axonal branches which are normally eliminated in development. It is interesting that direct anatomical and functional studies of the sensory periphery, behavioral testing and functional mapping of animals treated with the toxin capsaicin all fit this pattern (e.g., ref. 80).

That there is no clear evidence for a functional plasticity of the types described for the visual system [31] and now clearly demonstrated in adult animals [44], including rats [78], comes as a surprise for it had

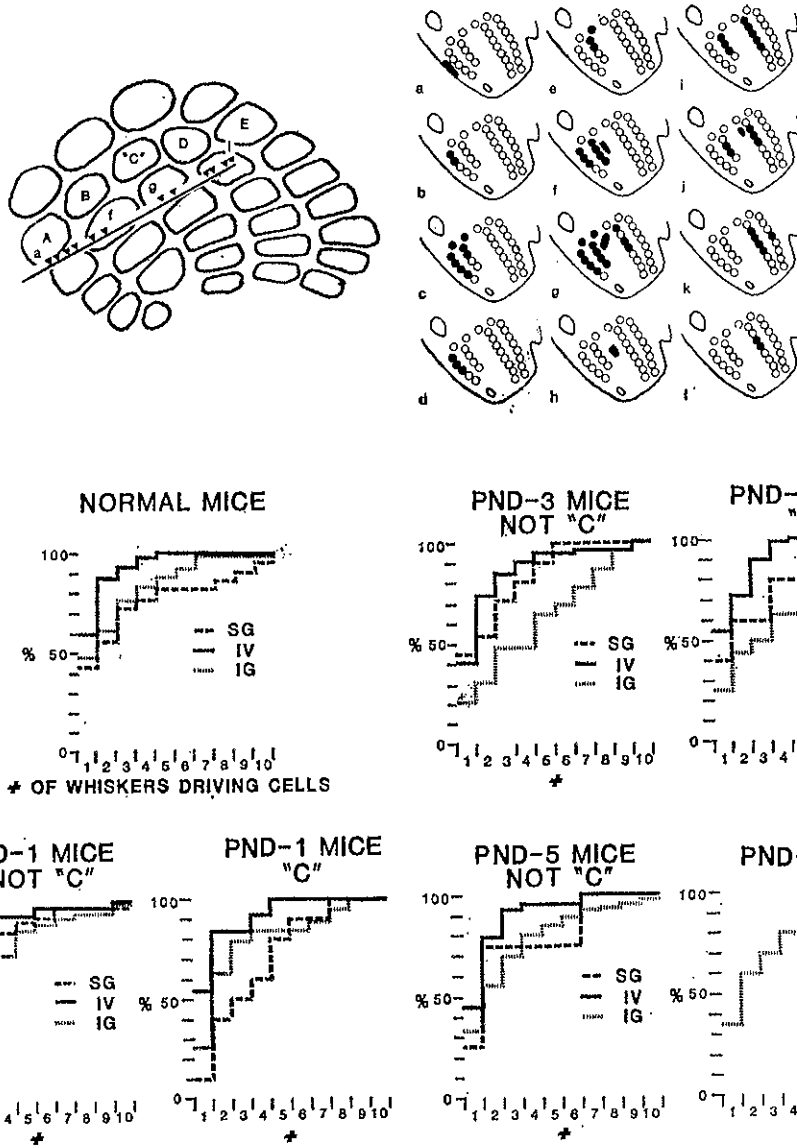

FIG. 9. *Top*: Receptive fields recorded from a mouse with Row C whisker damage on the fifth day of life. Although the map is topographically correct (electrode track to left) abnormal receptive fields are found and the cortex associated with the lesion is mostly electrically silent. Silent cells are consistent with a peripheral disconnection. Abnormal receptive fields could be explained by peripheral sprouting and/or a persistence of overlapping peripheral innervation believed to be characteristic of the newborn sensory periphery. *Bottom*: Cumulative histograms summarizing the number of units (%) found with varying numbers of whiskers driving each cell by layers. IV = layer IV, SG = supragranular layers; IG = infragranular layers. Not « C » are units outside the cortex associated with the damaged whiskers; « C » are units in the cortex associated with the damaged whiskers. The distribution in each case is basically the same, suggesting that whisker convergence is not a « plastic » property of the mouse cerebral cortex. (Data from ref. 62).

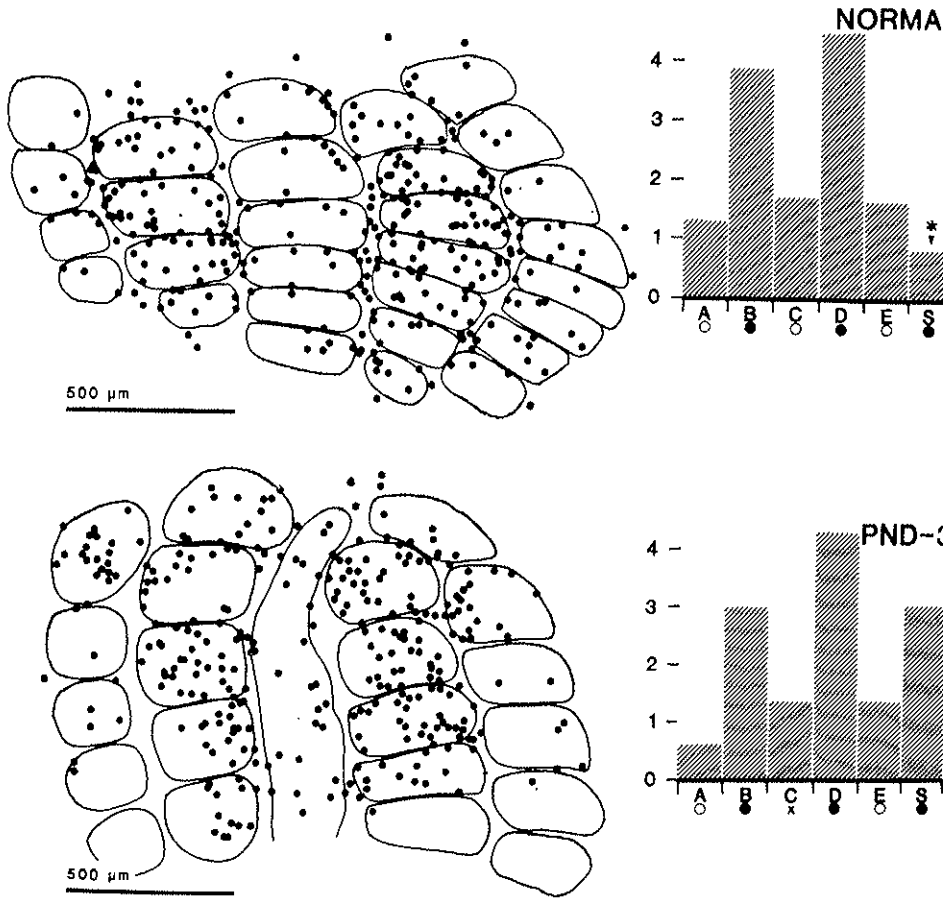

FIG. 10. Tritiated 2-DG maps showing the location of cortical neurons activated in a normal animal and an animal with a Row C lesion on PND-3. In the lesioned animal the active cells obey the architectural boundaries and are no more frequent (see density histogram to right) than expected from the normal animal. Open symbols under the histograms indicate the whisker groups which were clipped in the labeling experiment. (Data from ref. 22).

been presumed that the developing nervous system would be capable of greater plastic changes. However, it may well be that the phenomena being studied are based on different aspects of neuronal behavior; specifically the formation of axonal connections in young animals and modulation of synaptic drive in adults [79].

The Biochemical Plasticity

The histochemical stains for mitochondria succinic dehydrogenase (SDH) [46] and cytochrome oxidase (CO) [85] have proven to be excellent anatomical markers in the rodent somatosensory system and this and other systems in other forms (see Fig. 11). However, it is worth remembering that in her initial description of the CO method Wong-Riley emphasized the functional correlations that the histochemical method would provide [85]. She and Welt clearly showed that various manipulations of the whiskers would alter the relative staining intensity for CO in the somatosensory cortex in a manner that is specific for a particular whisker [86]. Unfortunately until recently there has been no assurance that these changes could be quantitated in any reliable way. One of the problems of the deafferentation experiments described above, as indeed the case for many experiments which depend on the substractive lesion strategy is that the normal inputs to the system are removed (e.g., refs. 21, 62). It thus becomes tedious to follow functional changes and difficult to make inferences from what remains after such a deafferentation. One way to evaluate the changes in the deafferented structures which have a functional correlate is to examine the biochemistry of those structures. In addition to the histochemical methods mentioned above there are a number of microchemical techniques which allow one to assay quantitatively the concentrations of enzymes, substrates and products from small anatomically identified parts of the brain [15]. To work out the necessary methodology in the rodent somatic system, to compare the results to the histochemical methods with an eye to quantitation and to assess tbe long-term functional changes in this system, we assayed the entire somatosensory pathway for a variety of enzymes related to aerobic glycolysis in adult animals subject to a variety of experimental manipulations.

A requirement of the microchemistry is tbat the tissues to be analyzed be unfixed and quickly frozen to prevent the modification of materials to be assayed (see Fig. 11). Normally this would pose serious problems for precise localization of molecules, but it was found that freeze dried cryostat sections of the mouse brain can be illuminated in such a way as to permit the accurate localization of relevant structures, including individual barrels [15]. From these, small samples can be dissected selectively for a variety of enzymatic analyses frequently of several materials from the same tissue samples (see Fig. 11). The normal levels of a number of enzymes have been determined in this way. In the first set of experiments levels of three different enzymes were measured in adult mice after division of the

infraorbital nerve. The levels of two mitochondrial enzymes fell within several weeks throughout the somatosensory pathway and paralleled changes in histochemical staining for other mitochondrial enzymes in similarly prepared animals (Figs. 11 & 12). Since all levels of the system show similar relative changes at similar time points we believe that the changes are most consistent with the altered activity hypothesis of Wong Riley although a trophic component to these changes cannot be ruled out [85].

Interestingly enough there is a recovery over longer survivals suggesting that the concentrations of these molecules will reequilibrate in spite of the continuing disconnection from the periphery (Fig. 12). In this regard the somatosensory pathway is different than the visual thalamus.

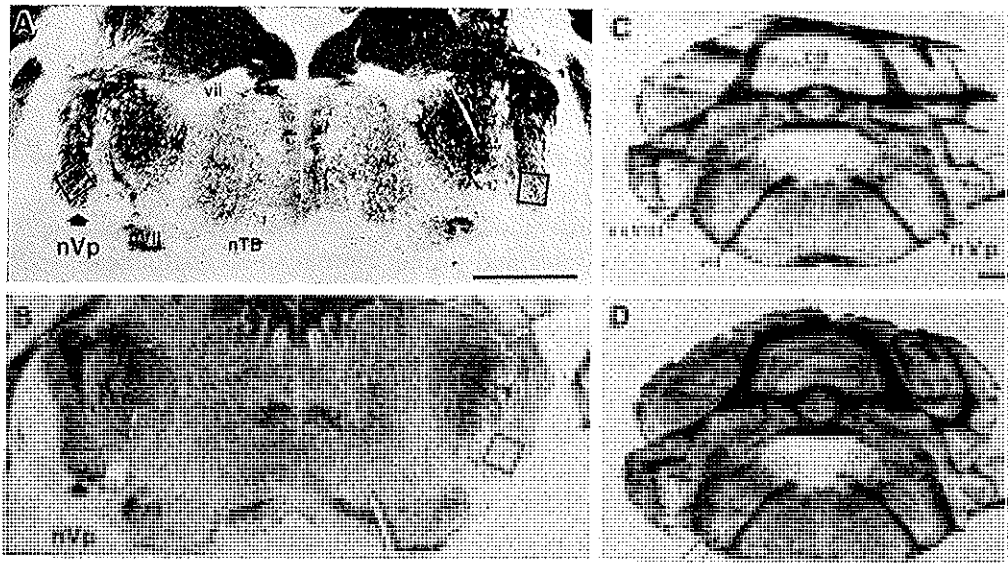

FIG. 11. Section through the principal nucleus of V in the mouse brain (nVp). A is a SDH (succinic dehydrogenase) stained section from an adult 6 weeks after division of the right infraorbital nerve. The stain in the right hand box is pale. B is CO (cytochrome oxidase) stained section from an adult 6 weeks after division of the right infraorbital nerve. The stain intensity is decreased in the box on the right. C & D are unstained cryostat sections through the pons of a mouse. Landmarks guide the removal of small tissue samples (arrows in D). vii = seventh nerve; nTb = nucleus of the trapezoid body; nvii = nucleus of seventh nerve; nVmot = motor nucleus of V; CB = cerebellum; nnVIII = auditory nuclei. Bars in A and C = 1mm. Even without stains it is possible to identify relevant structures and to dissect them under the microscope for microchemical analyses. (Data from ref. 96).

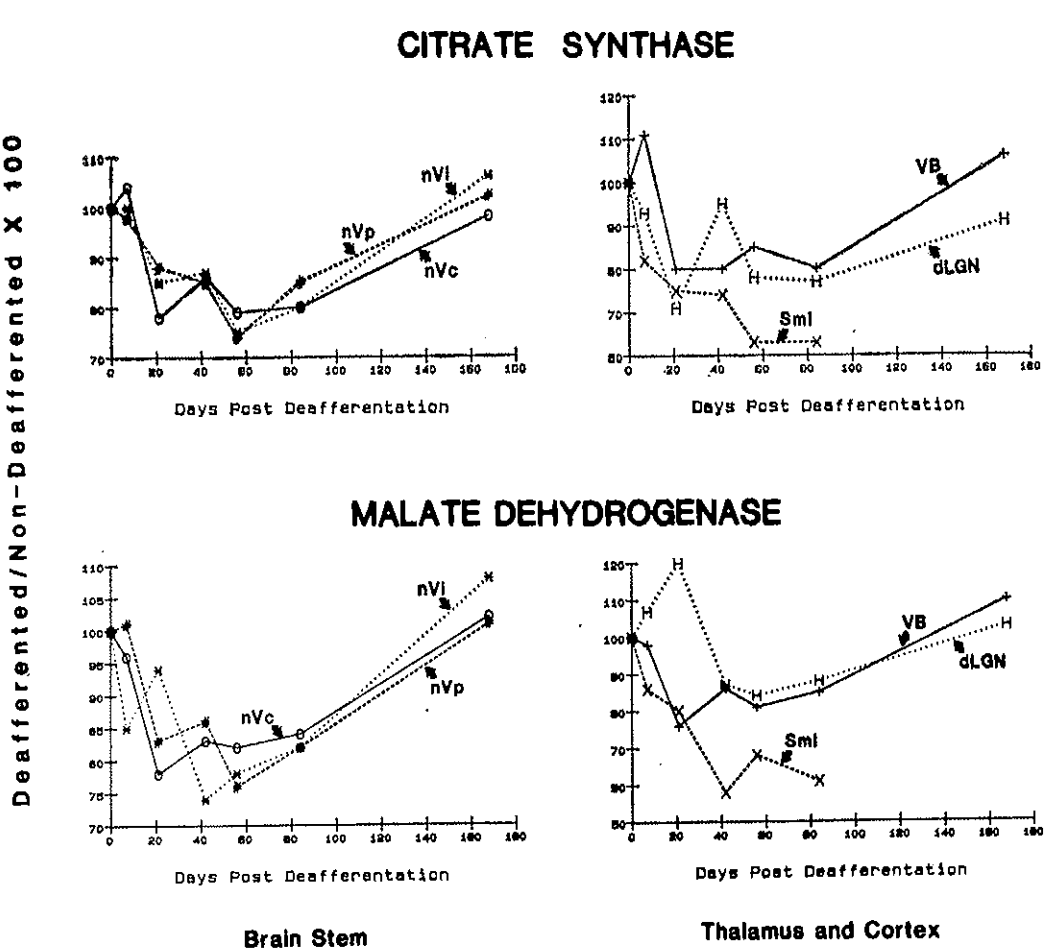

FIG. 12. Graphs showing biochemical changes in the brain demonstrated with microchemistry for mitochondrial enzymes. With deafferentation the levels of these enzymes fall throughout the pathway but do recover after half a year. There is a direct statistically significant correlation between the changes of these enzymes in the brainstem with each other and with photometric measurements taken from sections like those shown in Figure 11 A and B. (Data from ref. 96).

If the animals are enucleated the reduction in mitochondrial enzymes persists and the levels of activity of these appear to be modulated by other connections relaying in the nucleus some no doubt related to the remaining inputs from the intact eye [96].

That the biochemical modulation of the central nervous system in

deafferentation occurs is not surprising and is entirely consistent with a mechanism, either indirect or direct, which links the genome to the depolarization of the neurons integrated over considerable periods of time. With the exception of a slight increase which is statistically significant in the intact BTC of the deafferented animals when compared to normal unoperated controls — this is one of the major advantages of the quantitative histochemical approach — there are no differences in the intact somatosensory pathways in these animals [96]. This is in spite of behavioral observations that the animals will use the intact side of the face almost exclusively and certainly more than they would have normally to explore the world [74]. If the energy related enzymes could be regulated in response to increased metabolic demand as well as in deafferentation, one might, on a simple activity enzyme coupling model, have expected an increase throughout the intact pathway of the deafferented animals.

In a different experiment in which there was no deafferentation but a simple sensory deprivation by means of chronic whisker clipping a quite different result has been observed (Fig. 13). Rather than showing a decline in enzyme activities as might be expected from functional assays, the enzyme activities in the cerebral cortex associated with the clipped whiskers remain essentially normal. The levels in the intervening stations on the pathway have not yet been determined. In this case in contrast to the deafferentation experiments, the cortex associated with the intact whiskers shows a statistically significant increase in the levels of glycolytic enzymes. If the whiskers are allowed to grow back, thus reversing the deprivation, the enzyme levels return to control values [16]. Here the critical factor seems to be that both sides of the brain still receive afferent drive but the balance between this drive is distorted and the animal detects this in such a way as to increase the enzyme levels in the cortex associated with the intact whisker hairs (Fig. 13).

The phenomena described indicate that the adult nervous system is capable of remarkable metabolic plasticity in response to long term changes in neuronal activation. Not all neurons respond in this fashion in a simple way. Depending on the circumstances, presumably related to the use of existing axonal connections in different ways, this plasticity may be manifested by either a down or an up regulation of the relevant molecules. That these markers can be used to evaluate an aspect of function that in some instances is impossible by other functional methods, because the sensory inputs have been removed, is of more than passing interest. The quantitative correlation with histochemically demonstrable markers suggests

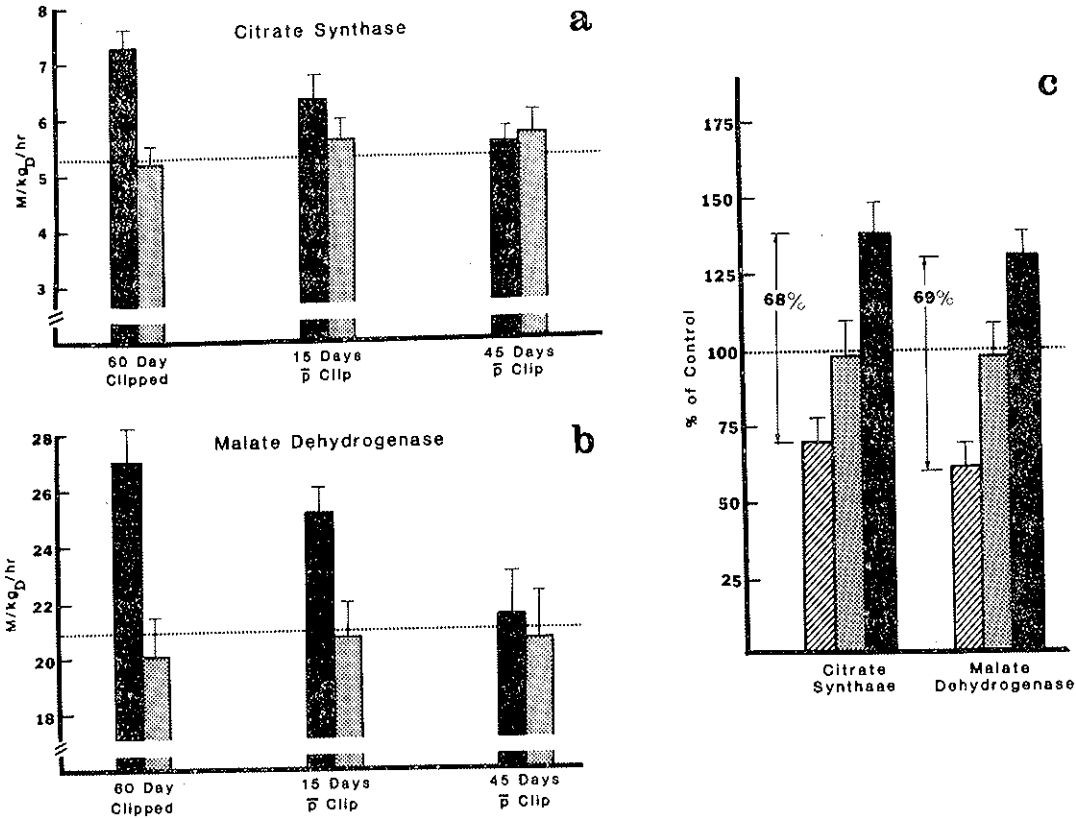

FIG. 13. Two mitochondrial enzymes 5 changes in the cortex when the whiskers have been clipped for one side of the face for 2 months. Stippled bars in a and b are from the cortex getting inputs from the clipped whiskers; solid bars are from the cortex for which the whiskers were not clipped. Increased enzyme levels are thought to be related to increased use of the intact whiskers. The enzyme levels return to normal after the whiskers grow back. c shows the extent of this metabolic plasticity. Cross hatched bars show levels after surgical deafferentation which depresses the levels of these enzymes nearly 70% with respect to their « increased stimulation » levels. For details see text. (Data from ref. 15).

that the approach could be used as a powerful adjunct to other methods of functional evaluation of the so-called plasticity of the adult nervous system.

Concluding Remarks

Studies of the organization, development and morphological, functional and biochemical plasticity of the rodent brain have been exceptionally instructive. The principal advantges are that the animals are altricial and have a well defined sensory periphery which is directly related to this periphery. The relationships greatly simplify the analysis and interpretation of experimental manipulations on the developing and mature nervous system.

Morphological studies indicate that remarkable changes in the pathway can be produced by manipulation of the sensory periphery. The changes appear to affect the pathway throughout but to differing degrees in a way that is consistent with the hypothesis that a signal from the periphery is being read by more central stations. The signal does not depend on the absolute numbers of input fibers nor does it necessarily depend on the peripheral specificity of those fibers or their bulk activity. It clearly requires direct contact of the nervous system with the periphery. The terminals of the relevant neurons clearly seek and find their targets. The manner in which they do so is inferred to be staged and competitive. These processes are reflected in the morphology of the relevant target cells.

Functional correlates of these fibers are relatively precise in terms of the part of the periphery to which they are related. There is surprisingly little in the way of functional plasticity that has been so far detected in the processing of information from the whiskers. Experiments now being done suggest that there may be a role of the intact periphery and sensory experience in the establishment of cortical function [41]. These studies have had to wait on the development of appropriate means for controlled stimulation of the sensory periphery [60]. Simple means of sensory deprivation have been available for some time [16]. Against a growing background of detailed intracortical connectivity, any morphological changes associated with functional changes, should they be demonstrated, should be clear. A paradox that remains is that the functional changes that are produced with deafferentation in adults seem more widespread than

those produced by similar manipulations in young animals [78]. The findings are consistent with the role of intact afferent inputs in providing inputs that are reasonably integrated by the brain and that, when these are removed in the adult, their modulable inputs are revealed. The inputs change with use presumably over existing pathways rather than over new pathways [44]. The developing nervous system on the other hand simply treats deafferentation as a deletion; the deletion does not lead to any really abnormal functional consequences [78].

That the chemistry of the adult nervous system can be dynamically and in some cases reversibly altered argues for the importance of ongoing functional activity in the maintenance of the "housekeeping" molecules. On one level this would seem to be a simple down or up regulation which is appropriate to the needs of the relevant neurons. It nonetheless indicates a remarkable degree of metabolic plasticity in the adult nervous system. The dramatic differences in the biochemistry of adult brains in which the animals have been deafferented as opposed to simple chronic and reversible sensory deprivation suggest that there are pathways which distinguish between an absent and an impaired sensory periphery in adults. The factors responsible first for the differences in the metabolic control and second for neuronal activity presumably in relation to the two different circumstances are now of great interest. They can with current technology be expected to be understood at the genomic level within the foreseeable future.

Acknowledgements

This paper summarizes work with many colleagues whose contributions have been substantial. I thank Ms. Margo Gross for secretarial assistance and Ms. Judy Decker for help with the illustrations. The work was supported by Grants from the National Institutes for Health (P01 NS17763), the McDonnell Center for Studies of Higher Brain Function and the McKnight Foundation.

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ORGANISMIC AND PHARMACOLOGICAL FACTORS INFLUENCING RECOVERY FROM BRAIN INJURIES

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INTRODUCTION

In this review several issues will be discussed that are of central importance to understanding brain organization and "plasticity". *First*, I review the question of whether there are intrinsic factors that permit the organism with brain or spinal cord injuries to recover from the damage. *Second*, I examine the question of whether neuroplasticity is limited to early stages of development or whether adaptation of the brain to injury is a phenomenon that also occurs in the mature organism. I will also review some of the literature on the pharmacology of recovery and discuss what can be done to promote or enhance recovery from severe brain injuries.

The last few years have seen an explosive growth of interest in, and research on, neuronal response to traumatic damage. Yet, only about a decade ago, those conducting research on recovery of function were often seen as hopelessly pursuing curious "anomalies" of behavior or aberrant neuronal activity that had no bearing on understanding nervous system organization or functions. Aside from a handful of individuals, the generally believed dictum that, "in the nervous system, nerve paths are fixed and immutable, everything may die, nothing may regenerate" [69], kept most neuroscientists from becoming involved in the study of recovery from brain injury. Why pursue an area of inquiry when there was no hope of finding any answers? Why look for growth and plasticity where none could occur?

The recent and growing concern about enhancing or promoting brain

plasticity is due to several factors. One factor which may not be of direct interest to the basic scientist, but which nonetheless plays an important role in shaping the direction of research, is economic and social pressures. The number of people who suffer from CNS injuries each year can be counted in the hundreds of thousands in just the United States alone. And these figures do not include the victims of stroke, Alzheimer's, or Parkinson's disease. Patients and their families have pressured policy-makers to encourage research in this area and scientists are attracted to sources of financial support for their work.

Second, once conclusively demonstrated, the phenomena of injury-induced synaptogenesis and recovery from brain injury, appeared to be intrinsically very interesting. What is the basis for aberrant growth in the developing, mature, or senescent brain? Are the principles of injury-induced growth similar to growth and differentiation of nervous system seen in early development? Are there specific manipulations or factors that can enhance and maintain "anomalous" neuronal growth? Is the new growth beneficial or detrimental to behavioral recovery?

Third, and finally, prevailing concepts of nervous system function had become too static. Tracing fixed pathways throughout the brain and spinal cord using classic, anatomical techniques or creating specific lesions and measuring the behavioral deficits, has not added very much to our understanding of brain function.

The concept of neuroplasticity — a cautionary note

The *concept* of neuroplasticity itself is one with important theoretical and practical implications; yet despite the frequent use of the term in the experimental literature, its meaning is often obscure, or it is taken for granted that the reader will know what is meant by the term without it being clearly defined. Indeed, there seems to he little overall consensus about what "plasticity" means, or the conditions under which it is likely to be observed in the central nervous system.

The following citation taken from the preface to a recent volume on "Central Nervous System Plasticity and Repair" [5], reflects how general the use of the term "plasticity" has become and how it is applied to a wide range of anatomical and physiological phenomena.

> "One of the most important problems of biomedical research is the understanding of the details of the mechanisms of nervous system *plasticity* and repair. The *plastic properties* may be expressed at

> the cellular and synaptic level... Chemical, physiological and neurobehavioral evidence shows that *neuronal plasticity* is governed by genetic information (intrinsic factors) and environmental influence (extrinsic factors) overlapping in different ways. This double type of regulation is crucial in the determination of neuronal plasticity, as it is well known that environmental tissue reaction and therapeutic treatment may contribute to recovery of functions, possibly accompanied by anatomical recovery (italics mine)".

The problem and confusion about the term "plasticity" comes from the fact that the word has multiple meanings and it can be applied to different phenomena and to different experimental manipulations made upon either the whole organism or its parts, such as the brain or spinal cord. For example, a neuroanatomist might refer to the "plasticity" of regenerating axons in response to injury in a distant part of the brain. A neurochemist might use "plasticity" to refer to alterations in neuronal membrane receptor binding sites. An electrophysiologist might use the same term but apply it to the modification of firing rates of visual system neurons that have been altered by enriched or deprived sensory experiences. Thus, the response of the cells and their eventual recovery following deprivation could be attributed to the "plasticity" of the neuronal population under study.

On a more global level, the term "plasticity" may be applied to the way in which an organism adapts to its environment and "learns" new ways of coping. For example, an infant who learns to recognize the face and odor of its mother can be said to be demonstrating "plasticity".

In his now classic book of 1949, Donald Hebb [36] described an example of this type of "plasticity" which is still accepted today. "Any frequently repeated, particular stimulation will lead to the slow development of a 'cell-assembly', a diffuse structure comprising cells in the cortex and diencephalon, capable of acting briefly as a closed system. A series of such events constitutes a 'phase sequence — the thought process'" (page xix [36]).

Thus "plasticity" is obviously a useful descriptive term, but it cannot stand on its own as an explanatory concept because, as we have seen, almost any change in the nervous system can be seen as an example or an illustration of the phenomenon. Although there are conceptual pitfalls to be avoided, "plasticity", as I will use the term, can have descriptive meaning and usefulness in a clinical and laboratory setting. I will limit my use of the word "plasticity" to those phenomena that can be, in whole or in

part, related to recovery from brain damage. However, it should be mentioned that some kinds of "plasticity" are maladaptive, such as when new nerve collaterals lead to motor spasticity or to inappropriate behaviors (The reader may want to see Finger and Stein [19] for a more thorough discussion of this issue).

As we proceed with this review of a rapidly changing and exciting field of research, it soon will become clear that there really is no single or monolithic explanation for functional recovery from CNS injuries. In the last 10 years, investigators have begun to realize: (a) that many different events other than the locus of injury can contribute to deficits following brain injury [75] and (b) that there are many physiological and environmental factors that contribute to functional recovery after brain injuries [19].

Plasticity and recovery of function after lesions in early life

Neuronal sparing and reorganization after fetal or neonatal brain damage

Hans-Lukas Teuber, a distinguished neuropsychologist, once stated: "If I'm going to have brain damage, I'd best have it early rather than late in life". Teuber's remark reflects a generally held conviction that brain lesions suffered during early development are less likely to result in permanent impairments than similar damage inflicted at maturity.

The notion that recovery of function after cerebral injury may be age-dependent, can be traced, in part, to the work of Margaret Kennard of Yale University [45]. She performed her experiments on monkeys and apes in the late 1930's and early 1940's. Kennard made bilateral lesions in the motor cortex areas 4 and 6 in monkeys that were 3 weeks to 11 months of age. She then examined them throughout their development with different measures used to evaluate motor and limb performance. Kennard found that the earlier the lesions were made, the greater the degree of functional recovery of the limbs contralateral to the lesions. However, some of the monkeys that showed initial sparing very early in life, began to show signs of spasticity as they grew older and they also evidenced mild deficits in purposive behaviors such as walking or grasping objects.

Even though the monkeys with early motor cortex lesions were not completely recovered, they were much better than the monkeys injured a

few months later in life. Thus, the animals with early lesions could walk and climb, feed themselves and grasp objects correctly. Such behaviors as these were never seen in the monkeys with damage inflicted when they were already juveniles.

Although Kennard thought that the recovery she observed was due to spontaneous reorganization of function (i.e. the intact areas of the brain changed their functions to "take over" those functions of the damaged areas), it is now generally thought that early onset of brain injury results in less impairments because during development, not every CNS structure has become committed to the mediation of specific behaviors. In other words, there is differential rate of maturation so that in the case of specific, anatomically defined structures, the region may be too immature to have a function "localized" in it.

In a novel set of experiments designed to test this hypothesis, Patricia Goldman [30] sought to determine how development and experience can alter a subject's response to brain damage. She created lesions in monkeys immediately after they were born and then compared their performance to monkeys given lesions in the same brain regions, but much later in life (that is, as juveniles several years of age). Fifty-day-old rhesus monkeys were given bilateral aspiration lesions of the dorsal frontal cortex. In adults and juveniles, when this part of the brain is injured bilaterally in a single operation, the subjects show severe impairments in their ability to learn spatial tasks [30, 31].

Goldman was able to demonstrate that animals operated upon at 50 days of age were virtually unimpaired on spatial learning when they were tested at one year of age. The results of this experiment, taken in context with the earlier findings of Kennard, would seem to suggest that Teuber's rather glib remark represents a certain fundamental principle of neuronal or cerebral plasticity; namely, that early lesions result in less behavioral deficits than similar damage inflicted later in life.

Because she was interested in a developmental analysis of the monkey's behavior, Goldman continued to test her animals at 6-month intervals until they were juveniles or adults. Her subsequent findings were surprising. She was able to demonstrate that the monkeys with bilateral, frontal cortex lesions who initially showed complete sparing of function, gradually deteriorated as the testing continued. In other words, the animals "grew into" the deficits that are typically associated with lesions of the frontal cortex in adults. Goldman interpreted her findings to suggest that the onset of the behavioral deficits appeared as the "need" for a

mature frontal cortex "committed" to the mediation of spatial behavior became manifest.

This view suggests that the functions of certain CNS structures may change over time and that damage which occurs in early life may be just as devastating as that which occurs at maturity. To test this hypothesis further, Goldman performed a second experiment in which she removed the orbitofrontal cortex of infant monkeys and then tested the animals as juveniles and later again, as adults. She found that initially, the monkeys with damaged orbitofrontal cortices were very impaired on the spatial tasks when compared to their intact and age-matched counterparts. But more importantly, these monkeys showed very significant deficits compared to the animals with dorsolateral frontal cortex lesions during the initial stages of testing. Again, Goldman tested her monkeys well into later life and found that the group with orbitofrontal lesions got significantly better as they matured. In fact, the deficits in spatial performance actually disappeared. Why this change? Goldman and her colleagues felt that the orbital region was *initially* committed to the mediation of spatial behaviors, but as other CNS structures matured, they came to replace the capacity of the orbitofrontal cortex to mediate spatial behavior. Again, this suggests that the functions of brain regions may not be "fixed" and that they can change as a consequence of the organism's developmental status.

This latter point is made even more dramatically in a study by Patricia Goldman and Thelma Galkin [31]. These investigators developed a procedure by which they could remove a fetal monkey from its mother's womb and perform intrauterine brain surgery upon it on days E104 or E106. The fetus was removed from the uterus and an opening was made in the calvarium to expose the cerebral surface. The dorsolateral frontal cortex was then removed by gentle, subpial aspiration. In this case the lesions were extended just anterior to the arcuate sulcus. Following the brain resection, the dura was closed and the fetus was returned *in utero*, and the membranes, muscles and fascia of the uterine wall were resutured. The uterus was then returned to the peritoneal cavity and the abdominal wall closed.

One hundred and fifty nine days after the surgery the fetuses were delivered by caesarian section. The animals were then periodically tested over a 2.5 year period to assess them for their cognitive capacities. Behavioral testing actually began at 12 months of age in the animal given bilateral lesions of the frontal cortex at day E106. This animal was tested

on a battery of 4 tests that were designed to evaluate visual discrimination and spatial abilities. The testing protocols were then repeated at 6-month intervals until the monkey was about 2.5 years of age.

Goldman and Galkin's data were dramatic. On all of the behavioral tests, the monkey "displayed no noticeable abnormality or neurological symptoms". Its nursing and feeding behavior was unremarkable *as was the development of sensorimotor agility and coordination.* This animal was also able to learn spatial tasks better than normal monkeys of the identical age.

The spatial learning and visual discrimination performance of the monkey given surgery at day E106 was then compared to other animals operated on at 50 days of age postnatally and also to monkeys given the same surgery at 1.5-2.0 years of age. The surgery at 50 days of age, did result in some sparing of function in comparison to the older animals, but these animals did not perform as well as the monkey who received surgery during its gestation. Thus, the older the animal at time of surgery, the less the likelihood of behavioral sparing following bilateral, single-stage surgery.

What might account for the dramatic instance of sparing seen in the monkey operated upon as a fetus? When the brain of this animal was carefully examined microscopically, several very interesting features were observed. First, as Figure 1 shows, the animal developed ectopic gyri and sulci *not seen* in unoperated animals of the same age. These new sulci may have been instrumental in mediating the restitution of function when the monkey was tested some 12 months after surgery. Even more important was the finding that the animal had a normal-appearing medial dorsal nucleus of the thalamus. That is, the number of neurons remaining intact in the thalamus after the lateral surface of the cortex was removed, was the same as in an unoperated control. This finding was particularly interesting because frontal cortex lesions in more mature animals normally cause extensive retrograde degeneration; the fibers from the medial dorsal thalamic nucleus die when their axons are destroyed by the cortical lesions.

Goldman and Galkin speculated that the behavioral sparing was due to the possibility that the thalamocortical neurons had not yet occupied their normal terminal positions in the frontal cortex. As a result, their terminal tips were not destroyed by the lesion. Therefore, these fibers were able to grow into other brain areas in the absence of their

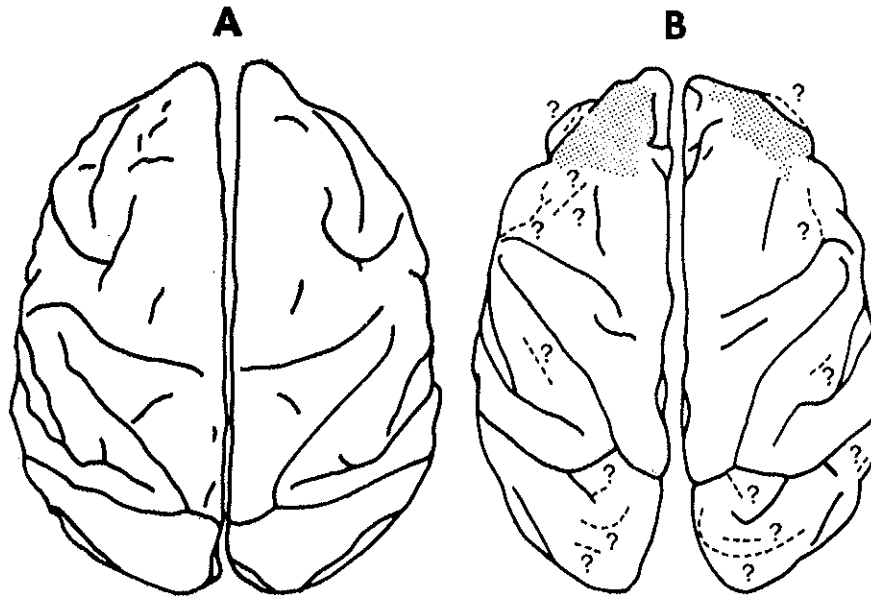


FIG. 1. A. Dorsal view of a monkey brain 2.5 yrs. of age, non-operated. — B. Dorsal cartoon of a monkey brain operated on fetal day 106. The stippled areas show the extent of the frontal cortex removals. The dotted lines and question marks show the location of abnormal and ectopic culci found in the brain of this animal (redrawn from [31]).

appropriate targets (see recent review by Cowan, *et al.* [12] for detailed discussion).

One physiological explanation for the behavioral recovery might also be that neurons that would otherwise "die back" as development proceeds, remain intact and grow into adjacent areas of the brain that are still capable of mediating the functions lost by the cortical lesions [12, 13]. This is important because we now know that we are born with a greater complement of neurons and their processes (dendrites and axons) than are required for "normal" functioning. As maturation proceeds, "unneeded" neurons "die off". Thus, if injury occurs early in life and reduces the neuronal population, the "excess" would no longer be present and the signal to "die back" would not occur. These remaining neurons would then quite possibly be used to "take over" the functions of those cells that were lost as a result of damage.

Many of tbe articles in this volume support this notion. In one specific experiment designed to test this view, Land and Lund [47] took

one-day-old rat pups and injected one of their eyes with horseradish peroxidase (HRP). By injecting the HRP into the eye, the researchers were able to trace the axons into the superior colliculus. They then repeated the same experiment with adult rats and compared their results to those obtained from neonatal animals. In the one-day old pups the projections from the superior colliculus to the ipsilateral retina, were deemed to be heavy. In contrast, the rats injected with HRP as adults had a much more restricted HRP labelling.

By examining rats of different ages with this technique, Land and Lund were able to show that the projection from retina to colliculus shrinks, and by postnatal day 10, the adult pattern of innervation is established. To go one step further, these workers showed that *if one of the rat's eyes were removed at birth, the retraction process was completely blocked.*

Here, then, we have an interesting example of injury-induced plasticity that could account for some of the sparing or recovery of function seen in young victims of brain injury. An injury in one part of the brain prevents the naturally occurring "dying back" of neurons that would normally be lost as development proceeds. Now, when behavioral sparing is seen in infant subjects after brain injury, we must give consideration to the possibility that the "plasticity" may be due to the preservation of pathways that would normally die in the intact subject.

There is also evidence that true neuronal regeneration may occur after brain injury in neonatal mammals. In one series of experiments, Kalil and Reh [41] examined the effects of cutting the pyramidal tracts in adult and infant hamsters with surgery beginning 2-20 days after birth. The pyramidal tract lies on the ventral surface of the hamster's brain (making it relatively easy to cut) and its fibers only descend to terminal sites in the brainstem and spinal cord.

At three months of age, the hamsters were injected with tritiated proline into the left sensorimotor cortex ipsilateral to the lesion so that fibers could be traced to the brainstem and spinal cord. Kalil and Reh first showed that, at no age, did labelled axons grow through the lesion site. This demonstrates that the pyramidal tract was completely severed and that axons did not regenerate across the cut. However, there *was* massive new growth emanating from the severed axons beginning about 1-3 mm rostral to the cut. Most of the regenerating fibers crossed to the opposite side of the brain and descended for a distance of up to 7 mm into the cervical spinal cord.

The pathway followed by the regenerating axons was "completely abnormal" (in comparison to normal hamsters), but their pattern of termination in the dorsal column nuclei and cervical spinal cord was normal. By injecting the cortex with radioactive tracers at 2-day intervals, Kalil and Reh were able to estimate the rate of regeneration at about 1 mm/day. The extent of regeneration was age-dependent and it declined markedly if surgery was performed during the first week of postnatal life. By 20 days of age, there was still evidence of regeneration, but it was much more limited.

By then injecting tracers into the sensorimotor cortex contralateral to the lesion, the authors were able to show that there was also some anomalous "sprouting" from the intact side of the brain over to the damaged side (or possibly sparing of fibers that would normally have died back). Adult hamsters with pyramidal lesions show impairments of digital functions, (i.e. use of forepaws) from which they never recover, but the same lesions created in neonates did not lead to any forepaw deficits, suggesting that the axonal regeneration led to functional recovery.

These data, taken in conjunction with Goldman's work [30, 31], indicate that early brain damage may very well have different consequences than when the same types of injuries occur in more mature subjects. The processes which mediate recovery (or delayed appearance of functional deficits) may reflect actual, age-dependent regeneration of damaged axons or injury-induced sparing of neurons that would normally "die off" as maturation proceeds.

The frontal cortex is not the only part of the brain that seems to adapt to early injuries. Sparing or sprouting of neural pathways in the visual system may account for the behavioral recovery from early visual cortex damage in cats that has been carefully studied hy Peter Spear and his colleagues [79]. These investigators removed visual cortex areas 17, 18 and 19 in newborn kittens and compared the animals to those who had received damage to the same visual areas as adults. Behaviorally, cats with neonatal lesions of these areas can learn simple brightness and photic frequency discrimination that adult cats with similar lesions are incapable of doing. Such cats can also learn two-choice form and pattern discriminations, but those with brain damage inflicted in adulthood cannot.

In the neonates, lesions of areas 17, 18 and 19 result in a very suhstantial, transneuronal degeneration of the retinal ganglion cells of the X-cell layer which is much *more* severe than when similar damage is created in adult cats. Unlike adults, however, there is a marked increase in the

number of anomalous projections to the surviving neurons in the lateral geniculate nucleus (LGN). The LGN cells show electrophysiological responses to visual stimulation that are quite similar to those of normal neurons in intact animals. In turn, surviving cells in the LGN show a very significant (a 10-fold increase) anomalous projection into the postero-median lateral suprasylvian gyrus (PMLS); a projection which is never seen after lesions of areas 17, 18 and 19 in adult cats.

Spear was later able to demonstrate [78], that PMLS neurons receiving the anomalous inputs had normal receptive field properties and binocularity when lesions of visual cortex were created after the first day of birth. In marked contrast, cats with visual cortex lesions as adults showed none of these receptive field characteristics when recordings were taken in the PMLS region. Spear suggests that the anomalous inputs to the PMLS enables those cells to develop receptive field characteristics that are equivalent to those normally reserved for the visual cortex. This "plasticity" of function, in which a portion of cortex adjacent to the zone of injury takes over the functions of the damaged zone, appears to be limited to the early stages of development. As Spear states: "the *visual system* is very plastic neonatally. Pathways between structures can be enhanced and, in at least two cases, this enhancement is due to an active invasion of excessive inputs. Furthermore, these anomalous inputs can form synaptic connections that are of physiological significance" (page 240). Despite the neuronal plasticity and better recovery seen after early lesions, it is important to note that vision is *not* completely normal.

The question of just how much behavior actually recovers after early lesions is still under much debate (but see [3, 20] for excellent reviews of much of this important literature). This is particularly the case when one attempts to examine plasticity of function in children with brain damage and this is discussed in the following section.

Sparing and function after early brain injury in children

Some authors have argued that in humans, brain lesions occurring early in life are also more likely to lead to sparing of function than when the injury occurs later in development. Such sparing occurs in particular with respect to the preservation of linguistic capacity after damage to the left, or supposedly dominant hemisphere of the brain. Most of us would generally agree that adults suffering left hemisphere, anterior cortical injuries, are very likely to develop varying degrees of aphasia and related

language disorders that are dependent upon the locus and the extent of the injury. In contrast, children with left hemisphere injuries in the same general areas as adults, usually develop speech as well as the ability to understand spoken language. The sparing of function is maximal when the injury occurs *before two years of age*. The older the child is at the time of the injury, the less the sparing of function that is observed. By the time the child is 12 years of age or older, the adult pattern of language disorders begins to emerge.

What might account for these differences in recovery and "plasticity" between the young and adult central nervous system? We have already discussed the possibility that there may be more neuronal "sprouting" or preservation of neurons after injury to the immature brain. Another hypothesis to account for the sparing of language functions in young subjects has been offered by the Canadian neuropsychologist, Brenda Milner [54]. Milner suggests that lateralization of cortical functions (i.e., left hemisphere dominance for language) develops as a part of the maturation process. In other words, at birth, *both* hemispheres are capable of mediating language (but perhaps not to the same extent). Milner's hypothesis is that as development progresses, the left hemisphere would come to inhibit the language mechanisms of the right hemisphere. If a child suffers a lesion of the left hemisphere at birth or before two years of age, for example, the right hemisphere is "disinhibited" and can mediate language capacity. There is some evidence for this view. When sodium amytal is injected into the right carotid artery, language functions are disturbed in children who have had injuries to the left side of the brain. There is also some evidence that damage to the right side of the brain results in a greater risk for temporary language disturbances in children than in adults.

Despite the better recovery of language capacity seen in children with left hemisphere brain injuries, there apparently is a price exacted for the "plasticity". Milner gave extensive batteries of cognitive tests to patients who had early brain injuries, and she found that although they *did* show considerable sparing of language, the functions of both the right and left hemispheres were depressed. In other words, there was a "blunting" of verbal and nonverbal IQ scores in these patients as they grew older. Milner speculated that the right hemisphere suffers from a "crowding" of too many functions into the remaining, intact hemisphere. Thus, not only does structure/region/hemisphere "A" have to mediate its normal behavioral functions, but it must now handle the functions of area "B" lost to the injury. Milner's observations can be compared to the experiments of Patricia

Goldman discussed earlier. Remember that she found that her monkeys with lateral frontal cortex lesions, were initially spared on spatial tasks, but later "grew into" a severe spatial performance deficit as they matured.

There are also other studies on humans which bear directly upon this question. Eric Lenneberg [49] has cited interesting cases of growing into a deficit following damage to the parietal lobes in very young children. These lesions will retard the growth of the long bones of the contralateral limbs. A parietal lesion in adults will very often result in hemiplegia, but when the lesion occurs in infancy, there is no measurable effect for at least 3 months — *and often for much longer*. At first, then, all four of the patient's limbs move normally and symmetrically and *growth is entirely unaffected*. It seems that up until about 3 months of age, growth of muscle and motor control is mediated by brainstem and thalamic structures. Only as the cortical centers (parietal region, for example) begin to exert an influence on behavior, do the abnormal signs begin to emerge. Since the infant is moving normally during its first three months, the subsequent abnormalities are not caused by disuse atrophy.

Initially the clinical signs are hardly observable, but they do become worse and worse as the child learns to walk. Not only is there retarded bone development, but signs of spasticity and abnormal reflexes begin to emerge. As Lenneberg states: "One may say that the child with perinatal cerebral injury only gradually 'grows into his symptoms', and that both lesions and symptoms have their own ramified consequences, *often affecting distant structure years after the primary injury*".

Goldman's data taken from neonatal monkeys and Lenneberg's case histories on describing the long-term effects of brain lesions in children emphasize an important theoretical point that should be considered in assessing the outcome of early brain injuries. The idea is that as development proceeds, the functions associated with specific brain areas may change. For example, in one recent review, Isaacson and Spear [38], point out that the long-term effects of early brain damage (in human children or in animals) are very hard to evaluate because "the mental and behavioral changes may not bear resemblance to the effects of comparable lesions later in life" (page 92). Thus, primary lesion effects *and* secondary effects (e.g. changes in vascularity, alterations in levels of neurotransmitters and/or corticosterones) will have different consequences than when the same changes occur in the mature CNS.

Isaacson and Spear also argue that comparisons of an organism's performance at different stages of development are not justified because

the brain is "specialized" to do different things at different ages. Taking this one step further, they suggest that different measures of behavior at each stage of development are required to assess the long-term effects of brain injury.

The issue of whether sparing of behavioral function is more likely to occur after infant lesions, seems to depend, at least to some extent, on the individual investigator's commitment to the doctrine of strict cerebral localization. For instance Hecaen, Perenin and Jeannerod [37] tested 56 children with cortical lesions on the St. Anne's Hospital neuropsychological Battery. These workers found mutism and aphasia in almost all of the children tested under 10 years of age. Nonetheless, almost all of the children showed considerable improvement or complete recovery (depending upon the extent of the damage) of speech, but persistent deficits in writing or oral expression at least for the periods of time during which they were tested. Based on these findings, Hecaen *et al.* conclude that "there exists a region of brain tissue specially evolved for the representation of language". Hecaen *et al.* see the localization and lateralization of language as an "invariant" characteristic of nervous system organization and would then have difficulty accounting for cases of recovery or sparing when these specific regions are lost.

From a similar perspective, Fletcher, Levin and Landry [22] recently argued that when the absence of gross aphasia or other cognitive disturbance is observed after left hemispherectomy in children, it is because investigators typically do not perform a detailed follow-up examination designed to factor out subtle disturbances in speech or language (e.g. deficits in reception or expression of language or deficits in visuospatial perception). These critics attack the sensitivity of the tests used to measure behavior after brain injury and unless impairments are found at some point in testing, they argue that the test is insensitive to the deficits. From this perspective, virtually no demonstration or evidence for recovery would ever be accepted because it can always be claimed that the "underlying deficit" is never revealed. In this context, any form of therapy leading to improved behavioral performance could be seen as simply "masking" the permanent impairments. This orientation may come from the fact that in the clinic, neuropsychologists often see children with very diffuse damage to the brain caused by intraventricular hemmorage, hydrocephalus, anoxia, malnutrition or complications associated with very low birthweights. When damage is very diffuse, it is difficult to make direct comparisons to those cases (especially in the animal

literature) of highly focalized injuries such as those produced in the laboratory. With diffuse cerebral injuries it is not surprising to find that verbal and motor skills are likely to be impaired in later life, probably to some extent because of reduced sensory interactions with the environment during most of the child's development.

The fact is, that even after *complete hemispherectomy early in life*, the capacity for normal language may be totally spared in some individuals. For example, in one report by Aaron Smith of the University of Michigan [76], he examined a 29-year-old man who had a total left hemispherectomy at the age of 5-1/2 years. This patient had been followed and examined repeatedly for more than 20 years. The man was a successful industrial executive who had completed his studies for a graduate degree and was continuing to do work in library science. Smith's patient had a verbal IQ score of 126 and above average scores on performance IQ as well. Other cases of similar dramatic sparing or recovery from extensive damage have also been reported [77].

While recovery of function following lesions in very young subjects appears to be much more likely than when damage occurs in adults, there is some evidence that the period of "plasticity" may extend into the second decade of life. Teuber [84] carried our a long-term follow-up of American Korean War veterans who were examined on speech performance, motor functions and visual field integrity among other measures. Teuber's patients were separated into groups who had received their injury between 17-20 years of age and 26+ years of age. The follow-up examination was made some 20 years after these patients were initially diagnosed. Teuber's data show that some sparing from the effects of brain wounds even occurs in patients 17 to 20 years of age at the time of their injury. The percentage of patients showing improvement in motor performance was over 58% in the 17-20 year-old group, 41% in the 21-25 year-old group and about 26% in the 26+ group.

In summary, the experimental and clinical data show that, under certain conditions, young organisms are capable of greater behavioral sparing from brain injury than their older counterparts, although the specific mechanisms and parameters that control the recovery have yet to be defined with any certainty. It does seem clear that injury-induced neuronal growth may occur more easily in the developing brain or that there is more "preservation" and rerouting of neuronal elements than is seen in the adult CNS.

In humans, the issue of whether early brain damage results in more

behavioral "plasticity", is much more controversial. In the last part of this decade, the general consensus seems to be that there are many environmental, biochemical and anatomical factors that play a role in determining the outcome of early hrain damage but few, if any of them, are well understood. In addition, both researchers and clinicians have not been able to agree on the extent to which recovery following early brain injury does occur, nor have they been able to agree on the most appropriate tests to measure the recovery (see [76], for discussion).

Despite the inability to reconcile the disparate findings, interpretations of test data and theoretical views, there are still very interesting avenues of research to pursue. Whether or not deficits occur or persist after early brain damage, there is *still* the question of whether it might be possible to facilitate recovery by appropriate intervention. This is a field of inquiry that is only now just beginning to receive some attention. In a later section of this chapter, I will present some recent findings that bear directly on this issue.

Although the prognosis for the adult brain-damaged subject is usually more guarded, there is now a considerable body of evidence to indicate that earlier views of central nervous system "stability" in adults may have been inaccurate. The view that, with maturity, the brain becomes "fixed" and highly localized in its functions has prevented many clinicians from considering the possibility that recovery of function is, indeed, quite possible in the adult subject. In this next section, I discuss some of the variables that influence and modify the plasticity of adult, mammalian brain.

Plasticity and recovery of function in the mature organism

Although the idea that neuronal remodeling after injury to brain structures is not new (it was postulated by the German biologist, Exner in 1885), almost fifty years would pass before substantial attempts would be made to study the process and relate it to functional recovery. In 1941, for example, Weddell [89] and his colleagues damaged the cutaneous afferent nerves in fully mature rabbits and showed that "sprouts" from intact nerve fibers invaded the deafferented regions of the spinal cord. Along with the reafferentation, they observed increased restoration of tactile sensations. Although this was quite interesting, the first significant work demonstrating that sprouting could occur *within the central nervous system itself* did not appear until 1958 with Liu and Chambers of the

University of Pennsylvania [52]. These investigators cut some of the dorsal root ganglia on one side of the spinal cord in adult cats. They then waited several months to permit removal of the axonal debris that normally follows such injury and then they cut a single, intervening or adjacent dorsal root and its matching root on the opposite side of the cord. They then used another group of cats to denervate the spinal cord partially by unilateral section of the corticospinal tract. They again waited for absorption of degenerating axons and then sectioned a pair of dorsal roots. The cats were killed 4 days later and prepared for histological examination. When they examined the spinal cords of the experimental animals, Liu and Chambers found that the degeneration products were heavier on the side of the cord that was initially denervated. They interpreted this finding to indicate that the remaining nerve fibers proliferated their terminals as a response to the deafferentation caused by the sectioning of the dorsal roots. Thus, they thought that the remaining, intact, neurons had formed new projections to replace those lost by the initial injury. Although these results were quite exciting, there was still no conclusive proof that a similar phenomenon could occur in the brain itself.

Some of the first, solid, evidence for anomalous growth in the brain came from the experiments of Geoffrey Raisman [67]. Raisman selected the septal nucleus of the adult rat for his studies because this structure receives afferents from two distinctly different parts of the brain. Thus, there are fibers that originate in the hippocampus and arrive in the septum by way of the fimbria. Fibers whose cell bodies originate in the hypothalamus also arrive in the septum via the preoptic part of the medial forebrain bundle.

Raisman's strategy was to cut off one set of afferents to the septum and then examine the response of the other afferent system to the denervation.

Based on his synaptic classification scheme, and using stains selective for degenerating neurons and their terminals, Raisman was able to show that in normal, adult rats, axons from the hippocampus have their terminals primarily upon the dendrites of the septal neurons. Once he had established the normal projection patterns of afferents to the septum, Raisman was ready to examine the question of what changes occur in this pattern after injury to one of the systems projecting to the septum.

First, he created a lesion in one of the pathways and then waited several months for axonal debris to be absorbed. Next, he created a *second lesion* in the *remaining*, intact pathway (for example, if the fimbria carry-

ing fibers from the hippocampus was damaged first, he would then make the second lesion in the medial forebrain bundle carrying fibers from the hypothalamus).

Since Raisman knew the exact pattern of the septal afferents from previous experiments, this second lesion, followed by the same, selective stains for degenerating fibers and their terminals, would show whether or not the system that had been spared at the time of the initial surgery had sprouted or proliferated new terminals to replace those that had been lost earlier. Indeed, Raisman's findings conclusively demonstrated that even in the adult brain, there is a clear indication of anomalous growth (but not regeneration). He found that when the fimbria was damaged in the first operation, fibers from the hypothalamus sprouted new endings which then established new synapses on the dendritic sites evacuated by the dying hippocampal neurons. And when the hypothalamic pathways were damaged first, the hippocampal fibers sprouted new terminals which established their synapses on the cell soma of the septal neurons. The initial lesion thus precipitated a major reorganization of the synaptic inputs to the septum.

Other workers repeated these experiments, but used histochemical fluorescent markers to determine whether the newly sprouted fibers were of same or different neurotransmitter class [55]. The fibers coming from the hippocampus to the septum are cholinergic while those arriving from the hypothalamus are noradrenergic. Thus, when one system "sprouts" and grows into a deafferented zone, the neurotransmitters may not be the same as in the "normal" condition. When changes like this do occur, it is important to ask whether the re-established contacts may, indeed, be functional. In other words, can recovery of function be mediated by a "replacement system" that uses an entirely different neurotransmitter than is found in the intact CNS?

There are even cases reported where neuronal rearrangements arise from fiber systems outside of the damaged brain. These fibers are thus capable of growing over long distances *in the adult subject* and establishing contacts where none may have existed before. For example, it has been found that fibers from the superior cervical ganglia of the sympathetic nervous system grow into the hippocampus from about 2 weeks to a month after the anterior hippocampus has been damaged. The newly sprouted fibers, when treated for histofluorescence, show all of the characteristics of catecholamine fibers. Such fibers are *not seen in the normal hippocampus*. The sprouting of these peripheral fibers into the brain

appears to be due to a signal that is given off by the damaged hippocampus although the granule cells of this structure must be intact for this response to occur. Recently, Turner *et al.* [87] reported that they were able to isolate a growth-promoting factor specific to the hippocampus. Thus, only neurons derived from hippocampal tissue would remain viable when maintained in a cell-culture medium containing the neurotrophic substance derived from the hippocampus itself.

This growth stimulation "at a distance" is very puzzling because it would certainly seem more logical for neighboring fibers to sprout into the deafferented areas, *especially if the neighboring fibers contain the same neurotransmitter*! The adaptive significance of this long distance, "heterotypic" sprouting is not well understood. In fact, some workers have argued that heterotypic reinnervation (i.e., reinnervation with a different neurotransmitter) may in fact cause maladaptive behaviors such as spasticity or other inappropriate responses.

One interesting possibility is tbat the "targets" of reinnervation, i.e. the postsynaptic sites, could influence the kinds of neurotransmitters released by the anomalous, new terminals. In other words, could the environment of the new terminal determine what type of neurotransmitter the nerve cell will subsequently manufacture and release? While this idea may seem far-fetched, there *is* evidence that, during development, "transmitter choice" can be modified. In one study Furshpan *et al.* [26] cultured ganglion cells in the presence of heart cells and then examined their transmitter release and their electrophysiology. These investigators showed that the neurons would "switch from adrenergic to cholinergic transmission depending upon the fluid medium that was provided by the heart cells".

There is also evidence to suggest that, during normal development, the choice of which transmitter can be released from a neuron terminal, is influenced by the presence of glucocorticoid hormones [16].

In the adult organism with brain damage, the processes stimulating anomalous growth may be similar to those seen during normal development and maturation (e.g. the injury-induced release of neurotrophic factors, cell adhesion molecules and glucocorticosteroids). Thus, the extent to which adaptive, functional recovery occurs could be related to the degree to which the environment of the anomalous axons can change the type of neurotransmitter released (this would assume that the "output" activity of the reafferented zone would be more like the normal if the appropriate neurotransmitter were being released by the presynaptic inputs).

More recently Carl Cotman and Manuel Nieto-Sampedro [9] have argued that synaptic reorganization following brain injury follows very specific principles, despite the fact that such growth can be elicited by different stimuli. According to these investigators, the most direct way to induce synapse turnover is by direct damage to the central nervous system and provided that a sufficient number of afferents are removed to elicit the synaptic response. As Cotman and his students have shown, a unilateral ablation of the entorhinal cortex (the structure which provides the major extrinsic input to the hippocampus) will cause the loss of almost 60% of the total number of synapses in the dentate gyrus of the hippocampus. *This lost input can be totally replaced within three days after the lesion.* In this case, the proliferation of new terminals comes from crossed fimbria fibers whose cell bodies originated in the *contralateral entorhinal cortex.*

The new fibers are electrophysiologically active and may mediate the same functions as those fibers that were lost by the injury. According to Cotman and Nieto-Sampedro, the synaptic sprouting follows three general rules. First, the new synaptic sprouts restore the lost inputs caused by the original entorhinal lesion. Second, the afferents will reinnervate a denervated zone only if the terminal fields of the two systems overlap. Third, the reactive growth *only causes a quantitative increase of previously existing connections. Qualitatively new pathways are not created during lesion induced synaptogenesis in the adult organism.* Obviously, this view is not shared by those who have demonstrated heterotypic reinnervation as described above.

There is now increasing evidence to show that there may actually be more capacity of the CNS to show injury-induced axonal growth than previously thought possible. While many researchers are now prepared to accept the fact that intact fibers can enlarge their terminal fields to replace synapses that have been lost, there is less agreement about "true" axonal regeneration or growth over relatively long distances. Typically, after a CNS injury, scar tissue forms and the growth of transected axons is quickly aborted only a short distance from their damaged tips. However, in a recent series of studies Aguayo [2] has shown that such aborted growth need not be the case and that such stunting is due to "environmental factors surrounding the regenerating neurons".

To test this hypothesis Aguayo and his colleagues excised pieces of sciatic nerve and then transplanted one end of this tissue into the brain parenchyma of adult rodents. The axons within the peripheral nerve

sheath degenerate rapidly, but the Schwann cells remain in appropriate alignment along the length of the transplanted nerve shaft. Thus one end of the nerve shaft is inserted into the brain (e.g. medulla oblongata), while the other end is inserted caudally into the transected spinal cord to form a neural "bridge".

Aguayo then used the enzyme, horseradish peroxidase (HRP) to label neurons on either side of the transected cord. Such transport could only occur if axons grew from the medulla through the sciatic nerve sheath bridge and into the caudal portion of the spinal cord. By taking careful measurements, Aguayo was able to show that CNS neurons are capable of growing from 3-5 cm, thus "approximating those long projection fibers in the brain and spinal cord". Aguayo's studies are important because they show that the growth of axons is actively inhibited, probably by glial reactions or the release of growth suppressing substances in the brain itself.

Nonetheless, there are "other" environmental conditions which can be manipulated to enhance neuronal growth very significantly. As Aguayo stated: "The marked regrowth of central axons following the substitution of the central environment by that of peripheral nerves indicates that the limited growth of CNS axons is not necessarily the result of a primary inability of the differentiated mammalian neuron". At the present time, Aguayo has not yet reported that the extended fibers are capable of mediating behavioral recovery, but this could well be the next step in a program of research concerned with functional restitution.

In fact, previously long-held convictions about the "stability" of the adult Central Nervous System are rapidly changing. Many investigators are now more willing to consider the possibility that synapse loss and replacement may occur in the intact mammalian brain as a part of the normal life process. We now know that natural renewal of synapses in the adult CNS can occur in at least several brain areas (see Cotman and Nieto-Sampedro [9], for a detailed review). For example, in the olfactory system, bipolar neurons as well as synapses in the olfactory bulb undergo a cycle of death and replacement every 10 to 20 days. It has been suggested that such turnover may he a part of the process that "repairs small damage caused by 'wear and tear' of nerve endings, in the adaptation of the brain programs to the current state of the body as reported by peripheral feedback, and perhaps as one of the cellular correlates of learning and experience" (page 389 [7]).

Just a few years ago it would have been inconceivable to think that

neurogenesis might occur in the adult mammalian brain, but there is now new evidence to suggest that it might be so. No doubt, the suggestion of neuronal turnover will be highly debated because of its controversial implications for concepts of cerebral organization, but such findings are consistent with the fact of synaptic remodeling and turnover in the brain; notions that would also have been ridiculed a decade or so ago.

Is Neurogenesis Possible in the Adult Central Nervous System?

Another general dictum that has shaped research in neurobiology for many years is that neurons in the central nervous system of warm-blooded animals are only generated during prenatal, or very early in postnatal, development. While this is probably true for the vast majority of neurons, there is now exciting new evidence that neurogenesis may also be occurring in warm-blooded, adult animals.

Some of the most creative and interesting research in this area comes from the laboratory of Fernando Nottebohm at Rockefeller University, who did pioneering work in mapping out the circuitry of song in the canary [58, 59]. The brain nuclei which appear to be critical for the organization of normal singing are the nucleus hyperstriatum ventrale, pars caudalis (HVc) and the nucleus robustus of the archistriatum (RA), in the left hemisphere of the male. Unilateral damage to these structures will markedly alter singing, with injury to the left hemisphere having more profound effects than on the right.

Based on these anatomical and behavioral studies, Nottebohm was able to establish two very interesting and convergent lines of data. First, he and his group knew that canary singing is seasonal, with increasing song activity in Winter and Spring, a fall-off in Summer, and then development of a new repertoire in the Fall. Based on this observation, the Rockefeller group decided to perform anatomical evaluations on the HVc and RA during the Spring and 5 months later, in September when singing was markedly reduced [59]. The investigators found that the "song control nuclei" were measurably smaller than they were in the Spring (as indicated by measuring the length and branching of dendrites and axons in the HVc and RA). Nottebohm speculates that, under the control of seasonal, hormonal influences, there is growing and shedding of synapses in the adult organism. Since the changes in CNS circuitry are seasonal, this growth and retraction of nervous tissue can be said to be under environmental control.

A seemingly chance finding led Nottebohm and his students to an even more dramatic discovery of neuronal "plasticity" in the adult organism. Goldman and Nottebohm [32] injected adult canaries with tritiated thymidine in order to determine if neuronal proliferation was occurring in the HVc of adult canaries. With each day of the thymidine treatment, about 1 percent of the identified cells in HVc were labelled. The newly proliferated neurons appeared to migrate into the HVc from the ventricular zone next to the nucleus.

One very important question to ask is whether the newly formed neurons are integrated into the functional circuitry of the HVc. To provide an answer, Paton and Nottebohm [62] waited about 30 days until after tritiated thymidine was injected into adult male canaries and then used microelectrodes to record intracellular potentials from neurons in the HVc. At the end of the recording the microelectrodes were used to deliver horseradish peroxidase iontophoretically into the neurons. Of the several dozen neurons which had responded to auditory stimulation, seven were found to be labelled with the tritiated thymidine (thymidine is only taken up into the DNA of newly forming cells). Of the seven that were labelled, four had responded to the auditory stimulation. These data were taken to indicate that neurogenesis can also occur, and under limited conditions, such neurons can play a functional role in the warm-blooded, adult vertebrate.

Neurogenesis has also been reported in mature rats by Kaplan and his colleagues at the University of New Mexico. For example, in one study Michael Kaplan and James Hinds [42], injected mature rats (3 months of age) intraperitoneally with ^{3}H-thymidine and allowed them to survive for 30 days. The brain tissue of the rats was then processed for autoradiographic analyses. Neurons were considered labelled if they contained 5 grains over the nucleus of the cell — a level that Kaplan claims is considerably above background. According to these criteria Kaplan and Hinds found labelled neurons in the olfactory bulb and in the dentate gyrus of the hippocampus (an area, it will be remembered, that is very active with respect to synaptic remodelling). With the aid of the electron microscope, the researchers were able to note that the labelled cells did *not resemble neuroglial cells* and instead, meet the criteria for being designated as newly formed neurons.

In a subsequent report, Kaplan [43] used electron microscopy to study neurogenesis in the visual cortex of 3-month old rats. At this age, the animals are young, but fully capable of reproducing, a criterion com-

patible with designating the rats as "mature". ^{3}H-thymidine labelling was again employed and the animals were allowed to survive for an additional 30 days to permit incorporation of the labelled material into the brain. The autoradiographs provided evidence of labelled neurons in layer IV of the visual cortex. Subsequent electron microscopic analysis showed that the labelled cells had fully developed axons, dendrites and synaptic profiles. The number of these newly differentiated neurons was estimated to be about 1 in 10,000 or 0.11% of cells in layer IV.

The research of Nottebohm and that of Kaplan demonstrate that injury to the brain is not a necessary condition for the initiation of adaptive or "neuroplastic" phenomena to occur in the adult CNS. Yet the possibility that the brain can generate new neurons to replace those that have been lost as a result of injury, even if such replacement is very limited, opens important new avenues for research that have yet to be considered as worthy of serious attention. For example, where, if they do occur at all, do new neurons come from? One possibility that could be explored is that, under the right conditions (as yet not known), glial cells in or near the area of injury could be transformed into neurons or at least assume properties characteristic of neurons. Such transformed cells might then become integrated into the anatomical or biochemical matrix mediating the functional recovery.

Admittedly, this idea is far-fetched, but there are some data on environment-induced cell transformations that bear on this question and that merit further consideration. For example, adrenal chromaffin cells (from the adrenal medulla), when located in their proper "context" are seen as smooth and rounded cells containing large amounts of epinephrine [24]. If rat chromaffin cells are removed from the adrenal gland and cultured in a solution containing Nerve Growth Factor, the cells undergo morphologic and biochemical transformation into normal appearing adrenergic neurons [16].

If adrenal chromaffin cells taken from 5 to 7 week old donors, are transplanted into the third ventricle of the brain, there is some successful transformation (but it is not as great as when fetal brain tissue is transferred — this will be discussed later). Nonetheless the chromaffin-cell "grafts" show large dopamine fluorescence and are capable of reducing some of the deficits that occur following nigro-striatal damage [23]. It would be interesting to note whether more effective transformation and/or integration into the host brain would have been accomplished if the

transplants had been placed directly into the damaged area, where neurotrophic factors are likely to be released (see below).

Whether the appearance of "new" neurons is due to transformation or genesis remains the subject for more research. But we now have evidence that, even in the adult brain, there is far more "plasticity" than one would have ever imagined only a few years ago. From a conceptual as well as a practical point of view, the recognition that the brain is capable of such adaptive response to injury (or disease), will enhance the likelihood that the means will be found to promote such plasticity more actively. Or inhibit it where it could lead to deficits.

Sprouting and sparing of neurons after brain damage in adults

Despite all the excitement generated by research on synaptic modelling, anomalous growth in the CNS and even neurogenesis in the adult brain, there is still much that needs to be learned about how these phenomena relate to *functional recovery* in brain damaged organisms, or to learning and remembering in intact subjects. While few will any longer deny the presence of anomalous or collateral sprouting in the adult CNS, clear correlations showing that sprouting mediates restoration of functions lost as a result of lesions are still difficult to obtain.

However, there is some evidence to demonstrate that sprouting or proliferation of new fibers into the dentate gyrus of the hippocampus following entorhinal cortex lesions may be responsible for restoring adequate spatial performance in adult rats. Much of this work has been done by Oswald Steward and his students at the University of Virginia Medical School.

In one study, Loesche and Steward [53] created unilateral or bilateral lesions of the entorhinal cortex in adult rats and compared the performance of these animals on a spatial alternation task to normal controls. Loesche and Steward reasoned that if anomalous sprouting occurs in response to CNS injury, and if behavioral recovery also occurs, the time course of the two events should be correlated. In other words, if it were to take 10 days for sprouting to be observed after a unilateral lesion, there should be no behavioral recovery for at least 10 days — if the two events are related. A group of animals with bilateral lesions was included in the experiment; the investigators hoped to show that such animals would not recover because the nerve fibers that would normally sprout, would be totally eliminated.

Loesche and Steward confirmed their hypotheses. The animals with bilateral entorhinal cortex lesions were unable to learn the task and they showed a severe deficit that lasted throughout all of the training. The rats with *unilateral* lesions showed an initial deficit that persisted for about 10 days and gradually began to disappear over time. Another group of rats with unilateral lesions given 10 days postoperative recovery *before they started training*, performed as well as normal controls. Thus the authors of this experiment were able to demonstrate a correlation between the time necessary for anomalous sprouts from the contralateral hemisphere to begin to function and the behavioral recovery they observed. Finally, Loesche and Steward cut the dorsal psalterium (the fiber path carrying axons from the contralateral entorhinal cortex to the denervated hippocampus) in the recovered rats and found that in doing so, they created as severe a deficit as if the entorhinal cortex lesions had been made bilaterally.

Other experiments from this same group have demonstrated that the neuronal reinnervation can have electrophysiological and biochemical consequences. For example, microelectrode recordings from the molecular layer of the hippocampal dentate gyrus show a change in excitability in comparison to recordings made in non-brain injured controls. The graded potentials *shifted to the outer molecular layer* of the hippocampus when the *contralateral entorhinal* cortex or commissural fiber system was stimulated. Graded potentials are never seen in response to commissural stimulation in normal animals and thus it can be inferred that the shift, which resembles the short-latency response *in form*, is due to the sprouting of new terminals from the contralateral hemisphere moving into a previously deafferented zone.

More recently Steward [82] has shown that the deafferented dentate gyrus shows more active metabolic activity in response to the injury. After a unilateral entorhinal cortex lesion, adult rats were injected with ^{3}H-2-deoxyglucose (2-DG), an analog of glucose that is taken up by active neurons, but not metabolized. Autoradiograms were then used to visualize those areas of the CNS that actively incorporate the 2-DG, and this incorporation taken as a measure of metabolic activity. Steward showed that, within 8-10 days after the EC lesion, the deafferented zone has a greater density of the label; a finding consistent with the time necessary to visualize collateral sprouting into this region.

In subsequent experiments, Steward [82] examined the incorporation of ^{3}H-labelled leucine, a precursor of proteins, into the sprouting

neurons. The leucine was injected from 6-20 days after the initial EC lesions and then the rats were killed 30 minutes after the injection. The markedly increased incorporation of the leucine in the deafferented zone, along with the findings of increased 2-DG incorporation, led Steward to believe that functional processes in the neurons deafferented by the contralateral lesions, are stimulating and perhaps guiding, the new growth. They also suggested that glia may be playing an important role in the re-afferentation process.

In a similar study, Gage, Stenevi and Dunnett [27], created selective lesions of the aminergic and cholinergic pathways to the hippocampus and tested their adult rats on an alternation learning task in a T-maze. Bilateral lesions of the supracallosal afferents to the HC result in a marked reduction of noradrenergic, serotoninergic and cholinergic innervation and a severe deficit in alternation performance. But, within six weeks of surgery, marker levels of these neurotransmitters had returned to normal, and there was a concomitant behavioral recovery of alternation learning which correlated significantly with the serotoninergic reinnervation of the hippocampus.

Pritzel and Huston [66], also looked at the relationship between sprouting and recovery of function following chemotoxic lesions of the substantia nigra. Unilateral lesions in this brain region typically result in stereotyped, ipsiversive and conversive turning behavior that usually disappears spontaneously about one week after injury. The behavioral recovery that Pritzel and Huston observed, correlated in time with the proliferation of projections from the intact substantia nigra. They speculated that already existing, sparse connections proliferated into the ventromedial thalamus of the damaged hemisphere. The authors noted that in the intact animal, this thalamic region only receives fibers from the ipsilateral substantia nigra.

One question that needs to be more thoroughly explored, is whether newly sprouted terminals may be a part of the physiologic processes required to *initiate recovery* of function, without playing any part in the *maintenance* of the recovered behaviors. Alternatively, the development of new fibers, in some circumstances, could parallel functional recovery, without actually taking part in the recovery process *per se*.

In one recent study, Ramirez and Stein [68] trained groups of adult rats on a spatial learning task and then subjected the animals to unilateral entorhinal cortex (EC) lesions (lesions that were very similar to those employed by Steward in his studies), unilateral EC lesions followed by

transections of the dorsal psalterium (to eliminate decussating fibers from EC to the dorsal hippocampus), or bilateral EC lesions. The intended purpose of the study was to replicate the proposed relationship between sprouting and behavioral recovery that had been reported by Loesche and Steward and described above.

In brief, Ramirez and Stein were able to show that, following EC lesions, the intact EC projections proliferated in the dentate gyrus of the hippocampus. However, the rats with unilateral EC lesions *and* dorsal psalterium transections, were able to perform the spatial learning problem as well as intact controls. Ramirez and Stein were unable to detect any behavioral impairments when the rats were tested as early as two days after the surgery had been completed. Thus, in this series of experiments, sprouting clearly occurred in the dentate gyrus of the hippocampus, but it did not seem to be related to the rate or extent of behavioral recovery on a task that is known to be sensitive to limbic system damage.

Thus, while one can argue that neuronal sprouting may play an important role in initiating behavioral recovery, the specific fiber connections may not be required to maintain the adaptive behaviors once they have been established. This generalization is probably more applicable to neural systems which mediate complex, learned behaviors rather than those associated with sensory functions. Nonetheless, it would be important to test this assumption empirically. This could be accomplished by creating an initial lesion, observing behavioral recovery, correlating the recovery with evidence of anomalous sprouting and then cutting the new fibers. If the sprouted pathways are *necessary* to sustain the recovered behavior, the deficits should immediately reappear and *be permanent*! We will have to await further research on this question before we can fully understand the contributions of anomalous growth in tbe central nervous system to behavioral recovery following brain injuries.

Thus, despite the fact that there is now clear evidence for *sprouting* in the mature central nervous system, there is still only a relatively small literature demonstrating an unequivocal relationship between the new growth *and behavioral recovery.*

Another fascinating study deserves mention in the context of analyzing the relationship between neuronal alterations in response to injury and subsequent behavioral recovery. Dineen, Hendrickson and Keating [15] examined the morphology of the retina and retino-cortical projection areas in two monkeys that had received total bilateral striate cortex removals as adults. The animals were tested for visual capacities that

included measurements of flux discrimination, luminance discrimination and pattern discrimination. The behavioral tests revealed that the monkeys had completely recovered their ability to make visual discriminations, despite total visual cortex removals. They were adept at orienting to visual targets and to reaching "fairly accurately" for them. Upon completion of the behavioral testing the animals were killed and their brains prepared for histological examination using both light microscope and electron microscopic analyses. As a result of the lesions there was a 30% loss in the parafoveal retina due to retrograde transneuronal degeneration. There was also a significant loss of neurons and a change in axonal distribution in the dorsal lateral geniculate nucleus of the thalamus (dLGN). Despite the heavy loss of neurons in the dLGN, Dineen and his colleagues [15] did observe islands of neurons and neuropil within the dLGN. Their most striking finding, however, was found in the *pregeniculate nucleus*, where there was an actual enlargement of retinal terminals. Could this enlarged projection have contributed to the restoration of visual functions seen in these monkeys?

Dineen *et al.* suggest that three factors may play a significant role in behavioral recovery after striate cortex removal in the adult primate. First, they suggest that older animals show *less retrograde degeneration* after visual cortex lesions than do younger animals. This means that there may have been more neuronal sparing from the retina to the CNS in the animals that were studied in their experiment since they were at least 6 years old at the time of surgery. Second, the spared islands of neuropil observed in the dLGN may be involved with residual, subcortical visual input and that these islands remain even after massive destruction of the visual cortex. Third, the increased projections to pregeniculate areas may play the same role in recovery of functions as the anomalous, entorhinal cortical projections are presumed to play in the recovery of spatial behaviors we discussed earlier.

The findings of Dineen and his colleagues may help to explain some unusual reports on delayed recovery from lesions of visual cortex seen in the human clinical literature. For example, it is well known that patients with visual cortex lesions very often develop scotomas. Patients with such a disorder have gaps or defects in their ability to detect targets that move slowly across their visual field. Most clinicians have accepted the notion that brain injury causes permanent scotomas or blind spots.

This idea has been questioned by Poppel, Held and Bolling [65] who were able to show that, under careful testing conditions, patients

could demonstrate residual vision and even make discriminations within the blind spot itself. Even more interesting is the finding of Zihl [90] at the Max Planck Institute of Munich showing that with sufficient training, the scotomas of some patients with visual cortex lesions could be made to shrink. The improved vision could still be observed up to one year after the training had been discontinued.

Perhaps the shrinking of the scotomas seen by Zihl or the residual vision reported by Poppel and colleagues is due to the spared islands of neuropil noted by Dineen *et al.*, as well as the enlarged projection field they observed in the pregeniculate nucleus of the thalamus. Of course, a certain amount of caution must be taken in interpreting these findings because there is still only limited behavioral evidence yet available to draw strong inferences about the relationship between sprouting, anomalous growth and behavioral recovery after cerebral injuries.

The main point we can take from the studies so far discussed is that there is far more adaptive "plasticity" in the central nervous systems of adults than was imagined only a decade or so ago. Anomalous growth in the CNS is now a well established fact, even though such growth may not lead to behavioral recovery. The task-at-hand is now to use what is known about anomalous and collateral growth to *facilitate* recovery by directing that growth more effectively. To accomplish this task we will need a better understanding of the processes of neuronal organization as well as a grasp of the developmental and environmental factors that influence this organization. Given the extraordinary progress that has been made in only the last 10 years, it is probably safe to speculate that we are very close to this goal.

Sprouting is one aspect of neuroplasticity that has captured the imagination of many neuroscientists, but it is only *one aspect* of a complex and multifaceted phenomenon that can be related to recovery from brain injury. In other words, there are cases in which complete recovery from brain injury has been observed, but which *cannot* be attributed to anomalous growth induced by the brain injury. For example, is it realistic to assume that sprouting mediates recovery from bilateral lesions of the brain? In this case, *both* the lesion-target structure *and* the contralateral homologue are eliminated. Where would the new fibers originate? Where would they terminate to mediate the functional recovery? When may anomalous growth actually impair functional recovery or be maladaptive? These are but a few of the questions that must be addressed but that have not yet received critical analysis by researchers in this field.

For the clinician who is faced with the immediate urgency of dealing with usually nonspecific, often diffuse brain injuries due to trauma, bleeding, ischemia, penetrating wounds, etc., the relatively clean and carefully proscribed lesions that are produced in the laboratory, may not effectively mimic the "real world" situation. The models of recovery based on sprouting over highly defined pathways may eventually help us to understand the principles of cerebral organization, but they may not be appropriate to the clinical situation — at least at present.

One of the critical questions we must ask, then, is whether there is recovery of function in mature subjects that may occur in situations in which anomalous growth, as an explanation, is unlikely. More important for the clinician, perhaps, is the question of whether recovery or sparing of function is as evident in adult subjects as it is in younger patients. Finally, if such recovery is possible then what are some of the conditions under which it can be made to occur and how can such conditions be used to help the patient suffering from brain damage?

Variables affecting the outcome of injury to the central nervous system in adults

Examples of "Endogenous recovery"

Most clinicians are aware of reports scattered throughout the neurological literature, of cases in which patients have almost miraculously recovered from the effects of very severe damage to the brain. But, as noted earlier, such instances of recovery were considered as anomalies, unusual events, defying "pat" descriptions, lying outside the realm of explanation and traditional neurological dogma, and of no particular consequence to prevailing theories of brain function. The mounting evidence of neural plasticity in *adult subjects* and a better understanding of how brain-damaged organisms can adapt to their injuries, is beginning to change the idea that "once development has ended, everything is fixed, that functions are genetically determined, and that once a structure is injured, the function it mediated must be irretrievably lost". Yet, history has a curious way of repeating itself, and to demonstrate the point, this discussion should begin with a quote from John Hughlings Jackson [39]. In describing the sequelae of brain tumors to a group of physicians he stated:

> "Before I speak of the several classes of symptoms in cases of intracranial tumors... I have to make a statement which may surprise some of you. It is that occasionally there are no *symptoms in these cases*. And frequently when symptoms are present, they are *insignificant* in comparison to the size of the tumor."

Since Jackson's time there have been studies showing that slowly inflicted damage, such as that which might occur with a tumor, causes less impairments than when the damage occurs more suddenly, as in the case of a penetrating wound or stroke. When no symptoms appear, such patients may not realize that something is wrong with their nervous system. Jackson was aware of this curious phenomenon and it was he who first discussed the concept of "momentum of the lesions" in 1879 and applied it to the observation that slowly induced neuronal injury is less likely to cause behavioral impairments than injury of rapid onset [40]. Jackson's view was not generally well received in the neurological community (for a discussion of the history of localization the reader is referred to [19]) because his observations did not fit with prevailing localization theory.

In the laboratory, it is possible to examine the effects of slow growing lesions more carefully. One of the most dramatic instances of functional sparing after slowly induced CNS lesions was provided by the neurosurgeon, John Adametz [1]. Adametz was interested in testing the hypothesis that the critical functions of sleep and respiration were localized in the reticular formation of the brain stem (BSRF). He chose this area because just a few years ago prior to his own work, Lindsey and Magoun at UCLA had presented their evidence demonstrating that focal damage to the reticular formation would cause adult cats to fall into deep coma and eventually die. Based on their work, it was then assumed that tbe BSRF was the "center" for cortical and thalamic arousal and further, that this center was essential for the maintenance of conscious experience.

Adametz used 80 adult cats in his experiments. One group was given a large, electrolytic lesion of the entire midbrain reticular formation in a single operation. When this was done, the cats fell into a deep coma and even though intensive postoperative care was provided to save them, most of the animals never regained consciousness and died within a few days after surgery.

A second group of fully mature cats was subjected to the same electrolytic lesions, but instead of damaging the BSRF at once, this group of animals had a *series* of smaller lesions made 1-2 weeks apart. In all, the cats received more than 8 separate surgeries so that when the operations

were completed, the entire BSRF was destroyed. The outcome of this "seriatum" surgery was dramatic. The cats arose shortly after each operation and were soon walking about, feeding and eating. These cats actively pursued mice, climbed ladders, groomed themselves normally and had sleep-wake cycles typical of cats with no brain damage.

Clearly, in this situation, the specific-lesion, specific-symptom classification scheme did not work, despite the fact that by giving the animals multi-staged surgeries, one might have expected greater surgical stress and postoperative trauma. Since Adametz's studies, the serial lesion phenomenon has been extended to many different species tested in a wide variety of behavioral tasks (see [19, 21] for detailed reviews). With respect to complex, or "higher order behaviors" involved in learning, memory and cognition, the serial lesion phenomenon has proved to be quite robust. For example, at Clark University [81] we created bilateral, single-stage lesions of the frontal cortex or hippocampus in fully mature, male rats. These animals were then compared to non-injured controls or to aged-matched conspecifics with identical lesions that were created in two stages. In this latter condition, a lesion was made on one side of the brain and then 30 days later, a second identical lesion was created in the opposite hemisphere. All of the rats were given 2 weeks of postoperative recuperation and then they began testing on a standard *series* of learning and performance tasks known to be highly sensitive to the specific deficits caused by the lesions we made.

After all behavioral tests were completed the animals were killed so that their brains could be examined for the extent of the injuries. As Figure 2 shows, there were markedly different effects of the lesions. In comparison to intact controls, the rats that had received *simultaneous*, bilateral lesions of either hippocampus or frontal cortex, showed the typical, long-lasting behavioral deficits on the spatial and visual discrimination tasks. In contrast, the rats *with the same amount of cerebral injury* inflicted in two operations, spaced 30 days apart, were basically *unimpaired* on the same behavioral measures. Clearly, the time between the two operations had a determining influence on the outcome of the experimentally induced injury. In the first case, the symptoms produced by the single-stage surgery conformed to expectations based on prevailing views of structure-function relationships in the adult brain. But what of the animals with serial lesions? Their performance was basically asymptomatic!

The time between surgeries seems to be an important factor in

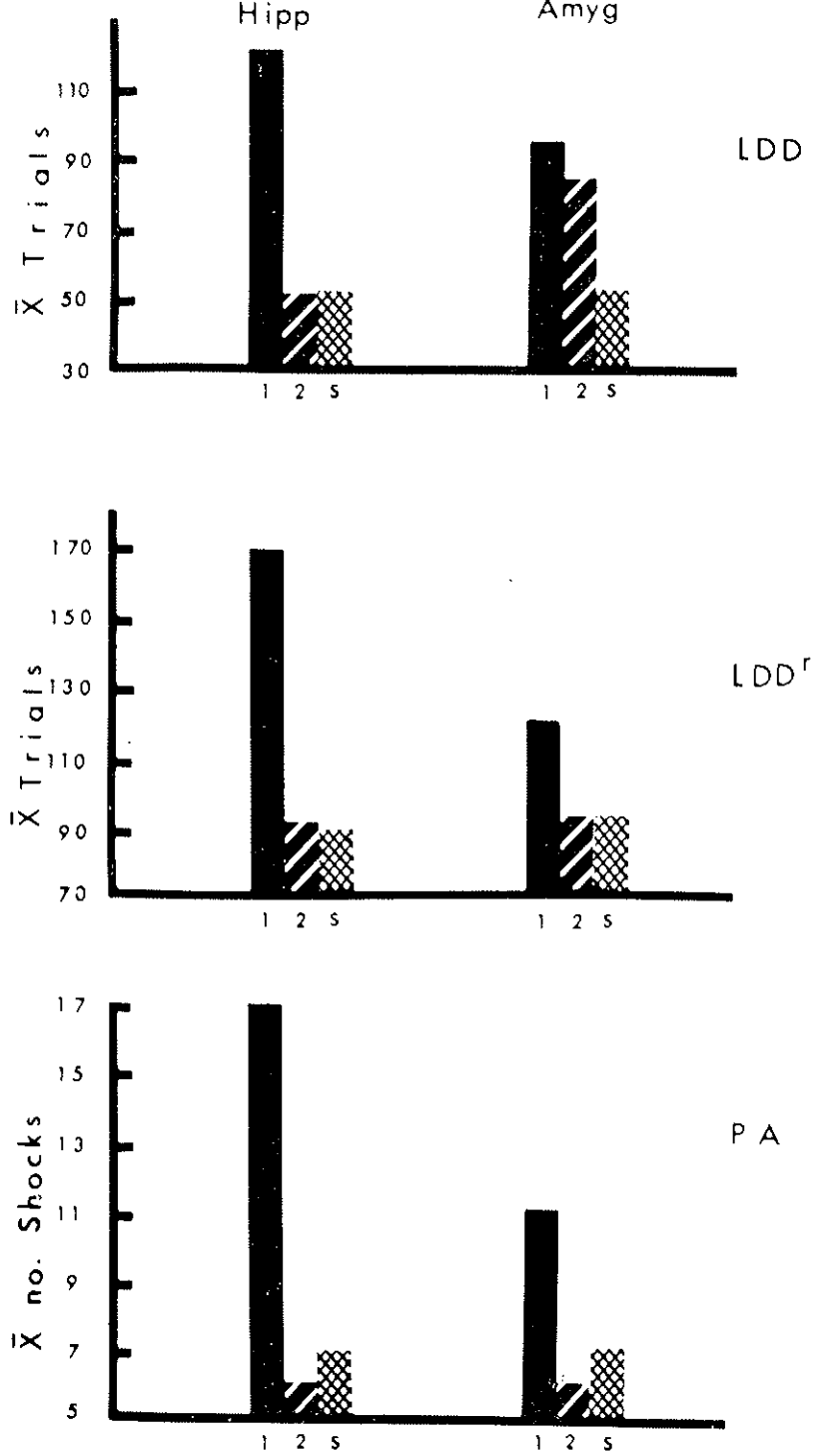


FIG. 2. Postoperative performance of rats with one- or two-stage lesions of the hippocampus or the amygdala on light-dark discrimination (LDD); light-dark discrimination reversal (LDD^r); and passive avoidance learning (PA). The rats with one-stage lesions are indicated by the solid bar; those with two-stage lesions by the diagonal bar. Sham-

determining the extent of the sparing produced by the "serial" lesions. In one study Patrissi and Stein [63] examined this question by aspirating the frontal cortex of rats in one- or two-stage bilateral operations. We then compared the animals to normal controls on a spatial alternation task used in our previous experiments. In all, we used 5 groups of fully mature, male rats. The groups had the frontal cortex removed either in one, simultaneous, bilateral operation, or with an interval of 10, 20, or 30 days between the first and second surgery. When testing was completed, the animals were killed for histological verification of the locus and size of the lesions.

As expected, the rats with one-stage bilateral lesions showed very significant and long-lasting deficits in learning ability. Those rats given only a 10 day interoperative interval were also very impaired but (see Figure 3) they were still able to perform much better on the spatial alternation task than the animals with one-stage operations. In comparing the performance of the animals with 20 and 30 day interoperative intervals, we saw that their learning scores were practically identical to those of the unoperated controls. Clearly, the time between surgery is an important variable in determining the behavioral sequelae of brain lesions. It is interesting that the minimal, "interoperative" time necessary to observe behavioral sparing seems to correlate well with the minimum time it takes to observe collateral sprouting after unilateral injury and with the time required for optimal levels of neurotrophic substances to be released by glia in the damaged brain [11]. However, I should also note that the relationship between the serial lesion phenomenon and anomalous growth is, at the very best, only circumstantial. Given the fact that all subjects have bilateral lesions at the time behavioral testing begins, it is difficult to imagine where the new fibers might be arising and where they might be terminating in the absence of the critical target issue. Nonetheless, there is some evidence to suggest that progressive brain damage does accelerate axonal sprouting in the adult rat [74]. Thus, Scheff *et al.* made unilateral lesions of the entorhinal cortex in 90-day-old rats in one or two stages. As will be remembered from our previous discussion, a unilateral EC lesion removes nearly all of the input from the EC and stimulates the proliferation of afferents from the septal nucleus to innervate the synaptic spaces in the dentate gyrus that are vacated by dying EC neurons. Using a stain selective for acetylcholinesterase, Scheff and his colleagues were able to demonstrate a 30% increase in the spread of new collaterals in those animals given 2-stage

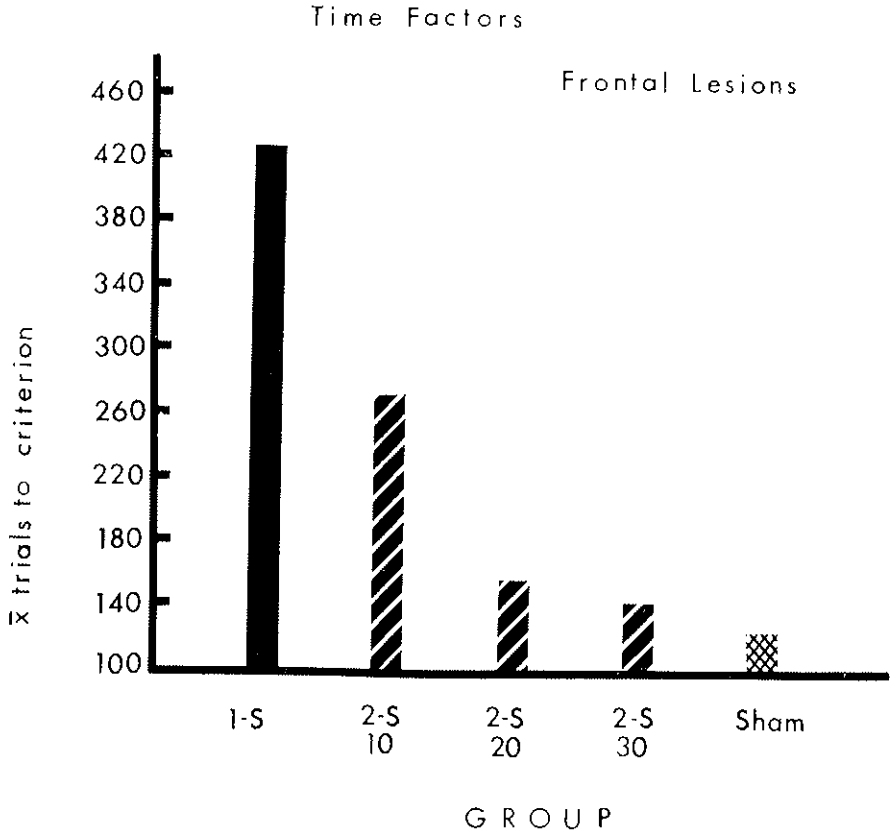


FIG. 3. Recovery of spatial alternation performance in rats with one- or two-stage lesions of the frontal cortex. The black bar represents the performance of rats with one-stage aspirations (1-S); the diagonally striped bars show the performance of rats with 2-stage lesions separated by a 10-day interoperative interval (2S-10); a 20-day interoperative interval (2S-20) or a 30-day interoperative interval (2S-30). The hatched bar to the right of the graph represents performance of the sham-operated controls.

lesions. In contrast, in the one-stage group, no growth was seen for at least 4 days postoperatively and the proliferation of the septal fibers was not nearly as intense as it was in the two-stage group.

If "seriatum" lesions somehow result in more sprouting or in enhanced survival of neurons, what might be the mechanism(s) by which such sparing might occur? Until very recently, there were no explanations and very few clues available to researchers who wished to pursue this question. However, within the last few years, Cotman and his colleagues [9, 10, 11]

have provided very interesting and provocative new data that may shed some light on the mechanisms underlying functional recovery.

These investigators had been long concerned with determining the neurochemical factors that could stimulate (or retard) injury-induced synaptogenesis in the brains of adult subjects. To study this question directly, Cotman and his collaborators aspirated portions of the entorhinal cortex in neonatal, adult or aged rats and filled the cavity with saline-moistened Gelfoam sponge. The animals were then allowed to survive for varying periods of time after surgery and were then killed so that the contents of the Gelfoam sponge and the brain tissue surrounding the wound cavity could be evaluated for the presence of neurotrophic or neurotoxic substances.

The Gelfoam or brain tissue "extracts" were then applied to dissociated cultures of neurons taken from embryonic rat brain, and cell survival and extent of neurite outgrowth was measured. Cotman *et al.* reported that neurotrophic activity was much higber in extracts taken from brain-damaged subjects than from intact controls. Ablation of the entorhinal cortex also caused a significant increase in neurotrophic activity in tissue surrounding the wound area. This increase was time dependent, and appeared to peak at 10-15 days following the injury. By approximately 20 days post-lesion, neurotrophic activity had declined to baseline levels. Cotman *et al.* reported that injured brain extracts were able to support the survival of neurons taken from various regions of the brain although the "highest" trophic titer was on hippocampal neurons, followed by septum, striatum and thalamus. Tbis is consistent with the hypothesis that injury-induced growth factors act preferentially on neurons connected to the injured area.

Cotman *et al.* speculate further [11] that reactive astrocytes are the source of most of the trophic activity that these glial cells secrete and that such neurotrophic substances may be vital for the survival of neurons in culture. They state that: "perhaps molecules associated with CNS injury activate glial cells, which in turn produce neurotrophic activity".

Recently Muller and Seifert [56] reported that embryonic, hippocampal neurons in culture fail to survive in tbe ahsence of glial cells which produce a diffusible neurotrophic factor that supports both survival and neurite outgrowtb. The neurotrophic substance can be isolated and applied to hippocampal cells in serum-free culture and they too, will survive and grow.

A number of years ago, I speculated that glial cells, and in particular, reactive astrocytes, may play an important role in promoting recovery, rather than impairments, following brain injuries. I also suggested that "neurotrophic substances" such as the Nerve Growth Factor (NGF), may alter the glial response to injury and initiate the recovery process [81]. For example, in one experiment we conducted, rats received bilateral radio-frequency lesions of the caudate nucleus. The animals were then given unilateral, injections of 250 μg of NGF directly into the damaged caudate. The contralateral hemisphere was injected with an equivalent volume of buffered isotonic saline. Thus, each animal served as its own control. The rats were allowed to survive for 0, 10, 20, 30 or 60 days after the lesions and were killed for histological evaluation of reactive astrocytosis. Here, we used the Cajal gold sublimate stain for reactive astrocytes and then counted and measured the number and size of the astrocytes on the NGF- and saline-treated sides of the brain.

We were able to demonstrate a time-dependent increase in both the size and number of injury-induced reactive astrocytes in the NGF-treated, damaged caudate nucleus. In brief the NGF treatment resulted in significantly larger numbers and size of these glial cells beginning at 20 days after the injury. However, by 60 days postsurgery, the interhemispheric differences in astrocytosis had disappeared. That is, there was an initial increase in the size and number of these cells and then a gradual decline to control levels.

Although we did not do any behavioral evaluations in this particular experiment, we noted that the appearance of enhanced astrocyte activity was related to the appearance of sprouting in other experiments (e.g. [53]), and to the time required for behavioral recovery to become manifest (see [63]). Since we had no biochemical data, we could only speculate that the NGF treatments stimulated the glial cells to secrete neuron-sustaining or growth promoting factors that would aid in the functional recovery. Cotman's recent findings [9] and those of Muller and Seifert [56] now seem to provide some support for this speculation, although the specific molecules involved have not yet been identified.

It is also important to recognize that, in a two-stage operation, neurons and glia on the uninjured side of the brain also play a role in the recovery process. For example, after a unilateral injury there are increases in microglia and blood monocytes on the side contralateral to the injury. It is also known that after unilateral injury, sprouting fibers from the contralateral hemisphere can grow across the midline to prolifer-

ate into those zones that have been deafferented by the ipsilateral lesions. Under these conditions, one could argue that glial cell release of neurotrophic factors may serve to stimulate axonal sprouting and provide the substances which help to guide and direct the newly-formed terminals to their appropriate targets.

Currently, the role of glial cells in the release of trophic substances that can enhance repair and regeneration of CNS tissue, has become the focus of renewed interest and investigation (see [35]) but the specific molecular bases for neurotrophic support remain to be identified. The important point is that a growing number of investigators believe that it now may be possible to stimulate and promote regeneration and repair of damaged brain tissue in an active manner (see [25] for timely review of this question). This change in perspective represents a radical departure from the long-held conviction that nothing can be done to facilitate recovery from brain injuries using pharmacological or biochemical techniques.

Substances that promote Behavioral recovery from brain injuries

Although there has been good progress being made towards understanding and manipulating CNS plasticity in laboratory experiments, much less has been accomplished for the clinical treatment of patients with severe brain damage. Only a few years ago, for example, Brailowsky [7], in reviewing the pharmacological treatments for nervous system injury, wrote: "Clinicians concerned with the physical therapy and general rehabilitation of persons with incapacitating brain lesions rarely make use of drugs to alter the course of the disease, and in general, the pharmacological management of these patients is only symptomatic" (page 187).

I think it is safe to say that, until only a very few years ago, most work in neurology and neuropsychology had been concerned primarily with the identification and description of symptoms and deficits that are caused by CNS injuries. Perhaps this view made sense in the light of the commonly held belief that, in the adult mammalian brain, everything was static — that once injured, there was no regrowth of neurons and no possibility for significant, functional readjustments.

But now that functional plasticity in the central nervous system has been repeatedly and consistently demonstrated, the view that nothing can be done after injury is giving way to renewed interest in the possibility

that behavioral recovery from brain damage may be facilitated or enhanced by pharmacological or surgical manipulations. With so many excellent reviews on CNS plasticity and recovery currently available [5, 9, 11, 19, 25, 38, 74], I will primarily limit my discussion to work which has recently been conducted in our laboratory at Clark University.

For the most part, we have used neurotrophic substances to promote behavioral recovery from acute and focal injuries to the brain. The model that we apply usually consists of creating specific brain lesions using mechanical or neurotoxic agents, application of intracerebral or systemically introduced "agents" which we wish to test, a series of behavioral tests known to be sensitive to the types of brain injuries we created, and finally, histological evaluation of CNS tissue for gliosis, sparing or sprouting of neurons. In all cases, subjects who receive the experimental treatments are compared directly to appropriate brain-damaged but non-treated controls, or to intact animals who provide us with our "baseline" or normal, data.

Within this context, one of the most interesting possibilities for promoting recovery from brain or spinal cord injuries is through the replacement of damaged neurons (or glia) by fetal brain tissue grafts (see especially the chapter by Björklund, this volume). In brief, implants of fetal brain tissue cells have been reported to be effective in overcoming some of the consequences of motor system or nigrostriatal injury such as performing on an elevated narrow runway [18], sensorimotor neglect [25], stereotyped rotational behaviors [6] and disordered spatial performance [46].

In our laboratory we became concerned with the question of whether implants of fetal neurons could be used to offset some of the cognitive and learned impairments that often accompany acute, bilateral removals of the frontal neocortex. In the many species from mice to monkeys that have been studied, lesions in this area of the brain result in severe, and often permanent, impairments in spatial and visual discrimination learning. Based on these findings, we reasoned that it might be possible to replace neurons lost as a result of the brain tissue extirpations in fully mature rats, and further, that the implants would enhance the behavioral recovery of post-surgically acquired cognitive and spatial performance.

Thus, in our first experiment, four groups of adult rats were used. One group received bilateral aspirations of frontal cortex, followed seven days later by implantation of approximately six cubic mm of frontal brain tissue taken from rat embryos at day E20 (see [46] for details). A second

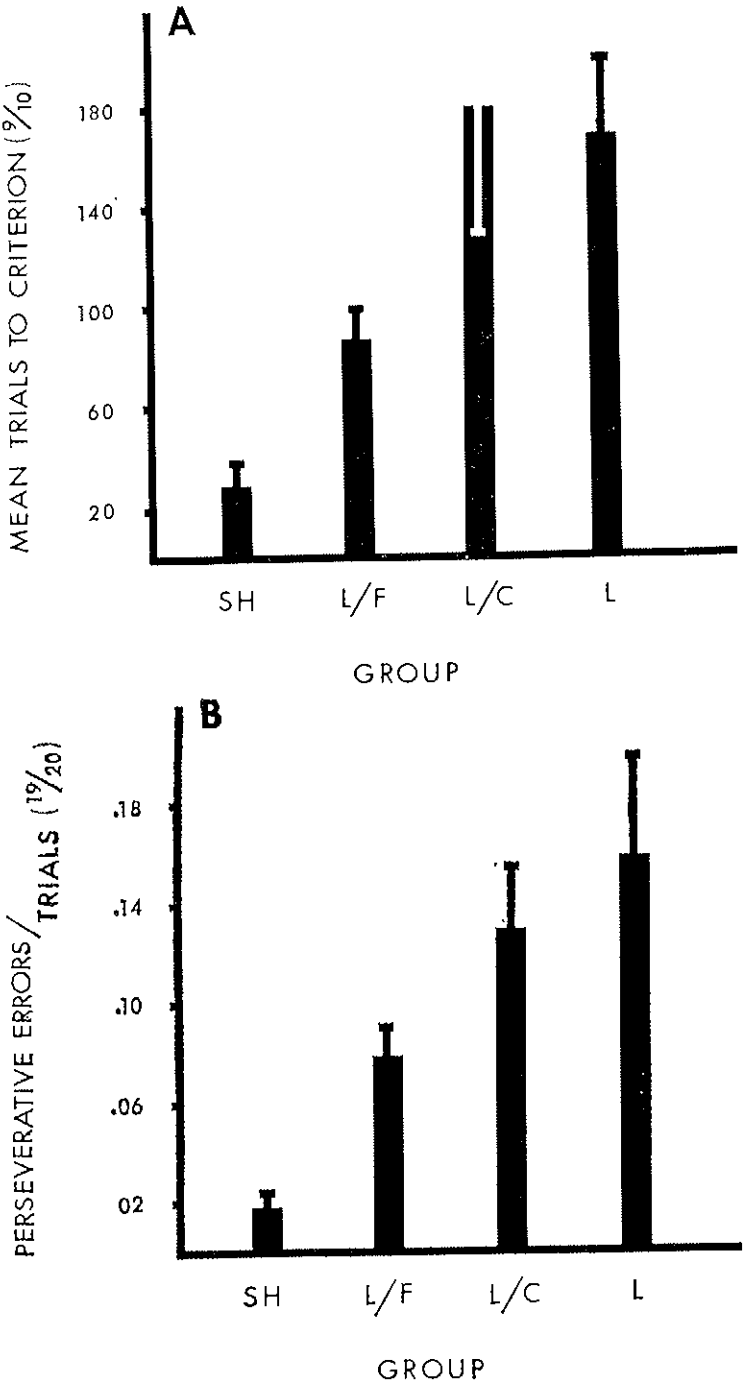


FIG. 4. A. Trials to criterion in a spatial alternation task for adult rats with bilateral lesions of frontal cortex given implants of fetal frontal cortex (L/F); fetal cerebellar tissue (L/C) or no transplants (L). The left bar represents the performance of the sham-operated controls. — B. Shows the performance of the same animals in terms of their perseverative errors on the spatial alternation task.

group of rats had the same lesions followed by implants of approximately the same volume of cerebellar tissue taken from the same embryos. The third group were age-matched rats with just frontal cortex lesions, and the fourth group consisted of intact controls.

Within four days after the fetal tissue had been implanted into the adult, brain-damaged hosts, all of the animals began testing on the spatial alternation problem. It is worth noting that our experiments differ from those of many of our colleagues because we begin behavioral testing very soon after the implants, whereas others usually wait 2-6 months before evaluating the effects of the transplants (see Björklund, this volume for details).

Our animals were given 10 trials per day, 5 days a week to assess their response to brain injury and to evaluate the therapeutic effects of the implants. When a rat made 19/20 correct choices over two consecutive days of testing, or when it had completed a total of 30 sessions, behavioral testing was terminated.

With respect to postoperative acquisition of spatial alternation, we found that implants of embryonic frontal cortex significantly reduced the number of trials required for the rats to reach what we established as a measure of learning (see Figure 4). In contrast, the rats which had received implants of cerebellar tissue directly into the wound area, were as impaired as those in the group with lesions alone. In addition, four of the six rats with cerebellar transplants and three of those without implants, were never able to reach the most stringent criterion we employed; whereas only one of the rats which had received frontal tissue failed to achieve this criterion. It is important to note that, although the rats with implants of embryonic frontal cortex showed a clear improvement on every measure of postoperative performance, they never performed as well as intact controls.

After the behavioral testing several of the rats in each group were used to determine whether reciprocal, anatomical connections had formed between the host and implanted tissue. First, we observed that all of the rats with cerebellar transplants had rejected them completely. In contrast, the frontal tissue implants had shown an approximate 6 to 10-fold increase in size during the time the rats survived. Thus we were only able to evaluate the connections in those rats given frontal cortex implants. To accomplish this, we injected very small quantities of the retrograde marker enzyme, horseradish peroxidase (HRP), directly into the transplant, and then carefully examined the adjacent cortex and dorsomedial nucleus of

the thalamus for HRP labelling of neurons. We also performed routine Nissl stains for neurons and glia so that we could examine cells in the transplant for their intrinsic organization and continuity with the host brain.

Our examination of the brain tissue revealed that the transplants formed continuous bridges connecting the two hemispheres or formed separate grafts, each adhering to the host cortex. As might be expected, there was glial scarring at the points of attachment to the host brain. In this experiment, light-microscopic evaluation of Nissl-stained sections indicated that there was virtually none of the internal order or laminar arrangement of neurons that characterize the frontal area of the normal rat (see Figure 5).

Inspection of the HRP-injected transplant tissue showed that there were a few labelled neurons present in the host cortex and that they entered the transplant at points of continuity. In some cases axons in the host would extend relatively long distances to penetrate into the implant (see Figure 6). HRP-labelled neurons could also be seen in the medial dorsal and anterior thalamic nuclei (Figure 5D). The areas of host brain which projected fibers into the implant were those known to have connections with the medial portions of the normal frontal cortex [51]. It should be emphasized, however, that the labelling we observed was not extensive, despite the fact that these animals had shown significant behavioral recovery.

In order to get a better idea of the connections between the transplant and host, Dr. Elliott Mufson of Harvard University has collaborated with us in applying immunocytochemical stains for specific neurotransmitters to the brain tissue. We used animals with E20 frontal cortex implanted one week after the medial frontal cortex had been aspirated. The animals were then allowed to survive for 2 months and were then killed for histology. First, as Figure 6A shows, neurons within the transplant show clear reaction for acetylcholinesterase. By carefully examining the juncture between transplant and host under the light microscope, we could clearly observe small numbers of reciprocal cholinergic fibers crossing between the two types of tissue. Although all of the transplants survived, showed good revascularization (Figure 6B) and all showed intense staining for AChE, not all of the transplants had reciprocal connections. In some cases, the axons at the juncture grew parallel to the medial wall of the lesion or turned in and swirled back into the transplant.

Nonetheless, we feel that cells within the transplants are metabolically

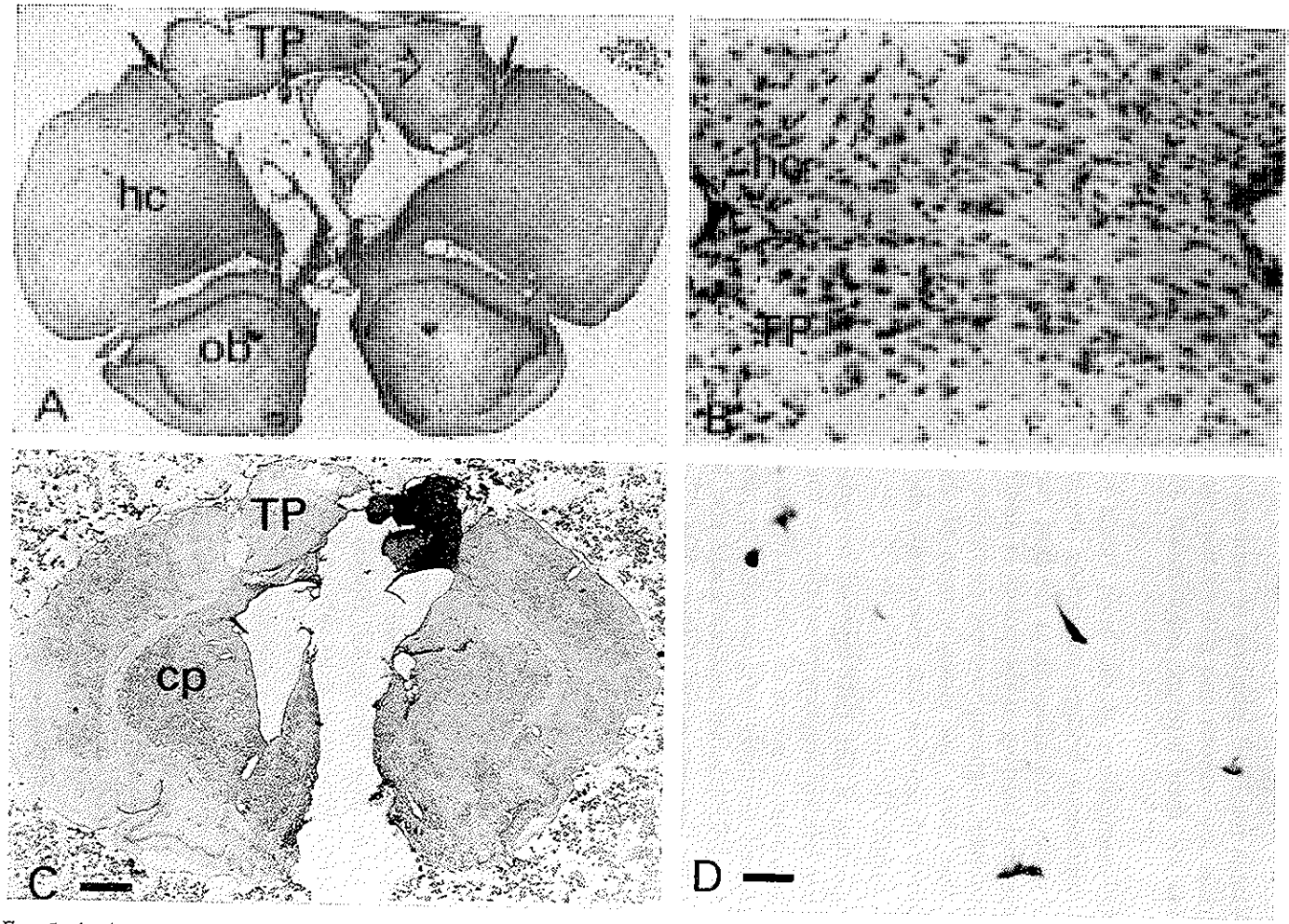


FIG. 5. A. A coronal section of rat brain showing the position of the fetal brain tissue transplant (TP) in the anterior/medial region of the frontal pole. The micrograph was taken 78 days after the implantation. The black arrows indicate points of attachment between the transplant and the host tissue. B. Shows the area of attachment between host and transplant in another animal. The host cortex is seen in the upper part of the micrograph (hc) and the transplant is in the lower part of the figure (TP). C. Shows a coronal section counterstained with cresyl-violet and a transplant injected with HRP. The injection site is black and there is

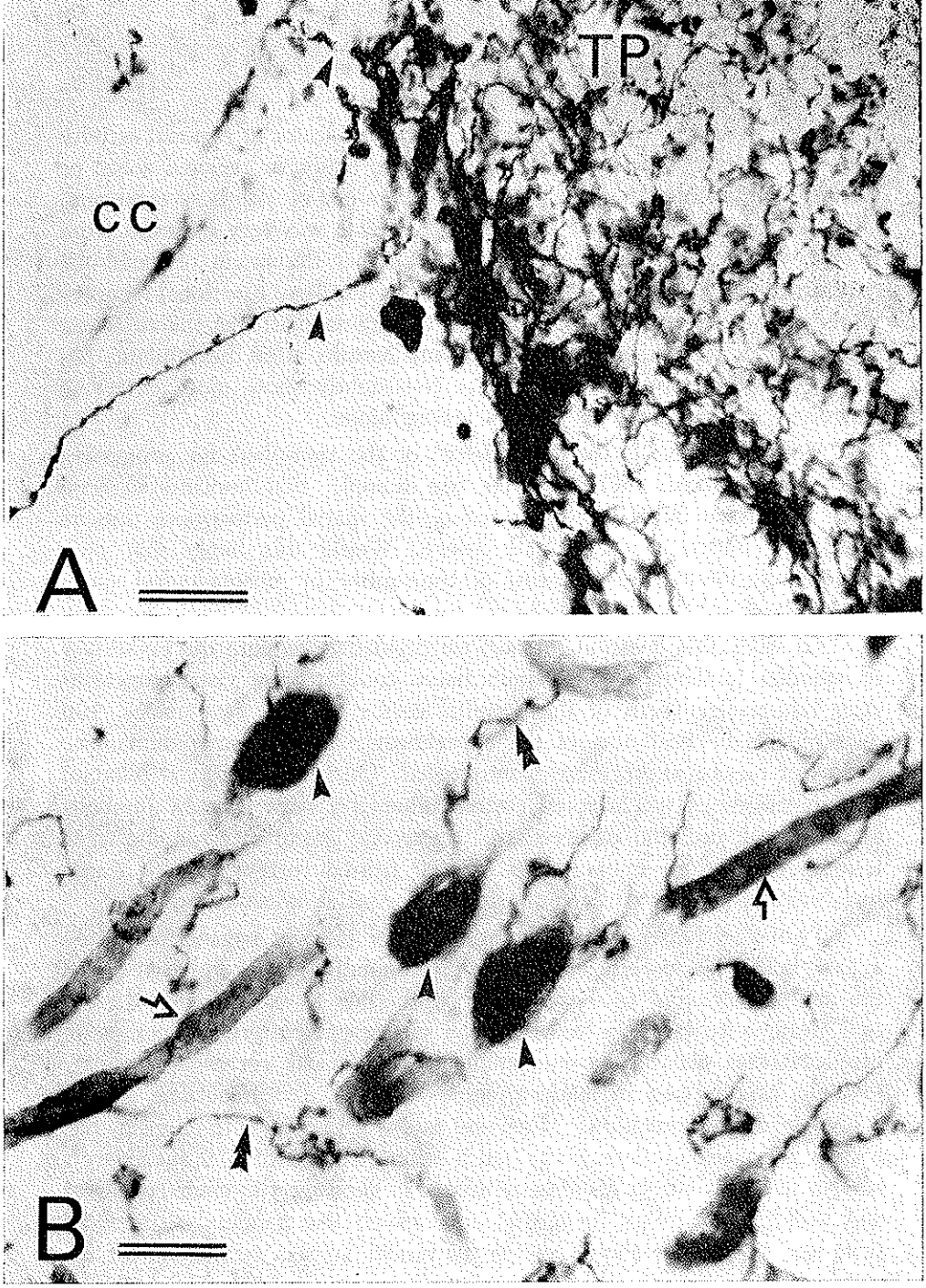


FIG. 6. A. Bright field photomicrographs of transplant tissue processed for the enzyme acetylcholinesterase (AChE). This micrograph shows the AChE-positive fiber network within a transplant (TP) bordering the corpus callosum (cc) of the host brain. Note the AChE-positive fibers (arrows) coursing between the transplant and the host brain. Bar = 100 μm. B. Transplant tissue stained for the enzyme AChE showing AChE-positive neurons (arrows), fibers (double arrows) and esterase-containing blood vessels (open arrows). Bar = 50 μm. Photomicrograph courtesy of Dr. Elliott Mufson,

active and used cytochrome oxidase stain as an indicator of regional activity We were able to observe cytochrome oxidase bands or regional zones of activity comparable to those seen in intact areas of the host cerebrum, although the organization of the transplant tissue was very different from that seen in the normal frontal cortex. Whether the extent of metabolic or neurotransmitter activity can be related specifically to functional recovery is yet to be determined, the main point here is that: (a) the cells are viable and active and remain so for many months after the implant; (b) on average, animals with transplants consistently show more extensive functional recovery than those with lesions alone.

How important is the "specificity" of the transplant for promoting functional recovery? We observed, for example, that implants of fetal cerebellar tissue failed totally to reduce the spatial performance deficits caused by injury to the frontal cortex. Would this imply that only homologous tissue (e.g. in terms of transmitter or morphological specificity) could enhance recovery?

To study this question further, we decided to explore the possibility of whether implants of fetal brain tissue could restore visual functions after bilateral removals of the occipital cortex in adult rats (males, 100 days old at the beginning of the experiment). In this experiment, four groups of rats ($n = 9$/group) were created. One group received bilateral suction lesions of the occipital cortex followed 7 days later by implants of E20 fetal, occipital cortex tissue. A second group received the same lesions followed by implantation of E20 frontal cortex; a third group had occipital lesions only and the fourth served as intact controls.

Two weeks after the implantation, all of the rats began training on a two-choice, black-white brightness discrimination task, which required the animals to avoid mild footshock by running to one or the other of two escape hatches. This task (and the subsequent pattern discrimination problem) was chosen because animals with visual cortex lesions are severely handicapped in learning these kinds of discriminations despite a high level of motivation. Thus, the rats were given 8 trials each day, 5 days per week until they attained a criterion score of 6/8 successively correct runs. A correct response consisted of choosing the white (positive) and avoiding the black (negative) door which was randomly alternated from one side of the choice box to the other. In this manner, all of the rats received a minimum of twenty testing sessions. We predicted that implants of fetal occipital cortex should facilitate the postsurgical acquisition of the brightness discrimination and that implants of the embryonic

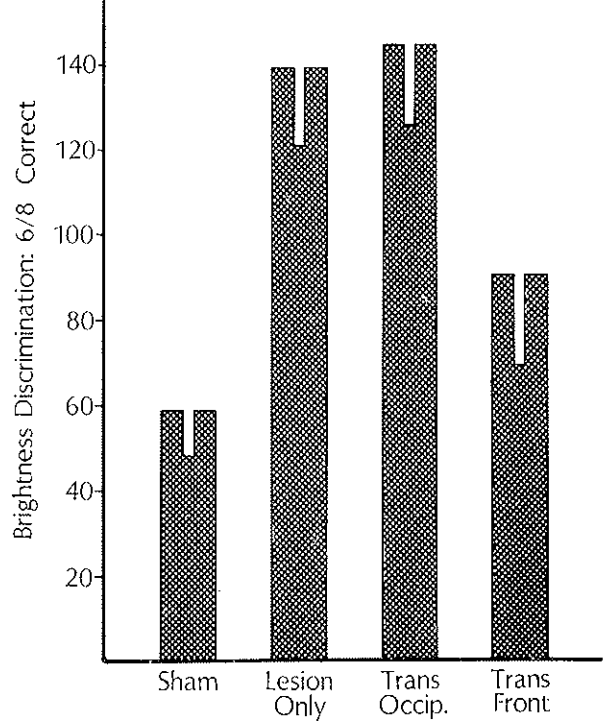
frontal tissue would render the rats just as impaired as the lesion-alone controls.

Although it was unexpected, we found that the implants of frontal cortex into the damaged occipital area facilitated learning of the brightness discrimination task, while implants of the occipital cortex had no beneficial effects. In fact, the rats that had received the implants of embryonic occipital cortex, were as severely impaired as lesion-alone group. Once again, the intact controls performed significantly better than all three groups with lesions on every measure we applied (Figure 7). Our individual comparisons among the groups revealed that the rats with frontal cortex transplants attained the criterion of 6/8 correct responses significantly faster than the lesion-alone group or the rats receiving the implants of fetal occipital cortex. The animals given the frontal tissue implants also showed less perseveration to the incorrect side than the other brain-damaged groups; in fact, they perseverated less to the incorrect side on 18 of the 19 days on which they were tested.

Further evaluation of our behavioral data revealed that none of the rats with occipital cortex lesions benefited from the transplants when it came to performance on the pattern discrimination. All of the animals with lesions were severely impaired and showed no recovery of their ability to distinguish horizontal from vertical black and white stripes. Nonetheless, this first attempt to enhance functional recovery from lesions of specific sensory cortex by means of transplants has demonstrated two important points. First, that fetal tissue transplants can mediate some degree of behavioral recovery following damage to a specific sensory system. Second, the tissue transplants need not be homologous to the injured system to mediate behavioral change.

In fact, and for reasons that are not completely clear, embryonic frontal tissue was more effective in facilitating the limited recovery than homologous, embryonic occipital cortex. We can speculate here that embryonic, frontal tissue of the same chronological age as the occipital implants, may not be as differentiated. If this is the case, the frontal cortex implants might be capable of secreting more of the neurotrophic factors that are required to maintain the metabolic functions that help the survival of neurons in the area of damage.

Based on our histological examination of the host and transplant tissue, we do not think that specific, neuronal connections between the two structures are mediating the functional recovery. In using wheatgerm agglutin-HRP injections into the transplants or adjacent host



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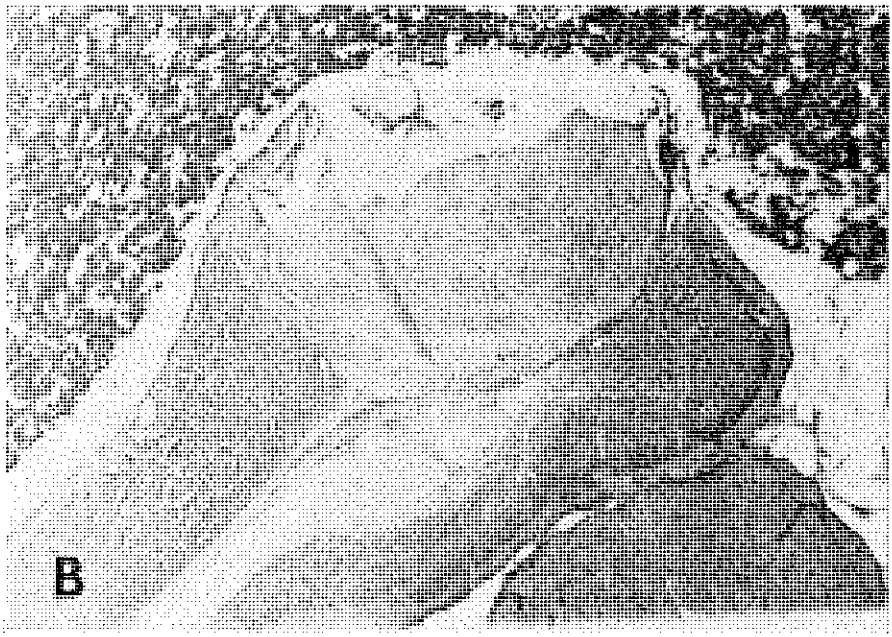


FIG. 7. A. Performance of adult rats with bilateral lesions of the occipital cortex given transplants of fetal occipital or frontal tissue directly into the area of the lesion. The bar on the extreme left of the figure shows the performance of the sham-operated controls. — B. Shows a coronal section (10X) of a frontal tissue transplant that has grown into the

tissue, we could find no evidence of unambiguous anterograde or retrograde transport of this substance. Using Nissl staining techniques, we also counted neurons and glia in the lateral geniculate nucleus of the thalamus in order to determine if there was significant sparing of neurons in those animals that showed behavioral recovery. Here too, we were unable to find significant differences between the lesion-alone group and the animals with either embryonic frontal or occipital implants.

We also analyzed the number and types of cells (both neurons and glia) in the transplants themselves. We counted healthy neurons, seemingly hypertrophied neurons, those that were in various stages of chromatolysis, neurons that appeared to be "healthy" but had arrested development, and glial cells. Except for the fact that the occipital transplants had significantly more glial cells, we could not detect any other unambiguous differences between the occipital and frontal cortex implants.

Yet, the behavioral effects of these two types of transplants were significantly different. Once again, we must conclude that the beneficial effects of brain tissue implants are probably due to their capacity to secrete neurotrophic substances rather than to their ability to reform specific connections that replace those that are lost as a result of injury or disease.

How transplants work to promote functional recovery is still the subject of much discussion and controversy. In one recent paper, Cotman *et al.* [11], stated: "(Transplants) serve primarily in a modulatory manner. They provide an additional source of neurotransmitter (and perhaps trophic factors) delivered in quantity to appropriate receptors" (page 6). In contrast, Björklund *et al.* [6] write that: "The innervation patterns formed in the host brain from the implanted neurons are remarkably specific. The original innervation patterns can be restored with very high precision" (page 59). It may be trite to say that much more research will be necessary before some of these important issues are resolved, but that clearly appears to be the case at the present time.

Although the implantation of fetal brain tissue into the damaged adult nervous system has become almost routine, there are many parameters related to the *effectiveness* of the tissue in mediating behavioral recovery. One question that might be raised is whether transplants are effective as therapies for brain injuries regardless of focus and type of the injury. Another important concern is whether embryonic neuron transplants could be used to slow the degenerative processes that occur in aged subjects or help such individuals overcome the effects of specific and acute damage to the brain such as stroke, space-occupying tumors or ischemia.

Recently, Gage and his colleagues [28] examined the effects of transplants on age-related behavioral impairments in non-injured, old rats. These investigators demonstrated that suspensions of dopamine-containing cells injected into the striatum of senescent rats could result in a significant improvement in motor coordination in tasks such as balancing, climbing, clinging and walking. In addition, they made injections of embryonic brain tissue, rich in cholinergic neurons, directly into the hippocampus of aged rats and then tested these animals for their ability to perform a spatial memory task. Gage *et al.* found that the non-injected old rats showed profound impairments in motor and memory performance in the Morris water maze and there was no evidence of any improvement over time.

In contrast, the rats with injections of embryonic, cholinergic cells showed gradual, but significant improvement in remembering spatial locations that they used to climb out of the tank of water in which they were forced to swim. These results are very encouraging because they imply that suspensions of embryonic cells can be successfully implanted in the aged brain, can survive and slow the appearance of behavioral deficits typically associated with neuronal loss in the aged.

In our laboratory, we were concerned with whether implants of embryonic neural tissue could promote behavioral recovery in aged rats that had also suffered brain damage. We decided to employ the same lesion and testing parameters that we had used successfully in our previous research (see [46]) and examined the effects of implanting fetal frontal cortex into 24-month-old rats who had received bilateral lesions of the frontal cortex. Briefly stated, and in marked contrast to our results with young but mature animals, the implants of fetal tissue into old, brain-damaged rats, were completely without effect. When we examined the brains, our histological analyses revealed that the transplants survived in only 2/12 cases. Thus in the situation where there was both significant aging *and* brain damage, the transplants conferred no benefits.

In another study conducted in collaboration with Bruno Will and Christian Koelche in Strasbourg, France, we examined the effects of transplants on behavioral recovery from bilateral, dorsal hippocampal lesions in adult rats. In this experiment fetal hippocampus (E18-20) or fetal frontal cortex (E18-20) was implanted directly into the cavity created by aspiration of the dorsal hippocampus and overlying neocortex. Transplants were made 7-8 days after the initial surgery and all animals began behavioral training 2 weeks later on an 8-arm radial maze designed to measure spatial and working memory. The rats were tested for their ability to enter each

arm of the maze to obtain some food without repeating an entry into any one of the 8 arms. The animals were again tested 6 weeks and 6 months after the initial training in order to assess both long-term and short-term effects of the transplants.

In this situation, the behavioral results were disappointing. None of the groups with transplants were able to learn (or remember) the radial maze better than rats with lesions alone. While transplants could be seen in the lesion cavity, none of them appeared to be as viable or as large as those seen in other areas of the brain we studied (e.g. the frontal or occipital cortex). While many factors could be called upon to explain our negative findings in this case, the failure to obtain recovery should serve as a cautionary note in considering the clinical applications of embryonic, brain tissue transplants.

First, if the transplants, for whatever reason, do not work in some cases, it obviously represents a certain risk to consider using them for treatments of all brain disorders. In addition to the initial injury, there is the need for additional cerebral manipulation to insert the tissue into the host brain. If the transplant is not successful, there is the additional risk that the new cells could begin to die and release unneeded neurotoxic substances into the already wounded brain.

Second, in the treatment of degenerative, age-related or autoimmune disorders there is a risk that the disease conditions affecting the host tissue could also ravage the newly implanted cells.

Third, in the case of human patients with extensive, neuronal loss or injury, the quantity of embryonic cells needed for replacement might be far in excess of what is required to re-establish appropriate connections, — if such connections are needed to mediate functional recovery. In this context, it is important to note that many human behavioral disorders involve many brain systems, and under such conditions it would be difficult to know where and how many fetal cells to inject.

Although fetal tissue implantation research is already an invaluable tool for neuroscience research and holds much promise for therapeutic treatment of brain injury, there may be other alternatives holding equal promise but with less risk for the patient [25]. Obviously, one such approach would be to inject substances systematically that could aid the recovery process. Intravenous, intraperitoneal, or intramuscular injections or even intraventricular administration of, for example, neurotrophic factors are one such possibility.

Since our laboratory has been concerned with recovery from brain

injury for almost two decades, we became very interested in recent reports demonstrating that gangliosides, a group of sialic acid-containing glycosphingolipids, may be important in promoting anatomical and behavioral plasticity in brain-damaged subjects.

For example, nervous tissue is particularly abundant in endogenous gangliosides [48], and when applied to cell cultures containing developing neurons, gangliosides increase cell survival and stimulate neurite outgrowth (see [14, 71, 72] for examples). Following damage to peripheral or central nervous system, exogenous ganglioside administration has been shown to stimulate sprouting into denervated structures [8, 33, 77]. Gangliosides also seem to play a role in development of the nervous system where their presence and quantity appears to correlate with the outgrowth of dendrites and the establishment of neuronal connections [4, 17, 89]. One of the more important and beneficial features of gangliosides is their potential use as a therapeutic agent in the treatment of brain injuries. Unlike Nerve Growth Factor, which needs to be injected intracerebrally to affect recovery (see [34, 80] for details), gangliosides have the ability to pass the blood-brain barrier in small amounts [61, 83] and are incorporated into neuronal brain membranes and facilitate the effects of neurotrophic substances in culture [50].

With respect to behavioral recovery, much less is known about the effects of exogenously administered gangliosides. However, there is evidence that chronic, systemic injections can improve the rate of postsurgical recovery from septal lesions [60], unilateral lesions of the entorhinal cortex [44], or lesions of the nigrostriatal pathway [86]. For the most part, behavioral studies have relied upon either unilateral lesions or relatively simple measures such as activity, spontaneous alternation in a T-maze or stereotyped rotational behavior.

In our experiments [73], we decided to address the question of whether systemic injections of GM1 ganglioside (provided by Fidia Pharmaceuticals) could enhance recovery from the often *severe and very long-lasting* learning impairments that accompany bilateral, electrothermic lesions of the caudate nucleus in adult rats. In a spatial reversal task, animals with caudate lesions tend to perseverate previously learned responses; i.e. they are not capable of switching from going to the left to going to the right in a maze when reward (or punishment) contingencies are changed.

Three groups of rats were formed in which one group served as sham-operated controls (who were given intraperitoneal saline injections for 14

days), one group received bilateral caudate lesions followed by 14 days of i.p. saline injections, and the third group received caudate lesions followed by 14 days of 33 mg/kg of purified GM1 gangliosides administered i.p. Postoperative testing began 9 days after surgery had been completed.

Each animal was trained to avoid shock by running to its initially non-preferred side in a two-choice discrimination-learning apparatus. When the animals learned to escape (or avoid) shock in 9/10 trials for two subsequent days, the correct side for shock/avoidance was reversed. In this manner, the animals underwent a continuous series of spatial habit reversals for 30 days of testing or 300 trials. Ninety days postoperatively the rats were retested on the same task for 14 days. We measured escape, avoidance and perseverative behaviors for all animals.

On each measure of learning we observed that the brain-damaged rats with chronic ganglioside treatments did significantly better than their untreated counterparts (see Figure 8 A, B). However, the treatments did not permit the rats with caudate lesions to perform as well as the intact controls. Ninety days after surgery, when the animals were all retested, the differences between the groups remained; the ganglioside-treated rats showed better retention than untreated animals.

Our results showed that even after massive, bilateral brain injuries (see Figure 9), exogenous administration of GM1 ganglioside could substantially improve postsurgical performance of a cognitive task. By what mechanism(s) did ganglioside treatments produce their effects? In this study, the extensive, bilateral damage we created in the caudate nucleus made it very difficult to assess the specific anatomical alterations that could have mediated the behavioral recovery we observed. Consequently, we decided to use a unilateral lesion that would provoke a specific and easily measurable "deficit" that had been shown to be altered by ganglioside treatments. Thus, with unilateral lesions, we could study the possibility that anomalous sprouting from the contralateral hemisphere, or enhanced survival of remaining neurons projecting to the damaged zone, would be implicated in the recovery.

Toffano and his colleagues [86] had already shown that ganglioside treatments can attenuate amphetamine-induced, stereotyped turning in rats with unilateral transections of the nigro-striatal pathway. These authors also showed that tbe treatments would restore dopamine uptake and tyrosine hydroxylase activity on the damaged side of the brain [86]. We wanted to replicate and extend Toffano's work and also show that the neuronal morphology of the injured hrain could be affected by ganglio-

side treatments [72]. Specifically, we wanted to measure ganglioside-induced behavioral recovery following hemitransection of the nigrostriatal pathway and then determine whether neuronal sparing or repair could be demonstrated with horseradish peroxidase technique. In this experiment, we unilaterally transected the nigrostriatal pathway in adult rats and gave daily intraperitoneal injections of 30 mg/kg of GM1 ganglioside i.p. There were six groups employed in the study, including saline injection controls. The groups consisted of brain-injured or sham operated control rats that were permitted to survive for 3, 15 or 45 days after surgery. The

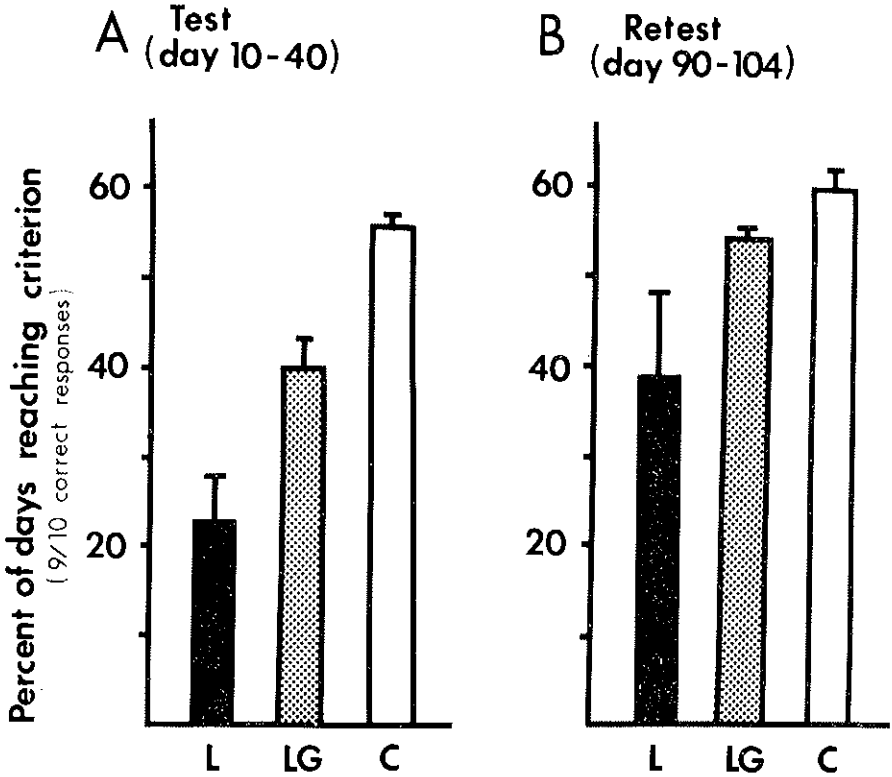


FIG. 8. Behavioral performance of animals with lesions of the caudate nucleus given i.p. injections of saline (C) or GM1 gangliosides for 14 days after surgery. The graphs show the rat's ability to learn to solve a spatial reversal task to avoid footshock 10-40 days after injury and then again at 90-104 days after treatments. The black bar represents the lesion alone group; the hatched bar shows performance of rats with lesions followed by ganglioside treatments and the open bar shows performance of the controls.

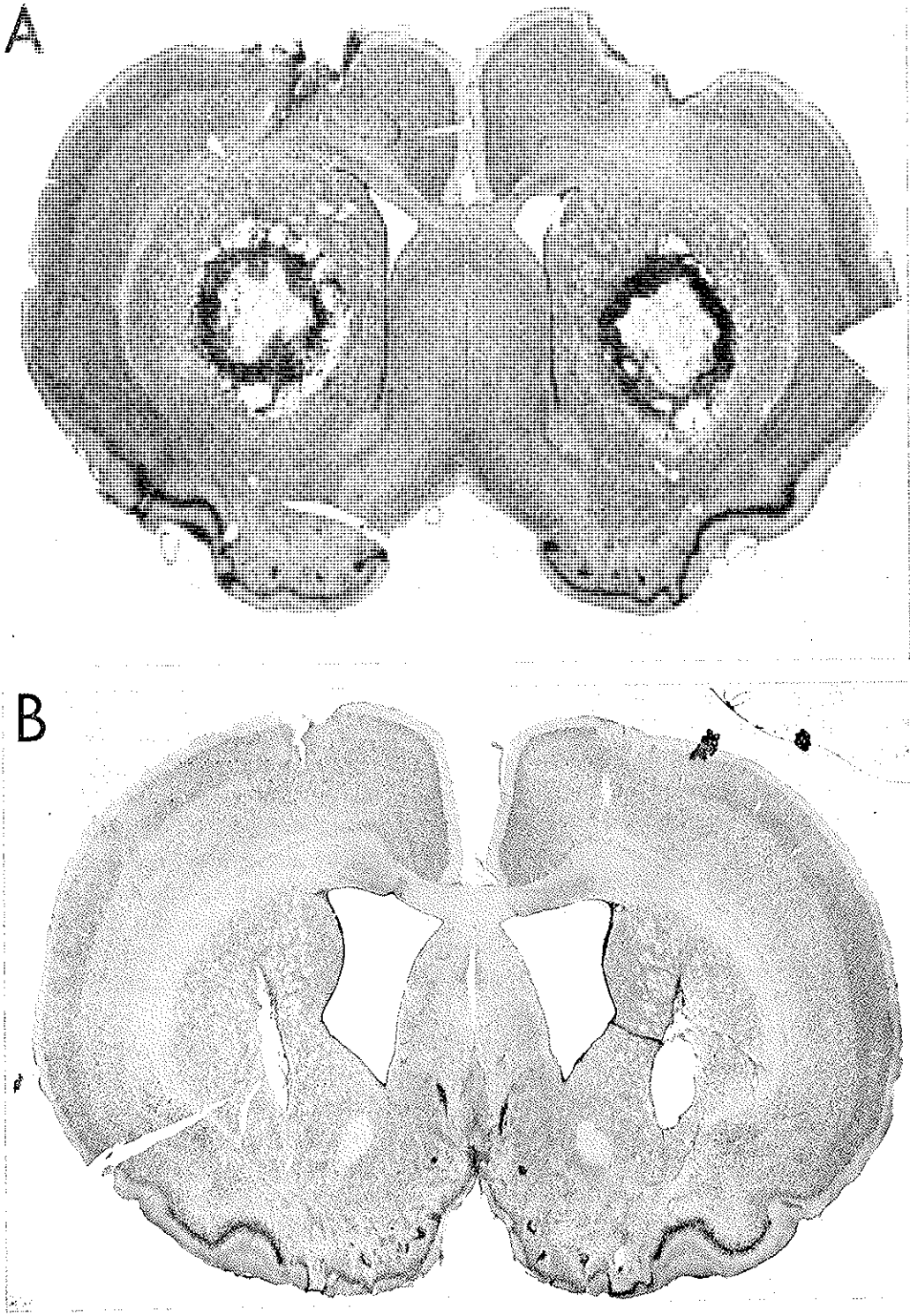


FIG. 9. A. A photomicrograph of the brain of an animal that received bilateral radiofrequency lesions of the caudate nucleus. This picture was taken 5 days after the injury. — B. Photomicrograph taken

day before they were killed, the animals were given a unilateral injection of wheat-germ agglutin-HRP directly into the caudate nucleus.

Rotational behavior was measured by harnessing the animals to a mechanical rotation counter that could distinguish between direction of movement. Each animal was placed in a spherical "rotimeter" for two hours following intraperitoneal injections of d-amphetamine sulfate or apomorphine.

Analysis of the rotational behavior showed that ganglioside treatments significantly decreased amphetamine- or apomorphine-induced rotation (see Figure 10). With respect to amphetamine, a significant reduction in rotation was seen within two days after nigrostriatal transections in the animals receiving GM1 treatments. This difference between the treated and untreated groups was still apparent 12 days later, and disappeared by 40 days after the injuries. This was due to the fact that the untreated rats also showed a decline in amphetamine-induced rotations over time. In contrast, animals given GM1 and then challenged with apomorphine were not initially different from saline-treated rats; however, by 42 days after the treatments, there was a marked increase in rotation in the saline-injected animals and no change from baseline in the GM1-injected rats. Apparently, the former group "grew into" the deficit whereas ganglioside-treated animals did not. The data from both the amphetamine- and apomorphine-treated rats can be taken to indicate that chronic ganglioside treatments facilitate recovery of function following nigrostriatal lesions by either restoring baseline levels of dopamine neuro-transmission (GM1-reduced rotation following amphetamine injections) or by alternating postsynaptic receptor sites (GM1-induced reduction in rotation following apomorphine injections), or both. The results of both experiments suggest, but do not conclusively indicate, that the recovery of function we observed is mediated by structural changes in neurons that project from the brainstem to the striatum.

We reasoned that if ganglioside treatments enhanced either the sparing or sprouting of neurons following nigrostriatal transections, we should be able to see: (a) greater numbers of neurons in the ipsilateral ventral tegmental area (iVTA), or the ipsilateral substantia nigra, pars compacta (iSNc); (b) enhanced "sprouting" of neurons in the contralateral substantia nigra, pars compacta (cSNc)). As mentioned above, we used horseradish peroxidase (HRP) labelling to assess the morphological changes. The pressure injections of HRP were made directly into the caudate nucleus ipsilateral to the nigrostriatal transection. The HRP is transported via retrograde,

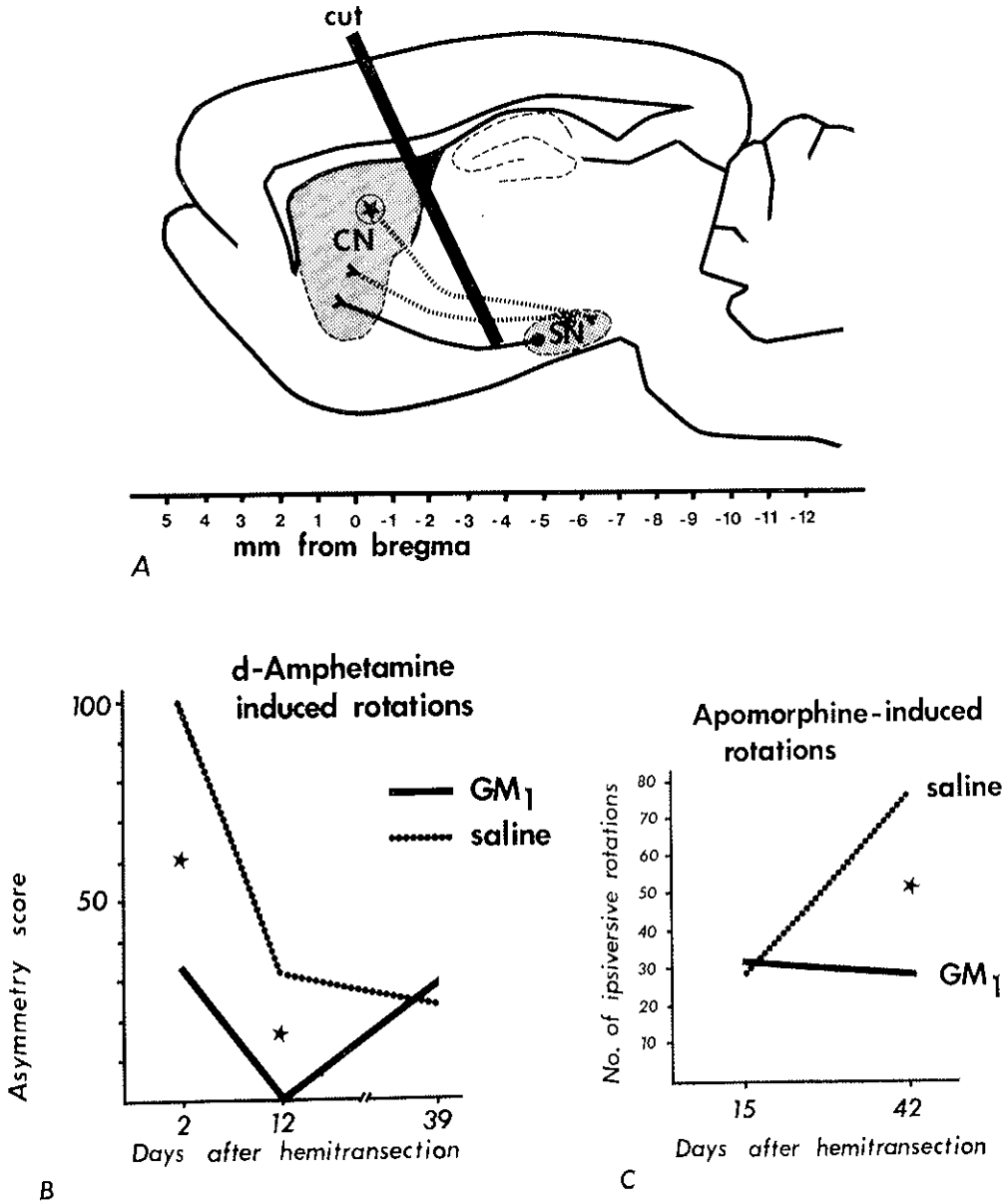


FIG. 10. A. Cartoon of sagittal section of the rat brain showing the transection of the nigrostriatal pathway. — B-C. Amphetamine-induced and apomorphine-induced rotations in rats with nigrostriatal lesions given injections of GM1 or saline following the injury.

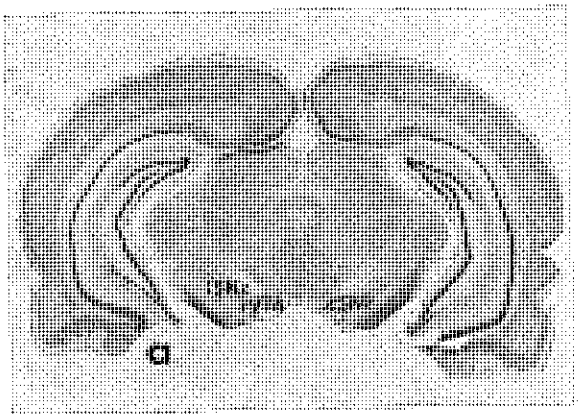
axonal transport to the neurons of structures that project directly to the caudate such as the iVTA and iSNc. There is also a sparse projection from the contralateral SN. Thus, the number of HRP-positive neurons in the structures that afferent the CN can be taken as a measure of neuronal sprouting or sparing, when the cell counts are compared to normal rats or to those with nigrostriatal transections given injections of saline instead of GM1.

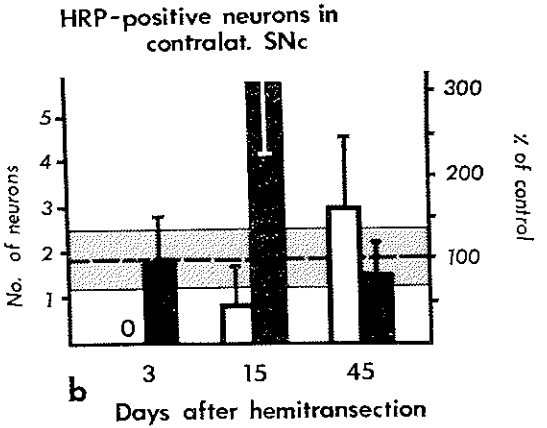
First, within three days after surgery, both GM1-treated and saline-treated rats showed a major loss of connections to the caudate nucleus (Figure 11). However, the contralateral connections from the cSNc were retained in the GM1-treated rats and completely lost in the lesion controls. By 15 days postsurgery, significantly more HRP-labelled cells could be seen in GM1-treated rats than saline-treated counterparts. In fact, the number of HRP-labelled neurons actually exceeded that of the unoperated animals in the iVTA and cSNc. By 45 days after surgery, the HRP labelling of neurons in the areas we examined was the same for both the GM1 and saline-treated animals. Figure 12 is a montage of photomicrographs showing the labelling in GM1-treated and saline-treated rats at different times of postsurgical survival.

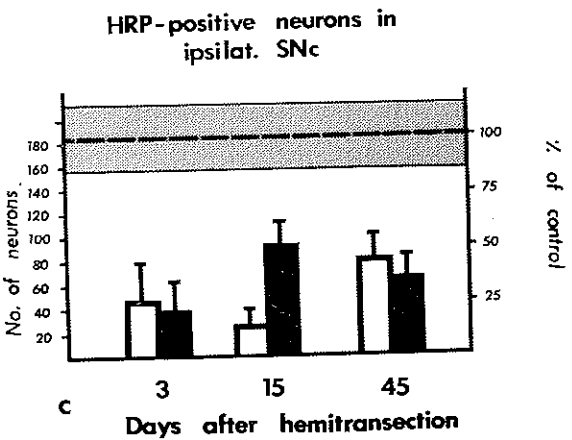
Although we tend to favor the hypothesis that ganglioside treatments enhanced lesion-induced sprouting in the nigrostriatal system, there are other hypotheses which can explain our data. For example, one such possibility is that the hemitransections we created spared some of the cells of origin of the nigrostriatal pathway and the GM1 treatments promoted their survival. We did, in fact, note a highly significant increase in HRP labelling in the GM1-treated rats, but this labelling had returned to the same level as saline-treated, hemitransected rats by 45 days after the injury. This increase in labelling followed by a decrease would suggest that the GM1 produced alterations in axonal transport rather than sprouting per se.

Recent evidence [86] tends to support this possibility. Toffano's group found that ganglioside treatments are ineffective in elevating tyrosine hydroxylase activity in the denervated striatum after total, rather than partial lesions of the substantia nigra. In a different set of experiments, Fass and Ramirez [18a] found that gangliosides enhance behavioral recovery of activity following entorhinal cortex lesions but their anatomical analyses showed an actual decrease of sprouting in their ganglioside-treated animals. Thus, the gangliosides may work to promote survival of neurons rather than by promoting new growth.

In summary, then, there is now fairly compelling evidence that ganglio-







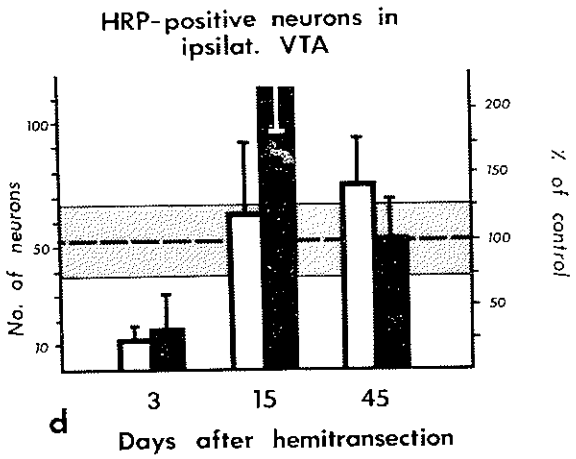


FIG. 11. a. Coronal section through the level of the substantia nigra showing HRP-labelled cells which were counted in the ipsilateral substantia nigra (iSNc) and ventral tegmental area (iVTA) and in the contralateral substantia nigra (cSNc). — b-d. Represent counts that were made at 3, 15 or 45 days after the nigrostriatal hemitransection White bars represent animals treated with saline; black bars, animals treated with GM-1. The horizontal grey bars show neuron counts in intact animals (average and s.e. of the mean).

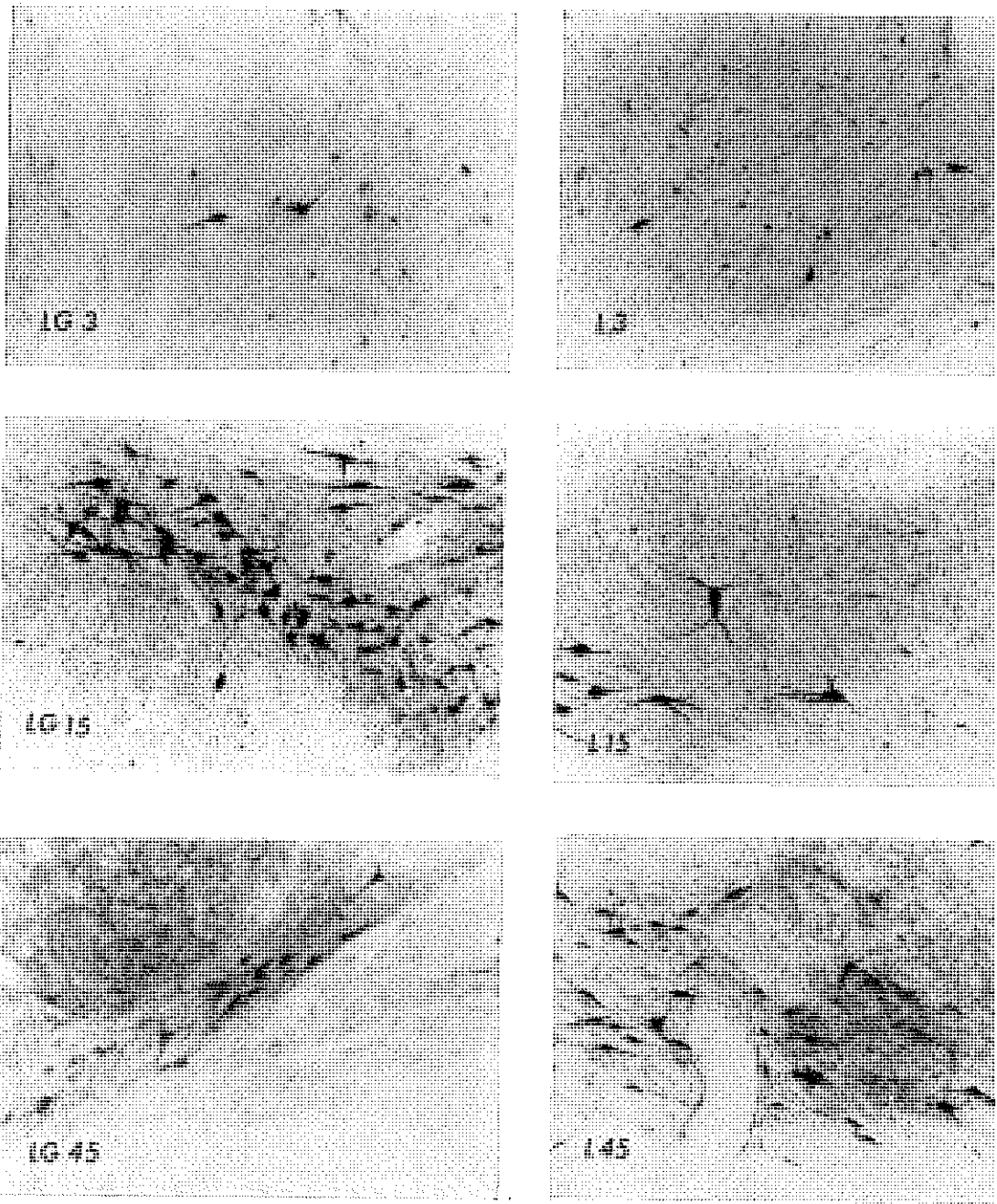


FIG. 12. Representative photomicrographs of HRP-labelled cells in substantia nigra, pars compacta, ipsilateral to the HRP injection. The micrographs on the left are X50 magnifications of SN cells in saline-treated rats with the same transections of the nigrostriatal pathway. Only a few labelled cells can be seen in either group by postoperative day 3. By 15 days there is heavier labelling in the ganglioside-treated rats than in saline-injected counterparts. By 45 days survival both groups show heavy labelling of neurons.

side treatments do in fact, promote neuronal plasticity and behavioral recovery although there may be different mechanisms by which this substance can alter the response to CNS injuries.

While not every damaged neural system may be amenable to ganglioside treatments, at least there is sufficient evidence for recovery to warrant more research into the specific parameters required to promote complete functional recovery. For example, would gangliosides combined with "neurotrophic" substances such as those isolated from brain-wound areas, be more likely to promote functional recovery than either substance alone? Should gangliosides be combined with fetal tissue implants to enhance the extent of functional recovery? Would gangliosides be more effective if they were injected directly into the zone of injury or into areas whose cells afferent the damaged zone? Would aged subjects be as likely to show ganglioside-induced recovery from acute or degenerative neuronal loss as younger counterparts? These are a few of the kinds of questions that could lead to exciting and important research in this area.

Conclusions

Throughout this chapter, I have attempted to focus on one major theme — that the central nervous system is far more capable of responding adaptively to injury than has been previously imagined. In working with tissue prepared for anatomical analysis, it is easy to get the impression that everything under the light of the microscope is static and unchanging; that a detailed analysis of the brain's fixed circuitry would reveal to us all that we need to know about function in a precise, quantifiable and objective manner. But the brain is far from being fixed and immutable; rather it should be seen as a system constantly in a state of flux. As organisms progress from one end of the developmental spectrum to the other, the "rules" governing organization or the response to injury and disease may change, but this is not cause for pessimism. Rather, we should see the fact that we do not know all of the answers as an exciting challenge for future research.

The key point of all of the experiments that I have reviewed in this chapter is not that there are complex and difficult questions that have yet to be answered, but rather that there is great hope that we can eventually unlock those secrets that stand as the last impediments to providing respite and hope to the victims of severe nervous system injuries. Almost daily, we see in the scientific literature, new examples of techniques or

potential therapies that appear to be effective in promoting some degree of anatomical and behavioral plasticity following brain damage. One fact is now certain. We can no longer accept the outdated view that the adult nervous system is incapable of adapting to injury or that there is no real hope for the victims of brain and spinal cord injuries.

ACKNOWLEDGEMENTS

I would like to thank Arthur Firl, Randy Labbe, Gary Dunbar and Bernhard Sabel for the many contributions that they have made to the research conducted at Clark University. The preparation of this review and the research has been made possible by the support of the American Paralysis Association, the National Institute of Mental Health Contract #R1MH39514A and Fidia Pharmaceuticals, Abano Terme, Italy. Dr. Elliot Mufson of Beth Israel Hospital has graciously provided the photomicrographs and many of the anatomical analyses of the transplant experiments. Dr. Mufson was supported in this work by an ADRDA Faculty Scholar Award. I would also like to thank Ms. Crystal Pantazis who has tirelessly typed the various versions of the manuscript.

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TRANSPLANTATION STUDIES OF THE DEVELOPING VISUAL SYSTEM: A REVIEW

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INTRODUCTION

The many detailed anatomical studies of the past 25 years have shown that the patterns of interconnections of neurons in the central nervous system while complex are nevertheless quite predictable and precise. This precision is further emphasized by the consistent physiological response patterns which can be elicited from specific neuron types, and by the uniform behavior patterns elicited by certain clearly defined stimuli. These apparently rigid patterns arise as a result of a developmental program in which factors external to the individual neurons are important in determining their final organization and function and many instances have been demonstrated in which modulation of such factors during development substantially alters anatomical connections and associated physiology and behavior. How far neural development can proceed without particular extrinsic cues and exactly how such cues modulate neural development is a central problem of neurobiology. At one extreme, the problem can be approached at the cell and molecular level to define molecular controls of neurite outgrowth, specific cell adhesion patterns, recognition molecules and others. However, neurons function as communities and while molecular approaches define the necessary substrates for development, they may be less helpful in advancing our present understanding of how functional assemblages of neurons are formed. At the other extreme, then, developmental neurobiology is closer as a discipline to that of population

biology. Most developmental studies in fact lie somewhere between these two extremes.

There are many ways of studying neural development. These include defining the developmental process in the intact animal, examining the effects of perturbations, making computer simulations of development, and studying the properties and interactions of single neurons or groups of neurons explanted *in vitro* or transplanted to other brain regions or animals.

I will devote this review to a discussion of the last, specifically with respect to our work on transplantation of embryonic rat central nervous system, mainly of regions subserving visual function, to the brains of newborn rats. For such studies to address developmental questions, a set of conditions must be observed.

First, it is important to know a lot about the normal sequence of development of the system, so that the stage of development at the time the donor tissue is taken for transplantation is known as is that of the recipient tissue. In addition, some of the transient events which occur during development are clearly amenable to investigation using transplantation, and it is therefore valuable to know exactly when they are manifest.

Second, it is important to know whether the transplantation procedure significantly interferes with the developmental process. Is there massive cell death shortly after transplantation? Do particular cell types die as a result of the transplantation process? Can cell division continue in a normal sequence? Are the transplants rejected by the host or do they suffer other effects of being recognized as "non-self"?

Third, it is ideal for transplantation studies to have parallels with *in vivo* and *in vitro* experiments.

Fourth, it is important that the system studied has a number of features significant to its organization and function, which are relatively easy to assay after experimental manipulation.

Fifth, it is helpful if a normal functional pattern can be elicited either physiologically or behaviorally, since the ultimate goal of any developmental sequence is to provide an optimally working system.

Finally, while we have directed our attention principally to developmental issues, it is always important to bear in mind the relation of this work to the question of whether transplantation techniques may be of value in replacing damaged or defective nervous tissue and to the matter of immunological privilege in the central nervous system.

Normal Developmental Patterns

The development and organization of the visual system of rodents, including rat and mouse, have been studied in considerable detail. Briefly, retinal ganglion cells are generated between embryonic days E12 and 18 in rats. The first optic axons to reach the optic chiasm do so on E15 and can be traced shortly thereafter into the contralateral tract by which they reach the superior colliculus on E16 and with little delay form small numbers of synapses with tectal neurons [1, 26]. There is a gradual increase in synaptic numbers over the first postnatal week [30]. Retinal ganglion cells first receive synaptic input on postnatal day 10, an ERG develops between days 12 and 14 [58], and the eyes open on about day 14. At this point, there is an elaboration of synapses in the colliculus with more optic synapses, more synapses per terminal in a single electron microscopic section and more complex synaptic arrangements involving serial synaptic arrays [30]. This corresponds to the period in hamsters (Schneider - this volume) during which there is an increased complexity of optic axon terminal arbors.

The uncrossed optic pathway is first detected in the optic tract slightly later than the crossed pathway. It reaches the superior colliculus on E17 and ramifies over its whole area. This is followed by a period of retraction during the first postnatal week [25], to its normal patchy anterior location which occurs at a time when more than half of the retinal ganglion cells die [6, 8, 44].

The process of restriction of the uncrossed pathway can be partially reduced by unilateral eye removal before the retraction process [25] and also appears to be modified by injecting TTX into an eye [6]. The presence of transient projections in the developing rodent visual system is not restricted to the uncrossed pathway. There is suggestion that the topographic distribution of retinal axons upon tectum is more diffuse at earlier times [42]. There is also an exuberant callosal projection, the pattern of retraction being substantially modified by manipulation of subcortical optic pathways [9, 28] and finally there is a transient pathway from occipital cortex to spinal cord which disappears during the first two postnatal weeks [40, 56].

Although this discussion has been restricted to rat, studies on mouse [13], hamster [47, 55], cat [2, 19, 50], and monkey [45] indicate that many of these events occur quite generally.

The two sets of *in vitro* studies which are relevant to transplantation

experiments involve on the one hand the interactions between explanted lumps of retina and various regions of the brain, and on the other, the conditions necessary for retinal ganglion cell survival. Explant studies show that outgrowth from retinal explants is specifically directed towards tectal explants [7, 53, 54]. There is a semblance of spatial order between the origin and termination of outgrowing retinal axons [52] and the axons form functional synapses in the tectal cell masses. The ganglion cell survival studies (Nurcombe and Bennett [41] and this meeting) show that supernatant derived from tectal neurons has a specific trophic effect on retinal ganglion cells in dispersed cultures.

Transplantation of Embryonic Nervous System

Our present transplantation studies fall into three groups. In the first, we have examined whether tissue transplanted close to a region of the host brain with which it would normally connect (a) differentiates normally (b) makes normal connections and (c) makes connections which are functionally active.

In the second group, we have studied how differentiation is affected if transplants are placed in regions distant from those with which they would normally interconnect. In the third group of experiments, we have begun to investigate how to examine early development of transplant/host interconnections and the behavior of small groups of transplant cells embedded in a host brain.

These will be reviewed for the categories defined above and then discussed in more depth.

Transplantation Adjacent to Related Host Brain Regions

Differentiation. All the studies conducted so far show that the undifferentiated tissue taken at the time of transplantation subsequently develops many of its normal characteristic features. Thus retina taken at E14 or 15, at a time when few ganglion cells have undergone their terminal division, differentiates into a laminated structure typical of normal retina with at least qualitatively normal synaptic patterns in the plexiform layers and characteristic cell classes in the ganglion cell and inner nuclear layers [36, 39, 43]. Outer segments are present and can be demonstrated both electron microscopically and using antibodies specific

for receptor outer segments. With antibodies specific for cones, a small percentage of outer segments are stained, as in a normal intact retina (V. Lemmon, K. Rao and R. Lund, unpublished observations). If the retina is damaged during transplantation, if it is maintained *in vitro* for any period prior to implantation [32] or if it is dissociated and reaggregated before transplanting [33], there is a tendency to form rosettes rather than a continuous sheet, but even under these conditions, the laminae are still recognizable.

The laminar order of cortex and tectum is very much more dependent on the care with which the tissue is transplanted, but under ideal conditions it is possible to achieve a structure showing the laminar pattern of the intact region [4, 29]. Detailed studies on histogenesis have so far been limited to cortex, but they show that this does not seem to be affected by the transplantation procedure [21]. Examination of tissue shortly after transplantation shows that if young donor tissue is used and the transfer of tissue to the host is done with care, there is very little cell death in the transplant. We have also failed to find evidence of immunological rejection of transplanted tissues in these studies.

This section emphasizes, therefore, that much of the structural organization of several regions of the visual system can develop unimpeded by transplantation. The more careful the process of tissue transfer, the more likely it is that the tissue will develop a normal organization. Thus a major concern that pathological events may confound interpretation of results in developmental terms seems without foundation.

Connection patterns. In general, regions placed close to normal afferents or targets tend to make relatively normal connections. Thus tectum placed adjacent to tectum receives input from more than 40 different regions of the host brain, all regions normally projecting to tectum [16], while retina placed in a similar position, although receiving little or no afferent supply projects only to normally retinorecipient nuclei of the brainstem [35]. There are, however, several additional points to be made. First, the afferents to the tectum are not all of equal density, the heaviest being from regions which either develop late or project to the superficial layers. Second, the retinal transplant efferents are heaviest if the host optic projection is removed at the time of transplantation [35] and third, efferents from tectal transplants seem more restricted than those of the host tectum [57]. In each case, there appears to be a relation to the maturation of host pathways and in the case of transplant efferents, the

suggestion of a competitive interaction between host and transplant pathways for the same terminal sites.

A third point, derived from studies in which left or right tectum was placed on one or other side of the midbrain, is that laterality is not recognized by the transplants. This might be expected from a variety of experimental manipulations *in vivo* [10, 49]. Topography of retinotectal relations between transplant and host has not been clearly demonstrated and indeed present evidence would suggest that it is non-existent. This may be because transplants normally connect by several bundles with the host brain and even if the axons in each bundle were ordered in a logical fashion, they may interfere with one another as they attempt to map relative to polarity markers in the tectum. Another more expansive view of topography concerns the segregation of the cortex into areas, each of which has a characteristic set of connections. Is each region defined at an early stage and connections fit into predefined patterns or is perhaps the segregation of cortical areas a property of the afferents? We have begun to examine this by transplanting cortex from rat embryos aged E16 or earlier at an age when thalamic afferents have yet to arrive in various regions of host neonatal rat cortex [31].

In experiments in which donor cortex was placed on top of or embedded in host visual cortex [3, 4], we have found that thalamic afferents arising in the lateral geniculate do not innervate the transplants, although thalamic nuclei such as the lateralis posterior which innervate adjacent cortical areas often do project into the transplants. Callosal axons from the contralateral cortex innervate transplants in a manner similar to that of the underlying host cortex. Connection patterns are similar whether the transplant is derived from occipital or frontal cortex. Long distance efferent projections from transplants seem to be minimal unless the transplants have contiguity with the cortical white matter [11, 23].

Thus it would appear that regional specificity plays some part in determining patterns of interconnections between transplants and hosts. The relative density of innervation by individual pathways and the topographic distribution of axons may be disturbed in the transplantation procedure, perhaps because of mechanical factors, geometry of interconnecting pathways and timing.

Functional interrelations between host and transplant. This has presently been examined in two studies. In the first [15], tectal tissue from E16 rats was placed over the superior colliculus of neonatal rats and allowed to mature and make interconnections with the host brain. Stimulat-

ing electrodes were placed in the visual cortex and a recording electrode in the transplant. In 34 penetrations made through the transplants in 12 animals, 300 active units were isolated, about 12% of those tested being driven by cortical stimulation. The most effective region for driving cells was located along the border between areas 17 and 18a, the region from which the heaviest projection was found using anatomical techniques [16].

In the second study [51], we transplanted retina over tectum and a month or more later exposed the transplant and flashed a light at it, having previously taken the precaution of removing the host optic input. Gross potential responses were recorded from the transplants which were graded in magnitude according to the intensity of the light. Unit responses recorded from the tectum were highly specific. Some responded to light on with a short burst of activity, others with a cessation of spontaneous activity. Others responded to light off and yet others to ambient light levels. These have counterparts in the intact colliculus driven by the regular optic input [12, 18].

These results indicate that transplant cells are both driven by and themselves drive cells in the host nervous system. Whether such physiological activity can form the basis of a behavioral interrelation between transplant and host remains to be investigated.

Transplantation to Unrelated Host Brain Regions

Such experiments fall into a number of different categories from extreme cases in which the implant is placed in a completely foreign location such as retina in cortex or spinal cord to situations such as transplantation along the course of transient host projections and to regions where there is opportunity to make only a small number of normal afferent and efferent connections. These will be dealt with separately.

Transplantation to foreign locations. Retina placed in cortex [37] differentiates a laminar pattern similar to retina placed near the tectum and shows synaptic patterns typical of each plexiform layer. However, in the absence of an appropriate target, there is no indication of axons leaving the transplants and cell size histograms suggest that there are no ganglion cells present in the ganglion cell layer. A similar appearance is found in retina placed in the spinal cord [35], despite the expectation that the optic axons might find the primary somatosensory pathway to have features in common with the primary visual pathway and despite

positive findings for a comparable experiment performed in non-mammalian vertebrates [24].

Transplantation to the course of a transient pathway. It has been shown [40, 56] that the occipital cortex sends axons to the spinal cord in young rats, but that this projection disappears by two weeks postnatal. We were able to confirm the early presence of this pathway although it appeared to have a somewhat more restricted origin, deriving originally from the border zone between areas 17 and 18a. When tectal tissue was transplanted to the spinal cord at birth and the host tectum removed, cells persisted in this region of the cortex with projections extending into the spinal cord [48].

Transplantation to a region with little access to normal afferents and efferents. Cortex placed over the superior colliculus [22] lies close to two normal targets — superior colliculus and pretectum — which it frequently innervates. In addition these transplants show a number of projections not normally characteristic of cortex that go to regions such as the mesencephalic central grey matter. There are few host afferents to these cortical transplants and these are in low density. Some regions such as locus ceruleus and host cortex may be considered normal although they come from parts of the output in each case which would not normally innervate cortex. Otherwise afferents come from regions which would normally innervate tectum but not cortex. It should be emphasized, however, that these inputs are very small compared with those to tectal transplants placed in the same position [16].

This section shows, therefore, that transplants survive when placed in anomalous locations, but their capacity to form connections with host neurons seems to vary depending on the region transplanted. It appears that when connections are made which are typical of the region transplanted, they are of low density compared with the connections made between matching host and transplant.

Ways of Examining Developing and Embedded Transplants

Two major questions which have not been addressed concern the behavior of transplants embedded in the host brain and the early stages of maturation of transplants [16]. The last is extremely important in the present context, because it is clearly dangerous to propose developmental mechanisms while examining only the final stable outcome of an earlier experimental manipulation. Various approaches have been used.

One involves soaking the tissue to be transplanted in a solution containing a tritiated amino acid. While interesting results have been obtained this way [23, 38], there are limitations which relate to the mobility of the proteins into which the amino acid is incorporated. This results in diffusion of label from the transplant into adjacent host tissue and therefore permits clear identification of only the largest pathways. In addition, the label is diluted with time, limiting this approach to short survival experiments. A second approach [5] has taken advantage of antibodies to the two allelic forms of the antigen Thy-1, which is found on neuronal cell surfaces. Since different strains of mice express one or other allele, it is possible to demonstrate tissue from one strain transplanted into the brain of another with appropriate choice of donor and recipient using the appropriate antibody. The limitations of this approach for the present studies are that Thy-1 is only weakly expressed in immature nervous system, so limiting its application in developmental studies [46]. Furthermore, the antibodies are best demonstrated in unfixed tissue, which limits the resolution of study.

We have followed a similar strategy, however, by taking advantage of another cell surface antibody, M6, which is specific to neurons of the mouse CNS. It is heavily expressed early in development and can be demonstrated in paraformaldehyde fixed tissue both in light and electron microscopic preparations [27]. Studies presently in progress show that it is possible, using this technique, to examine cell migration and early axonal outgrowth patterns from transplant to host, as well as the efferent connections made by transplants embedded for some period of time in a host brain [14, 27].

The use of species-specific antibodies is clearly not limited to the combinations defined here, and with appropriate screening it should be possible to identify others which may be more satisfactory for other experimental circumstances. Indeed, recent work has identified an antibody to quail cells which permits them to be identified after transplantation to chick (C. Lance-Jones and C. Lagenaur, unpublished observations). The matter of immunological incompatibility does not seem to he a problem if immature animals are used as recipients, and recent studies have shown that mouse transplants survive in adult rat brains if the recipients are treated with cyclosporin [20].

It is clear therefore that this approach holds considerable promise for examining with greater sophistication the host-transplant interactions occurring during development.

Discussion

When we began working with transplantation procedures some 12 years ago, we knew very little about the conditions for success. We did not know what was the best donor or recipient age, whether transplants would die, whether cells generated pre- or post-transplantation only would survive, and whether there might be complications from tumorous transformations or immunological rejection. We also had to adapt standard labelling techniques for examining transplant/host interactions. At a biological level, we did not know whether indeed the preparation would be useful for addressing development questions. Work done over the intervening years in this and other laboratories has shown that the technique is a useful one added to the armamentarium of approaches used to examine neural development.

From the summary given here it is clear that embryonic neural tissue transplanted to ectopic positions in a developing host brain survives and develops much of the internal organization by which it would be recognized *in situ*. The connections formed with the host brain are elaborate and depend both on the donor region and the site to which the tissue has been transplanted. It is apparent that at least some host-transplant interconnections are functional. Pathological events are minimal and there is little or no evidence for immunological rejection. However, it is important to ask whether the work done so far has given any indication that this approach will permit us to learn more about the determinants of development, and to anticipate future research directions.

Perhaps the most important observation made so far is that transplanted tissue if placed close to a region of the host brain with which it will normally connect can make quite precise connections with that region. This occurs despite the age-difference between transplant and host and the abnormal course followed by the interconnecting axons. Thus, it would appear that *in vivo* there are special affinities between certain populations of neurons which are not overridden by temporal or spatial factors. There are nevertheless many subtleties which do appear to be affected by the conditions of transplantation. Thus the heaviest and most predictable afferent projection to tectal transplants placed over the tectum comes from the most immature pathway at the time of transplantation, suggesting that the vigor with which a pathway invades a transplant depends not only on special affinities but also on its state of maturation, determined perhaps by its intrinsic growth potential, how

many synapses it has already formed and other factors. Like the afferents, the density of host innervation by the transplant also seems to relate to the maturation of competing host pathways. This is well shown in the retinal transplant innervation of the superior colliculus which is much reduced if there is also an input to that colliculus from the host eye. It would be of some interest to know whether a transplant made at an earlier developmental stage when the host optic projection is less mature would innervate relatively more of the host neuropil. The interactions between transplant and host retinal afferents in the tectum and the effects of eye removal have a parallel in developing animals in those occurring between the inputs from each eye despite the fact that transplant and host afferents vary by 1 week in age while competing host afferents in intact animals are obviously the same age.

It appears, therefore, that the relative timing of events can play a part in how much of a particular region is innervated, but no evidence has been assembled to show that it can alter specificity of innervation.

Similarly the route followed by retinal transplant axons to the superior colliculus does not affect their ability to innervate the appropriate layer although it could cause disruption of such features as the regular topographic order. Experiments in non-mammalian vertebrates in which axons are forced to enter the tectum by an anomalous route often show re-establishment of normal topographic order, and this would appear to differ from the present study. This may be because in our experiments, the transplant connects with the host by many fiber bundles. It is possible that each bundle contains some semblance of order, but that because there are so many an overall map on the tectum becomes obscured.

A further feature which has emerged from these studies is that different axon populations placed in the same region appear to follow different substrates. For example, host retinal axons grow on the surface of tectal transplants and similarly retinal transplants emit axons, which tend to grow on the surface of the brainstem. In each case, this mimics the normal growth pattern of retinal axons, and contrasts with cortical and tectal transplants whose axons penetrate the brainstem. It is tempting to suggest that retinal axons, therefore, follow surfaces while the others follow some feature of the brain parenchyma. There is, however, a notable exception in that retinal transplants when placed in the aqueduct send axons radially through the midbrain to innervate the tectal surface, rather than grow along the aqueduct surface, or the physical defect caused by introduction of the transplant.

Transplants also provide an interesting preparation in which to study trophic maintenance in development. We have described one study in which a transient pathway was retained by placing target tissue in its course. We know also that in the absence of appropriate targets provided by adjacent host tissue, cells in the transplants can be lost. It will be interesting to know how this pattern of cell death compares with that occurring during normal development. A most important feature of this preparation compared with other experiments of similar intent is that the cells are transplanted without axotomy, and this may lead to a somewhat different response to the environment in which they are placed.

It is clear therefore that the technique of transplantation has permitted us to assess the importance *in vivo* of a variety of putative and real determinants of development. There are however many more issues which can be examined with increasing sophistication of approaches.

Most important will be to learn more about the early development of transplants and their first interconnections with the host. Deducing developmental mechanisms from examination of the end point can be misleading, especially in the light of the several demonstrations of transient projections which regress later in development.

Another important direction will be to apply and extend studies labelling specific cell populations. The use of species-specific antibodies coupled with interspecies transplantation, for example, allow demonstration of connections between transplants and hosts without resorting to traditional labelling techniques, which, apart from being inefficient, are problematic when applied to transplants incorporated in host brains or to very small groups of transplanted neurons.

A further approach worthy of attention is the manipulation *in vitro* of neurons with the subsequent transplantation serving as an assay for the effect of the manipulation. The feasibility of the approach has been demonstrated: the growing numbers of cell specific surface antibodies will allow examination of the effects of removal or modification of specific cells in a region.

Up to recently, the relatively small amount of neurophysiology undertaken on transplants has been somewhat superficial. The opportunity for examining in much more detail the functional capacity of the artificial circuits made between transplants and hosts may provide some interesting insights into afferent and target cell interactions. The correlation between physically reconstructed circuits and specific behaviors is a further area deserving attention. Most of the studies which have examined behavioral

recovery after transplantation are in systems where endocrine effects are involved or where the highly specific connections characteristic of the visual system are not necessary for normal function. Whether the intricate connections between component parts of the visual system can carry normal functions after transplantation should clearly be explored. The end-point of the developmental process is an optimally functioning system: any study examining the constraints on development should eventually pay attention to this.

Acknowledgements

The work described here is the result of a series of productive and enjoyable collaborations with a large number of colleagues. It has been supported by NEI grant EY05283.

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NEURAL TRANSPLANTS AND RECOVERY OF FUNCTION AFTER BRAIN DAMAGE

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INTRODUCTION

During the last few years evidence has accumulated that fetal neurons, implanted into the depth of the brain in adult rats, can reestablish damaged connections in the host brain and substitute functionally for elements lost or damaged as a result of a preceding lesion. This research work has led to the realization that, contrary to traditional views, the adult mammalian CNS has a potential to incorporate new neuronal elements into already established neuronal circuitry and that such implanted neurons can modify the function and behavior of the recipient. For a long time it was thought that the remarkable regenerative and functional potential of CNS tissue grafts that had been demonstrated in cold-blooded vertebrates reflected a fundamental difference in the regenerative properties of central nervous tissue between cold-blooded vertebrates and mammals. During the last few years it has become evident, however, that at least certain types of intracerebral neural grafts can perform just as well in developing and adult mammals as in developing or adult submammalian vertebrates.

Intracerebral grafting of neurons with monoamines or acetylcholine as their transmitter has particular relevance in the context of neurodegenerative diseases. In two neurodegenerative disease syndromes, Parkinson's and Alzheimer's diseases, in particular, the progressive and substantial loss of such types of neurons are likely to underlie some of the major clinical symptoms of the disease. Thus, in Parkinson's disease the severe motor disturbances can be related to a loss of some 80-90% of

the neurons in the nigro-striatal dopamine (DA) system. And in both Alzheimer's and Parkinson's diseases there is a strong correlation between the degree of dementia and the loss (or possibly atrophy) of cholinergic neurons in the basal forebrain projection systems, which is the source for the cholinergic afferents to the hippocampal formation and the neocortical mantle. There is experimental evidence in rats to support these relationships. Thus, interference with DA transmission (e.g., by administration of the DA-depleting drug reserpine) or selective lesioning of the nigro-striatal DA neurons (by the toxins 6-hydroxydopamine, 6-OHDA, or 1-methyl-4-phenyl-tetrahydropyridine, MPTP) induces sensorimotor impairments in rats or monkeys which can be said to be analogous to the disfunctions seen in Parkinsonian patients [14, 15, 33, 45]. Interference with cholinergic transmission, on the other hand (e.g., by cholinergic receptor blockade), or surgical lesions to the septo-hippocampal or basal forebrain-neocortical pathways carrying the cholinergic projection systems, cause severe learning and memory impairments in rats [see 2, 17 for review]. The analogies between such experimentally induced conditions and the clinical disease states with respect to neuropathological, neurochemical and behavioral changes have justified the use of animals with 6-OHDA lesions of the nigro-striatal DA pathway, on one hand, and surgical lesions of the septo-hippocampal or basal forebrain-neocortical cholinergic pathways, on the other, as experimental models for at least some aspects of the pathology involved in Parkinson's and Alzheimer's diseases.

In this chapter we will review briefly the results obtained by implantation of dopaminergic and cholinergic neurons in animals with lesions of the nigro-striatal or septo-hippocampal systems, i.e., in conditions that can be said to represent *analogous* models of Parkinson's and Alzheimer's diseases.

Grafting Techniques

Two principal techniques have been used to graft fetal CNS tissue to the previously denervated striatal or hippocampal regions. The first one involves the transplantation cavity in which the graft is placed in direct contact with the denervated striatum or hippocampus. In this procedure good graft survival is ensured by preparing the cavity in such a way that the graft can be placed on a richly vascularized surface (e.g., the pia in the choroidal fissure) that can serve as a "culturing bed" for the grafts [42]. The vessel-rich ventral surface of the fimbria-fornix lesion provides such

a "culturing bed". This cavity is in direct communication with the lateral ventricle which may allow the CSF to circulate through the graft cavity and thus probably help the graft to survive, particularly during the early postoperative period. In the case of the striatum we have adopted a two-stage surgical procedure [3, 9]. A cavity is first made in the dorsal parietal cortex to expose the dorsal surface of the head of the caudate-putamen. After a few weeks, when a new vessel-rich pia has grown over the surfaces of the cavity, the cavity is reopened and a graft is placed onto the exposed striatal surface.

The second technique we have used involves injection of dissociated cell suspensions into the depth of the brain [7, 11, 38]. In this technique pieces of fetal CNS tissue are trypsinized and mechanically dissociated into a milky cell suspension. Small volumes of the suspension can then be stereotaxically injected into the desired site using a microsyringe. A major advantage of this technique is that it allows precise and multiple placements of the cells. The technique also makes possible accurate monitoring of the number of cells injected by counting the density of cells in the suspension [13]. For the remainder of this chapter the first technique will be referred to as the "solid graft" technique and the second technique will be referred to as the "cell suspension" technique.

Cell Survival, Fiber Outgrowth and Biochemical Function of Intracerebrally Grafted Neurons

Using the cell suspension technique, we have successfully grafted embryonic brain cells to a wide variety of target sites in the host adult brain. In this section we will present examples of this method using (1) the ventral mesencephalon and (2) the septum-diagonal band region. The ventral mesencephalic tissue was taken from embryos at about 13-15 days of gestation. After the suspension had been made the cells were injected stereotaxically into a caudate-putamen which had previously been denervated by a 6-OHDA lesion of the ascending nigro-striatal bundle. The developing septal-diagonal band donor tissue was generally taken from embryos at about 14-16 days gestation. After the suspension had been prepared the cells were stereotaxically injected into the hippocampus of rats, which in the same surgical session were subjected to complete, aspirative lesions of the fimbria-fornix and the supracallosal striae, as previously described, in order to totally denervate the hippocampus of its cholinergic innervation from the septal-diagonal band region.

Nigro-striatal DA system

Over 90% of the embryonic substantia nigra grafts survive to form aggregated clusters of cells containing fluorescent DA-containing neurons [12]. These clusters usually have between several hundred and several thousand DA-containing cells. We have injected ventral mesencephalic cells in a variety of brain regions, several of which are primary target zones for the ascending DA system and other regions which are not primary target regions. We can conclude from this rather extensive material that fiber outgrowth, but not necessarily the cell survival from the embryonic suspension, appears to depend on the relatively specific regional interactions in the host brain. For example, the fibers growing out from grafted DA neurons placed in the striatum radiated processes extensively in this region which is a normal target zone for the ascending DA system. However, the fiber outgrowth was restricted to the region of the cell implantation when the grafted DA neurons were placed in the parietal cortex, globus pallidus, substantia nigra or lateral hypothalamus.

Thus, it seems possible that the same mechanisms operating during normal ontogenetic development to guide the developing neurons to their target zones are operating during the regulation of fiber outgrowth from intracerebrally grafted DA neurons. In contrast to normal development, however, the grafted neurons must be placed within about a millimeter of the striatal border in order for the DA-containing axons to be able to reach the striatum. We have found no evidence that the DA neurons can elongate their outgrowing axons and extend fibers through "foreign", non-target areas to ultimately reach their telencephalic target areas. However, in recent experiments, nigral grafts were placed in an occipital cavity overlying the superior colliculus, and a 2- to 3-cm long sciatic nerve was simultaneously grafted above the parietal cortex with one end placed to penetrate the denervated host striatum and the other encapsulating the nigral graft in the posterior cavity. DA axons were seen to grow from the nigral graft along the entire length of the sciatic graft to reach the host striatum [1]. Thus, the grafted DA neurons are capable of extending their axons over long distances, provided that the local regulatory factors are permissive and supportive of such growth.

Biochemical data from nigral suspension implants into the striatum confirm the histochemical observations that the implants can provide a substantial reinnervation of the surrounding striatal target tissue [39]. Specifically, rats with 6-OHDA lesions of the mesotelencephalic dopamine system (2-3 weeks before grafting) have in our experiments an

average DA depletion of 99% in the caudate-putamen and a 95% depletion in the remaining forebrain. The nigral cell suspension grafts implanted in the striatum restored striatal DA to an average of 13-18%, with only marginal effects in the remaining forebrain. The cell suspension grafts also provide substantial restoration of dopaminergic neurotransmission in the initially denervated caudate-putamen, as indexed by an increase of the metabolite 3,4-dihydrophenylacetic acid (DOPAC) in the striatum, from 5% to about 20% of normal, in the grafted animals. By calculating the DOPAC:DA ratios it was determined that the implanted DA neurons had a spontaneous turnover of the transmitter at rates that were on the average 50 to 100% higher than those of intact intrinsic nigro-striatal DA neurons, but similar to those previously reported for solid nigral grafts for the reinnervation of the dorsal caudate-putamen [40].

Septo-hippocampal cholinergic system

In previous studies, solid pieces of the embryonic septum-diagonal band area have been found capable of providing a new cholinergic innervation of the hippocampal region of adult recipient rats, previously denervated of their intrinsic cholinergic pathway from the medial septum and diagonal band which cross through the fimbria-fornix in route to the hippocampal formation [6, 8]. With this solid grafting technique the reinnervation of the denervated hippocampus was incomplete and the reinnervation that did take place took a long time to occur. By using cell suspension grafts of the septal-diagonal band region from 14- to 16-day-old rat embryos, we have been able to increase the rate of reinnervation of the denervated hippocampus as well as to increase the total area of the denervated hippocampus that is reinnervated by the acetylcholine esterase (AChE) positive fibers growing out from the grafted cells [5].

As with the ventral mesencephalic suspension grafts in the striatum, the survival rate in vivo is greater than 90% for the septal cell suspensions in the hippocampal formation. In a recent study we examined the time-dependent changes in the septal suspension implants as indexed histochemically by their expression of AChE and biochemically by determination of the cholinergic marker enzyme choline acetyltransferase (ChAT). AChE was first expressed in the grafted cells after the first post-operative week. The AChE-positive fibers began to extend into the hippocampus

by the third week. The most rapid phase of outgrowth occurred between 3 weeks and 3 months after grafting. Even more complete reinnervation remains even after 14 months. Thus, we have suggested that the graft-derived innervation of AChE positive cells and fiber staining is permanent. This general pattern and time course of fiber ingrowth is supported by the studies using ChAT as a cholinergic marker enzyme. ChAT activity is first expressed by 10 days after grafting and there is a subsequent 3-fold increase in ChAT over the next 3 weeks. By 6 months the ChAT activity is restored to near normal levels in all previously denervated segments of the hippocampus [4].

The functional activity of the grafted septal cholinergic neurons has been further analysed using measurements of acetylcholine (ACh) turnover in vitro [4]. Since ChAT is probably not a rate-limiting step in acetylcholine synthesis under normal conditions, the activity measures of this enzyme do not give a direct measure of the actual transmitter turnover rates. The (^{14}C)ACh synthesis from (^{14}C)glucose provides a better index of the functional state of the ACh transmitter system as a whole, and Sims *et al.* [41] have shown that the synthesis rates measured are similar in vitro and vivo. Thus using this method in vitro ACh synthesis measurements indicate that the ACh transmitter system in the newly established septo-hippocampal circuitry operates at a rate similar to that of the intrinsic septo-hippocampal circuitry.

Behavioral Recovery

Sensory-motor impairments after nigro-striatal DA bundle lesions

6-OHDA lesion of the nigro-striatal DA pathway is currently the most widely used experimental model of the principal neuropathology of Parkinson's disease, and the behavioral syndrome induced by such lesions has been thoroughly characterized in rats. A unilateral 6-OHDA lesion causes *asymmetric motor behavior* (both spontaneously and in response to DA releasing drugs, such as amphetamine, and DA receptor activating drugs, such as apomorphine), *asymmetric posture*, and *sensory inattention* (or sensory neglect) towards the stimuli applied to the side of the body contralateral to the lesion. Bilateral lesions result in a state of profound behavioral unresponsiveness, including *akinesia, bilateral sensory inattention, catalepsy, hunched posture*, and *aphagia plus adipsia* [see 33, 43, 45, for reviews].

In unilaterally 6-OHDA-lesioned rats, solid nigral grafts, placed in a dorsal cortical cavity or in the lateral ventricle, have been shown to reduce or completely abolish both spontaneous and drug-induced motor asymmetries [3, 9, 10, 20, 27, 37], but have no effect on the sensory neglect [3, 20]. As more extensive testing has been conducted on nigra-grafted rats with unilateral or bilateral 6-OHDA lesions [10, 18-23] it has become apparent that graft-derived recovery is dependent upon graft placement, in parallel with the known topographic organization of striatal function.

Thus, to summarize a range of observations in animals with both solid and suspended nigral grafts, reinnervation of the dorsal caudate-putamen is a requirement for amelioration of both spontaneous and drug-induced rotation [3, 10, 18, 20]; reinnervation of ventral and lateral caudate-putamen is required for reinstatement of sensorimotor attention and responsiveness on the contralateral side of the body [18, 19, 21]; and reinnervation of nucleus accumbens and/or prefrontal cortex is required for amelioration of the akinesia in bilaterally lesioned rats and normalization of the locomotor response to dopaminergic drugs [21, 23, 34]. The only behavioral deficit that has so far not been influenced in any substantial way is the severe aphagia and adipsia seen in bilaterally 6-OHDA-lesioned rats [19, 22].

The compensation of motor asymmetry induced by solid nigral grafts has been shown to be correlated with the degree of striatal reinnervation. Interestingly, restoration of as little as 3-5% of the normal striatal DA content is sufficient for complete compensation of amphetamine-induced turning behavior [3, 39, 40], and it has been estimated that some 120 surviving DA neurons (i.e., about 2% of the number present in the normal substantia nigra) are sufficient to produce a substantial compensatory effect on this behavior [13].

Learning and memory impairments after lesions of the septo-hippocampal pathway

The hippocampus has a special role in learning and memory, and bilateral fimbria-fornix lesions in rats are known to result in severe impairments in both working memory [36] and spatial memory [35]. This lesion disrupts several major afferent and efferent connection systems of the hippocampal formation. Nevertheless, since similar effects are obtained by pharmacological blockade of cholinergic transmission [25,

44], it appears that lesioning of the cholinergic septo-hippocampal pathway contributes importantly to the memory impairments seen after fimbria-fornix transection.

The ability of the fetal septal grafts to reverse these impairments has been studied with tests of spatial learning and memory in different types of radial mazes. In the eight-arm radial maze [32] rats with solid septal grafts showed a positive linear trend in maze performance over days of testing, but did not differ significantly from non-grafted rats with lesions. However, potentiation of cholinergic transmission by pretreatment with the choline esterase inhibitor physostigmine produced a significant enhancement of maze performance in the grafted group, but not in the lesioned control group, and in some cases the grafted rats performed as well as the non-lesioned control animals. In another study [24] using a T-maze forced-choice alternation test, 7 of 9 rats with solid septal grafts and 4 of 5 rats with septal cell suspensions learned the task, some of them up to the level of the control rats. The remaining rats with septal grafts, and a separate group of rats with locus coeruleus grafts, performed at chance level, similar to the rats that only received the fimbria-fornix lesion. In this study there was a positive and significant correlation between performance of the grafted rats and the amount of graft-derived AChE-positive staining in the previously denervated hippocampal formation. This result has subsequently been replicated by Low *et al.* [31] in experiments with cross-species (mouse to rat) septal suspension grafts in fimbria-fornix lesioned rats.

Together these three studies strongly suggest not only that the grafts can partially ameliorate lesion-induced deficits in maze learning tasks in fimbria-fornix lesioned animals, but that the behavioral recovery shows some specificity for the septal grafts which provide a cholinergic reinnervation of the deafferented hippocampus. Interestingly, the effect of the septal grafts seems specific also with respect to different components of the lesion-induced behavioral syndrome. Thus, it has been found that those septal-grafted animals which showed improvement in spatial learning remained impaired with respect to the marked hyperactivity and hypoexploration which is characteristic for the fimbria-fornix lesioned animals.

In a more recent study, Kelly *et al.* [30] investigated the magnitude of lesion-induced alterations in the hippocampal formation as reflected in the local rates of (^{14}C)-2-deoxyglucose (2-DG) utilization, and the degree to which this index of functional activity could be normalized

following reinnervation by solid septal grafts. Six months after transection of the septo-hippocampal pathway by a unilateral fimbria-fornix lesion the 2-DG utilization was reduced by 30-50% throughout tbe ipsilateral hippocampal formation of the non-grafted animals. Fimbria-fornix lesioned rats that had received septal grafts displayed a significant recovery in hippocampal 2-DG use, to within the normal range, as compared to the rats with lesion alone. In addition, the changes in 2-DG utilization were significantly correlated with the density of AChE staining in adjacent sections from the same brains ($r = 0.75$; $p < 0.01$), thus suggesting a relationship between the cholinergic reinnervation from the septal grafts and the restoration of functional glucose utilization. These results strongly suggest that the cholinergic component of the grafts is functioning at the biochemical level and influencing, or normalizing, the function of the deafferented hippocampal formation.

Mechanism of Action of Intracerebral Neural Implants

The combined morphological, biochemical, electrophysiological and behavioral data summarized above show that implanted embryonic nerve cells in some cases can substitute quite well for a lost intrinsic neuronal system in mammals. The intracerebral implants probably exert their effects in several ways. The functional effects seen with grafts placed into one of the cerebral ventricles, such as in the studies of Perlow *et al.* [37], Freed *et al.* [26, 27] and Gash *et al.* [29], are thus probably explained on the basis of a diffuse release of an active amine or peptide into the host CSF and adjacent brain tissue. In other instances, such as in animals with DA-rich grafts reinnervating the neostriatum or acetylcholine-rich grafts reinnervating the hippocampus, we believe that the available data provide quite substantial evidence that the behavioral recovery is caused by the ability of the grafted neurons to reinnervate relevant parts of the host brain. This is illustrated by the studies mentioned above showing that the degree of functional recovery in 6-OHDA-lesioned rats with nigral transplants is directly correlated with the extent of striatal DA reinnervation, and that the "profile" of functional recovery is dependent on which area of the striatal complex is reinnervated by the graft.

To what extent the intracerebral inplants can be functionally integrated with the host brain is, however, still poorly known and remains therefore an interesting question for further investigation. The chances for extensive integration may be greatest for neuronal suspension grafts

implanted as deposits directly into the depth of the brain, but even solid grafts inserted as whole pieces into the brain have in several cases been seen to become reinnervated from the host brain both in adult and developing recipients. Nevertheless, a recent HRP study [28] failed to detect any host afferents to intracortical solid nigral grafts despite the fact that these grafts had themselves formed extensive DA connections in the host striatum and had produced behavioral recovery. This suggests that implanted neurons can also function well in the absence of some, or perhaps even all, of their normal afferent inputs.

Available data, derived primarily from studies on monoaminergic and cholinergic systems, indicate that implanted embryonic central neurons can substitute to some degree for a lost set of afferents to a denervated brain region in adult rats, and replace a lost intrinsic neuronal system in normalizing the rat's behavior. This indicates a remarkable plasticity of the mature rat CNS in incorporating new neuronal elements into its already established circuitries. There is now abundant evidence that the adult CNS can reorganize and rebuild itself in response to damage (see [16] for review) and in some cases continued neurogenesis and incorporation of newly-formed neurons are likely to play a normal physiological role.

Neuronal replacement by neural implants is a striking further example of how the brain can allow new elements to be inserted and linked into its own functional subsystems. Obviously there must be definite limitations as to which types of neurons or functional subsystems can successfully be manipulated in this way. Neural implants would seem most likely to have behaviorally meaningful functional effects with types of neurons that normally do not convey, or link, specific or patterned messages, e.g., in sensoric or motoric input and output systems.

Conclusions

Intracerebral grafting to the adult vertebrate brain has developed in the last ten years to the point where two major applications of the general method can be identified as having been, and apparently will continue to be, profitable in the future. One possible application may be the direct clinical use of the method to ameliorate neurodegenerative disease states in humans. This application of the transplant method requires both the development of suitable animal models and extensive experimental research on such models in order to obtain sufficient experimental background knowledge. Another application of the neural grafting method-

ology is to basic research. Thus, the transplantation method can, for example, be used as an in vivo culture system to evaluate the role of different host environments on cell survivability and axonal elongation, and the host brain can be experimentally and selectively altered to address specific questions related to factors important for cell survival, axon elongation and functional recovery.

With the development of several model systems to the point where graft survival is greater than 90% and axon elongation is not only extensive but selective and specific in its pattern of innervation, the outlook for the development of other model systems is optimistic. However, many basic issues concerning the morphology and function of the intracerebral cell suspensions remain unresolved. This is particularly true for the exact mechanisms by which intracerebral grafts can restore function in the host CNS. To clarify this we will need to know more about the ultrastructural organization of the transplants; the efferent and afferent connections of the graft with the host brain; the full characterization of the types of cells that are grafted and ultimately survive in the host brain; and the question of whether the grafted cells are functionally integrated with the host brain or whether the grafts may act as an independent entity. In particular, it will be interesting to know whether the functions that appear to be restored to the organism with brain damage and subsequent intracerebral grafts are new functions unavailable to the organism previously or whether the functions are reacquired.

Intracerebral neural grafting has by now become established as a highly specialized field of research. It promises to be a very useful tool with which to experimentally address questions of neuronal cell survival and growth in vivo, and to explore problems related to mechanisms of regeneration and functional recovery after damage in the CNS, not least in conjunction with conditions of relevance for neurodegenerative diseases in man.

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ERRATA

Owing to exceptional delay in the mail, the following corrections were received after the printing was completed.

Page 123 — NATURAL, INDUCED AND REGULA*TIVE*. . . .

Page 138 — [44] . . . Histochemistry *85*, 335-338 (1986).

Page 342 — [52] Ontophyletics instead of Antophyletics

Page 345 — [113] . . . corpus callosum in mice