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Pathways of regenerated retinotectal axons in goldfish

I. Optic nerve, tract and tectal fascicle layer

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SUMMARY

This study investigates the order of regenerating retinal axons in the goldfish. The spatiotemporal pattern of axon regrowth was assessed by applying horseradish peroxidase (HRP) to regenerating axons in the optic tract at various times after optic nerve section and by analysing the distribution of retrogradely labelled ganglion cells in retina. At all regeneration stages labelled ganglion cells were widely distributed over the retina. There was no hint that axons from central (older) ganglion cells might regrow earlier, and peripheral (younger) ganglion cells later, as occurs in normal development.

The absence of an age-related ordering in the regenerated optic nerve was demonstrated by labelling a few axon bundles intraorbitally with HRP (Easter, Rusoff & Kish, 1981) caudal to the previous cut. The retrogradely labelled cells in retina were randomly distributed in regenerates and not clustered in annuli as in normals. Tracing regenerating axons which were stained anterogradely from intraretinal HRP applications or retrogradely from single labelled tectal fascicles illustrated the fact that the regenerating axons coursed in abnormal routes in the optic nerve and tract.

On the surface of the tectum regenerated fibres re-established a fascicle fan. The retinal origin of tectal fascicles was assessed by labelling individual peripheral, intermediate and rostral fascicles with HRP. The retrogradely labelled ganglion cells in the retina were often more widely distributed than in normals, but were mostly found in peripheral, intermediate and central retina, respectively.

The order of fibre departure from each tectal fascicle was revealed by placing HRP either on the fascicle's proximal or on its distal half. With proximal labelling sites labelled ganglion cells were found in the temporal and nasal retina, and with distal labelling sites labelled ganglion cells were confined to nasal retina only. Further, the axonal trajectories of anterogradely labelled dorsotemporal retinal ganglion cells were compared to those of dorsonasal retinal ganglion cells in tectal whole mounts. Dorsotemporal axons were confined to the rostral tectal half, whereas dorsonasal axons followed fascicular routes into the fascicles' distal end and reached into caudal tectum. This suggests that the fibres exited along their fascicle's course in a temporonasal sequence.

Thus in the tectum, fibres in fascicles restore a gross spatial and age-related order and tend to follow their normal temporonasal sequence of exit.

INTRODUCTION

In lower vertebrates regenerating retinal axons ultimately find their correct termination sites in the synaptic layer of tectum (Schmidt, 1978; Meyer, 1980; Stuermer, 1978, 1981; Fujisawa, Tani, Watanabe & Ibata, 1982; Gaze & Fawcett,

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1983; Cook & Rankin, 1984; Egert, Kalko & Stuermer, 1984), but travel through abnormal routes (Horder, 1974; Cook, 1983; Udin, 1978; Meyer, 1980; Dawney, 1981, 1982; Fawcett & Gaze, 1981; Fujisawa *et al.* 1982; Stuermer & Easter, 1984*a*).

The goal of this investigation is to determine where and to what extent regenerating retinotectal axons in fish deviate from their normal spatial order. These investigations extend and corroborate an earlier study on fibre order in regeneration (Stuermer & Easter, 1984*a*). The order of retinal axons in the normal retinotectal pathway in fish has been analysed extensively in the recent past (Scholes, 1979; Easter *et al.* 1981; Bunt, 1982; Stuermer & Easter, 1984*b*; Stuermer, 1984*a*; Rusoff, 1984), thus providing a basis for the evaluation of the deviations of regenerated fibre routes more precisely.

In the normal fish, retinal axons are ordered by their origin from retinal sectors such that dorsal, ventral, temporal and nasal axons, respectively, are grouped and confined to a fraction of the optic nerve and tract (Easter *et al.* 1981; Bunt, 1982). Throughout their path, retinal axons are also ordered by their origin from annular retinal regions, i.e. by their age (Bunt, 1982; Easter *et al.* 1981; Rusoff, 1984; Stuermer & Easter, 1984*b*). Axons of the continuously added ganglion cells at the peripheral retinal margin (Johns & Easter, 1977; Meyer, 1978) travel together in age-related fascicles in the nerve and tract (Easter *et al.* 1981; Stuermer & Easter, 1984*b*). In the tectum, fibre fascicles of dorsal and ventral retina are arranged in two fan-like arrays over the ventral and dorsal hemitectum, respectively (Attardi & Sperry, 1963). Within this fan old axons from central retina course in short rostrocentral fascicles and those from progressively younger and more peripheral ganglion cells course in progressively longer and more peripheral fascicles (Stuermer & Easter, 1984*b*). As they enter into the synaptic layer, fibres exit from each rostrocaudal fascicle in a well-defined sequence: temporal axons leave from the fascicle's proximal half, while nasal axons depart from the distal half (Stuermer & Easter, 1984*b*).

In view of these findings we investigated in a previous study (Stuermer & Easter, 1984*a*) the paths of axons during regeneration. We showed that regenerating fibres from dorsal and ventral retinal origin were mixed and not restricted to their normal positions in the optic nerve and tract. However, they segregated at the brachial bifurcation such that 80 % of the dorsal (ventral) fibres grew through their correct ventrolateral (dorsomedial) brachium and only 20 % through the incorrect brachium (Stuermer & Easter, 1984*a*). In the tectal fibre layer, regenerating fibres coursed in fascicles which were arranged in two fan-like arrays (Attardi & Sperry, 1963; Cook & Horder, 1977; Stuermer & Easter, 1984*a*). An analysis of the spatial order of fibres in the fascicle and synaptic layers revealed that regenerating fibres did not reoccupy their old paths in the tectum (Cook, 1983; Stuermer & Easter, 1984*a*). In those experiments the distribution of retrogradely labelled ganglion cells was determined after insertion of HRP into both the upper fascicle and the deeper synaptic layers at well-defined sites. While such procedures resulted in very orderly patterns in normal animals (Cook, 1983; Stuermer & Easter, 1984*a*; Easter

& Stuermer, 1984), ganglion cells were scattered widely over the retina in regenerates (Cook, 1983; Stuermer & Easter, 1984*a*).

However, in previous experiments when HRP application was restricted to fascicles alone (Stuermer & Easter, 1984*a*), in a small sample of animals, labelled ganglion cells were less widely scattered and were even clustered in annular retinal regions. This suggested that regenerating fibres of similar spatiotemporal origin might have reassociated to some extent as in normals.

This report extends these experiments and systematically examines the regional origin of retinal axons in the tectal fascicles. It further examines whether regenerating axons might regrow in a spatiotemporal order reminiscent of the annular growth rings seen in normal development, as suggested by Mansfield (1983). It also tests whether regenerating axons might regain their normal spatiotemporal association in the optic nerve and tract. Another question addressed in this report is whether regenerating fibres exit from tectal fascicles in the same temporonasal sequence found in normal animals.

Preliminary accounts of this work have been published as abstracts by Stuermer (1984*b*) and Egert *et al.* (1984).

MATERIALS AND METHODS

Adult goldfish *Carassius auratus*, 4–8 cm in length (and two at 14 cm), were maintained in aerated aquaria at room temperature (17–20 °C). For all surgical procedures, the fish were anaesthetized in 0·1 % aqueous solution of tricaine methanesulphonate. Optic nerves were sectioned intraorbitally with iridectomy scissors so that the cut edges of the nerve were visible. Normal and regenerated retinal axons were labelled with HRP (Type VI, Miles) at different survival times after sectioning the nerve.

Regenerating axons were labelled by cutting the optic nerve or both brachia of the optic tract and applying HRP to the stumps. A flap of cranial bone was removed to provide access to the brachia and the telencephalic lobes were aspirated. Smaller populations of retinal axons were labelled by inserting an HRP-coated needle through the regenerated optic nerve caudal to the previous site of the cut (Easter *et al.* 1981; Stuermer, 1984*a*). Axons from defined regions of the retina were labelled by applying HRP intraretinally to the dorsonasal, dorsotemporal, or dorsoperipheral retina as described in Easter & Stuermer (1984).

To label individual or groups of tectal fascicles, a dorsal bone flap was removed over the cranium to expose the tectum. The cranial fluid was aspirated, the fascicles were severed with a tungsten microneedle and a small crystal of HRP was placed at the damaged site. In two additional cases a micropipette filled with HRP was introduced into the fascicle and the superficial synaptic layers and the HRP injected by pressure.

Generally it should be noted that labelling of regenerating fascicles was difficult. Regenerating fascicles are poorly myelinated (Murray, 1976) and are therefore thinner. They were often very difficult to discern through the overlying *stratum marginale* (SM), with the exception of peripheral fascicles which travelled close to the pial surface. Because regenerated fascicles were difficult to see, we often involuntarily injured fibre processes in the synaptic layer SFGS (*stratum fibrosum et griseum superficiale*). HRP was then picked up by all of the fascicular axons, the extrafascicular axons, and by terminals, as shown by previous studies (Easter & Stuermer, 1984; Cook, 1983). Uptake of HRP by axon terminals in regenerates produced clusters of cells in retina at retinotopic sites (Cook & Rankin, 1984; Stuermer & Easter, 1984*a*; Egert *et al.* 1984), often surrounded by lightly labelled cells (Easter & Stuermer, 1984). Most ganglion cells outside the cluster can be attributed to fibres labelled in the *stratum opticum* (SO) (and to a minor extent from extrafascicular axons in SFGS or possibly from mislocated terminal arbors). The ventral

hemitectum was inaccessible and not labelled. After the operation the cranial opening was closed and the fish allowed to recover.

With each of the application procedures HRP was transported both anterogradely, labelling axons and terminals, and retrogradely to label axons and their cell bodies of origin in the retina. Whole mounts of retina and tectum, often including the optic tract and nerve, were prepared after 1 to 3 days of survival.

To prepare retinal whole mounts, the retina was detached from the pigment epithelium, fixed in 4% glutaraldehyde and phosphate buffer for 20 min, and reacted with o'dianisidine (OD) (Coleman, Scalia & Cabrales, 1976) or tetramethylbenzidine (TMB) (Mesulam, 1978). The OD-reacted retinae were dehydrated, cleared in xylene and mounted under Permount. In most cases, the TMB reaction product faded within 1 h (Stuermer & Easter, 1984*a*,*b*). Labelled axons and cells were immediately recorded by *camera-lucida* tracings as in previous publications (Stuermer & Easter, 1984*a*,*b*; Easter & Stuermer, 1984). When the label in cells faded before completion of the tracing, retinae were re-reacted in TMB to revisualize the HRP-containing cells. These re-reactions increased the amount of precipitate on the retinal surface. This precpitate obscured the labelled cells in photographs but did not impede their identification during microscopic examination, since they reside in a deeper focal plane and since the trained eye can readily distinguish between non-specific precipitate and labelled cells. *Camera-lucida* tracings were also preferred over photographic documentation, since only heavily labelled cells which are densely clustered and arranged in orderly patterns can be seen in reasonably sized photomontages (Mansfield, 1985) (see Fig. 2A,B).

To prepare tectal whole mounts, fish were perfused through the heart with saline, the lobes and the optic tracts and nerves were isolated and reacted unfixed in diaminobenzidine (DAB) (Adams, 1977; Fujisawa, Watanabe, Tani & Ibata, 1981), and then fixed for 2 h in 4% glutaraldehyde. In order to flat mount a tectal lobe, it was slit along the equator from the caudal pole almost to the rostral pole, flattened between slide and coverslip, dehydrated, cleared in xylene, and mounted with Permount. Since all tecta contain heavily stained blood vessels, fibres and terminal arbors were generally recorded by *camera-lucida* tracings. Whole mounts of tecta which had experienced surgical manipulations were of poor quality as compared to uninjured brains. However, they were adequately preserved to localize the HRP application sites, and in most cases to observe the resulting labelled fascicles. Occasionally the labelled axons could be traced to their terminal arborizations in the synaptic layer SFGS.

RESULTS

The spatiotemporal pattern of axon regeneration

To test whether regenerating axons grow towards the tectum in an orderly spatiotemporal pattern, in animals with previously sectioned optic nerves HRP was applied to both brachia of the optic tract at increasing regeneration times. The contralateral retina was examined for retrogradely labelled cells.

If regenerating axons regrow in the same order as during their normal development, the retinae of early regeneration stages should have labelled ganglion cells in an annulus centred around and close to the optic disc. With increasing regeneration times, central and increasingly more peripheral ganglion cell annuli should be labelled (Mansfield, 1983, 1985).

The retinae of 34 fish contralateral to the HRP application site were examined at 4 (n = 4), 5 (n = 2), 7 (n = 2), 9 (n = 2), 11 (n = 5), 13 (n = 1), 14 (n = 2), 15 (n = 1), 17 (n = 4), 19, 20, 21 (n = 1 each), 24 (n = 2), 25 (n = 2) and 32 days (n = 2), and 2 months (n = 2) after optic nerve section. The success of regenerative growth varied among animals (Stuermer & Easter, 1984). The earliest survival

times at which a few labelled ganglion cells appeared in the retina were 9 days (n = 1) and 11 days (n = 1). One retina at 14 days had most ganglion cells close to its centre (Fig. 1A). All other fish from 13 days onwards had numerous labelled ganglion cells which were mostly scattered over the entire retina (Fig. 1B,D,E,F). There were occasional additional clusters of labelled cells in discrete portions of

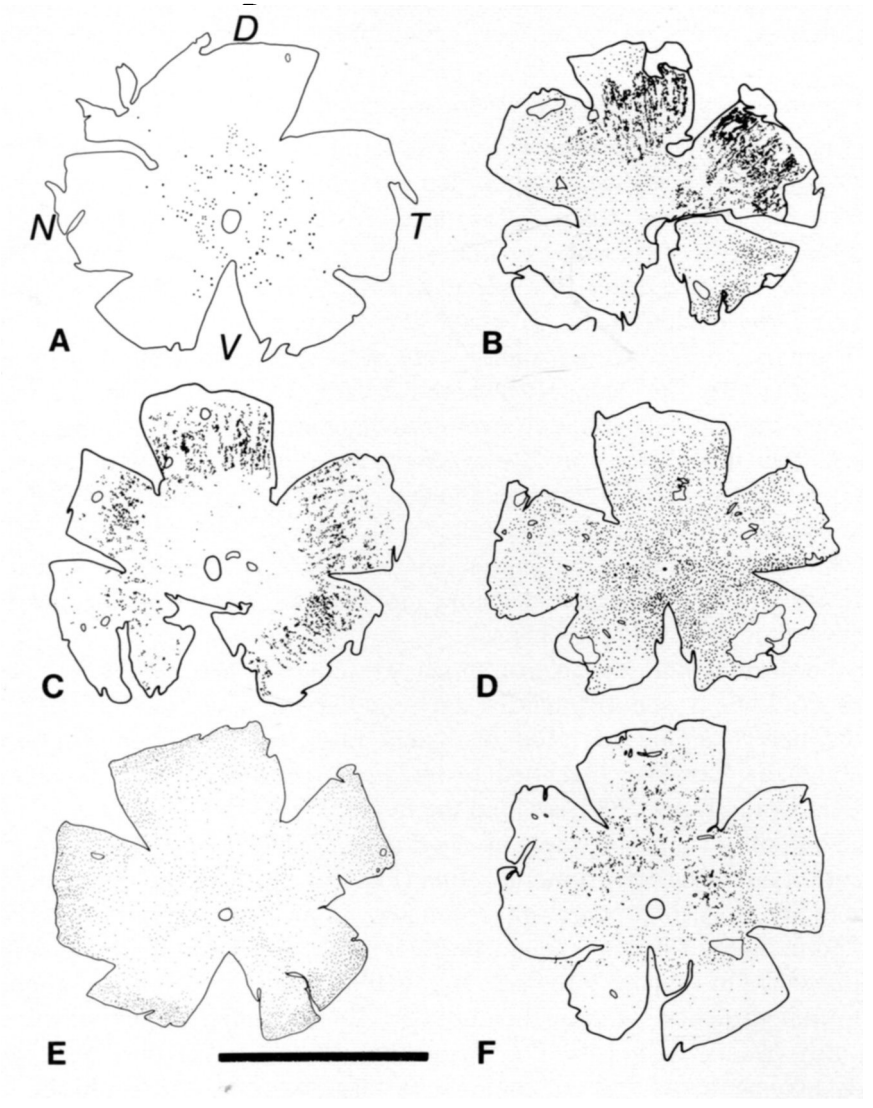


Fig. 1. *Camera-lucida* tracings of left retinal whole mounts, all in the same orientation. The black dots indicate the ganglion cells, retrogradely labelled from HRP applications to the contralateral optic tract at (A) 14 days, (B) 13 days, (C) and (D) 17 days, (E) 21 days and (F) 32 days after optic nerve section. Note the scattered distribution of ganglion cells in B,D,E and F. In (C) most ganglion cells are accumulated in a ring in midretinal to peripheral positions and only a few cells lie in the retinal centre. In (A) they are predominantly clustered in midretina. Abbreviations: *D*, dorsal; *V*, ventral; *T*, temporal; *N*, nasal. Calibration bar, 5 mm.

the retina (Fig. 1B,C). The positions of these clusters did not correlate in any obvious way with increasing survival times.

The results suggest that the spatiotemporal order in which regenerating axons reach the optic tract is random and therefore different from that in normal development. The occasional occurrence of clusters of labelled cells in certain parts of the retina may indicate that the axons of some retinal cell clusters, as a group, arrived at the brachia earlier than most other retinal ganglion cells.

Order in the optic nerve at later regeneration stages

To examine the spatial order of the regenerating retinal fibres caudal to the site of the earlier nerve cut, three experiments were performed.

(1) To test whether regenerating fibres restore an age-related order in the vertical dimension of the nerve as in normals (Easter *et al.* 1981), an HRP-coated needle was introduced into the nerve (Rusoff & Easter, 1980) in the posterior orbit, close to the chiasm.

In normals, the labelled ganglion cells were clustered in annuli or annular portions (Fig. 2B). In regenerates (nine fish of 2, 3 and 10 months after section) there were no annuli, but widely scattered ganglion cells instead (Fig. 2A,C,D). Thus, neither at early nor at late regeneration times were regenerating retinal fibres ordered by their origin from annular retinal regions in the optic nerve and tract.

(2) Axons filled by labelling a few adjacent fascicles in the dorsomedial hemitectum were viewed directly in whole mounts of the optic nerve and tract of normals and regenerates (n = 15).

On the normal side, the axons, which are joined in a common fascicle in the tectum, could be traced through the dorsomedial brachium, the tract (Fig. 3A,B) and the nerve as one (or two) consistent fascicles. On the regenerated side (Fig. 3C,D,E), however, labelled axons were distributed more widely in the brachium (Fig. 3D,E), the tract, and the nerve.

(3) The path of anterogradely labelled axons, which were filled from an HRP application in the dorsoperipheral retina (Fig. 10), through the nerve and tract is shown in Fig. 4. As in preparations labelled by fascicle lesions in the tectum, fibres travelled in highly abnormal routes throughout the width of the nerve close to the previous cut. On their way towards the tectum, most fibres became aligned into the normal direction of growth, although fibres changing their position were frequently observed (Fig. 4C,D). Regenerated fibres never displayed the same orderly arrangements and confinement to fascicles observed in normal animals (Fig. 5) (see also Stuermer & Easter, 1984*a*; Fawcett & Gaze, 1981).

Tectal fascicles

By 2 to 3 months regenerating fibres had restored a fan-like fascicle array over the dorsal and ventral hemitectum (Stuermer & Easter, 1984*a*) reminiscent of the normal fascicle array (Fig. 6). Deviations from the normal pattern were

evident (Stuermer & Easter, 1984*a*), like crossing of fascicles and fascicles crossing the equator.

To determine the retinal regions from which their axons originated, peripheral, intermediate, and rostrocentral tectal fascicles were individually labelled on the regenerated and normal sides of tecta (according to procedures in Stuermer & Easter, 1984*a,b*). The labelled sites were located in tectal whole mounts after processing for HRP (see Fig. 12A,B) and recorded in *camera-lucida* tracings.

The pattern of labelled ganglion cells in retinal whole mounts after labelling normal tectal fascicles was consistent with our earlier results in that labelled ganglion cells were restricted to a half-annulus in the ventral hemiretina which extended to the nasal boundary between the dorsal and ventral hemiretinae (Stuermer & Easter, 1984*b*).

Following the labelling of regenerated fascicles, retinae either exhibited annular labelled portions or scattered labelled cells. There was no hint that longer regeneration stages gave more orderly patterns than shorter ones, and we therefore treat them together.

Peripheral fascicles were labelled in five fish at 2 (n = 2), 4 (n = 1), and 12 (n = 2) months after sectioning of the optic nerve. In all of the retinae from these fish most labelled ganglion cells were in a partial annulus correctly positioned in the peripheral ventral hemiretina. The radial widths of the annular zones were wider than on the normal side and often labelled cells were also scattered in the vicinity of the partial annulus. Two typical examples, one at 2, the other at 4 months, are shown in Fig. 7A,B.

The labelled ganglion cell distributions in the 12-month regenerates were similar to and no more orderly than those of the 2- and 4-month regenerates (see Discussion).

When HRP was applied to *intermediate* fascicles (in 21 fish of 2–12 months after sectioning of the optic nerve), one group of fish between 2 and 8 months (n = 11) exhibited labelled ganglion cells in annular portions, appropriately positioned in the middle of the ventral hemiretina (Fig. 8A,B). Nine fish between 2 and 12 months had no annular region of labelled cells. Instead, there were clusters of labelled cells at retinotopically appropriate sites, and labelled cells distributed more widely in the ventrotemporal and ventronasal hemiretina (Fig. 8D,E,F). In some cases they reached into the dorsonasal retinal quadrant (Fig. 8C,F). In one fish, the labelled cells were in the correct ventral and in the incorrect dorsal hemiretina in nearly mirror-symmetrical distributions (Fig. 8E).

Taken together, these results suggest that age-related ganglion cell axons were correctly routed in peripheral and, in some instances, in intermediate fascicles in the dorsal hemitectum. In others, however, intermediate fascicles were composed of axons from ganglion cells widely distributed in the ventral and to some extent in the dorsal hemiretina.

Lesioning of *rostrocentral* fascicles was extremely difficult, because they were often hidden in the curved rostral tectal pole.

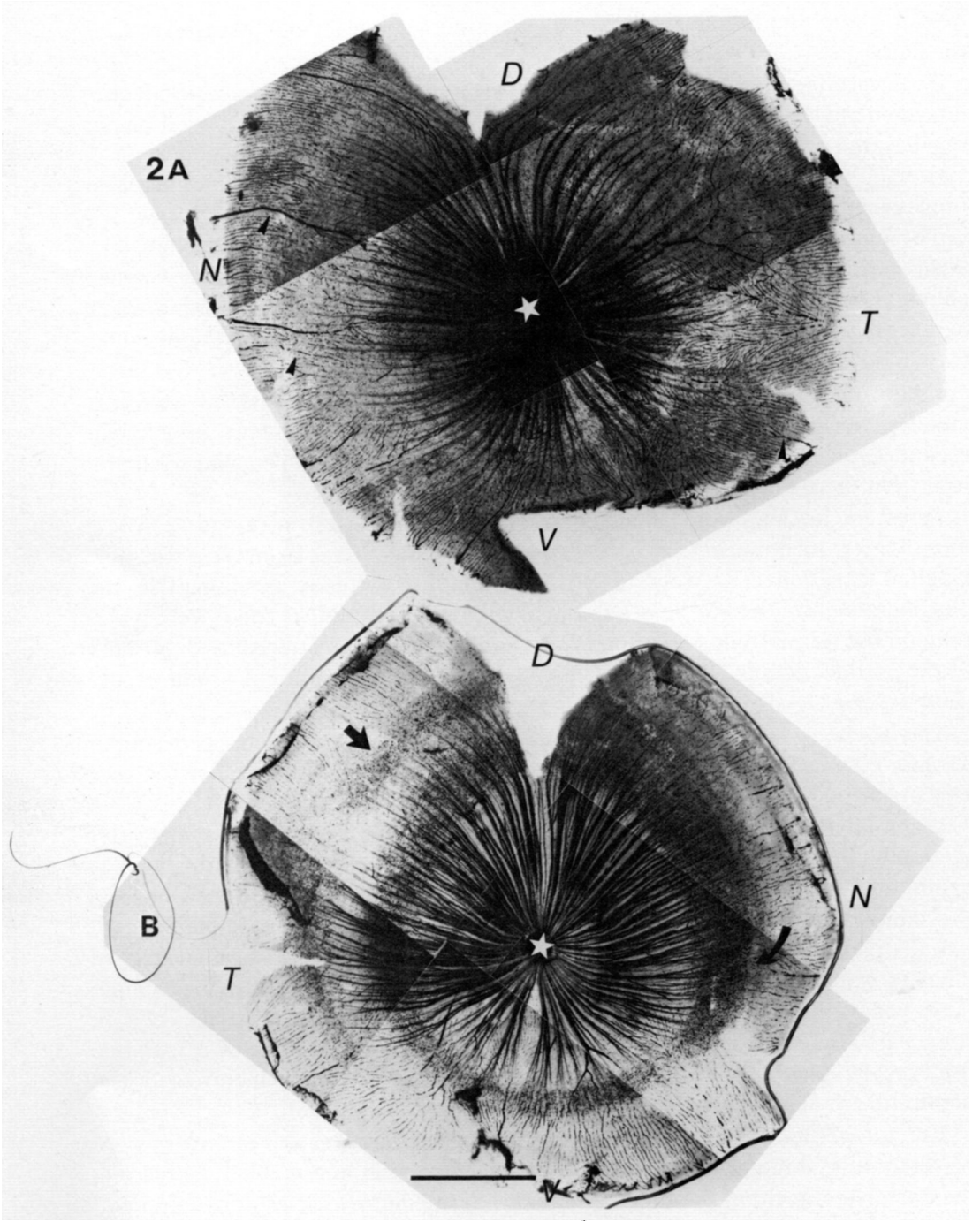
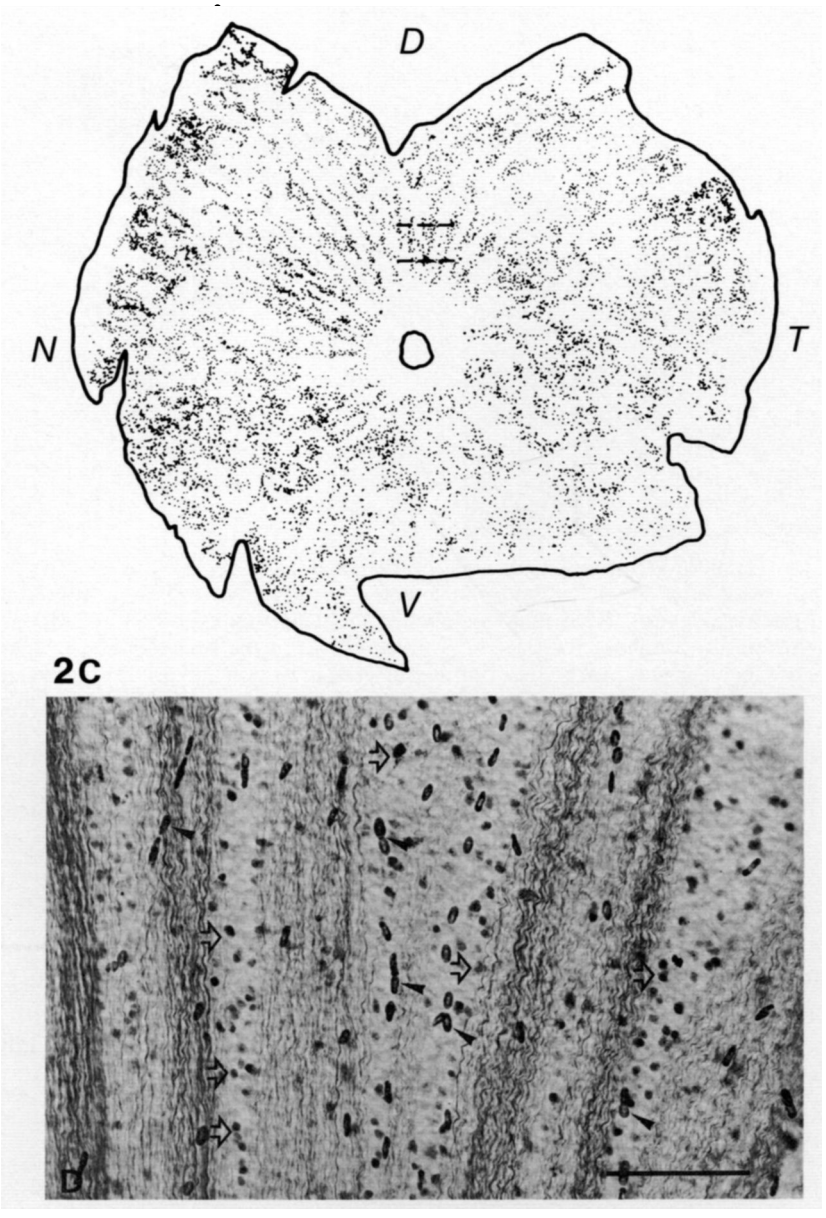


Fig. 2. (A,B) Photomicrographs of freshly OD-reacted retinal whole mounts showing ganglion cells and axons retrogradely labelled from vertical penetration of the optic nerve close to the chiasm with an HRP-coated needle. Ganglion cells are visible as black dots. They are partially obscured by the widely ramified blood vessels (arrowheads). (A) Labelled ganglion cells are scattered all over the retinal surface



after labelling regenerated axons caudal to the previous cut at 3 months after optic nerve section. A *camera-lucida* drawing of this retina is shown in Fig. 2C. (B) In the normal, labelled ganglion cells are clustered in an annulus in midretinal position (bent arrow) and in a partial annulus more peripherally (arrow).

(C) *Camera-lucida* drawing of the retina shown in (A). A photographic documentation of the labelled ganglion cells and axons in the region, indicated by dashed lines, is shown in Fig. 2D. Abbreviations in A,B,C as in Fig. 1; scale bar, 1 mm. The axons run in bundles radially directed towards the optic disc (*).

(D) Photomicrograph documenting the labelled ganglion cells and axons in the region marked in (C). The open arrows point to examples of individual or groups of labelled ganglion cells. The arrowheads point to blood cells. The irregular dark lines are labelled axons running towards the optic disc (bottom). Scale bar, 150 μm.

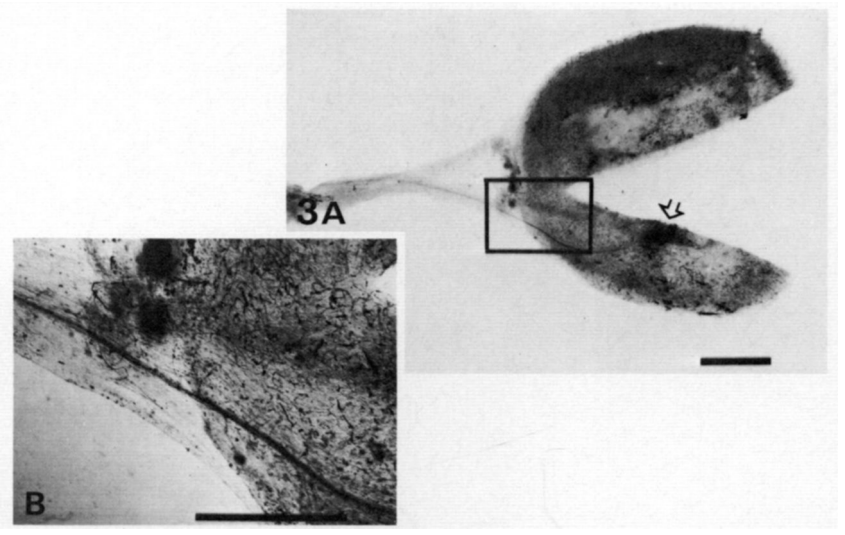


Fig. 3. (A–D) Photomicrographs of tecta of a normal and a regenerate showing the HRP application sites (open arrow) in dorsomedial tectal halves and the retrogradely labelled fascicular axons. Note that the labelled fascicular axons in (A) and (B) are tightly clustered throughout their tectal path as well as in the brachia. The labelled axons in the regenerate (C,D) are less tightly clustered in tectum and occupy almost the complete width of the brachium. The boxed areas of the dorsomedial brachium of the normal and regenerated optic tract are shown in higher magnification in (B), (D) and (E) to illustrate that fibres (open arrows in (D)) are tightly clustered in the normal, but widely scattered in the regenerate.

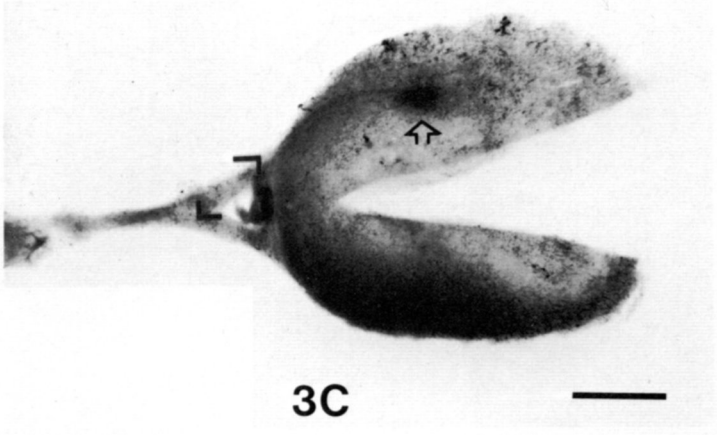
(E) This *camera lucida* tracing shows the path of the labelled fibres in the dorsomedial brachium of the regenerate more clearly. Fibres at different focal depths are drawn in one plane. Bars in (A) and (C), 1 mm; in (B) 0·5 mm, (D) 100 μm.

With local HRP application to a few lesioned fascicles or injections of HRP into the rostral tectum (between 2 and 12 months after optic nerve section), four retinae had partial annuli of labelled ganglion cells close to the retinal centre (Fig. 9A) and two other retinae had partial annuli in the ventral hemiretina and many cells scattered in the dorsal and ventral retinal halves. Two fish had labelled cells scattered all over the retina with no hint of an annulus.

When several fascicles were lesioned simultaneously ($n = 4$), two retinae had labelled ganglion cells clustered predominantly around the optic disc in both dorsal and ventral retinal halves (Fig. 9B). Two other retinae with relatively deep wounds at the site of the lesion had labelled ganglion cells scattered all over the retinal surface.

With the limitations of our labelling method in mind, we conclude that rostrocentral fascicles may be composed of axons from older, more central ganglion cells, and that additional errant axons from various retinal positions either joined them or crossed the labelling site.

A preference of peripheral ganglion cell axons for peripheral tectal fibre routes was confirmed by tracing anterogradely labelled axons in tectal whole mounts following intraretinal HRP applications in the dorsoperipheral retina (Fig. 10, top).



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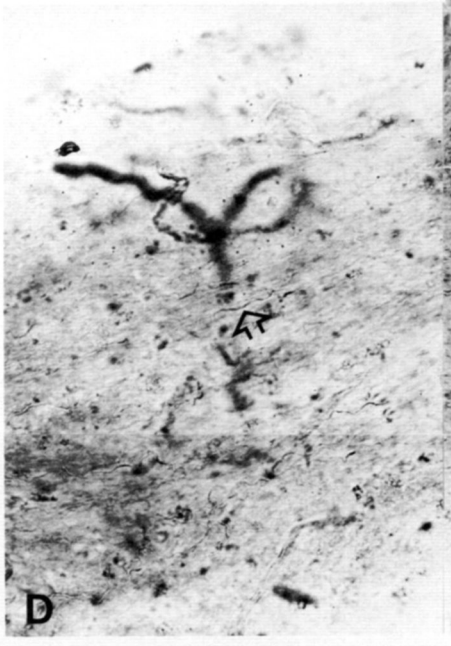

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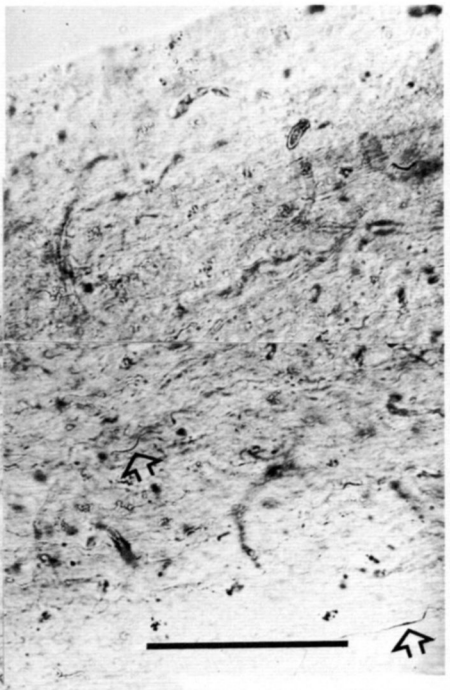

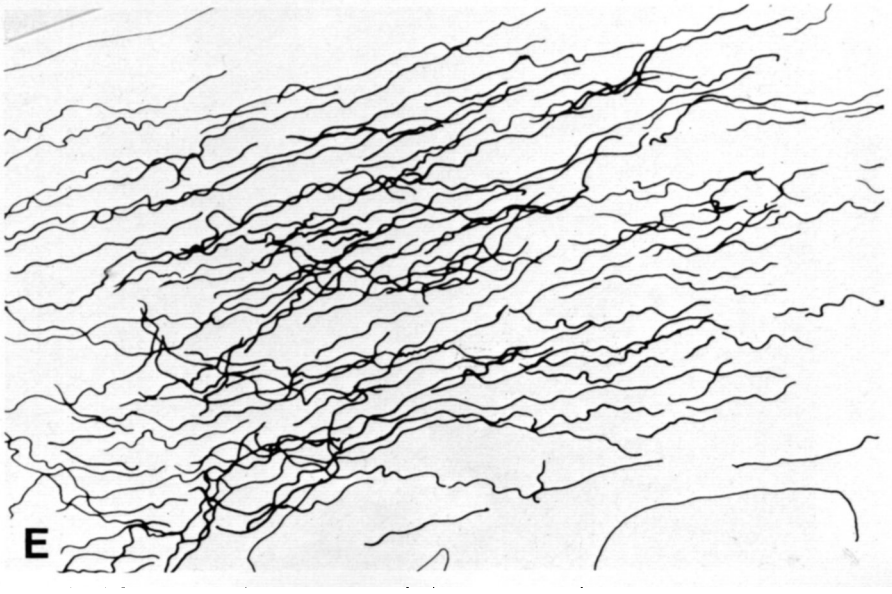

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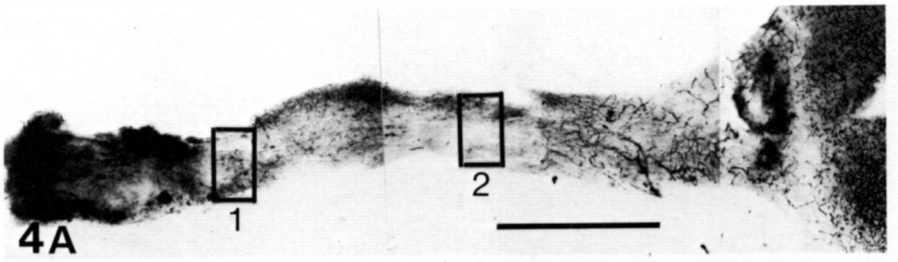
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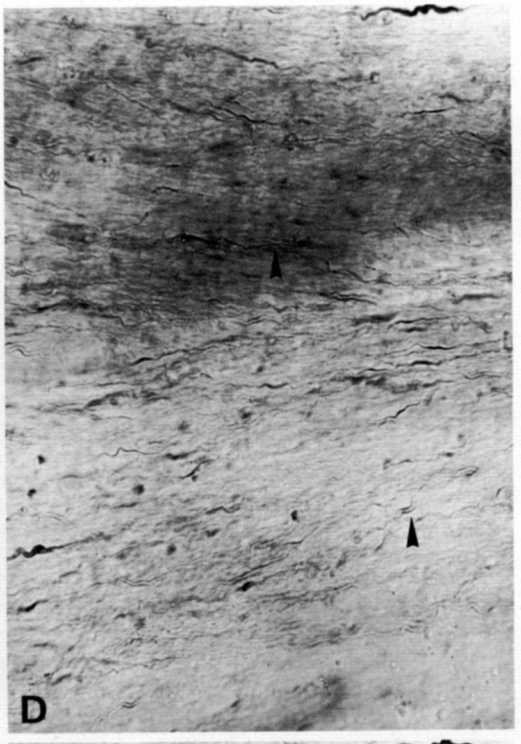
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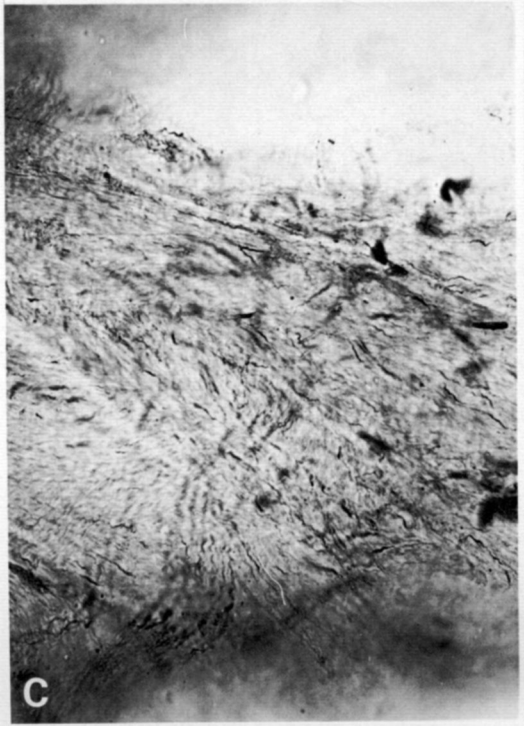
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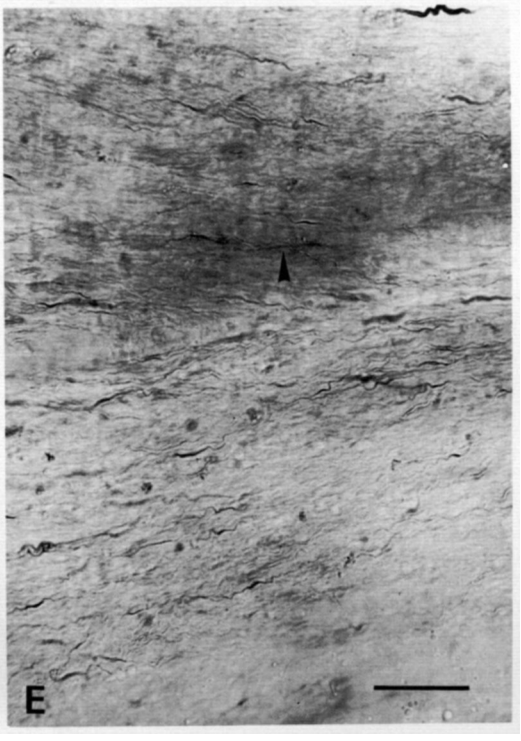

E

The vast majority of the labelled fibres entered the tectum through the correct ventral brachium (Stuermer & Easter, 1984*a*), and travelled in numerous peripheral fascicles (Fig. 10, bottom). Only a very few errant axons were noted in central tectum.

Order of fibre exit

Next we tested whether regenerating axons might exit over the proximodistal extent of individual fascicles in a temporonasal sequence similar to that in normals (Stuermer & Easter, 1984*b*). As shown above (Figs 7A, 9), labelled ganglion cells were located in both the temporal and nasal retina with HRP applications to the proximal half of a fascicle. In a series of 13 fish, HRP was applied to a fascicle's distal half.

Peripheral fascicles labelled distally at 2 months (n = 2) and 4 months (n = 2) had shorter annular portions in the periphery of the ventronasal retina (and sometimes, in addition, in the dorsonasal retina) (Fig. 11A,B). With distal labelling sites on intermediate fascicles (seven fish between 2 and 12 months) three retinae had partial annuli of labelled ganglion cells in midretina at ventronasal (Fig. 11C) and (in one fish) at ventro- and dorsonasal quadrants. The remaining four retinae (Fig. 11D) had a cluster of labelled cells at retinotopic sites and labelled cells scattered over the nasal retina. These results suggest that the sequence in which regenerating fibres leave intermediate and peripheral fascicles may be similar to that in normals. We were not able to label rostrocentral fascicles at two different sites, because they were difficult to reach.

The tectal whole mounts of a normal and a regenerate in Fig. 12A,B confirm the orderly fibre exit. The label sites in both were in the fascicle's distal half. All axons and terminal arbors caudal to the HRP application site were restricted to the caudal tectal half. Within this area, however, they terminated in widely separated locations in the regenerate (Fig. 12B) in contrast to the normal (Fig. 12A).

Further evidence that temporal fibres leave the fascicular routes before nasal axons came from experiments in which axons were labelled by intraretinal HRP

Fig. 4. Photomicrographs of regenerated retinal axons in a whole mount of the optic nerve and tract. HRP had been applied onto dorsoperipheral axons in retina (see Fig. 10 top), 2 months after optic nerve section.

(A) Low-power view of the complete nerve and tract extending between the left eye (to the left) and the right tectum (to the right). The boxed regions 1 and 2 are shown enlarged in (B),(C) and (D),(E), respectively. Scale bar, 1 mm.

(B,C) Enlargement of the boxed region 1 of (A) showing the regenerated fibres at the site of the previous cut at two different focal planes. Labelled fibres are diverted from their normal centrifugal direction of growth and course in abberrant routes throughout the width of the nerve, often perpendicular to the normal direction (arrow) and cross each other frequently (arrowhead).

(D,E) Enlargement of the boxed region 2 of (A) at two focal planes. Labelled fibres occupy the complete width of the optic nerve. Most have regained their centrifugal direction of growth although fibres changing their positions and fibres crossing other fibres are still frequent (arrowheads). Scale bar for B–E, 0·1 mm.

Fig. 5. (A) Photomicrograph of a whole mounted normal optic nerve and tract in the same orientation as in Fig. 4, showing a population of retinal fibres, stained from HRP application in dorsoperipheral retina as in Fig. 4 two months after optic nerve section. The boxed region is enlarged in B. Scale bar, 1 mm.

(B) Enlargement of the boxed region of (A), the normal HRP-stained fibres course in an orderly fashion, mostly aligned parallel to each other and clustered in fascicles towards tectum (to the right). Note the difference to the less-orderly fibres paths of the regenerated fibres in Fig. 4. Scale bar, 0·1 mm.

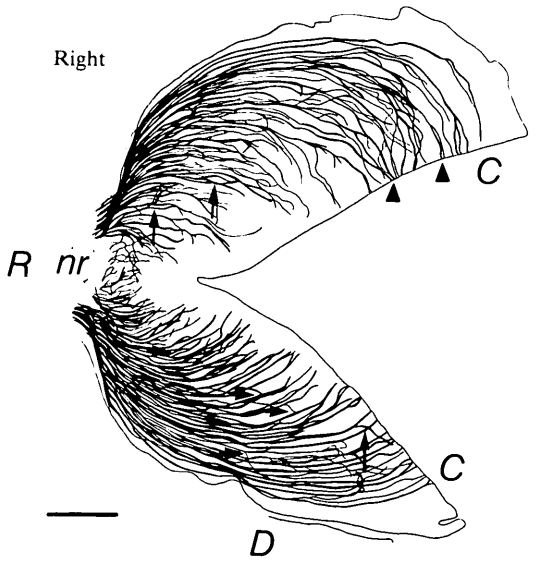


Fig. 6. This *camera-lucida* tracing of a right tectal whole mount illustrates the fan-like array made up by short rostrocentral, intermediate and long peripherocaudal fascicles at 3 months following optic nerve section. Note that several fascicles cross the tectal equator (arrowheads), and cross each other (small arrows). Abbreviations: *R*, rostral; *C*, caudal; *D*, dorsal; *V*, ventral; *nr*, nucleus rotundus. Bar, 500 μm.

applications at strictly dorsoperipheral, dorsonasal and dorsotemporal sites, and traced in tectal whole mounts (Figs 10, 13B,D).

In Fig. 10 the mass of anterogradely labelled dorsoperipheral fibres entered through peripheral fascicles, exited, and ended in terminal arbors within the ventroperipheral tectum. Labelled axons did not reach beyond the terminal arbor sites, suggesting that most dorsal retinal axons had exited from their fascicles correctly in the middle of the fascicles' proximodistal extent on the tectum.

With label sites close to the optic disc (Fig. 13A,C), the labelled axons (Fig. 13B,D) entered through rostrocentral, intermediate, and peripherocaudal fascicles. In the synaptic layers labelled terminal arbors were densely clustered in a zone between the centre and the periphery of the tectum (dashed lines in Fig. 13B,D) in generally appropriate retinotopic positions. In both tecta, most fibres entered through the correct ventrolateral and fewer through the incorrect dorsomedial brachium (Stuermer & Easter, 1984*a*).

With temporal labelling sites, labelled axons were restricted to the rostral tectum. Several of the peripheral axons in the rostral tectum made right-angled turns as they exited from their fascicles. Almost no axons were visible caudally to the zone occupied by terminal arbors in the rostroventral hemitectum (Fig. 13B).

Dorsonasal axons (Fig. 13D) travelled throughout the fascicles' full extent. Some prominent axons (arrows in Fig. 13D) were seen to exit close to the fascicles' distal end, then to change direction and course in extrafascicular routes towards the ventrocaudally located terminal arbor zone.

These observations indicate that regenerating axons from different retinal sectorial origins had left their fascicles at different sites. Temporal and nasal axons may share a common fascicle, but only the nasal axons remained in the fascicle as far as the distal end, whereas the temporal axons tended to leave the fascicle in its proximal half.

A detailed description of the routes of individual fibres, and particularly the tortuous path of misrouted fibres, is beyond the scope of the present report and will be described later. It should be noted that numerous fibres were found to cross

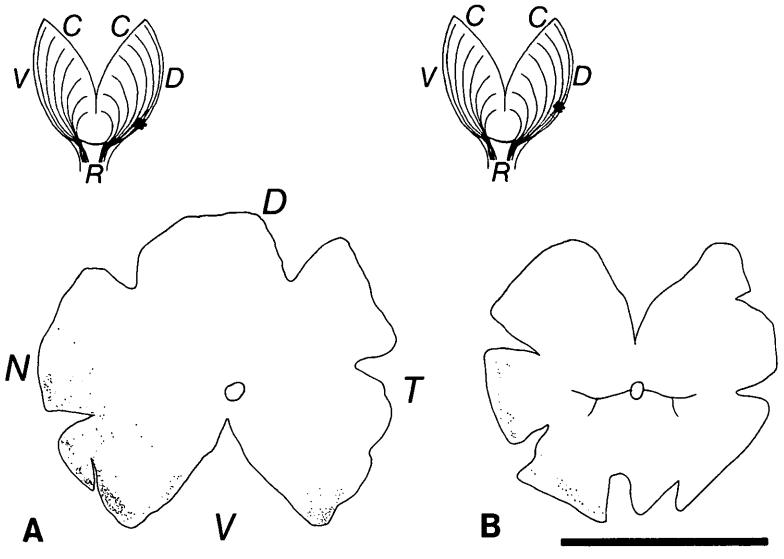


Fig. 7

Figs 7, 8, 9 and 11. *Camera lucida* tracings of left retinal whole mounts, all in the same orientation showing the distribution of retrogradely labelled ganglion cells after labelling a few fibre fascicles in the dorsomedial tectum. The labelling sites on tectal fascicles are indicated by a star in the schematic tectal whole mounts above each retina. The curved lines are representatives for tectal fascicles. Abbreviations are the same in all figures. *D*, *T*, *N*, *V* as in Fig. 1. Calibration bar, 5 mm.

Fig. 7. With labelling of peripherocaudal fascicles at 2 months (A) and 4 months (B) after nerve section, most ganglion cells were clustered in an annular zone in the periphery of the ventral retina and a few scattered cells are outside the annulus in (A). The more proximal label site in (A) gave an annular portion extending further into the temporoventral retina, whereas the more distal site in (B) gave a shorter annular portion confined to the nasoventral retina.

Fig. 8. Patterns of ganglion cells after labelling intermediate fascicles at 8, 2, 8, 5, 2 and 2 months, respectively, after optic nerve section. (A,B) A partial annulus resembling a normal distribution. (C,D) No annular portions are present. There are clusters of cells (arrowhead) in the ventrotemporal retina tectotopically to the HRP application site. The ganglion cells outside the cluster are scattered. (C) This retina is an example of the most extreme disorder in which the ganglion cells were widely scattered in the ventral and dorsonasal retina. (D) Scattered cells were confined to the ventronasal retina and most in midretinal regions. (E) Cells were arranged almost mirror-symmetrically in both the ventral and dorsal hemiretinae, predominantly in midretinal regions. (F) Cells in midretinal regions were not confined to the ventral hemiretina, but extended, similar to (C), into the dorsonasal retinal quadrant.

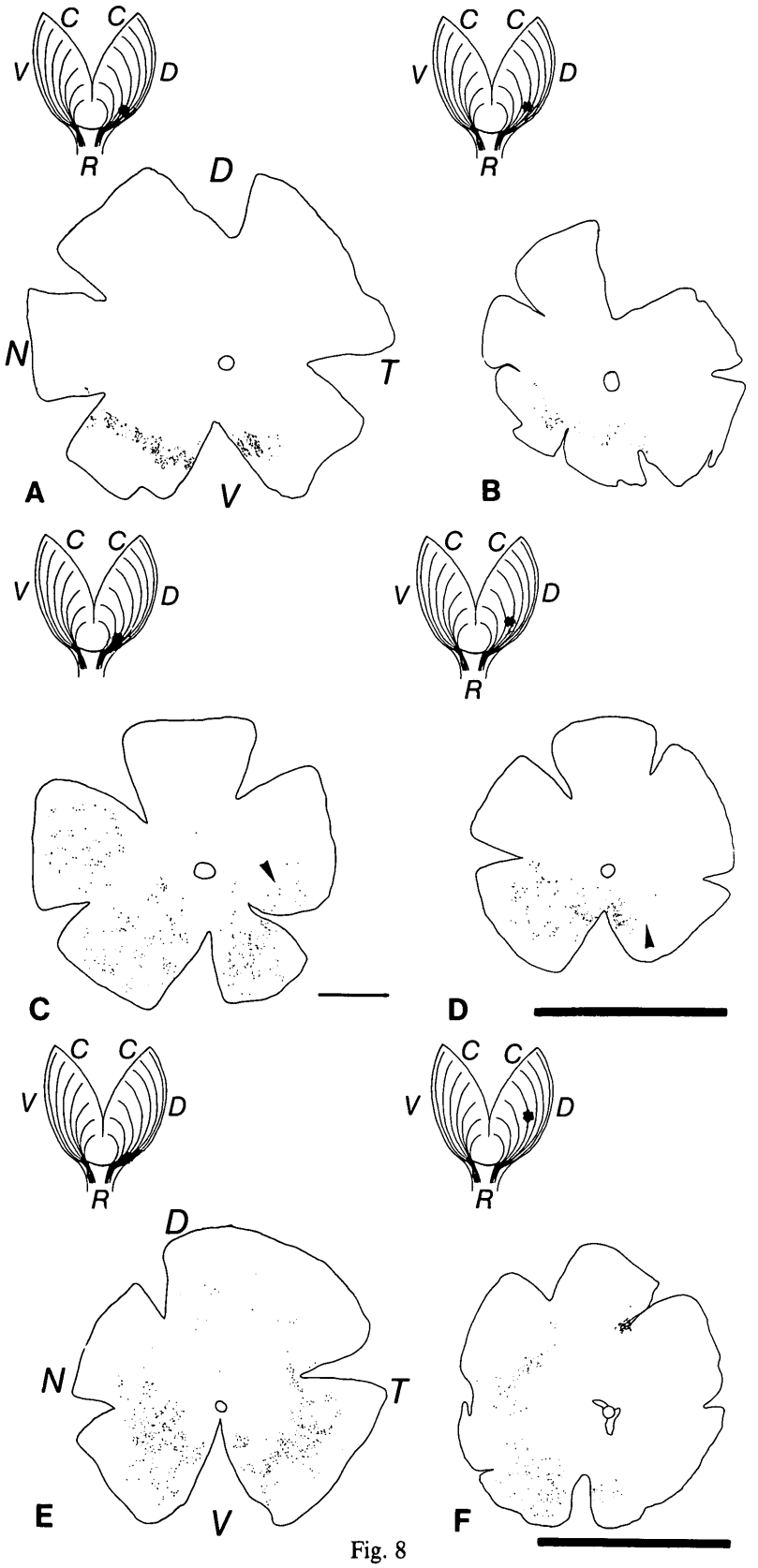


Fig. 8

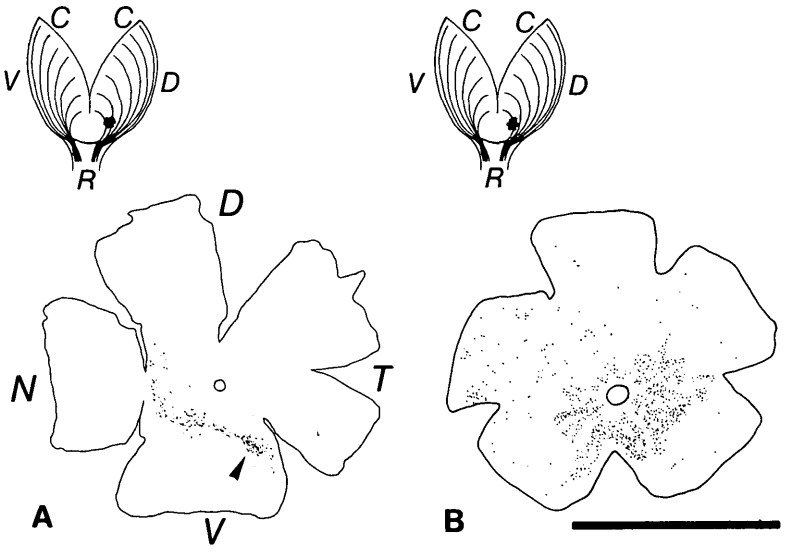


Fig. 9. (A) HRP was injected into the rostral dorsomedial tectum 2·5 months after nerve section. The resulting pattern in the retina resembles that of a normal animal (compare Fig. 5 in Easter & Stuermer, 1984). Note a cluster (arrowhead) of cells in the ventrotemporal retina tectotopically related to the injection site. (B) HRP was applied to several disrupted rostrocentral fascicles 3 months after nerve section. Ganglion cells were scattered, however, most were accumulated close to the optic disc.

the tectal equator and to enter the terminal arbor zone in the ventral hemitectum (Egert *et al.* 1984).

DISCUSSION

Sequence of axonal regrowth

The foregoing results show that axotomized axons do not recapitulate the normal sequence of axonal growth in which central ganglion cell axons grow towards the tectum before peripheral axons. Ganglion cells backfilled from regenerating axons were located at variable positions and appeared in various distributions at all survival times. We did not observe an orderly increase of labelled cells starting in the centre and moving to the periphery with increasing regeneration time. Moreover, occasionally ganglion cells in midretinal to peripheral positions were labelled (Fig. 1C) while none or only a few were in central retina, indicating that the axons of peripheral retinal regions grew prior to central ones in some preparations.

Our findings differ from those of Mansfield (1983, 1985) in which he claims that 'regeneration recapitulates the retinotopically specific sequence of innervation found in development'. However, as stated in Mansfield (1985) 'the sequence of axonal regrowth was not always reproducible when a second series of fish was tested'.

Our findings are consistent with those of J. E. Cook (personal communication). He found no difference between the times it took central and peripheral ganglion cells to reach the tecta of five goldfish in which HRP was applied to a deep transverse cut across the rostral tectum. In every case, retrogradely labelled cells were uniformly distributed over the entire extent of the retina 21–50 days after sectioning the optic nerve. Cook's results are complementary to our own earlier

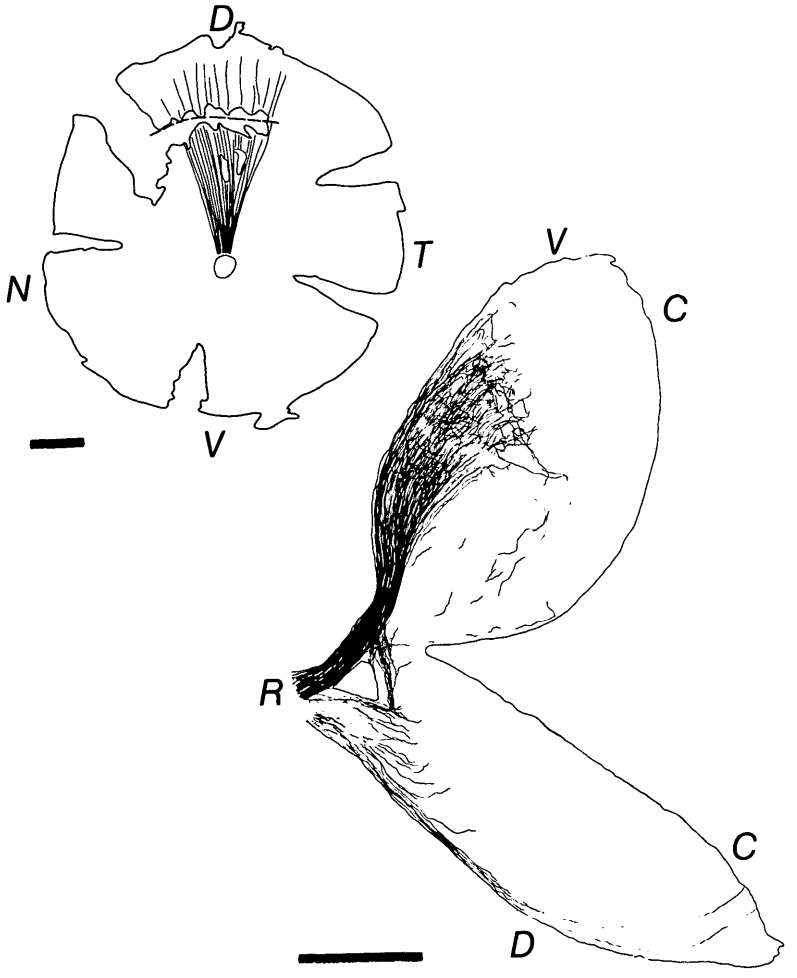


Fig. 10. *Camera-lucida* tracings of whole mounts of a left retina (upper left) and its contralateral tectum (lower right) of a regenerate 2 months after optic nerve section. HRP was applied in retina (dashed line) in the dorsal periphery. Black lines indicate the retrogradely and anterogradely labelled axons above and below the dashed line, respectively. The black lines in the tectal whole mount are the anterogradely labelled axons originating from the dorsal peripheral ganglion cells. Most axons enter rostrally through the correct ventral brachium and fewer through the incorrect dorsal brachium. Axons are largely confined to the peripheral half of tectum and only a few are seen in central tectum. The small forked axon endings in the ventral hemitectum represent terminal arbors. Bar below retina and tectum, 1 mm. Abbreviations as in Figs 1, 6.

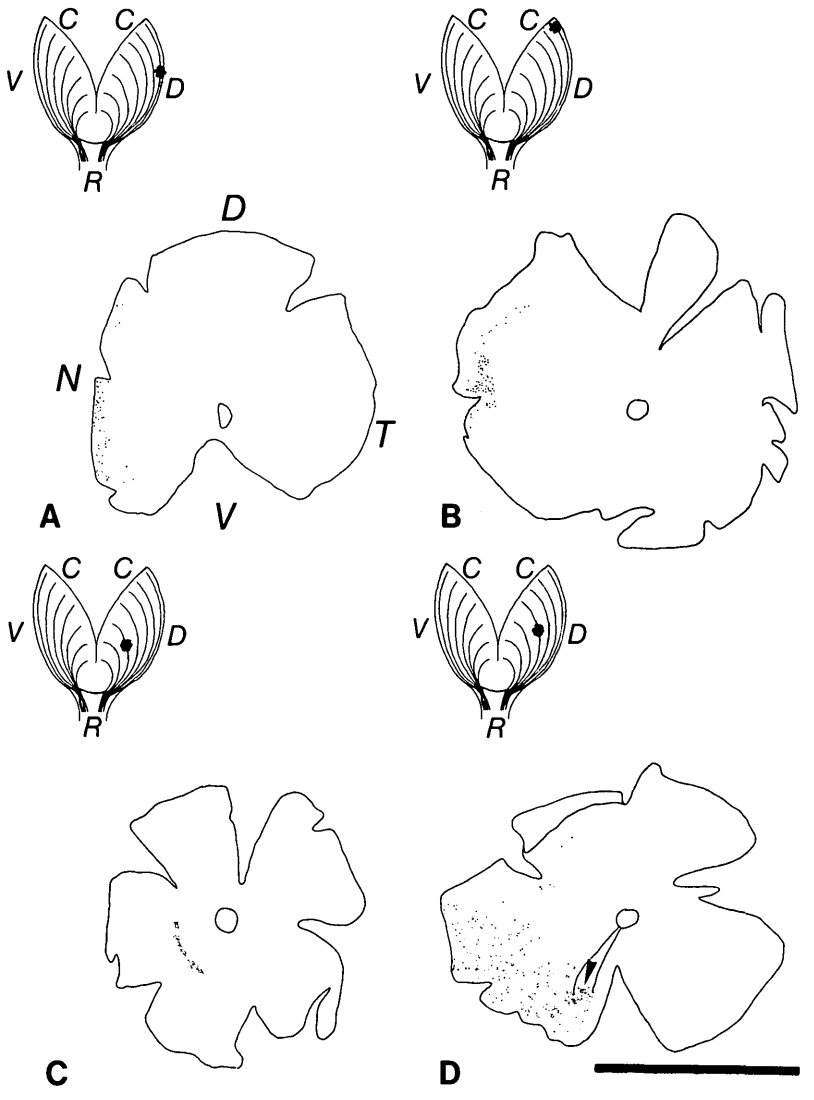


Fig. 11. Distribution of ganglion cells after labelling fascicles in the distal half of their tectal extent at various intervals, i.e. (A) 2·5 months, (B) 4 months, (C) 2 months, (D) 12 months after optic nerve section. In all cases the ganglion cells are confined to the nasal retina. (A) Peripheral fascicle labelling gave a short annular segment in the periphery of the nasoventral retina. (B) Labelling a peripheral fascicle at its extreme distal end gave ganglion cells in the peripheral nasal extreme of the ventral and the nasodorsal hemiretina. (C) Distal label sites on an intermediate fascicle gave a partial annulus in the nasoventral retina in midretinal position. (D) Example of a disordered pattern with distal label sites on an intermediate-to-peripheral fascicle. In addition to the tectotopically positioned cluster (arrowhead) in the ventronasal retina ganglion cells outside the cluster are scattered, however confined to the ventronasal retina. The two radial black lines are labelled axons.

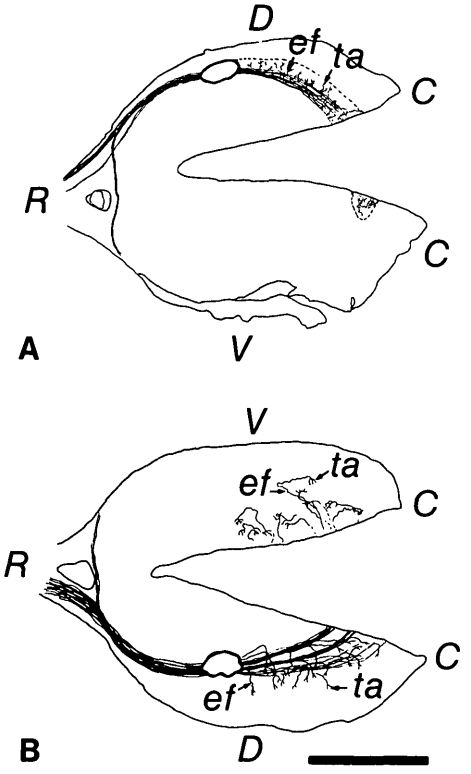


Fig. 12. *Camera-lucida* tracings of tectal whole mounts of a normal (A) and a regenerate of 4·5 months after optic nerve section (B) after labelling a fascicle in tectum. In both, the retrogradely labelled axons proximal to the HRP application sites (open circles) are joined in a fascicle. In (A) the anterogradely labelled axons distal to the HRP application site are also tightly clustered in a bundle. Axons are seen to exit individually from the bundle, travel caudally through short extrafascicular routes and deploy their terminal arbors next to each other in a partial annulus in the dorsomedial hemitectum. In the regenerate (B) the anterogradely labelled axons distal to the HRP application site are more widely dispersed than in the normal. The extrafascicular axons course from their point of exit from the fascicle layer into various directions, even crossing the tectal equator, and deploy their ventrolateral hemitecta. They are, however, still restricted to the caudal tectal half. Fibres in fascicles in SO and in extrafascicular routes (*ef*) and terminal arbor (*ta*) in SFGS below SO are drawn in the same plane. Abbreviations as in Figs 1, 6. Scale bar, 1 mm.

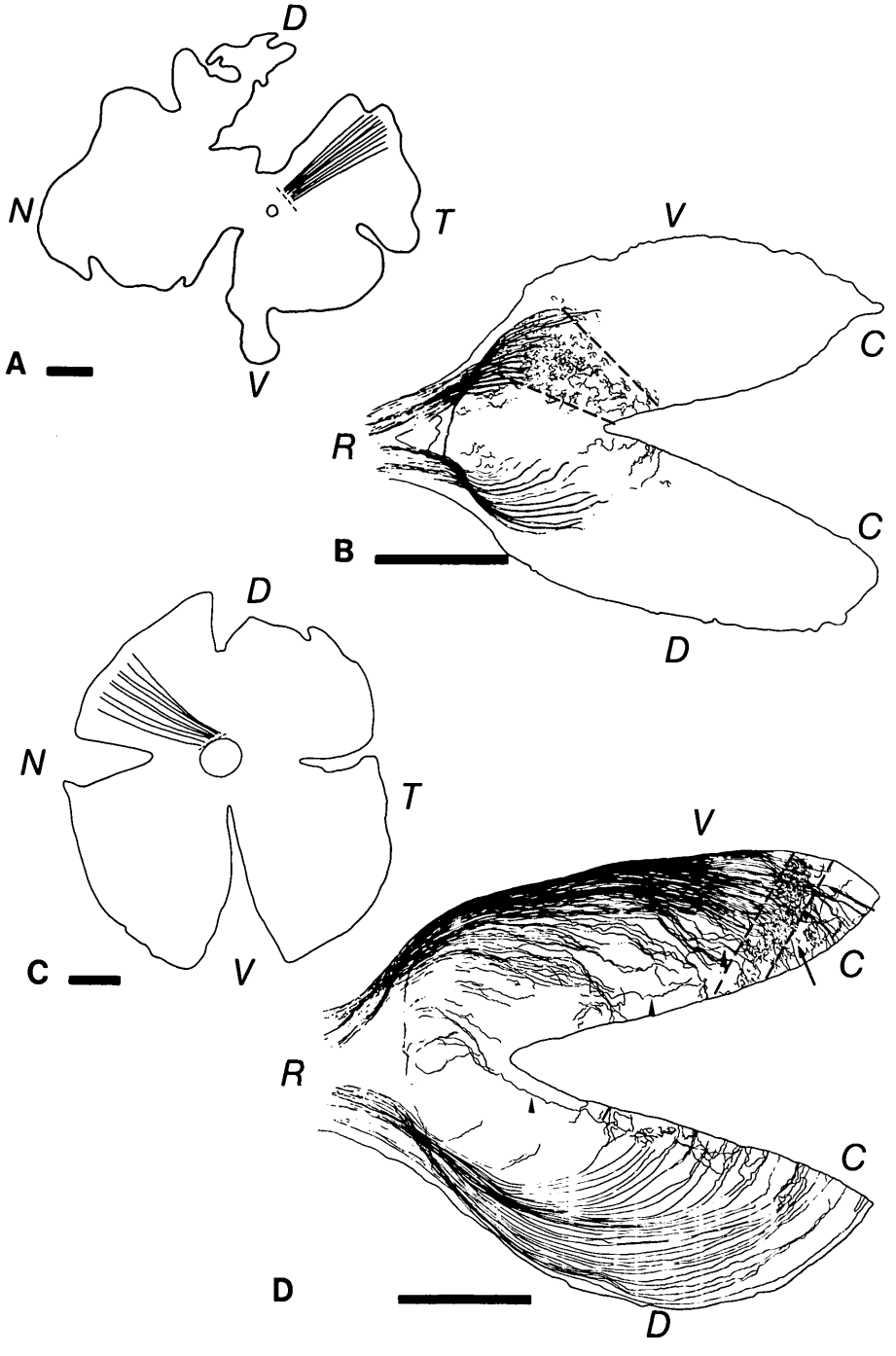
experiments in which HRP was inserted deep into the rostrodorsomedial tectum 10 to 110 days after nerve section. These insertions resulted in labelled ganglion cells widely distributed over the retinal surface.

We therefore propose that any such population of retinal axons may constitute the leading front during regenerative axon growth.

Optic nerve and tract

At the site of the cut the regrowing axons became drastically diverted from their normal lines of growth. Similar observations have been made on the regenerating axons of amphibians (Bohn, Reier & Soubeer, 1982).

As a consequence, the normal relative ordering of axons is not regained in the nerve stumps entering the tectum. This has been shown previously in sections (Stuermer & Easter, 1984*a*) and is confirmed here by the demonstration that axons from defined retinal regional origin occupied different positions of the nerve to their normal counterparts. The absence of a correct spatial order in the nerve and



tract was further substantiated by the finding that regenerating fibres which formed a single fascicle in the tectum occupied an abnormally large fraction of the nerve and tract. Moreover, the regenerating axons did not become layered in age-related order within the nerve as normal. This was shown by the absence of annuli or partial annuli in the retina after labelling subpopulations of axons in the nerve.

Evaluation of the method of fascicle labelling

Before discussing the fibre order in the tectal fascicle layer, a critical evaluation of the methods may be useful.

To identify the retinal origin of tectal fascicles, our local HRP applications should ideally have been confined to the fascicle layer. We knew from earlier experiments and a report by Cook (1983) that if, in addition to fascicles, deeper extrafascicular fibres and terminal arbor profiles in the synaptic layer SFGS were exposed to HRP, labelled ganglion cells were found to be widely scattered over the retina (Cook, 1983; Stuermer & Easter, 1984*a*). In advanced regeneration stages, HRP uptake produced clusters of labelled cells (Cook, 1983; Egert *et al.* 1984). With the more restricted lesions used here, labelled ganglion cells were less-widely dispersed, and, as shown in Figs 7, 8A,B, 9A, occasionally accumulated in correctly positioned annular-like zones. However, labelled ganglion cell clusters were also apparent at retinotopic sites in several instances (Fig. 8C,D). This implies that HRP penetrated too deeply. Therefore, in some cases ganglion cells labelled by the uptake of HRP in terminals and extrafascicular axons might mask more orderly arrangements of ganglion cells labelled from axons in fascicles alone. The risk of HRP pick up from extrafascicular axons and terminals was particularly high after the disruption of several fascicles in rostrocentral tectum, since this procedure created larger wounds than usual. However, labelling of terminals cannot have been excessive in these fish, since terminal labelling in rostrocentral tectum resulted in the widespread labelling of cells all over retina at early

Fig. 13. *Camera-lucida* tracings of whole mounts of left retinae (A,C) and their contralateral right tecta (B,D) of regenerates at 7 months (A,B) and 12 months (C,D) after optic nerve section. HRP was applied in the retina (dashed line) close to the optic disk, dorsotemporally in (A) and dorsonasally in (B). Black lines indicate the retrogradely labelled axons. The black lines in the tectal whole mounts are the anterogradely labelled axons originating from the dorsotemporal and dorsonasal ganglion cells, respectively. Broken lines indicate the zone in the synaptic layer SFGS in which the terminal arbors were clustered. Most axons enter rostrally through the correct ventral and fewer through the incorrect dorsal brachium.

(B) The temporal axons are only seen in fascicles in the rostral tectum. Those in the correct ventral hemitectum are confined to the fascicles' proximal part. Those in the incorrect dorsal hemitectum had exited from their fascicles but then reassociated in a plane deep to the fascicles in SO (arrowhead) to cross the tectal equator.

(D) The nasal axons travelled in fascicular routes into the fascicles' distal part. A few prominent axons in extrafascicular routes are shown (arrowheads) between their point of exit from the fascicle and the terminal arbor regions. Note that several axons in fascicles in the incorrect dorsal hemitectum cross the tectal equator and enter into the terminal arbor zone, some in caudorostrally directed routes (arrows). Abbreviations as in Figs 1, 6. Scale bar, 1 mm.

regeneration stages (Stuermer & Easter, 1984*a*) and in additional clustering of cells in peripherotemporal retina at advanced regeneration stages (Egert *et al.* 1984; C. A. O. Stuermer, unpublished observations).

Tectal fascicles

The reformation of a fascicle fan reminiscent of the normal fascicle pattern is a striking feature in regenerates. In the past, the appearance of an orderly fibre arrangement has led to the concept that regenerating fibres follow their old pathways on the tectum (Attardi & Sperry, 1963) or are guided by maintaining their normal neighbourhood relationships as they extend to their correct termination sites (Horder & Martin, 1978). The demonstration that most regenerated fibres were diverted from their normal routes, but still appeared to terminate in approximately their correct target area, speaks against these mechanisms.

By backfilling small subsets of tectal fascicles and determining the retinal regions in which their axons arose, our experiments revealed that the order of regenerating fascicles is a gross approximation of the original developmentally derived, age-related order of normal (Stuermer & Easter, 1984*a*). However, the precision of the normal ordering was never regained, either in early or in late regeneration stages.

The highest precision of correct fibre routing according to age was revealed by fibres derived from peripheral retina as they innervated peripheral tectum. This is possibly true for peripheral fascicles as a whole and not only for those most peripheral fascicles which might arise from a population of peripheral ganglion cells born after the cut and extending *de novo* (Johns & Easter, 1977).

If and to what extent new axons contributed to the peripheral fascicles of regenerates was not investigated by us. We assume that some new axons travelled together with regenerating ones. However, at least for the younger regenerates, their number was probably small for the following reasons: most of our fish were kept in crowded conditions and fed once a day. They did not increase noticeably in body size. Under these conditions, neurogenesis is sparse (P. Raymond, personal communication). In a recent experiment (E. C. C. Rankin, personal communication) three goldfish at 43·1 mm mean standard length had the right optic nerve cut, were pulse labelled with intraperitoneal [^{3}H]thymidine, and were then maintained on a high-growth diet for 382 days until they reached 68·7 mm standard length. A ring of thymidine-labelled cells was found only 100–200 μm from the ciliary margin in each of the six retinae.

Our fish, which had peripheral fascicles labelled one year after the optic nerve was cut, were kept in large tanks. They had gained at least 4 cm in body length, neurogenesis had probably occurred to the extent reported by E. C. C. Rankin (personal communication), Rankin and new axons had probably arrived in the tectum. The finding that peripheral fascicles of these regenerates derived from retinal annuli approximately ten times wider than the corresponding annuli in equivalent positions on the normal side, indicates that either regenerating axons mixed in with new axons, or that new axons growing towards the tectum aligned

with or next to regenerating axons did not take the same paths, and were not as precisely fasciculated as their counterparts on the unregenerated, i.e. normal, side.

The association of fibres into intermediate and rostrocentral fascicles from correct annular retinal regions was achieved only in some of the experimental cases.

To account for the occurrence of near normal annular distributions in the retina, one might argue that axons within the fascicle have traversed the cut together and retained their association throughout their centrifugal path. In the light of the disordered spatial arrangements of most regenerating fibres, however, it appears unlikely that they had occupied and retained normal positions while growing towards the tectum. It is then remarkable that they have recaptured fascicular routes in appropriate tectal positions. We never found annuli which were out of place with regard to the position of the lesioned fascicle.

The fascicles deriving from ganglion cells more widely spaced in the retina, originated from approximately correct regions, that is most cells were in mid-retinal regions after labelling intermediate fascicles, or crowded in central retina after disrupting rostrocentral fascicles.

Since each fascicle-labelling procedure was carried out in individual fish, we cannot judge whether the fascicle order was altogether better in some individuals and worse in others, or whether there was a variation of fibre order among fascicles within individual animals.

The most general conclusion derived from these results is that the axonal order of tectal fascicles in regenerates is far from being random, which thus agrees with Cook (1983). More specifically, fascicles in the dorsal hemitectum derived preferentially from ganglion cells of the ventral hemiretina and *vice versa* those in the ventral hemitectum from ganglion cells of the dorsal hemiretina (Egert *et al.* 1984; Stuermer & Easter, 1984). Moreover, fibres in fascicles exhibited a strong bias to become distributed over the tectum by age. They achieved this order despite the absence of a consistent spatial ordering by age or retinal sectorial origin in the nerve and tract.

Fibre exit from fascicles

This mode of fibre ordering at the fascicular level implies that axons have to go through successive rearrangements on their paths through the tectum to reach their retinotopic termination sites. The regenerating fascicles were sometimes recomposed of axons from temporal and nasal retinal ganglion cells in tight annular regions as in normals, and often from cells scattered more widely. Thus, along each fascicle's proximodistal extent, the regenerating fibres have to leave their fascicle according to their nasal and temporal retinal origin, respectively (Stuermer & Easter, 1984*b*). We provided evidence from labelling peripheral and intermediate fascicles that the distal parts of fascicles only contained fibres originating from nasal retina, suggesting that temporal fibres have left these fascicles in their proximal half.

Further, tracing of anterogradely labelled nasal and temporal axons suggested that temporal fibres generally departed before nasal fibres. In other words, fibres changed their priorities. Even when misrouted, they left their fascicular associates to enter at approximately correct sites into the synaptic layer SFGS.

These results were derived by tracing axons at relatively late regeneration stages. To determine how reliably ingrowing axons find their correct sites of exit at early regeneration stages, single fibre tracings in early and late regeneration stages are currently being undertaken.

It thus remains to be considered by what mechanisms regenerating fibres adopt certain fascicular paths and restore a fan-like fascicle array. It has been proposed that regenerating fibres are guided by glial channels or glial-engulfed remnants of old fibres in the tectum (Murray, 1976); or markers left behind by previous innervation (Schmidt, 1978). Pure mechanical guidance by glial channels alone appears unlikely, since fibres of any retinal origin could then follow any channel without creating any systematic order by age or position. Guidance by axonal remnants or markers left behind by previous innervations remains to be considered as a serious alternative (Schmidt, 1978).

It is equally likely that retinal axons are ordered by following tectal positional markers (Bonhoeffer & Huf, 1982; Harris, 1984; Holt, 1984).

If cues or markers guide retinal axons in tectum, we have to propose two sets: one to enhance correct retinotopic positioning of terminal arbors in the synaptic layer SFGS, and an additional and complementary set acting on the alignment of fibres in the fascicle layer in the SO (F. Bonhoeffer & A. Gierer, personal communication). The homing in of axon terminals in correct territories in SFGS might occur by gradients of markers in dorsoventral and rostrocaudal axes, as suggested from experimental work in embryonic chick (Bonhoeffer & Huf, 1982; Bonhoeffer & Gierer, 1984). In the SO, however, goldfish retinal axons are ordered by age and position, i.e. by the radial position of their parent ganglion cells in the retina. Such routes could theoretically be achieved by the influence of a differently organized gradient with a radial or cone-shaped distribution (H. Meinhardt, personal communication). Along these lines one might speculate that the natural age of tectal cells could serve as a positional cue for ingrowing retinal fibres. If regenerating axons remembered their original developmental age imposed on them by their parent ganglion cells, and could follow the crescent-shaped distribution of the age-related tectal cells, a typical curved fascicle path would result and consequently the axonal alignment by age.

In summary, we are confronted with the fact that regenerating fibres are not confined by order in time and space to defined pathways in the nerve and tract. This does not free them to course to their termination sites in tectum directly, but they are biased to restore an age-related pathway order instead. This mode of fasciculation, although less precise than in normals, bears consequences on the further path of axons from SO to progress to their termination sites in SFGS (Stuermer & Easter, 1984*a*). It appears that, as in the normals, fibres leave their fascicles in a temporonasal order. After entering the synaptic layer SFGS they

course through extrafascicular routes to their retinotopic sites of termination (Egert *et al.* 1984). In the normals these routes represent the trajectories taken by the axons shifting from previous to actual retinotopic termination sites (Easter & Stuermer, 1984) and they are very orderly. Regenerating axons establish novel and often tortuous routes (Egert *et al.* 1984) through which even misrouted axons can ultimately arrive at their proper targets.

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