

# Axonal Pathfinding During the Regeneration of the Goldfish Optic Pathway

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## ABSTRACT

Retinal ganglion cells in fish and amphibians regenerate their axons after transection of the optic nerve. Fiber tracing studies during the third month of regeneration show that the axons have reestablished a basically normal fiber order in the two brachia of the optic tract; axons originating in the ventral hemiretina are concentrated in the dorsal brachium, axons from the dorsal hemiretina in the ventral brachium. Attardi and Sperry (*Exp. Neurol.* 7:46-64, 1963) first suggested that the reestablishment of the fiber order reflects pathfinding by the regenerating axons. Recently, however, Becker and Cook (*Development* 101:323-337, 1987) have claimed that the fiber order observed at later stages of regeneration is due to secondary axonal rearrangements and that the initial brachial choice is random.

In order to evaluate whether regenerating axons are capable of navigating in the optic tract and brachia and on the tectum, the present study examined the pathway choices and the morphology of regenerating axons en route to their tectal targets in goldfish. Subsets of axons were labeled at various time intervals (2 to 30 days) following an optic nerve crush, by intraretinal application of the lipophilic fluorescent tracer 1,1-dioctadecyl-3-3'-3'-tetramethylcarbocyanine (DiI). After a survival time of 18 to 72 hours (to allow for diffusion of DiI along the axons), the experimental animals were perfused with fixative and their right and left optic pathways (nerve, tract, and tectum) were dissected free and separated at the chiasm. Fluorescently labeled axons were traced in whole-mounted pathways. Pathway choices were examined at the brachial bifurcation where axons from ventral and dorsal hemiretinae normally segregate.

DiI was found to label axons reliably up to their growth cones, even at the earliest stages of regrowth. The pathway choices of the axons were nonrandom. The majority of the ventral axons reached the appropriate, dorsal hemitectum through the appropriate dorsal brachium of the tract. Dorsal axons reached the ventral hemitectum mainly through the ventral brachium. This suggests the presence of specific guidance cues, accessible to the regenerating axons. Differences in the complexity of the growth cones of the regenerating axons (simple in the nerve and tectal fiber layer, complex in the tract and the synaptic layer of the tectum) provide further evidence for specific interactions between the regenerating axons and their substrates along the pathway. These results argue that regenerating retinal axons in fish are capable of axonal pathfinding.

**Key words:** axonal guidance, growth cones, retinotectal system

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Following transection of the optic nerve, retinal ganglion cells in fish and amphibians regenerate their axons and reestablish an orderly terminal map on the tectum (see Gaze, '70). Based on previous work in goldfish (Meyer, '80; Schmidt and Edwards, '83; Cook and Rankin, '86; Stuermer, '88a,b), two stages of regeneration can be distinguished: 1) an early period (about 30 days postcrush in adult goldfish) during which the retinotectal projection is reestablished and 2) a late period (between 1 and 3 months postcrush in adult goldfish) during which the initially crude retinotopic map of terminals on the tectum is refined to near normal precision.

The late stage of regeneration has been documented extensively by a range of techniques (electrophysiology: Schmidt and Edwards, '83; autoradiography: Meyer, '80; retrograde axonal tracing: Cook, '83; Stuermer and Easter, '84a; Rankin and Cook, '86; Stuermer, '86; anterograde axonal tracing: Stuermer and Easter, '84a; Bernhardt and Easter, '86; Stuermer, '88a). Sharpening of the electrophysiological map has been correlated with a decrease in the sizes of terminal arbors and with their segregation after initial overlap (Schmidt and Edwards, '83; Cook and Rankin, '86). Whereas factors intrinsic to the retinal ganglion cells are thought to contribute to the process (it has been suggested that ganglion cells might be unable to sustain the initially exuberant terminal arbors; see Schmidt and Edwards, '83), there is good evidence that terminal rearrangements are also influenced by patterned electrical activity. Refinement of the map can be prevented by disrupting the firing pattern of ganglion cells by stroboscopic illumination (Schmidt and Eisele, '85; Cook and Rankin, '86) and by blocking action potential activity with tetrodotoxin (Schmidt and Edwards, '83). These results have weakened the belief in a strict chemospecific match between retinal ganglion cells and their tectal targets (Sperry, '63). Instead they have supported the importance of activity-mediated tuning of the retinotectal projection, consistent with models of the segregation of ocular dominance columns during the critical period of development of the kitten visual cortex (Hubel and Wiesel, '65; Stryker and Harris, '86), and for the elimination of polynuclear innervation of muscles (Changeux and Danchin, '76; see also Purves and Lichtman, '80). These revisions of the chemospecificity theory have been discussed at length in a recent review (Easter et al., '85).

The initial stage of regeneration has been studied in much less detail, and it is currently unclear what factors influence the outgrowth of the regenerating axons. Speculations about such factors have been based in part on inferences from studies of the late stage of regeneration. The establishment of fiber order (albeit degraded from normal) in the optic tract, brachia, and on the tectum after 2 to 3 months of regeneration suggests that regenerating axons have access to pathfinding cues in all of these parts of the pathway (Bernhardt and Easter, '88). This notion was first suggested in the pioneering study of Attardi and Sperry ('63), which claimed that normal fiber order was established *ab initio* during axonal regeneration in goldfish. In contrast, Becker and Cook ('87, '88) have recently proposed that initial axonal outgrowth as indicated by brachial choice is random. They suggested that the generally correct brachial selection observed at 2 to 3 months postlesion resulted from the selective loss of misrouted axon collaterals, proceeding in parallel with and controlled by the same mechanisms as the sharpening of the terminal map on the tectum (but see Cook and Becker, '88).

The argument for the randomness of initial axonal outgrowth is based essentially on the retrograde labeling of retinal ganglion cells from a cut brachium (Becker and Cook, '87, '88). At early stages of regeneration there was virtually no correlation between the brachium labeled and the distribution of the backfilled cells in the retina. (Normally, cells labeled from the dorsal brachium are confined to the ventral hemiretina, cells labeled from the ventral brachium to the dorsal hemiretina.) If each ganglion cell had only one axon, then the finding would indeed imply randomness of axonal outgrowth. Because of the extensive collateralization of regenerating axons (Murray, '82), however, the proportion of misplaced cells in the retina does not necessarily reflect the proportion of misplaced axons in the brachia (see Becker and Cook, '87; Bernhardt and Easter, '88). Suppose that each axon produces five collaterals, four of which select the correct brachium, and one the incorrect one. If either brachium is labeled with horseradish peroxidase (HRP), all the cells in both hemiretinae will be backfilled. The ratio (labeled cells in the wrong half of the retina/labeled cells in the correct half) will be 1/1, a misleading result since 4/5 of the axons selected correctly.

This argument shows that data based on retrograde labeling have to be interpreted cautiously. Anterograde tracing of regenerating axons is not subject to the same limitations since it visualizes the axons directly. Therefore it should allow us to distinguish between the two alternative interpretations: random outgrowth or axonal pathfinding.

There have been no detailed studies with current anatomical techniques of the trajectories of the regenerating axons en route to their tectal targets. The paucity of anterograde tracing studies probably reflects the fact that the commonly used axonal tracer HRP does not reliably label retinal axons during the first 2 to 3 weeks of regeneration (Murray and Edwards, '82; Becker and Cook, '87; personal observations). The present study has overcome the problem of tracing regenerating axons by using the recently described fluorescent tracer 1,1-dioctadecyl-3-3'-3'-tetramethylcarbocyanine (DiI; Honig and Hume, '86).

Using DiI as an anterograde tracer, I have examined the outgrowth of regenerating axons from the time at which the first axons crossed the site of an optic nerve crush to the time when they reestablished an orderly terminal field on the tectum. DiI was found to label axons and their growth cones reliably from the earliest stages of regeneration. The present results show that brachial choice by regenerating axons is degraded compared to a normal control, but not random. Differences in the complexity of the growth cones of the regenerating axons in the nerve, tract and its brachia, and on the tectum were observed. This suggests that regenerating axons are capable of responding specifically to different substrates. This capacity is proposed as a mechanism that contributes to axonal pathfinding in the regenerating retinotectal pathway.

## MATERIALS AND METHODS

### Experimental animals

Two size classes of goldfish were used. The data describing the outgrowth of the regenerating axons and the morphology of the tips of the regenerating axons are drawn from 83 pathways in 49 small fish, approximately 25 mm to 35 mm in standard body length. These animals were chosen because a series of preliminary experiments indicated that

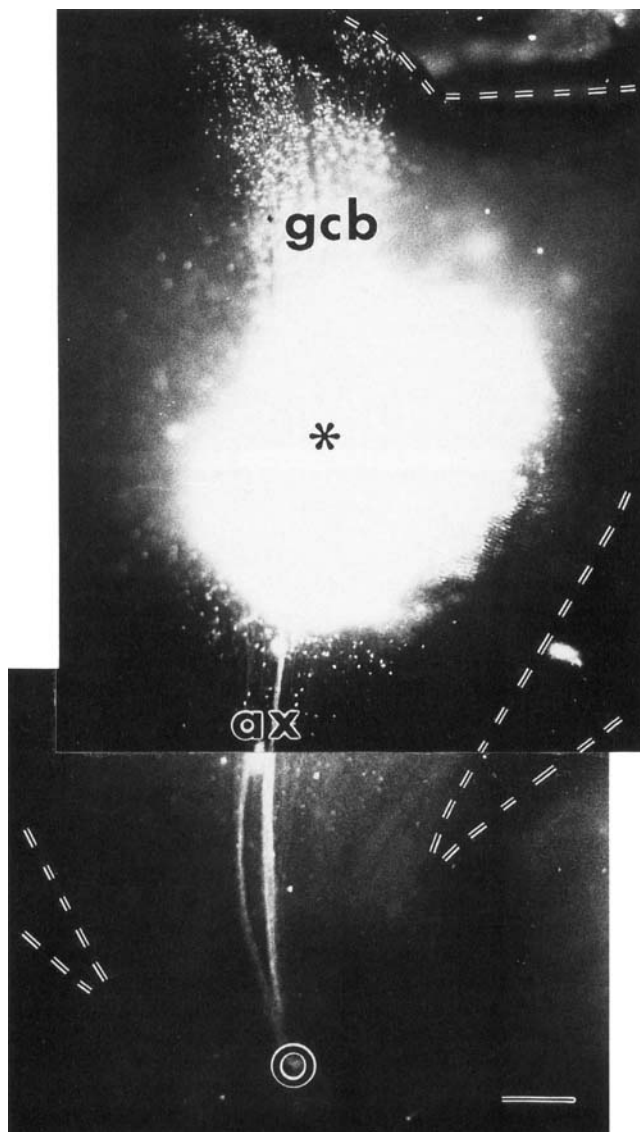


Fig. 1. Application site of DiI in a retinal whole mount. To flatten the hemispheric retina, four relaxing cuts were made, dividing the retina into quadrants. The micrograph shows the dorsal quadrant and includes the center of the retina, the optic disc (O). Ganglion cell bodies (gcb) and their axons (ax) are labeled. The gelfoam plug (asterisk) containing DiI (see Materials and Methods) is brightly fluorescent. Stippled lines indicate the retinal contour: the retinal margin is to the top, two relaxing cuts are to the left and the right. All the labeled axons that enter the optic nerve at the optic disc originate exclusively from the application site. Magnification bar is 250  $\mu\text{m}$ .

axons could be resolved with higher clarity on the tecta of small fish than in large animals. This is presumably due to the reduced thickness of the tectal cortex in small animals, in particular of the tectal stratum marginale and stratum opticum. In addition, the length of the pathway (retina to caudal tectum) is shorter in smaller than in larger fish (5–7 mm vs. approx. 10 mm or more). This obviated the need for prolonged survival times to allow for complete labeling of the entire length of the axon by the tracer. Since previous

studies of the brachial choice at early stages of regeneration (Attardi and Sperry, '63; Becker and Cook, '87; Hartlieb and Stuermer, '87) had used larger animals, brachial selection in 12 pathways of nine large fish, 55 mm to 65 mm in standard body length, were also examined.

### Optic nerve crush

The experimental animals were anesthetized in tricaine methanesulfonate (see Bernhardt and Easter, '86) and both the right and the left optic nerves were repeatedly crushed in midorbit with a fine watchmaker's forceps. Crushes were judged successful if a clear gap separated the distal (toward the eye) and the proximal (toward the brain) stumps of the nerve. The animals were then revived and returned to their home tanks. They were allowed to survive for various time intervals.

### DiI applications

At 2 to 30 days following nerve crush, the animals were reanesthetized. A pledget of gelfoam soaked in DiI in ethanol (4 mg/ml) was inserted through a stab wound in either dorsal or ventral retina (see also Bernhardt and Easter, '86). After a survival time of 18 to 20 hours (small fish) or approximately 72 hours (large fish), the animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer. The right and left optic pathways (nerve, tract, and tectum) were dissected free, separated at the chiasm, and prepared for whole mounts (see Stuermer and Easter, '84b). They were cleared in glycerol/phosphate buffer and coverslipped with 70% glycerol in phosphate buffer.

The location of the labeled ganglion cells was examined in retinal whole mounts prepared from several experimental animals. DiI applications labeled ganglion cells in a retinal wedge and their axons (Fig. 1), analogous to the pattern seen after a similar application of HRP (see Bernhardt and Easter, '86). The wedge, located at the dorsal (12 o'clock) or at the ventral (6 o'clock) radius of the retina, had an angular extent of 5 to 10 degrees and extended from the application site (approximately halfway along the retinal radius) to the retinal margin. The labeled axons originated exclusively from this wedge. Therefore, all the labeled axons traveling in inappropriate regions of the pathway are considered to be misrouted (see Results).

All data were recorded as photomicrographs taken with a Leitz Orthoplan microscope equipped for epifluorescence (rhodamine filter set). The tips of the regenerating axons were photographed, with either a 40 $\times$  dry objective or a 50 $\times$  water immersion objective, at one to three planes of focus. Some of the data on the growth cones of the regenerating axons are presented as ink drawings, obtained by tracing negatives projected in a photographic enlarger.

## RESULTS

The results are presented in three parts. First, the time course of regeneration in the small fish is described. Second, the pathway choices of the regenerating axons are examined. Third, the morphologies of the tips of the regenerating axons in different subregions of the pathway (nerve, tract, brachia, and tectum) are documented.

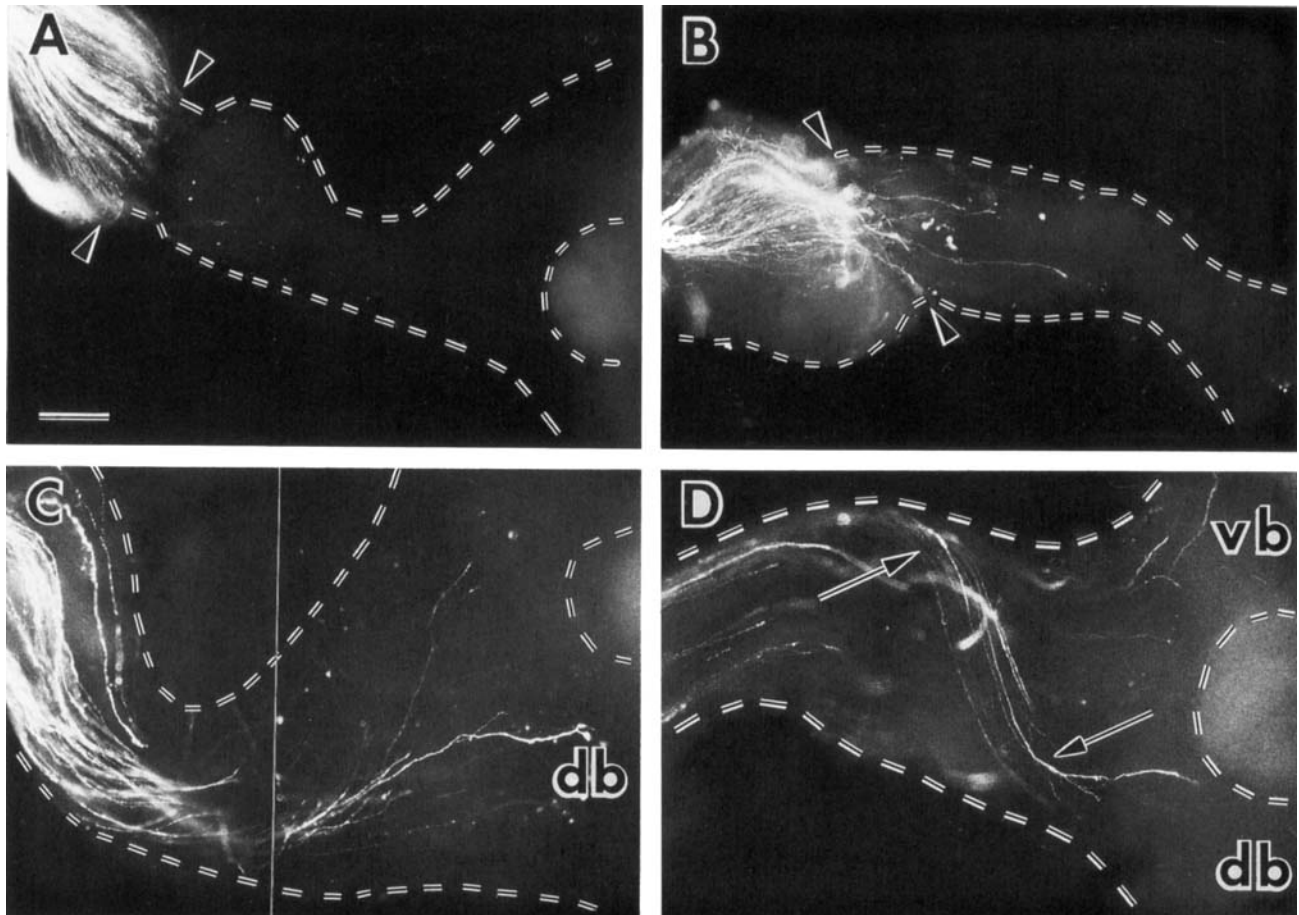


Fig. 2. Regenerating (ventral) axons in nerve and tract. Magnification bar in A is 200  $\mu\text{m}$ . **A:** Labeled axons have not yet crossed the crush site (between arrowheads); 4 days postcrush. **B:** In this pathway at 4 days postcrush, axons have crossed the crush site (arrowheads) and invaded the proximal nerve. **C:** Labeled axons, 4 days postcrush, have reached the brachial bifurcation of the tract. The leading axons are

heading toward the appropriate dorsal brachium (db). **D:** Leading axons in the brachial split region of the tract, 5 days postcrush. Axons have twice corrected their course (arrows) and head toward the appropriate dorsal brachium (db). Some axons have invaded the ventral brachium (vb).

### Time course of regeneration

In small fish the first axons regenerated into the deafferented portion of the pathway after an initial delay of 2 to 4 days following nerve crush. At shorter time intervals labeled axons were confined to the distal stump (Fig. 2A), and no labeled axons were seen in the proximal portion of the pathway, confirming that the nerve crushes had been successful.

Innervation of the optic pathway was reestablished between 2 and 7 days following nerve crush. At 2 to 4 days postcrush, individual axons had transversed the crush site and extended into the proximal nerve (Fig. 2B). At 4 to 5 days, the first regenerating axons reached the optic tract (Fig. 2C,D). Regenerating axons in the proximal nerve and in the tract generally lacked major collaterals. Short side branches were generally confined to the terminal region of the axons (see below). The dense fluorescent labeling in the neuroma that formed at the lesion site (Fig. 2B) precluded the direct observation of collateralization in this region. However, the present results suggest that the extensive col-

lateralization of axons previously documented in the regenerated optic pathway (Murray, '82) occurs mainly at the crush site.

Axons first invaded the tectum at 5 to 7 days post crush (Fig. 3). Between 10 and 30 days postcrush, regenerating axons reestablished a retinotopic projection onto the tectum. Initially, at 10 to 20 days of regeneration, axons restored a roughly defined terminal field (Fig. 4). The majority of the labeled terminals was clustered at a retinotopic location. However, the boundary of this terminal zone was not sharp since there were additional terminals outside the main cluster. Later, by 30 days, retinal terminals were confined to a dense band, occupying an appropriate retinotopic position on the tectum (Fig. 5).

In large animals regenerating axons were found on the tectum at between 15–18 days postcrush, in agreement with previous studies (Meyer, '80; Stuermer and Easter, '84a; Lowenger and Levine, '88). The growth rate of the regenerating axons was similar in both small- and large-size animals. It is estimated at 20 to 40  $\mu\text{m}$  per hour, similar to the

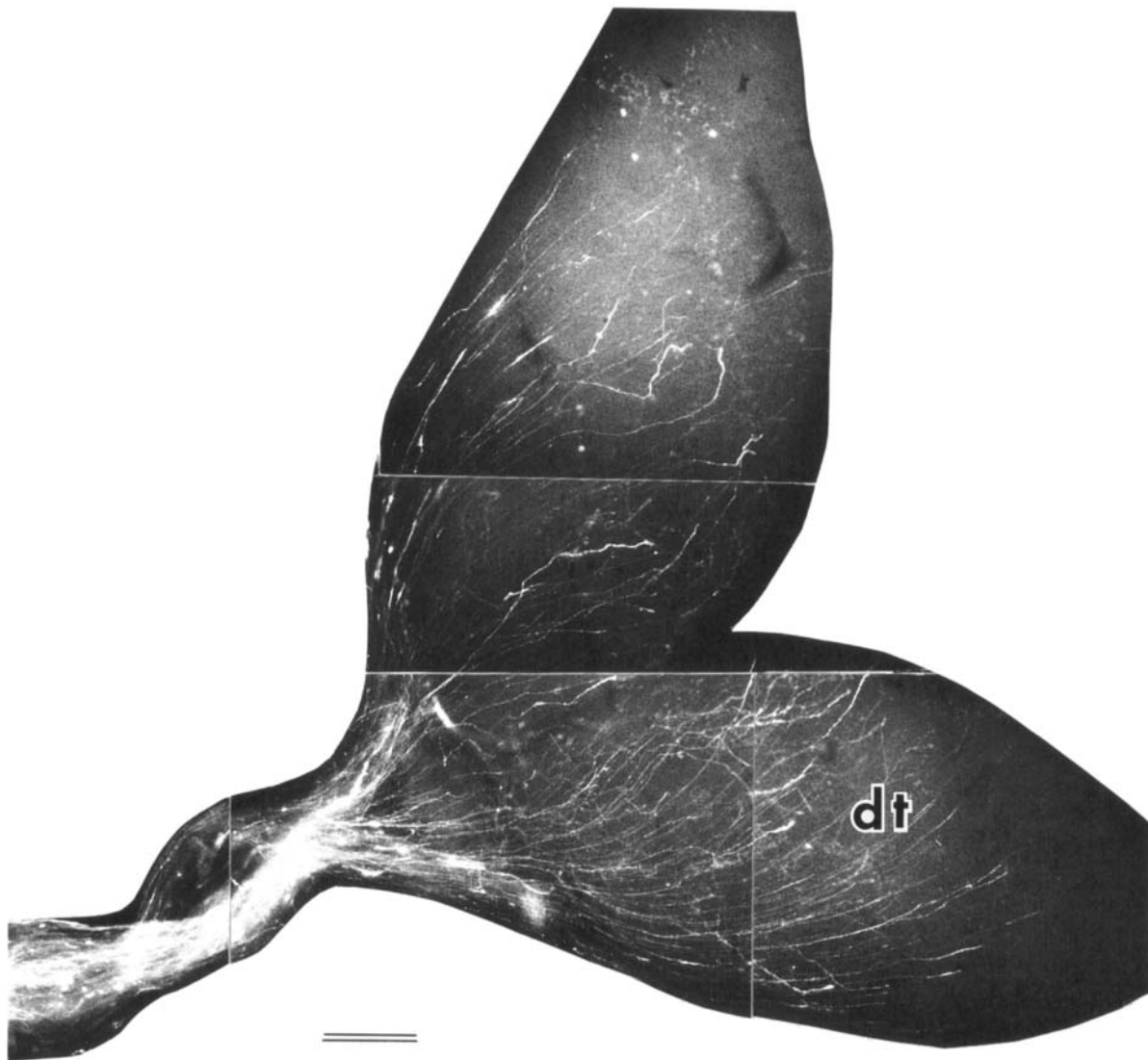


Fig. 3. Regenerating (ventral) axons on whole-mounted tectum, 5 days postcrush. The majority of labeled axons are found on the appropriate (dorsal) hemitectum (dt). Magnification bar is 500  $\mu$ m.

rate (32 to 52  $\mu$ m per hour) reported in the embryonic retinotectal pathway of *Xenopus* (Harris et al., '85, '87). The difference in the timing of the arrival of the axons on the tectum can be explained by the difference in the pathway length between the small and large fish (see Materials and Methods).

#### Pathway choices of regenerating axons

The pathway choices of the labeled axons were examined in the tract and its brachia and on the tectum. Dorsal and ventral axons are segregated in this portion of the normal pathway (Scholes, '79; Easter et al., '81; Bunt, '82; Stuermer and Easter, '84a; Bernhardt and Easter, '86; Fraley and Sharma, '86; Springer and Mednick, '86b), as well as in the

regenerated pathway at 2 to 3 months postcrush (Stuermer and Easter, '84a; Bernhardt and Easter, '88). Evidence for axonal pathfinding was obtained at very early stages of regeneration, before the axons had reestablished a terminal field on the tectum.

At 4 to 5 days postcrush, when only a few axons had reached the tract, it was possible to examine individual fiber trajectories. Two representative pathways in which ventral axons had reached the brachial region of the optic tract are illustrated in Figure 2C,D. In both pathways the leading axons are heading toward the dorsal (developmentally appropriate) brachium. The brachial selection is not simply due to fortuitous positioning of the axons in the tract, as shown by a comparison of the two pathways. Whereas in one pathway (Fig. 2C) the leading axons are heading straight

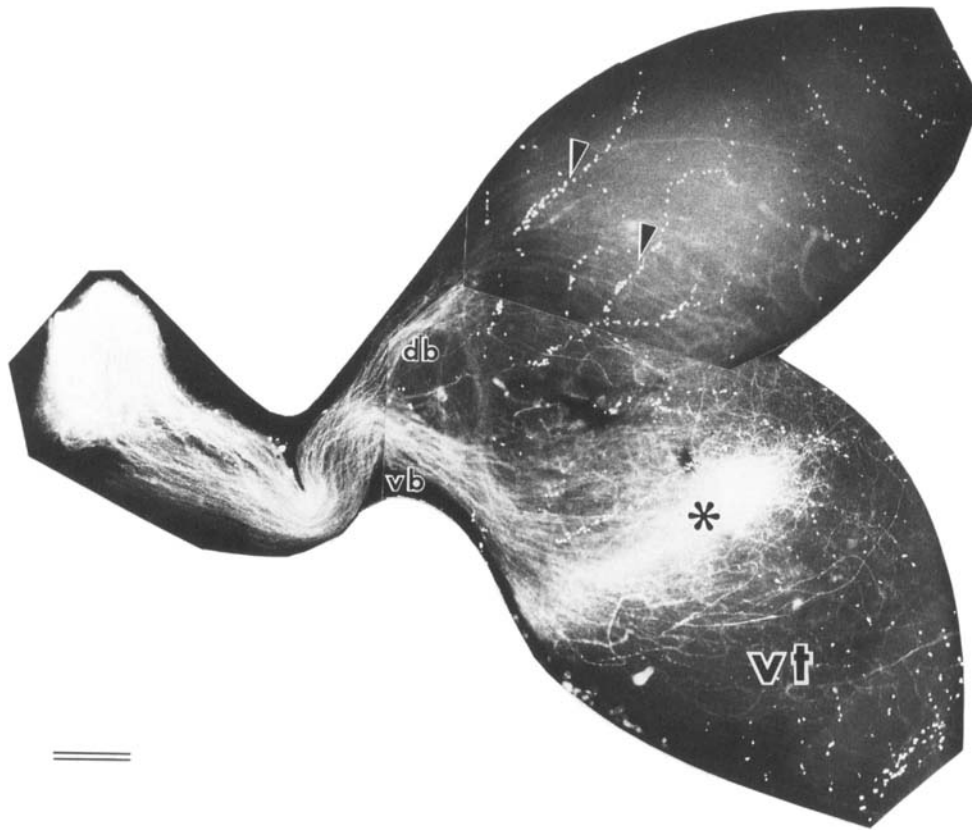


Fig. 4. Tectal whole mount, showing the reestablishment of a roughly defined terminal field (asterisk), 20 days postcrush. The majority of the labeled dorsal axons have reached the appropriate ventral hemitectum (vt) through the ventral brachium (vb) of the tract. A

minority of axons are found in the dorsal brachium (db). Speckles (arrowheads) are due to autofluorescence of erythrocytes in pial blood vessels. Magnification bar is 400  $\mu$ m.

toward the appropriate brachium, axons in the second one (Fig. 2D) have corrected their course. These axons, which at the nerve-tract boundary appeared to grow toward the ventral brachium, have executed two almost right-angle turns to approach the dorsal (appropriate) brachium instead.

Slightly later, a larger number of axons had progressed through the pathway. Their tectal innervation pattern, representatively illustrated in Figure 3 at 7 days postcrush, provided additional evidence for pathway selection. Figure 3 is a micrograph of a whole-mounted tectum split along the tectal equator. The labeled axons originate from ventral retina and their majority is found on the dorsal (appropriate) hemitectum. The selectivity of the tectal innervation at this early stage of regeneration is unlikely to reflect activity-driven interactions between retinal axons and their tectal targets, as it is seen prior to the establishment of a terminal field. It argues that the majority of the regenerating axons had reached the tectum by selecting the appropriate brachium.

Brachial selection was assessed systematically in 21 small fish in which axons had grown through the brachia (6–18 days postcrush). Because of the high density of labeled axons, it proved impossible to quantify selection by

counting labeled axons. Therefore, brachial choice was scored by visual impression. To objectively test the visual impression, labeling intensity was also measured using a photometer. The photometric measurements always confirmed the visual impressions. In 14 of the 21 pathways examined, brachial choice was clearly appropriate. In the remaining seven cases, selection was less obvious or inapparent. There was no example of preferential selection of an inappropriate brachium. Examples of selective and nonselective brachial choice are shown in Figure 6, which also illustrates that there was no obvious correlation between survival interval and degree of brachial selection. Selection was evident at 12 days postcrush in one pathway (Fig. 6A), but not in another pathway at 13 days postcrush (Fig. 6C).

Because previous studies (Attardi and Sperry, '63; Becker and Cook, '87; Hartlieb and Stuermer, '87) had examined the pathway choices of regenerating axons in larger goldfish, brachial selection in 12 pathways of large fish at 19–30 days postcrush were also examined. This is around the time when visually evoked electrical activity can first be recorded on the tectum but prior to the reestablishment of well-defined terminal fields (Schmidt and Edwards, '83). Brachial selection was obvious in nine cases and less clear in the remaining

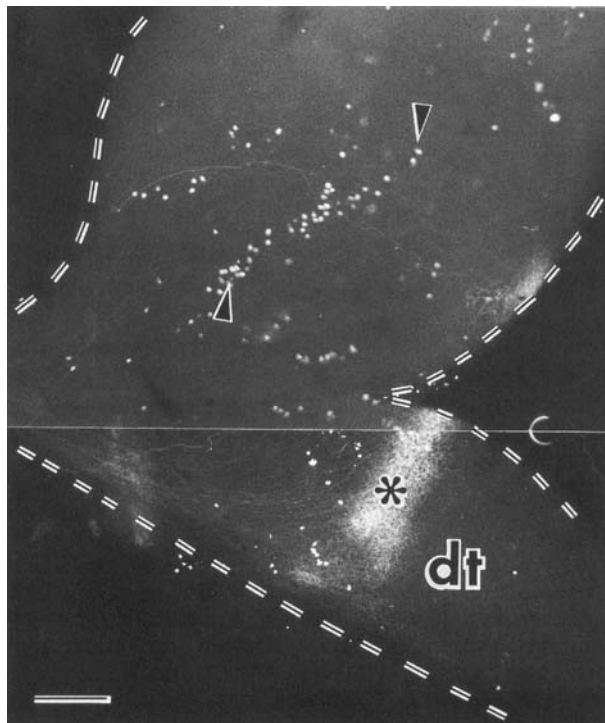


Fig. 5. Tectal whole mount, 30 days postcrush. The regenerated (ventral) axons have reestablished a well-defined terminal field (asterisk) on the appropriate dorsal hemitectum (dt). The relaxing cut, slightly dorsal to the tectal equator, separates some terminals from the main group. Speckles (arrowheads) are due to autofluorescence of erythrocytes in pial blood vessels. Magnification bar is 400  $\mu\text{m}$ .

three. Three representative examples of these pathways are shown in Figures 7 and 8.

In summary, I have found at least crude reestablishment of normal brachial selection at early stages of regeneration, that is, prior to the time at which well-defined terminal fields at retinotopic tectal sites are reestablished. Even though such selectively was not apparent in a sizeable minority of the pathways examined, there was no instance of obviously inappropriate brachial selection. This suggests that regenerating axons can rely on some kind of a guidance mechanism and do not choose their brachium randomly.

### Morphology of the growth cones of regenerating axons

The growth cones of the regenerating axons were examined in the nerve, the tract and its brachia, and on the tectum of small fish. Since there was no correlation between the retinal origins (dorsal or ventral) and the morphology of the regenerating axons, the following analysis applies to the pooled data of pathways labeled by DiI applications to either dorsal or ventral retina.

Two classes of growth cones could be distinguished—one simple, the other complex. Simple growth cones (Figs. 9, 11) were either bulbous (clavate, cf. Nordlander, '87) or lance-shaped (fusiform, cf. Nordlander, '87). Bulbous growth cones (Fig. 9A,B) lacked specializations. The lance-shaped growth cones (Figs. 9C, 11) were sometimes decorated with one or two thin filopodial extensions (Figs. 11A,B,D). Com-

TABLE 1. Terminal Branching Pattern of Regenerating Axons in Subregions of the Optic Pathway<sup>1</sup>

Subregion	No. of axons	No branching or one side branching		Two side branches or more	
		No.	% Total	No.	% Total
Optic nerve	16	15	93.75	1	6.25
Tract, brachia	14	7	50.00	7	50.00
Tectal SO	16	15	93.75	1	6.25
Tectal SFGS	19	10	52.63	9	47.37

<sup>1</sup>Number of branches longer than 10  $\mu\text{m}$  arising from the terminal 300  $\mu\text{m}$  of the regenerating axons.

plex growth cones were flattened and decorated with lamellipodial and filopodial extensions (Figs. 10, 12). Axons with short terminal side branches were often decorated with several complex growth cones (Figs. 10B, 12B-D).

The complexity of the growth cones varied consistently in the different subregions of the pathway (nerve, tract and brachia, tectal fiber layer, and synaptic layer of the tectum). This is illustrated by the ink tracings of growth cones in Figures 13 and 14. Simple growth cones were prevalent in the optic nerve (Fig. 13A) and in the tectal fiber layer (Fig. 13B), whereas complex growth cones were rare. In contrast, in the optic tract and its brachia (Fig. 14A) and in the synaptic layer of the tectum (Fig. 14B), growth cones were often complex.

Differences in the complexity of the tips of the regenerating axons were quantified by determining the number of terminal side branches. Table 1 scores the number of processes longer than 10  $\mu\text{m}$  arising from the terminal 300  $\mu\text{m}$  of the regenerating axon. Fewer than 10% of the growth cones in the nerve and tectal fiber layer had more than one side branch, whereas in the tract and tectal synaptic layer, approximately 50% of the tips had multiple side branches (two or more).

## DISCUSSION

The present study yielded information about the time sequence of regeneration and the pathway choices of regenerating axons. It also provided evidence for specific interactions between the terminal region of the regenerating axons and the environment. These three points are considered in sequence below.

### Time sequence of regeneration

DiI-labeled regenerating axons were first observed in the deafferented nerve stump after an initial delay period of 2 to 4 days following nerve crush. They reached the tectum at between 7 days (in small fish) and 15–20 days (in large fish). The results obtained in large fish agree with several previous studies using different techniques (electron microscopy: Murray and Edwards, '82; autoradiography: Meyer, '80; Stuermer and Easter, '84a; Lowenger and Levine, '88). There are no comparable data for small fish, but the present study shows that the axonal growth rates during regeneration are similar in both small and large fish.

Delayed onset of axonal reextension in the goldfish optic pathway has been reported previously. In an electron microscopic study of the early stages of axonal regeneration in the optic tract, Lanners and Grafstein ('80) first observed regenerating axons on the tectal side of the lesion site at the

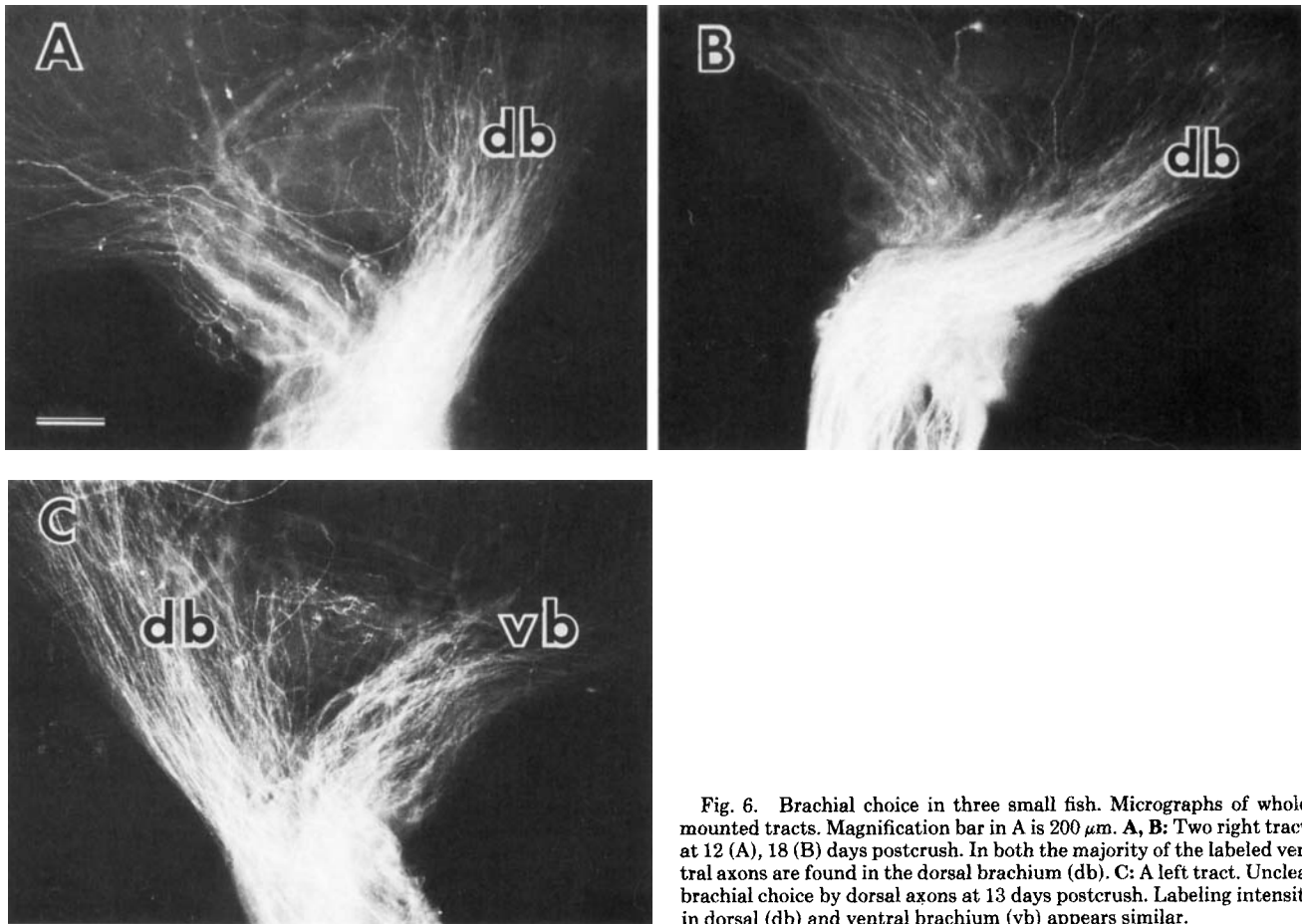


Fig. 6. Brachial choice in three small fish. Micrographs of whole-mounted tracts. Magnification bar in A is 200  $\mu\text{m}$ . **A, B:** Two right tracts at 12 (A), 18 (B) days postcrush. In both the majority of the labeled ventral axons are found in the dorsal brachium (db). **C:** A left tract. Unclear brachial choice by dorsal axons at 13 days postcrush. Labeling intensity in dorsal (db) and ventral brachium (vb) appears similar.

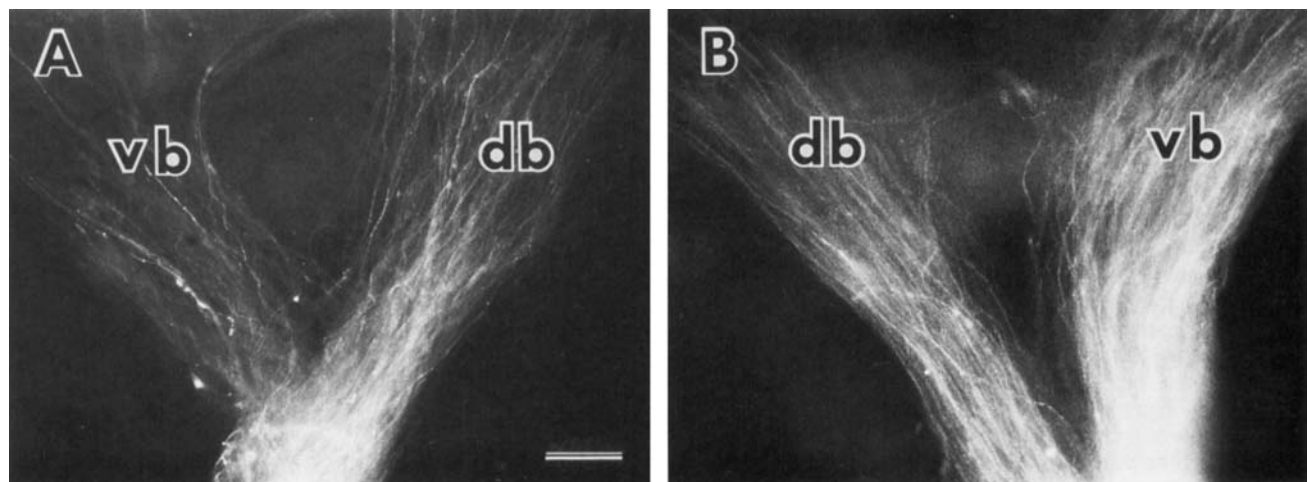


Fig. 7. Brachial choice in two large fish. Micrographs of whole-mounted tracts. Magnification bar in A is 200  $\mu\text{m}$ . **A:** Right tract. Ventral axons, 24 days postcrush, are more concentrated in the dorsal (db)

than in the ventral brachium (vb). **B:** Left tract. Dorsal axons, 22 days postcrush, are more concentrated in the ventral brachium (vb). A substantial number of errant axons is found in the dorsal brachium (db).





Fig. 8. Brachial choice of regenerating ventral axons in a large fish. Whole-mounted optic pathway, 22 days postcrush. Axons become

(asterisk). Most axons reach the dorsal hemitectum (dt) through the dorsal brachium (db); vb, ventral brachium. Magnification bar is 500  $\mu\text{m}$ .

third day of regeneration. Anterograde tracing of  $^3\text{H}$ -proline labeled retinal axons in the goldfish showed a 4 day delay in axonal reextension (Lowenger and Levine, '88). Similarly, in an extensive electron microscopic study of regeneration of the goldfish trochlear nerve (Scherer and Easter, '84) regenerating axons were first found in the deafferented nerve stump by 4 days postcrush.

#### Pathway decisions of regenerating axons

Examination of the trajectories of the regenerating axons in the tract and on the tectum indicates that axonal outgrowth is nonrandom during the initial stage of regeneration. A previous argument for the randomness of regenerative outgrowth in the retinotectal pathway has concentrated on the sizeable minority of misplaced axons (Becker and Cook, '87, '88). The current study considers the observation that the majority of regenerating axons chose the appropriate brachium, sometimes very strikingly so (see Fig. 8) as evidence for some navigational capacity of regenerating ax-

ons. This interpretation does not ignore the misplaced axons, which are discussed below, after additional evidence for axonal pathfinding during regeneration has been considered.

The finding of early brachial selection agrees with the conclusion reached by Attardi and Sperry ('63). But whereas these authors stressed the precision of the regenerated fiber order, the present study reveals a sizeable number of misrouted axons. This difference between the two studies may be explained partly by the increased resolution of the present fiber tracing technique when compared to the general silver stain used by Attardi and Sperry. An additional possible explanation is considered below. The present result also agrees with a short report (Hartlieb and Stuermer, '87) indicating consistent, albeit imperfect, brachial choice of axons labeled anterogradely with HRP as early as the fourth week of regeneration of the optic pathway of goldfish. Evidence for rearrangements of regenerating axons in the optic tract of goldfish was recently presented in an autoradiographic study (Lowenger and Levine, '88).

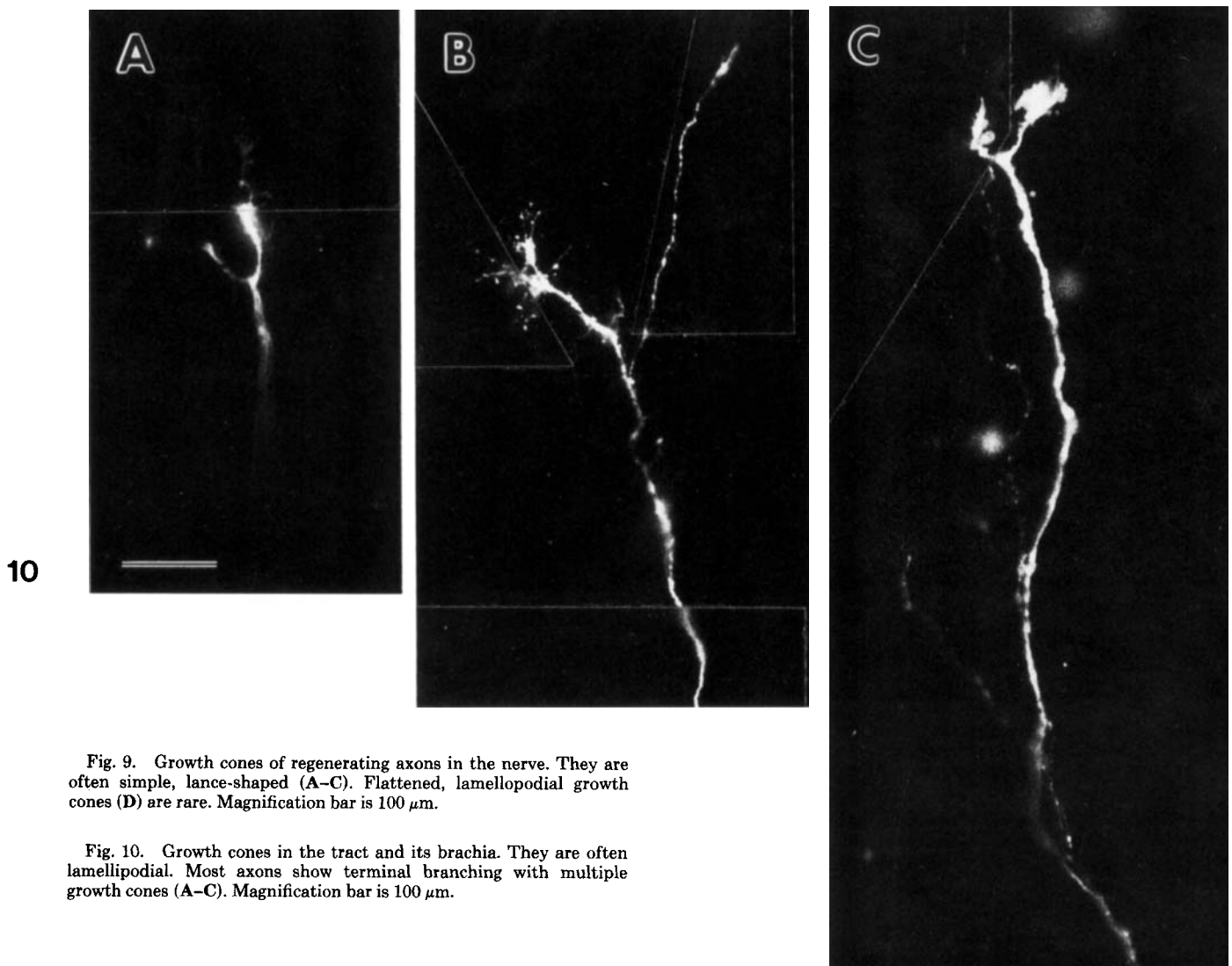
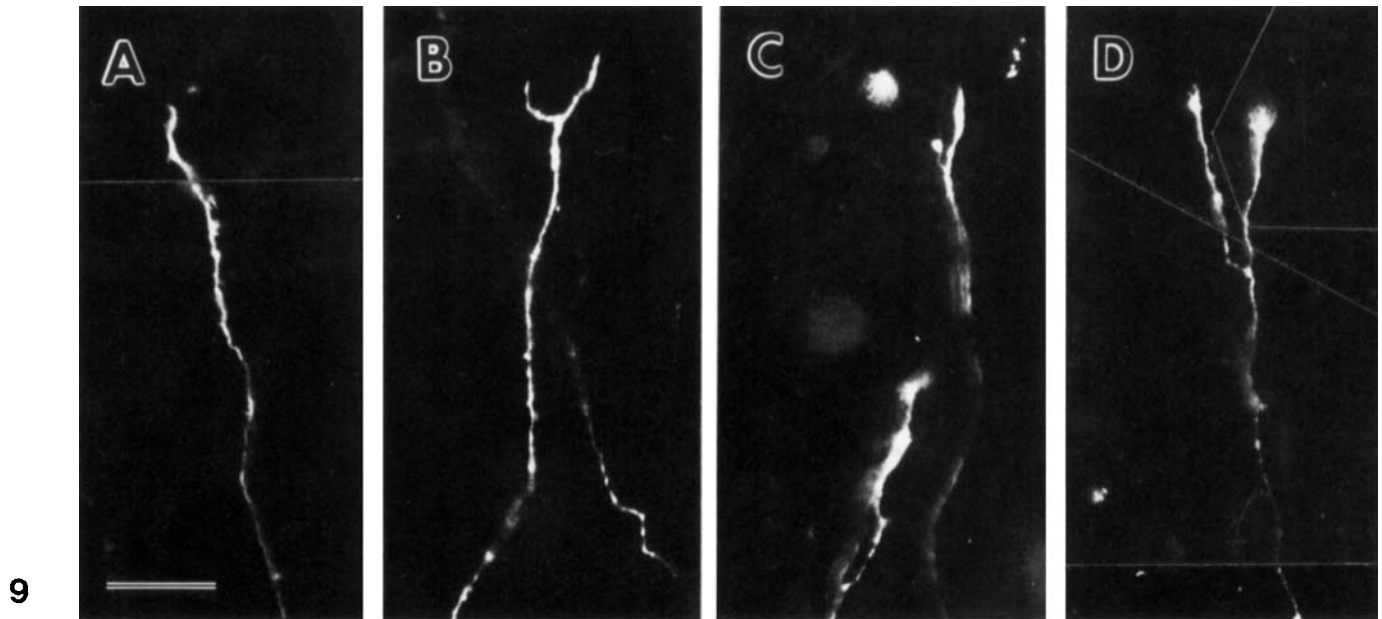


Fig. 9. Growth cones of regenerating axons in the nerve. They are often simple, lance-shaped (A-C). Flattened, lamellopodial growth cones (D) are rare. Magnification bar is 100  $\mu\text{m}$ .

Fig. 10. Growth cones in the tract and its brachia. They are often lamellopodial. Most axons show terminal branching with multiple growth cones (A-C). Magnification bar is 100  $\mu\text{m}$ .

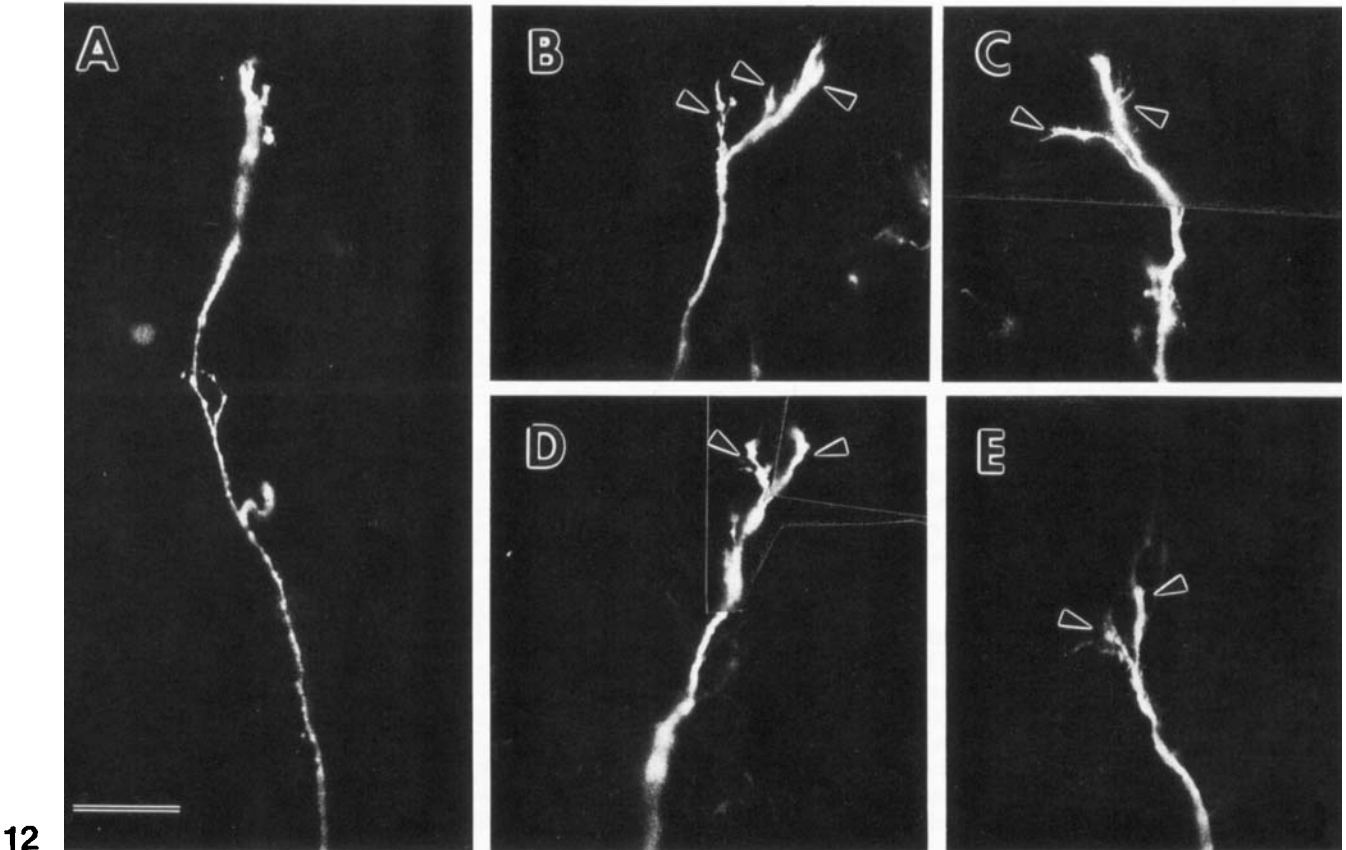
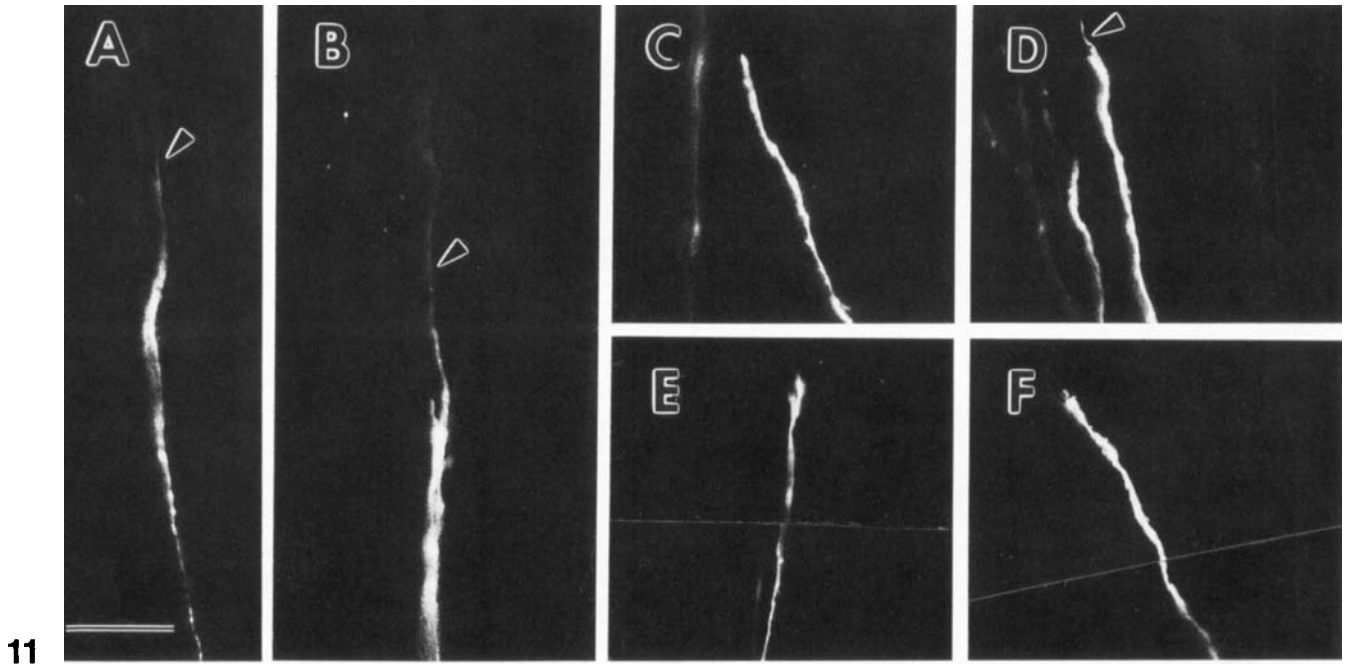


Fig. 11. Growth cones in the tectal fiber layer. They are lance-shaped (A-F), often tipped by one or two filopodial extensions (arrowheads in A,B,D). Magnification bar is 100  $\mu$ m.

Fig. 12. Growth cones in the synaptic layer of the tectum. They are often lamellipodial, decorated with spikelike extensions. Most axons show terminal side branches and multiple growth cones (arrowheads, B-E). Magnification bar is 100  $\mu$ m.

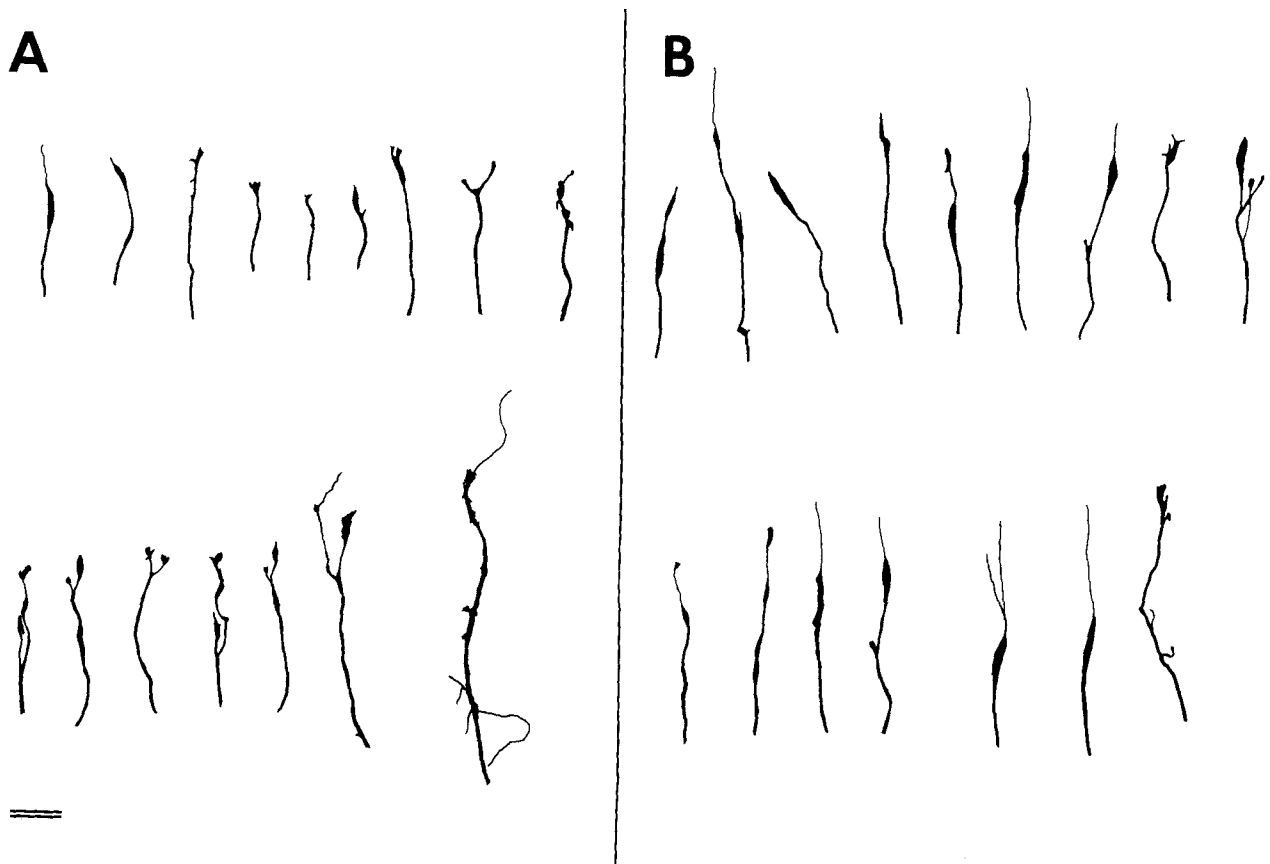


Fig. 13. Ink tracings of the terminal region of regenerating axons in the nerve (A) and tectal fiber layer (B). In these subregions of the pathway, the growing axons show little terminal branching and growth cones are simple. See text for analysis of branching pattern. Magnification bar is 50  $\mu\text{m}$ .

Pathfinding as documented in the present study is inconsistent with the conclusions, but not with the results, of a report by Becker and Cook ('87). They applied HRP to one brachium during the early stage of regeneration (during the fourth week postcrush in large goldfish) and found labeled ganglion cells scattered in both the dorsal and ventral hemiretinae, rather than confined to the corresponding hemiretina as in normal animals. This demonstrates that there were errant axons in the tract, but two considerations make this finding difficult to interpret.

The first has already been mentioned in the introductory material, but it is briefly recapitulated here. It derives from the extensive collateralization, at the crush site, of regenerating axons (cf. introductory material and Results; Murray, '82; Scherer and Easter, '84). If a particular ganglion cell had four collaterals in the appropriate but one collateral in the inappropriate brachium, this would normally be interpreted as axonal pathfinding. But this cell would be backfilled from an HRP-application to either brachium (see also Becker and Cook, '87; Bernhardt and Easter, '88), and thus give the misleading impression of a lack of brachial choice.

The second emerges from an analysis of the data presented by Becker and Cook ('87). During the fourth to sixth week of regeneration, the number per unit area (density) of backfilled ganglion cells in the inappropriate hemiretina

decreased, but the density of backfilled cells within the whole retina remained constant (Becker and Cook, '87; Fig. 11A,B). Collateral loss, the mechanism favored by Becker and Cook, certainly accounts for the decrease in the density of backfilled cells in the inappropriate hemiretina. But the constancy of density over the whole retina implies that the density rose in the appropriate hemiretina, and this suggests selective brachial choice, at least late in regeneration.

How can a claim for pathfinding by regenerating axons be reconciled with the abnormally large number of errant axons observed in the present, as well as in previous studies (Becker and Cook, '87, '88; Hartlieb and Stuermer, '87)? It has recently been proposed (Bernhardt and Easter, '86) that the normal fiber order in the optic pathway reflects a balance between nonselective and selective interactions between the axons and their environment. Nonselective interactions include the general tendency of growing axons to use predecessors as a substrate (Easter et al., '81; Grant and Ma, '85; Easter and Bernhardt, '87), a tendency for axons that grow out simultaneously to fasciculate with each other (Scholes, '79; Rusoff and Easter, '80; Bernhardt and Easter, '86), and the tendency of axons to grow along a subpial pathway (Rager, '80; Krayanek and Goldberg, '81; Cima and Grant, '82; Easter et al., '84; Silver and Rutishauser, '84). Selective interactions occur between subsets of axons and

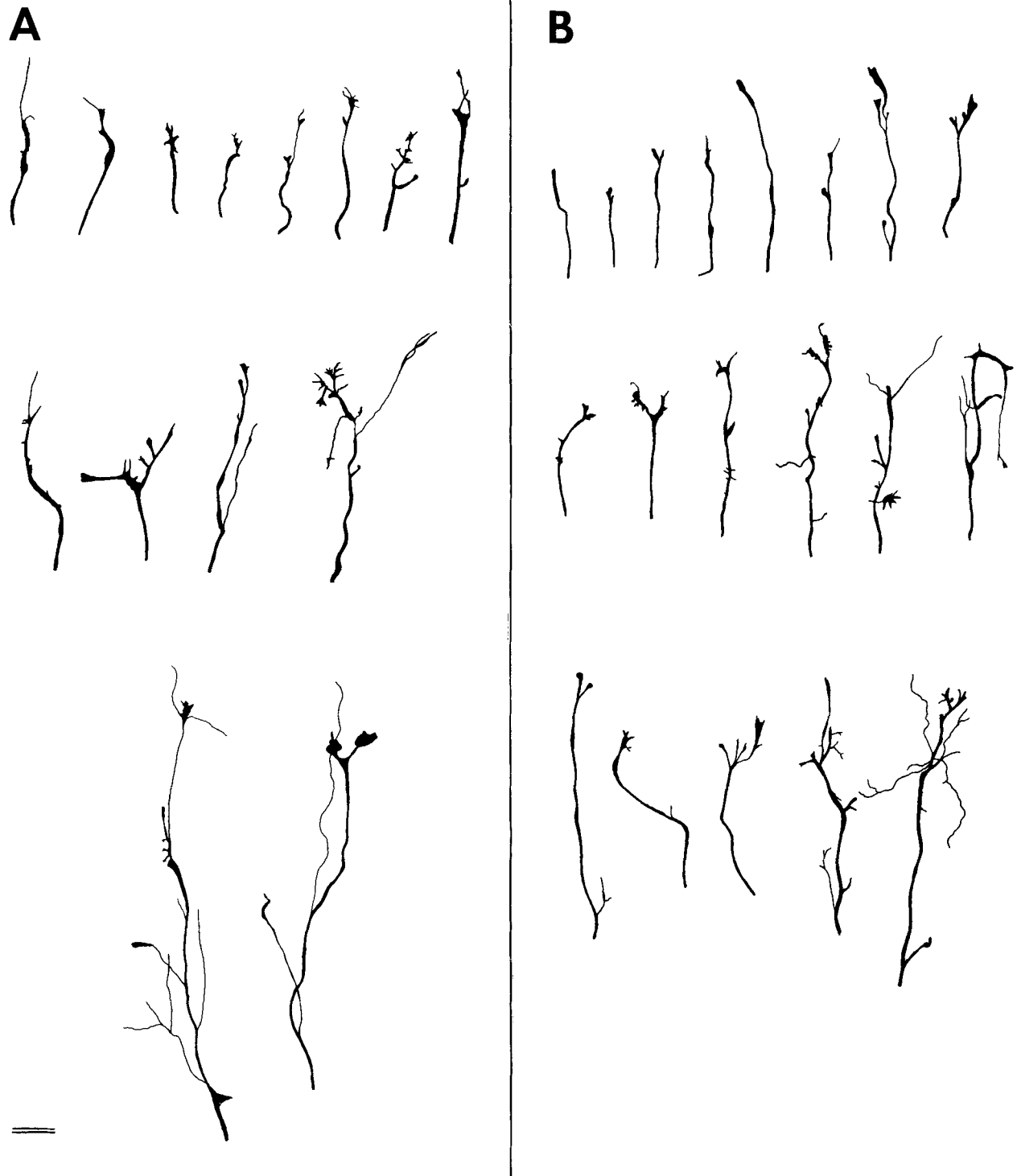


Fig. 14. Ink tracings of the terminal region of regenerating axons in the tract and brachia (A) and the synaptic layer of the tectum (B). In these subregions of the pathway, growing axons often end in multiple

branches and most growth cones are complex. See text for analysis of terminal branching pattern. Magnification bar is 50  $\mu\text{m}$ .

their environment. Evidence for selective interactions in the retinotectal system include the rearrangement of the fiber topography at the nerve-tract boundary (Scholes, '79; Bunt, '82; Bernhardt and Easter, '86; Fraley and Sharma, '86; Springer and Mednick, '86b) and the preferential extension of subsets of retinal axons in culture on subsets of tectal cells (Bonhoeffer and Huf, '82) and on subsets of axons of previously plated retinal ganglion cells (Bonhoeffer and Huf, '85). The abnormally large number of axons growing out simultaneously during regeneration might be expected to shift the balance in favor of the nonselective interactions, in particular between the axons themselves.

This notion is supported by another line of experiments, those in which the number of regenerating axons was artificially reduced. Normal precision in pathfinding by regenerating axons in the goldfish optic pathway has been reported (Busse and Stuermer, '87) if the number of regenerating axons is small. This also suggests an explanation for the apparently high degree of brachial selectivity observed by Attardi and Sperry ('63). Since specific tracing techniques were not available, these authors combined removal of a large sector of the retina with the section of the optic nerve. The course of the axons from the remaining intact retina was then visualized by a general silver stain. This approach greatly reduced the number of regenerating axons. The smaller number of regenerating axons might have had better access to specific guidance cues in the environment (cf. Bernhardt and Easter, '88). At the same time the possibility of nonselective interactions between the axons themselves would have been reduced.

### Interactions between the axons and their environment

DiI reliably labeled the whole extent of the regenerating axons. Therefore it was possible to examine the growing tips of these axons in different subregions of the pathway. The growth cones, ranging from simple, lancelike to more complex, flattened with lamellipodial and filopodial specializations, are similar to the growth cones in the regenerating optic pathway of *Rana pipiens* (Scalia and Matsumoto, '85). Retinal ganglion cell growth cones of comparable morphology have also been described in the *Xenopus* embryo (Harris et al., '85; Sakaguchi and Murphey, '85) and in the mouse embryo (Bovolenta and Mason, '87). These growth cones, which were labeled by application of a dye tracer to the cut axons and observed in fixed tissue, resemble active growth cones examined *in vivo* (*Xenopus*: Harris et al., '87). They are also similar to retinal ganglion cell growth cones observed in cell culture (Harris et al., '85). This similarity suggests that the growth cones observed in the present study are not distorted as a result of fixation or axotomy.

One previous study in the amphibian optic pathway did not correlate growth cone morphology with position along the pathway. This study differed from mine in important ways. Scalia and Matsumoto ('85) examined later stages of regeneration (2 to 15 weeks postcrush) and thus dealt with follower rather than with early growth cones. Work in other systems suggests that such growth cones are less complex than those of early axons (cf. Lopresti et al., '73; Tosney and Landmesser, '85). My study, in contrast, dealt with very early growth cones.

The finding of changes in growth cone morphology along an axonal pathway has precedents. In the embryonic *Xenopus* visual pathway, Harris et al. ('87) noted increased ter-

minial branching as axons approached their tectal targets. Recently, evidence for a comparable sequence of changes in growth cone complexity in the different subregions (nerve, tract and brachia, tectum) of this pathway has been presented (Holt, '88). Bovolenta and Mason ('87) described similar changes in the embryonic mouse visual pathway. There, the tips of the growing axons in the nerve were found to be simple with no branchlike protrusions. In the tract the growing axons generally showed a high degree of terminal complexity, with many growth conelike protrusions arising from the thickened axonal shaft. Within targets the axons showed extensive terminal branching. The complexity of growth cones in the embryonic spinal cord of *Xenopus* (Nordlander, '87) and in the embryonic chick limb (Tosney and Landmesser, '85) also changes consistently in specific regions of their pathways.

A possible interpretation of the present findings is similar to that offered in a study (Tosney and Landmesser, '85) of the growth cone morphology in the lumbosacral region of the chick embryo. There, the increase in size and complexity of motoneuron growth cones in specific subregions of the pathway (termed "decision regions") was suggested to relate to the presence of guidance cues. The increased complexity of regenerating axons, which I have observed in the optic tract and brachia (where axons segregate according to their dorsal or ventral hemiretinal origins) and in the synaptic layer of the tectum (where axons have to distinguish between retinotopically appropriate and inappropriate targets), is consistent with the idea that exploratory branching might be a mechanism by which regenerating axons sample pathway cues in regions where they face alternative pathway choices.

### CONCLUSIONS

The present study, in agreement with previous work (Attardi and Sperry, '63; Stuermer and Easter, '84a; Rankin and Cook, '86; Hartlieb and Stuermer, '87; Bernhardt and Easter, '88; Stuermer, '88b), indicates that axonal pathfinding is contributing to the reestablishment of the fiber order along the regenerated pathway of goldfish.

These results do not argue, however, that axonal pathfinding mediated by guidance cues specific to subsets of retinal axons is solely responsible for shaping the regenerated pathway. In fact, the present as well as previous results (Schmidt and Edwards, '83; Cook and Rankin, '86; Stuermer, '87) indicate that secondary events, acting after the reestablishment of a crude projection onto the tectum, control the segregation of initially overlapping terminal arbors. The sharpening of the regenerated map is apparently dependent on activity-mediated interactions between terminals (Schmidt and Edwards, '83; Cook and Rankin, '86) rather than on the recognition of specific cues.

Whether a secondary mechanism (acting subsequent to axonal outgrowth) is also sharpening the fiber order in the optic pathway is presently unclear. It has recently been proposed (Becker and Cook, '87) that selective loss of aberrant collaterals, mediated by activity-driven interactions at the terminal level, is responsible for the fiber topography observed in the tract and its brachia at 2 to 3 months of regeneration. A second study (Hartlieb and Stuermer, '87), however, has reported little influence of activity blockade (by application of tetrodotoxin to the retina) on collateral withdrawal. Recently, Cook and Becker ('88) have shown that

the fiber order observed in the brachia at 42 days after nerve cut is not affected by activity blockade during regeneration, even though such blockade prevents the refinement of the retinotectal map. Collateralization per se does not necessarily lead to the reestablishment of ordered connections; during the reinnervation of the extraocular muscles of goldfish aberrant collaterals persist indefinitely (Scherer, '86).

Evidence for axonal guidance has been presented in several studies of the embryonic development of the retinotectal system in fish (Stuermer, '87) and in amphibians (Holt, '84, '88; Sakaguchi and Murphey, '85; O'Rourke and Fraser, '86). In both, new neurons are added to the retina during postembryonic development (see Easter, '83). The fact that the new axons are systematically incorporated into the retinotectal pathway, apparently following the same rules as their predecessors (see Scholes, '79; Bunt, '82; Easter et al., '84; Bernhardt and Easter, '86; Fraley and Sharma, '86; Springer and Mednick, '86a,b; Bernhardt et al., 1988), suggests the continued presence of guidance cues. The present results indicate that regenerating axons, similar to new axons, are capable of responding to these cues. This notion agrees with a recent review (Holder and Clarke, '88) of axon regeneration following damage to the central or peripheral nervous system in several vertebrates. This survey suggested that the cues capable of guiding axons to their targets in postembryonic neurogenesis also operate during specific regeneration.

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