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Intrinsic changes in developing retinal neurons result in regenerative failure of their axons

(organotypic coculture/superior colliculus/axon regeneration/target innervation/Syrian hamster)

DONG FENG CHEN, SONAL JHAVERI, AND GERALD E. SCHNEIDER*

Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139

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ABSTRACT **The failure of mature mammalian central nervous system axons to regenerate after transection is usually attributed to influences of the extraneuronal milieu. Using explant cocultures of retina and midbrain tectum from hamsters, we have found evidence that these influences account for failure of regrowth of only a small minority of retinal axons. For most of the axons, there is a programmed loss of ability to elongate in the central nervous system. We show that there is a precipitous decline in the ability of retinal axons to reinnervate tectal targets when the retina is derived from pups on or after postnatal day 2, even when the target is embryonic. By contrast, embryonic retinal axons can regrow into tectum of any age, overcoming growth-inhibiting influences of glial factors.**

The rate and extent of axonal growth are influenced by intrinsic properties of individual cells (1, 2) as well as by the substrate through which the axons must navigate (3). During the last decade many studies have focused on the involvement of the extraneuronal milieu in the failure of maturing central nervous system (CNS) axons to regrow over long distances. David and Aguayo (4), utilizing peripheral nerve grafts implanted in the CNS, and Schnell and Schwab (5), applying antibodies to neutralize the effects of oligodendrocyte-associated inhibitory proteins, have shown that some axotomized neurons can be induced to reextend their axons for considerable distances. Regenerating axons from the axotomized retinal ganglion cells (RGCs) extended along the entire length of peripheral nerve grafts, and some penetrated the superior colliculus (SC) up to 500 μm from the end of the graft. A common interpretation of these findings is that, given the appropriate environment, all neurons should be able to regenerate their exons. However, despite the impressive regrowth of cut axons documented in these studies, the regenerative capacity is expressed by a limited population of neurons (5–9): many transected axons do not regrow into the peripheral nerve graft or regenerate through an area where glial cell inhibitory proteins are neutralized with antibodies.

To reexamine the problem of regenerative failure, we have used the primary visual system of the developing hamster as a model, employing organotypic explant cocultures of retina and tectum. This paradigm enables independent variation of the developmental stage of each tissue and allows us to separate the contributions of source and target tissue in influencing the extent of axonal regrowth and target reinnervation.

During hamster development, RGC axons leave the eye by embryonic day 10 (E10) and reach the rostral edge of the SC by E14 [day of mating = E0, and day of birth = E16 = postnatal day 0 (P0)]. The axons grow rapidly during this early stage of *elongation*, maintaining a simple, unbranched morphology as they invade the tectum. At about P0, they shift into a second mode of growth, referred to as the *arborization* mode, as they begin to emit collateral branches and to elaborate terminal ramifications (10–12).

Following axotomy made at the level of the rostral midbrain prior to P4, RGC axons are able to regenerate back into the tectum; if the cut is made on P4 or later, most retinal axons fail to reinnervate the tectum (13, 14). This regenerative failure could be attributed to maturational changes in the CNS environment or to developmentally regulated alterations in the growth capacity of retinal axons. These possibilities are not easy to differentiate *in vivo*, because both the afferent cell and the terrain through which their axons extend are changing simultaneously, making it difficult to separately control for maturational changes which occur in ganglion cells from those which occur within the tectal target. To circumvent some of these problems, we have developed a coculture paradigm in which retinal explants are grown adjacent to tectal tissue derived from hamsters aged E13 to adult. We have succeeded in mimicking the regenerative failure of retinal axons, using this coculture setup, and provide evidence that much of the precipitous decrease in the ability of postnatal retinal axons to reinnervate explants of the midbrain tectum does not derive from the target environment but occurs as a result of maturational changes in the retina itself.

MATERIALS AND METHODS

Embryos were obtained by cesarean section from timed pregnant Syrian hamster dams which had been overdosed with barbiturate anesthesia; prior to sacrifice, animals younger than P6 were anesthetized by hypothermia, and P8 or older pups were anesthetized by Nembutal injection. Brains were dissected out into ice-cold Gey's balanced salt solution (GBSS) (GIBCO) enriched with glucose (6.5 g/liter), and meninges were removed. Coronal slices through the SC, the inferior colliculus, and the visual cortex were cut with a McIlwain tissue chopper at a thickness of 300 μm and stored in GBSS for 2–3 hr at 4°C to allow for tissue equilibration and to rinse out proteolytic enzymes leaking from damaged cells. The eyes were dissected out. The retina was separated from other ocular tissues and cut in half through the optic disc. Half of the retina was abutted against a tectal slice with the optic disc next to the dorsal edge of the tectum. Tissues were placed on the microporous membrane of a Millicell well (Millipore) which was inserted in a six-well culture plate. Excess GBSS was suctioned off and 1 ml of SF21 hormone-supplemented serum-free medium (15), just enough to cover the explanted tissue, was added to each well. Cultures were maintained in a humidified CO_2 incubator at 37°C for 5 days and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The number of regenerating axons was sampled by applying four

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Abbreviations: CNS, central nervous system; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; E*n*, embryonic day *n*; P*n*, postnatal day *n*; SC, superior colliculus; RGC, retinal ganglion cell.

*To whom reprint requests should be addressed.

equal-size crystals of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes) in a standard manner to the retinal explants (16). The dye was allowed to diffuse at room temperature for 2–8 weeks and then visualized and photographed on a Nikon inverted microscope using fluorescent illumination and a rhodamine filter. The labeled retinal axons which penetrated tectal slices were counted. The length of the axons was measured by tracing the total length of the axon extending into the target from the retina–tectum interface.

Viability of the explanted tissues was assessed by examining the morphology of retinal and tectal neurons. Slices were fixed after 5 days of incubation, and tiny crystals of DiI were inserted into retinal explants as noted above, or into tectal explants, to label nearby neuronal cell bodies within the injected tissue.

The development of oligodendrocytes and myelinated fibers in cultured tectal tissue was determined by immunostaining with the monoclonal antibody Rip (17). Isochronic cocultures of retinal and tectal explants taken from E14–P8 hamsters were incubated for 5 days and fixed as described above. Following cryoprotection and embedding in Tissue-Tek O.C.T. compound (Miles), cultures were cut on the cryostat parallel to the surface of the tectal slice, at a thickness of 30 μm. Sections were incubated overnight at room temperature with the monoclonal antibody Rip or only in Dulbecco's modified Eagle's medium as a control and then were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG for 2 hr. Sections were mounted on gelatin-coated slides and coverslipped.

RESULTS

Growth Patterns of Retinotectal Axons in Coculture Correspond to *in Vivo* Observations. In the first set of experiments, cocultures were prepared from retinal and tectal tissue of the same age (isochronic cocultures). The standard application of DiI to retinal explants allowed quantitative sampling of regenerating retinal axons. Although the absolute number of labeled axons varied from one culture to another, the mean values for each age were similar. Extensive labeling of neurites was observed intraretinally in all cases examined, indicating good viability of the retinal explants. For isochronic cocultures prepared from embryonic animals (E13–E15) ($n = 30$), retinal axons extended through the superficial tectal layers. The morphology of these axons was simple and unbranched (Fig. 1 *A* and *D*), as seen for elongating fibers *in vivo*. When both retinal and tectal explants were derived from P0 animals ($n = 15$), retinal axons were observed emitting short collaterals in the tectum, and some preterminal branching was also visible (Fig. 1 *B* and *E*). This initiation of collateralization/arborization matches the normal developmental sequence described for retinotectal projections growing *in vivo*.

In each of the isochronic cocultures ($n = 42$) prepared from animals aged E13 through P0, growth of retinal axons into the tectum was extensive, the number of labeled axons averaging 82.7 ± 13.0 as assessed with the use of our sampling technique. In contrast, when isochronic cocultures ($n = 32$) were prepared from brains of animals aged P2 or older, the average number of labeled retinal axons invading tectal tissue was markedly reduced to 12.2 ± 2.6 fibers per tectal slice (Fig. 1 *C* and *F*). Fibers invading the tectal slice were confined primarily to the superficial layers even after 5 days of incubation, and significantly greater branching and arbor formation were observed than in the embryonic tissues. These observations indicate that starting with tissues from hamsters on P2, retinal axons exhibit a drastic reduction in their capacity to reinnervate the tectum.

Regenerative Failure of Retinofugal Axons Is a Function of the Age of the Retinae, and Not of the Target Tissue. To determine whether the sudden decline in regenerative capacity described above was due to maturational changes in the retinal neurons or whether it resulted from an age-related alteration in the target environment, we examined heterochronic preparations, in which retinae from animals of selected ages (E14 through adult) were cocultured with tectal explants, all of which were obtained from E14 animals. The rationale for choosing E14 as the age of the target tissue in these heterochronic cocultures was that the E14 tectum presents a permissive substrate for axon elongation *in vivo* as well as in isochronic coculture preparations. Retinae harvested from animals aged E14 through P0 all showed extensive fiber growth into E14 tectum. However, when the retinal explants were derived from animals aged P2 or older, little growth was observed from the retina into the embryonic tectum (Fig. 2). This result indicates that the presence of an immature target environment cannot reverse the regenerative failure of "older" retinal cells.

Quantitation of the results from the isochronic and heterochronic cocultures (Fig. 2*C*) illustrates a 10-fold decrease in

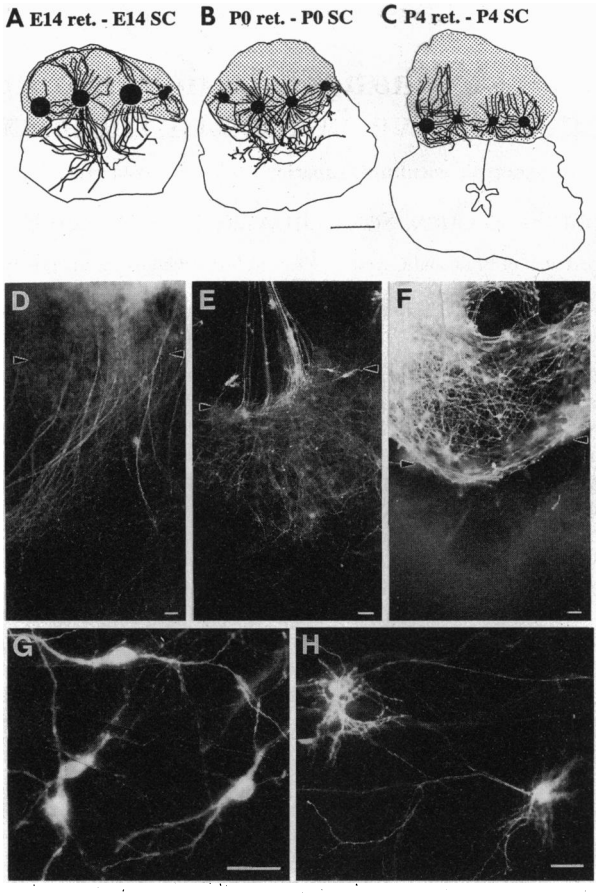

FIG. 1. Pattern of retinofugal axons growing into tectal explants in isochronic cocultures is similar to that obtained *in vivo*. Camera lucida drawings (*A–C*) and epifluorescence photomicrographs (*D–F*) represent DiI-labeled retinal axons in cultures prepared from E14 (*A* and *D*), P0 (*B* and *E*), and P4 (*C* and *F*) animals. The retinal explant was placed against the dorsal edge of the tectal slices. Arrows point to the boundaries of retinal (above) and tectal (below) explants in each panel. Axons from E14 retinae can be observed (*A* and *D*) coursing through the entire midbrain, with little evidence of arbor formation. If the retinae and tectum are harvested from P0 animals (*B* and *E*), retinal axons innervate the tectum, and beginnings of collateral formation and arbor elaboration are visible. If, however, these explanted tissues are derived from P4 animals (*C* and *F*), retinal axons fail to innervate the tectal slice but instead grow along the interface between the two tissues. Retrogradely labeled neurons within tectal (*G*) and retinal (*H*) explants from P10 animals look healthy and demonstrate that the cultures are viable after 5 days of incubation. This is true for all ages examined. (In *A–C*; bars = 1 mm; in *D–H*; bars = 25 μm.)

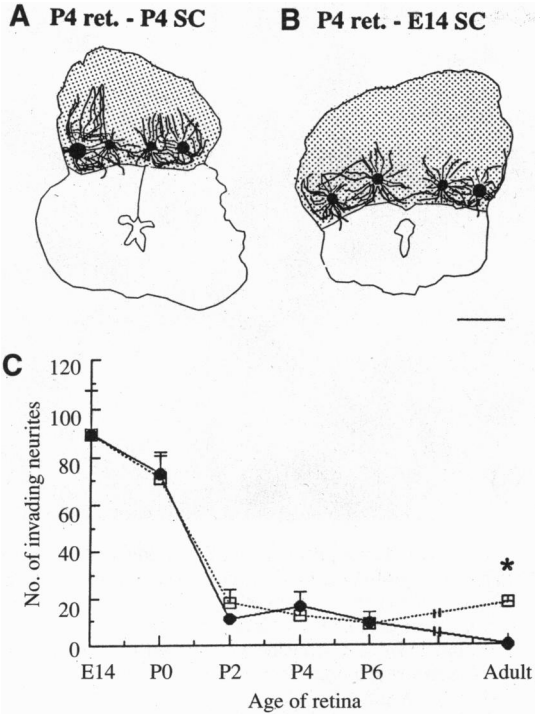

FIG. 2. Failure of retinofugal axon regeneration cannot be overcome by challenging older retinal explants with an immature target. (*A* and *B*). Drawings of labeled axons from P4 retina growing into P4 tectum (*A*) or into E14 tectum (*B*). There is a marked decrease in numbers of retinal axons, from P2 or older animals, which can invade the tectum (*A*). This is so even when P4 retinae are cocultured with E14 tectum (*B*). (Bar = 1 mm.) (*C*) Quantitative analysis of numbers of retinal axons which grow into tectum: numbers of invading neurites in isochronic cocultures prepared from animals aged E14 to adult are plotted in the solid line, and counts of neurites from retinal explants of various ages but all cocultured with E14 tectal slices are depicted by the dotted line. There are no significant differences for retinal axon ingrowth between the retinal explants cocultured with isochronic tectum and with embryonic tectum, with the exception of the adult retina/embryonic tectum combination (see text) ($P < 0.0001$, Student t test). Star indicates significant difference. Values are means and SEM of results from at least six cocultures.

the average numbers of neurites which enter the E14 tectum from the older retinae when compared with the numbers of fibers entering E14 tectum from E14 retinae. These results are consistent with observations from the first set of experiments, in which we had used isochronic cocultures of E14 (through P0) tissues or of P2 (and older) tissues. There was no significant difference between the ability of these retinae (E14 to P6) to innervate older tectum as compared with E14 tectum, implying that the composition of the tectal environment is not the determinative factor for the altered innervation capacity of retinal axons.

Parenthetically, it should be noted that a consistent difference was observed in the response of adult retinal cells challenged by adult tectal tissue (in the isochronic series) or by embryonic tectal tissue: a small but significant number (18 ± 2.2, $n = 6$; this is 22% of the average number of labeled embryonic retinal axons which enter the tectum) of axons from adult retinae succeeded in invading embryonic tectum, whereas no axons crossed over from adult retinae into adult tectum (0 ± 0, $n = 6$). This indicates that a minority of adult retinal cells (probably no more than about 20%) retain the capacity to regenerate their axons, given a favorable environment.

In sum, results of the above two sets of experiments suggest that the failure of retinal axons to regenerate, as defined by their ability to regrow into the tectal target, does not reflect an altered CNS environment; it appears that these older retinal axons are developmentally regulated for a failure to elongate in the target milieu.

Developmentally Regulated Changes in the Tectal Environment Do Not Account for the Inability of Retinal Axons to Invade Target Tissue. A third series of experiments was undertaken to determine the role of target tissue age on the regenerative capacity of retinal axons. E14 retinae were cocultured with tectal slices taken from hamsters aged E14 to adult. The embryonic retinal axons successfully penetrated tectal slices derived from all ages of hamsters examined (Fig. 3). No significant differences were found in numbers of DiI-labeled axons from E14 retinae penetrating tectal slices of different ages (Fig. 3*C*). We conclude that a maturational alteration in the tectal environment cannot account for the regenerative failure of the older retinal axons.

A somewhat surprising finding was that large numbers of E14 through P0 retinal axons were able to invade adult tectal tissue, despite the presence in this target of differentiated

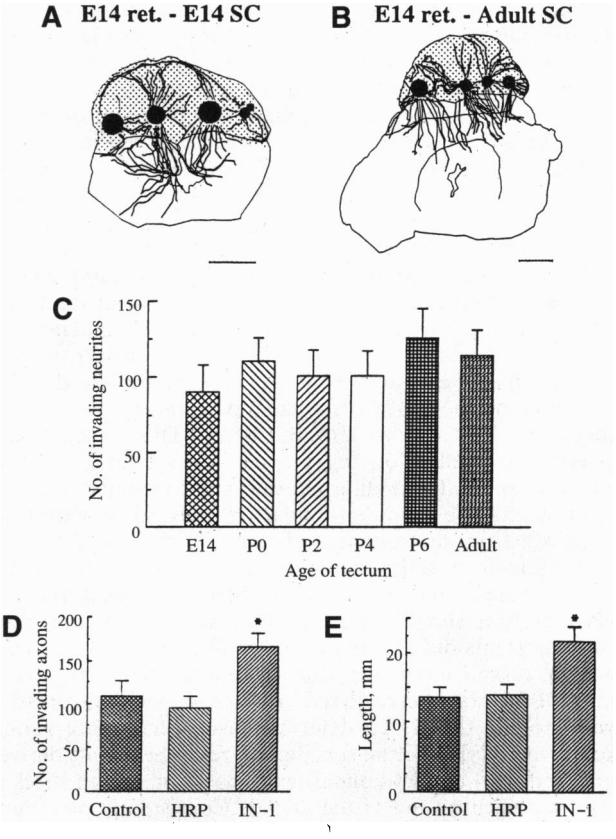

FIG. 3. Tectal slices from all ages support afferentation by retinal axons, as illustrated by camera lucida drawings made from E14 retinae cocultured with E14 tectum (*A*) and with adult tectum (*B*). (Bars = 1 mm.) Quantification of these observations (*C*) documents that there is no significant reduction in the average numbers of neurites which cross over from the retinal explant into the tectum for any age of tectum tested. *D* and *E* represent the quantitative results in experiment using IN-1 neutralizing antibody against oligodendrocyte inhibitory protein that obtained a significant increase in the number (*D*) and the length (*E*) of retinal axons (E14) innervating adult tectal slices ($P < 0.001$, Student t test). Supernatant (30%, vol/vol) from IN-1-producing hybridoma cells, or from control cells that produce an antibody against horseradish peroxidase (HRP) was added to cocultures of retinae from animals aged E14 and adult tectum. Cultures were maintained at 37°C for 5 days. The result illustrates that this inhibitory protein does have an effect to limit the axonal growth of embryonic retinal neurons, but it is not enough to cause the regenerative failure. Star indicates significant difference. Values are means and SEM of results from at least six cocultures.

oligodendrocytes and myelinated fibers. One possible explanation of this result is that embryonic or P0 retinal axons do not have receptors for the oligodendrocyte inhibitory protein described by Schnell and Schwab (5). However, in studies using the IN-1 neutralizing antibody against the inhibitory protein, or a control antibody against horseradish peroxidase (5), a significant increase in the growth of retinal axons into adult tectal slices was observed when the IN-1 antibody was added to the medium in which E14 retinae ($n = 12$) or P0 retinae (data not shown) were cocultured with adult tectum (Fig. 3 *D* and *E*). This result implies that embryonic to P0 retinal axons do, indeed, have receptors for the protein; nevertheless, they are able to traverse large territories of oligodendrocyte-containing tectal zones.

Explanted Tissues Are Viable and the Developmental Sequence of the Appearance of Oligodendrocytes and Myelin Continues Along a Close-to-Normal Schedule Under Coculture Conditions. To determine whether the growth of the tectal slices approximates normal development, we examined the sequence of appearance of oligodendroglia with the use of the monoclonal antibody Rip (17). For isochronic cocultures derived from P0 animals, Rip-positive oligodendrocytes were evident in deep tectal layers after 5 days in culture (equivalent age of coculture at the time of fixation = approximately P5 *in vivo*). Increasing numbers of these cells were present, now also in the more superficial tectum, if the midbrain tissue was harvested from P4 animals and maintained in culture for 5 days. And finally, immunopositive axons could be observed in the retinorecipient zones of the SC derived from P8 animals (equivalent culture age when fixed after 5 days of incubation = P13 *in vivo*). Thus, the development of oligodendroglia follows a close-to-normal schedule (18) in cultured tectal tissue.

The slices examined by trypan blue or Nissl staining showed no difference in the viability of tissues derived from animals of different ages. Retrogradely labeled retinal and tectal neurons had normal-looking and well-differentiated dendritic processes at all ages examined (Fig. 1 *G* and *H*). Finally, detailed inspection of the mature retinae revealed morphologically identifiable RGCs (Fig. 1*H*) and dense, DiI-labeled fibers, tipped with healthy-looking growth cones, emerging from retinae (Fig. 4). These data indicate good viability of RGCs under our culture conditions and a vigorous capacity for axon outgrowth from the retinae. It is unlikely that the failure of innervation from older retinae into tectal slices of any age resulted from a lack of viability of the older retinal tissue.

However, it should be noted that axons from the older retinal explants did approach the tectal tissue, but they consistently turned away from the target, preferring to course along the interface between retina and tectum without crossing over into the target. To determine whether mature retinal axons avoided the tectum specifically, retinal explants derived from both E14 and P4 animals were cocultured with slices of inferior colliculus and visual cortex. Consistently, we found that E14 retinal axons grew readily into these CNS slices, whereas P4 retinal axons stopped right at the edge of the retina or grew along the border of the retina. Even coculturing P4 retinal explants with embryonic retinal explants could not induce P4 axons to cross over into the embryonic explant, although vigorous axonal growth from the E14 retina was observed to invade the P4 retina. The result suggests that the failure of older retinal axons to regenerate is not due to changes in their target environment, nor is it due to an altered environment within retina; rather, it results from a programmed loss of the ability to grow axons by mature retinal neurons.

DISCUSSION

We have shown that (*i*) starting with P2-derived tissue, most retinal axons in explant culture exhibit a precipitous decline in their ability to reinnervate isochronic tectum—this failure cannot be overcome by confronting the axons with a more immature target nor by neutralizing the oligodendrocyte inhibitory protein; (*ii*) a small population of cells in the adult retina retains the capacity to innervate embryonic targets; and (*iii*) axons from embryonic retina successfully penetrate tectal targets of any age, despite changes in the tectal glial environment during CNS maturation. We conclude that developmentally regulated alterations occurring in the retina critically influence the regenerative capacity of its efferent axons.

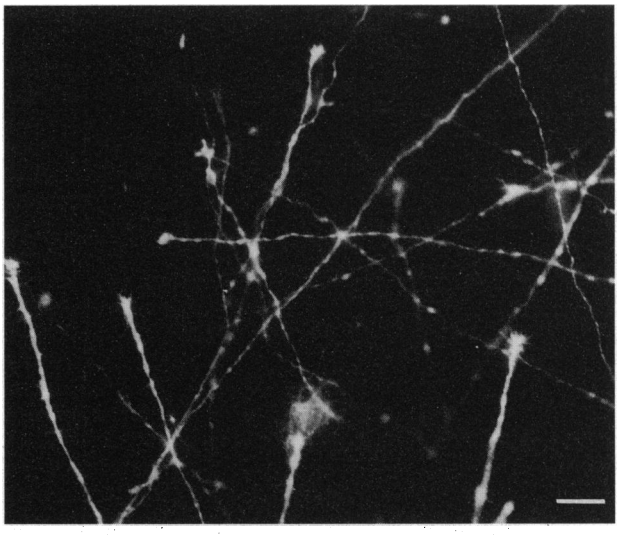

FIG. 4. DiI-labeled axons growing within retinal explants from P10 animals. This morphology and filopodia decoration of the growth cones (arrows) support the statement that the failure of tectal innervation by axons from older retinae is not due to decreased viability of the older explants. In fact, considerable growth of retinal axons is noted but these axons are now unable to enter and grow in the tectal substrate. (Bar = 25 μm.)

Prior studies have implicated extraneuronal factors in controlling the growth capacity of axons in the mammalian CNS (3). For example, the growth of developing axons can be promoted by diffusible factors from the glial environment of the embryonic target. With maturation, it is widely believed that the nonneuronal milieu of the CNS no longer facilitates, but actively inhibits, the growth of axons (19, 20). This inhibition has been attributed to the loss of local cues within the axon growth pathway, to changes in target cells, to maturing astrocytes, and to proteins found on oligodendrocytes which inhibit axon growth (21–23). Thus, the prevailing hypothesis is that the success or failure of regenerative growth depends upon environmental cues encountered by regenerating axons. Our findings challenge the sufficiency of this hypothesis. Changes in the CNS environment cannot account for the growth failure of most postnatal retinotectal axons; rather, our results demonstrate that starting on P2, it is the retinal axons which exhibit a marked change in their capacity to reinnervate their central target.

A possibility that must be carefully considered is that the decreased number of axons from older retina into tectal targets results from a decreased viability of mature retinal cells in culture. Although Nissl or trypan blue staining showed a significant number of necrotic cells within both the retina and the tectal explants, there was no indication of a dramatic increase in cell death in the older tissues. In fact, many healthy looking, retrogradely labeled RGCs could be observed within the retinae explanted from animals of all ages. Naturally occurring cell death *in vivo* peaks between P6 and P10 (24, 25), later than the time when retinal axon regeneration begins to fail (P2–P4). To further demonstrate that the regenerative failure of retinal axons was not due to the death of RGCs, we examined neurite outgrowth and cell death in dissociated cultures of retina after RGCs were back-labeled from tectum with DiI. The results showed a decline of RGC axon outgrowth

by the age of P2, similar to that in the cocultures, while RGCs remained alive and the number of viable cells stayed constant (unpublished data). Thus, programmed cell death cannot account for the regenerative failure of RGC axons. Observations from *in vivo* experiments (26) and our *in vitro* experiments (unpublished data) show that application of neurotrophic factors can enhance the survival of RGCs, but this did not reverse the regenerative failure of retinal axons postnatally. These findings further support the idea that the capacity for RGCs to regenerate their axons after a lesion is independent of cell death after axotomy.

The decrease in axon outgrowth can also not be ascribed to an increase in size of the retina, with a consequent decrease in density of RGCs and a resultant decrease in total number of retinal axons labeled by our standard-sized DiI crystals: the relative change in size of the hamster retina is much greater between P2 and adult than between P0 and P2. The decline in numbers of retinal axons is most dramatic within the first 2 days after birth, with little change in numbers observed after P2. In addition, Wikler *et al.* (24) showed that RGC densities in hamster retina increased from birth to P5, when regenerative failure of retinal axons starts to emerge; then, a gradual decrease in RGC densities was observed over the period P6–P10.

We cannot completely rule out the possibility that as retinal axons mature, they begin to express a receptor or a transduction mechanism which allows them to recognize an inhibitory signal on CNS substrates (27, 28), or perhaps they downregulate a receptor molecule which would permit recognition of a permissive substrate in the target. In a preliminary experiment, we observed that the presence of a monoclonal antibody against the α6- or β-integrin (two subunits of laminin receptors) (29–31) in the medium containing E14 and P0 isochronic cocultures did not alter the retinal axon growth pattern (unpublished data). However, this result does not exclude the possibility that other receptor molecules may be involved.

On the other hand, a limited population of mature RGCs does retain a capacity for axonal regeneration. This is consistent with reports of other investigators (32, 33), who have used a similar coculture setup to induce axonal growth from adult retinal explants into fetal midbrain sections and have documented functional retinotectal connections limited to the retino-recipient areas of fetal midbrain slices. This is also consistent with the observation that *in vivo*, a subset (generally 1–10%, not exceeding 20%) of adult RGC axons will regenerate if provided with appropriate environmental conditions (6–9), such as peripheral nerve grafts (3, 4), suitable neurotrophic factors (34), or antibodies which neutralize inhibitory proteins (5).

An important aspect of our results is that the events relating to axon growth observed in coculture correspond to observations obtained *in vivo*. Our studies as well as those from other laboratories have shown that CNS slices under culture conditions preserve their morphological characteristics and continue to differentiate (35, 36). The experiments using the Rip antibody demonstrate that the developmental sequence of appearance of oligodendrocytes and myelinated fibers continues along a close-to-normal schedule in culture. Also, retinal axons shift from an elongation to an arborization growth mode in P0 isochronic cocultures as they do *in vivo*. And finally, the majority of axons from retinae placed in culture at P2 or later fail to regrow into central targets. Since it requires close to 48 hr for the retinal axons to begin invading the tectum in culture (unpublished data), the actual age of the tissues at the time when reduction or failure of target innervation occurs would be around P4. These findings match the observations *in vivo*, where failure to innervate tectal tissue is observed when optic tract axons are transected on P4 or later, but not when they are cut earlier (13, 14). Thus, the coculture model is well-suited for further investigations into the mechanisms of axonal growth in the retinotectal system and for the analysis of cellular and molecular factors involved in regulating this growth.

In conclusion, we have provided evidence that developmentally programmed changes in afferent neurons determine the ability of their axons to regenerate into a central target. The molecular basis of such changes remains to be worked out (15, 37, 38).

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