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A Comparison of the Normal and Regenerated Retinotectal Pathways of Goldfish

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ABSTRACT

This is a light and electron microscopic study of the retinotectal pathway: intact and after regeneration of the optic nerve. The spatiotemporal pattern of axonal outgrowth and termination was studied with the methods of proline autoradiography, horseradish peroxidase (HRP) labeling, and fiber degeneration.

The spatial order of optic fibers in the normal and regenerated pathways was assessed by labeling small groups intraretinally with HRP and then tracing them to the tectum. The labeled fibers occupied a greater fraction of the cross section of the regenerated than the normal optic tract. At the brachial bifurcation, roughly 20% of the regenerated fibers chose the incorrect brachium vs. less than 1% of the normals. In tectum, the regenerated optic fibers reestablished fascicles in *stratum opticum*, but they were less orderly than in the normals. The retinal origins of the fibers in the fascicles were established by labeling individual fascicles with HRP and then, following retrograde transport, finding labeled ganglion cells in whole-mounted retinas. Labeled cells were more widely scattered over the previously axotomized retinas than over the normal ones. A similar result was obtained when HRP was applied in the tectal synaptic layer. All of these results indicate that the pathway of the regenerated optic fibers is less well ordered than the intact pathway.

Both autoradiography and HRP showed that the regenerating optic fibers invaded the tectum from the rostral end, and advanced from rostral to caudal and from peripheral to central tectum, along a front roughly perpendicular to the tectal fascicles. Synapses of retinal origin were noted electron microscopically in the tectum at the same sites where autoradiography indicated that the fibers had arrived. No retinal terminals were seen where grain densities were at background levels. Fiber ingrowth and synaptogenesis apparently occurred simultaneously. The synapses were initially smaller and sparser than in normals, but were in the normal tectal strata and contacted the same classes of post synaptic elements as in normals.

Key words: regeneration, fiber growth, order and disorder, optic nerve

The reestablishment, following section of the optic nerve, of a topographic map of retina onto tectum has been well documented in both amphibians and fish (Gaze, '59; Maturana et al., '59; Attardi and Sperry, '63; reviewed by Gaze, '70; Jacobson, '78). Such studies related the retinal positions of the ganglion cell bodies to the tectal positions of their terminal arbors, but relatively few commented on the pathways taken by the regenerated axons (exceptions are Horder, '74; Udin, '78; Meyer, '80). Recently, horseradish peroxidase (HRP) has been used to trace relatively small

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groups of axons in both normal and regenerated pathways (Dawnay, '79, '81; Fujisawa, '81; Fujisawa et al., '81, '82; Fawcett and Gaze, '82; Gaze and Fawcett, '83). Of these, only the studies by Dawnay dealt with teleost fish, a group in which the axonal pathways are strongly ordered according to their retinal origins (Scholes, '79; Rusoff and Easter, '80; Easter et al., '81; Bunt, '82, Bunt and Horder, '83; Stuermer and Easter, '83). We have extended this approach to examine more locations in the visual pathway. Here we describe how accurately the regenerating axons reestablish the old pathways and where they make mistakes.

In addition, we describe the spatiotemporal relation of axonal outgrowth and synaptogenesis. These two related phenomena have been studied in the development of the retinotectal pathway of chicks (Crossland et al., '75; Rager and von Oeynhausen '79; McLoon, '82), and there is some disagreement over the issue of whether the fibers make synapses immediately upon arrival, or wait. Retinotectal synaptogenesis has been described in *Xenopus*, both during development (Gaze et al., '79) and during regeneration (Ostberg and Norden, '79) but not related to axonal outgrowth. In fish, one study by Murray ('76) dealt with these two processes, but only secondarily. Our results show that synapses and new axons appear roughly simultaneously along a common front in tectum.

MATERIALS AND METHODS

Adult goldfish *(Carassius auratus)*, 5–8 cm standard length, were maintained in aerated aquaria at room temperature (18–25°C). During all procedures likely to be painful, such as eye enucleation, optic nerve section, intraocular injection, intratectal insertion of HRP, and sacrifice either by decapitation or transcardial perfusion with fixative, the fish were anesthetized in a 0.1% aqueous solution of tricaine methanesulfonate.

Optic nerves were sectioned intraorbitally with iris scissors. If both cut edges of the nerve were visible, and no significant bleeding occurred, the animal was used. Otherwise it was not used in these experiments.

Tissue was prepared for electron microscopy with standard methods. The fish were decapitated, and the brains were removed from the skull and fixed in chilled 5% glutaraldehyde/4% paraformaldehyde solution in cacodylate buffer (pH 7.4–7.6) (Karnovsky, '65). The tectum was chopped transversely into smaller fragments, which were postfixed in cacodylate buffered 2% OsO_4 for 2 hours, rinsed, dehydrated through ethanol, cleared in propylene oxide, and embeded in Araldite. Semithin sections (1–2 μm) were stained with toluidine blue and observed light microscopically. Ultrathin sections were mounted on formvar-coated one-hole grids, stained with uranyl acetate and lead citrate, and observed in a Philips 200 (Freiburg) or 300 (Ann Arbor) electron microscope.

A series of five to 20 ultrathin transverse sections, up to 500 μm wide, were cut from the dorsal half of each tectum, usually including the medial boundary. Most sections included the entire region from *stratum marginale* (SM) to *stratum periventriculare* (SPV); all included the external synaptic layer (S) (Landreth et al., '75) and *stratum fibrosum et griseum superficiale* (SFGS). The boundaries between S and SM (superficially) and SFGS and *stratum griseum centrale* (SGC) (deep) were determined ultrastructurally with the following criteria. The groups of large, myelinated, nonoptic axons (Laufer and Vanegas, '74a; Stuermer, '78) separate S and SM. The SFGS-SGC boundary was established with reference to three features. The first was the abundance of prominent radially oriented dendritic and glial processes, which are common to SGC, but largely absent in SFGS (Murray, '76). The second was the presence of myelinated axonal profiles (intact in normals, and degenerating soon after optic nerve section), much more common to SFGS than to SGC (Meek, '81a). Finally, SFGS contains substantially more cell bodies than SGC (Vanegas et al., '74), particularly in tecta deprived for months of retinal afferents. The boundary was also located by assuming that the radial width of SGC, from its lower boundary at *stratum album centrale* (SAC), had not changed after eye enucleation.

Electron microscopic photomosaics, assembled from prints of magnification ×9,800, covered 30–50 μm mediolaterally and included the complete radial width of stratum opticum (SO) and SFGS. Retinal terminals were usually identified on the photomosaics, but some required electron microscopic reinspection of the photographed area at ×15,000–20,000 magnification.

To label retinotectal axons and terminals with HRP, 10 μl of a 50% aqueous solution of HRP (Miles, Type 6) was injected into the vitreous of one eye. The fish were perfused transcardially 2–3 days later with 10 ml of 0.7% NaCl, followed by a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer (pH 6.9, 0.08 M). The brains were removed and immersed in fresh fixative for another 2 hours and then were sectioned transversely on a vibratome at 100 μm. The sections were collected in phosphate buffer, processed for HRP with tetramethyl benzidine (TMB) as the chromogen (Mesulam, '78), and then mounted on microslides and coverslipped.

To radiolabel retinal axons and terminals, 10–15 μl of 2,3-^{3}H-proline (Amersham-Buchler, specific activity 30–60 Ci/mmole) was injected intraocularly 1 or 2 days prior to sacrifice. Some brains were processed for electron microscopy and embedded in Araldite. Others were fixed in 10% formalin and embedded in paraffin. Araldite sections (2 μm) and paraffin sections (10 μm) were mounted on gelatinized glass slides and coated with nuclear track emulsion (Kodak NTB3 or Ilford L4). After 2 weeks exposure, the autoradiograms were developed with Kodak D19, lightly counterstained with toluidine blue, dehydrated, coverslipped, and examined by dark- and brightfield light microscopy.

In order to relate silver grains visible-light microscopically to retinal terminals distinguishable only electron microscopically, the optic nerves of some animals were cut 24 hours after proline injection. This interval allowed the terminals to be radiolabeled prior to sectioning the nerve, which produced darkened, degenerating optic terminals soon thereafter (Murray, '76). Araldite sections (2 μm) were prepared for autoradiography, and an adjacent series of ultrathin sections was examined electron microscopically. Photomosaics of the light microscopic autoradiograms were prepared. Electron micrographs of precisely positioned adjacent regions were scored for dark terminals and in some cases, photomosaics of electron micrographs were prepared.

The general question of spatial order in the visual system was investigated in several experiments in which HRP was applied to a small part of either the retina or tectum.This local application labeled a restricted group of axons or terminals with HRP, which was then transported both anter-

ogradely to label the axons and retrogradely to label the cell bodies of orgin.

To make the retinal applications, the cornea and lens were removed surgically, a group of axons was cut near the optic disk, and a crystal of HRP was applied to this region. The lens and cornea were returned.

The tectum was labeled in either of two ways. A part of the dorsal cranium was removed to expose the tectum. An individual fascicle, or a few neighboring ones, were severed with a tungsten microneedle, and a crystal of HRP was applied to the cut portion. Care was taken not to damage the tectum deep to SO. The cranium was then replaced. This method labeled the severed fascicles without labeling axons or terminals in deeper layers. The other method was to poke a small hole, 50–100 μm in diameter, through the tectum to the ventricle. Then a few crystals of HRP were inserted into the hole. This procedure was intended to label axons, including those in SO, and terminals. After 2–3 days the contralateral retina was removed, fixed in 4% glutaraldehyde, reacted with TMB (Mesulam, '78), and whole mounted for light microscopic examination. The TMB method was used because of its great sensitivity, particularly essential for the tectal labeling. The reaction product faded in most cases within an hour; to obtain a permanent record, the retinas were either sketched immediately through a drawing tube or photographed at ×125. The nerve, tract, and tectum were either reacted, unfixed, for HRP (Fujisawa et al., '81), and flat mounted on gelatinized slides, or, following fixation in buffered glutaraldehyde, sectioned transversely on a cryostat and reacted for HRP, using diaminobenzidine (DAB) as a chromogen (Adams, '77) to reveal axons.

To label many and perhaps all retinal axons, the optic nerve was cut and HRP was applied to the stumps. Two to 3 days later, the retina and tectum were removed and processed for HRP.

RESULTS

Spatiotemporal pattern of regeneration in tectum

In normal fish, intraocular injections of HRP or tritiated proline labeled the contralateral tectum in five strata: S, SO, SFGS, SGC, and SAC, confirming many earlier reports (Grafstein, '67; Landreth et al., '75; Schmidt, '79; Springer and Gaffney, '81). Labeling with HRP revealed clearly that retinal afferents within SFGS were stratified into layers (Fig. 1) as previously noted by others (Leghissa, '55; Grafstein, '67; Sharma, '72; Landreth et al., '75).

Axonal regeneration. Light microscopic autoradiography and HRP labeling were used to examine the time course of early optic fiber regrowth into the tecta of 41 fish. The rates of regeneration depended on the temperature of the water (Springer and Agranoff, '77), which ranged from 18°C in winter to 25°C in summer. Since the summer group contained a greater number of animals (N = 25), including those which had received HRP (N = 5), the time course of their regeneration will be discussed in detail, the autoradiography first. Unless otherwise stated, each time point applies to a single fish.

The tecta of four fish examined at 8 days (two) and 9 days (two) were unlabeled.

At 10 days, three fish showed no label, but in one fish, silver grains were detected in the extreme rostral tectum, in the dorsomedial and ventrolateral brachia of the optic tract.

At 12, 13, and 14 days the label extended more caudally in the brachia, over about the rostral third. In very rostral tectum, silver grains were seen over both SO and SFGS. Thus, in a reconstructed tectum viewed from its superficial aspect, the pattern of label was crescent-shaped, thickest rostrally, open caudally.

At 16 and 18 days, the brachia and adjacent SO and SFGS were labeled over roughly the rostral half (Fig. 2a). The labeled zone was therefore still crescent-shaped, open caudally, but it was larger than before, with longer arms and a thicker region rostrally.

By 24, 25, and 26 days, the brachia were labeled over the entire rostrocaudal extent, and the SO and SFGS were labeled more caudally than before (Fig. 2b). All sections still contained more label in the periphery than near the equator, the boundary between dorsal and ventral hemitecta. Very caudally, equatorial regions were still unlabeled.

After 30, 31, 32, and 34 days, the SO and SFGS were labeled everywhere with a normal pattern (Fig. 2c). In two of the four cases, however, the density of label in the caudal tectum was lighter than rostrally.

In summary, the retinal axons reinvaded the tectum over the period 10–34 days, with the front of the invading fibers moving both from rostral to caudal and from peripheral to equatorial regions.

This same pattern was seen in the tecta labeled with anterogradely transported HRP. Survival times examined were 14, 16, 18, 20, and 28 days.

The second (colder) group of fish labeled with radioactive proline showed the same pattern, but longer survival times were required to achieve comparable grain distributions. Tecta examined at 6, 7, 8, 9, 10, 11, and 12 days were unlabeled. Later stages, examined at 13, 14, 17, 23, 26, 27, 28, 31, and 42 days all were labeled, but only the last of these was entirely labeled. The tectum was invaded over 13–42 days vs. 10–34 days in the warmer group.

Synaptogenesis. The dual approach, with light microscopic autoradiography and electron microscopy, was used on eight fish to determine when the invading retinal axons formed synapses. If synaptogenesis occurs immediately, then any region labeled in an autoradiogram should have synapses in an electron micrograph. If it is delayed, the zones labeled earliest in the autoradiograms should not contain synapses.

Regenerated retinotectal terminals (Fig. 3) were identified by their degenerative darkening (Murray, '76), a consequence of the second transection of the regenerated optic nerve a few days before sacrifice. The possibility that they resulted from the initial transection was ruled out by examining a tectum 14 days after the contralateral eye had been removed. No such terminals were seen, as the coded tracing in Figure 4a illustrates. We conclude that no degenerating terminals remain at 14 days or more after optic nerve section. The survival times given below all refer to the interval between the two transections of the nerve. This series of experiments was carried out at the lower temperature, so the regeneration was slow.

At the earliest times (13, 14 days) the density of silver grains over the tectum did not exceed background, and no darkened terminals were found electron microscopically.

At 17 days, rostral sections were densely labeled near the brachia, but not centrally (Fig. 5) Electron microscopic examination of these regions revealed abundant dark terminals peripherally (Fig. 4b) and practically none centrally (Fig. 4c). Therefore the presence of silver grains correlated

Abbreviations

C	Caudal
CELL	Cell body
CL	Cluster
D	Dorsal
DEN	Dendrite
DM	Dorsomedial brachium of optic tract
F_1, F_2, F_{3a}, F_{3b}, F_4	Tectal strata with inputs from optic fibers
LC	Linear connector
m	Mitochondrion
N	Nasal
NR	Nucleus rotundus
PHA	Partial half annulus
R	Rostral
RT	Retinotectal terminal
S	external synaptic layer
SAC	*Stratum album centrale*
SFGS	*Stratum fibrosum et griseum superficiale*
SGC	*Stratum griseum centrale*
SM	*Stratum marginale*
SO	*Stratum opticum*
SPV	*Stratum periventriculare*
T	Temporal
TL	*Torus longitudinalis*
V	Ventral
V_1, V_2	Vesicle-containing profiles
VL	Ventrolateral brachium of optic tract

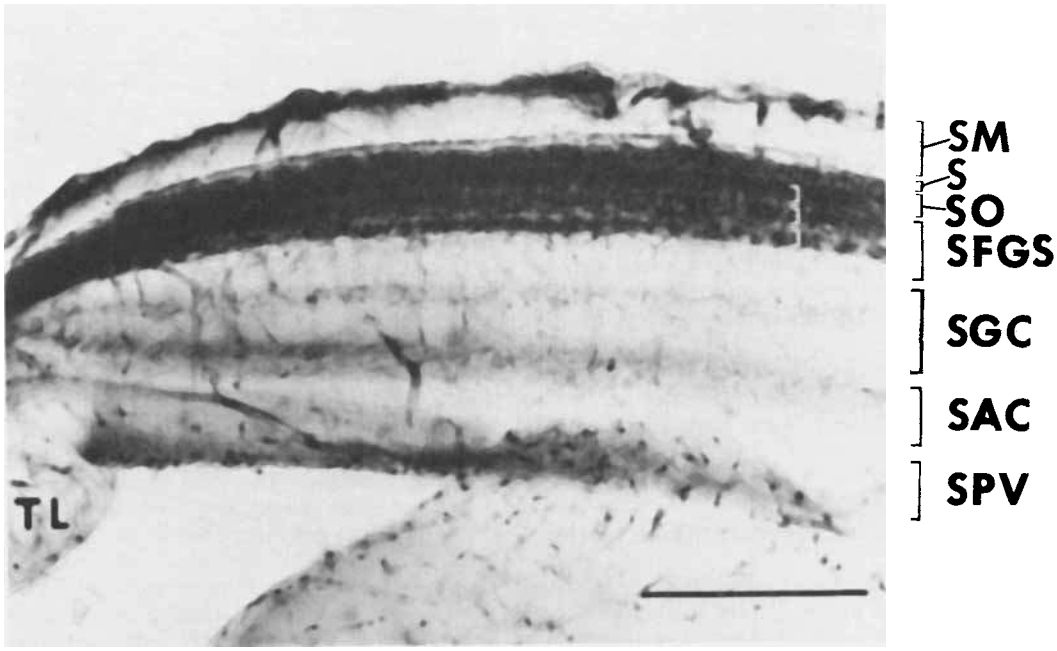

Fig. 1. Transverse section of tectum from an animal that had received an intraocular injection of HRP 48 hours prior to sacrifice. The HRP-TMB reaction product delineates the five retinal recipient strata. Note the three substrata within SFGS, indicated by white brackets. TL, Torus longtudinalis. Bar = 200 μm.

positively with the abundance of retinal terminals. The correlation was equally evident more caudally, where the silver grains and the degenerating terminals were seen only peripherally. Neither grains nor dark terminals were found in the most caudal tectum.

The spatiotemporal correlation between autoradiographic labeling and synapses was further substantiated in the fish that survived 20, 22, and 24 days. All layers of SFGS were labeled in rostral tectum. The label was densest at the periphery and less dense toward the equator. Sections from midway along the rostrocaudal axis were labeled peripherally and lightly labeled centrally. The dark terminal profiles had this same spatial distribution. The more caudal tectum had grains and dark terminal profiles only at its edges.

The tecta of long term regenerates (42 and 64 days) were densely labeled everywhere. The numbers of dark terminals had increased substantially (compare Fig. 4b, c with d).

Fig. 2. Photomosaics of darkfield autoradiograms from the right tectal lobes of three animals with regenerating left optic nerves. Tritiated proline was injected 48 hours before sacrifice. Survival times, since the original optic nerve section, were (a) 16, (b) 24, and (c) 30 days. The rostrocaudal level of each section is indicated in the small diagrams (lower right), in which dorsal is up and rostral to the right. In the autoradiograms, dorsal is up and medial to the right. Silver grains label areas containing regenerating retinal fibers. Bar = 1 mm.

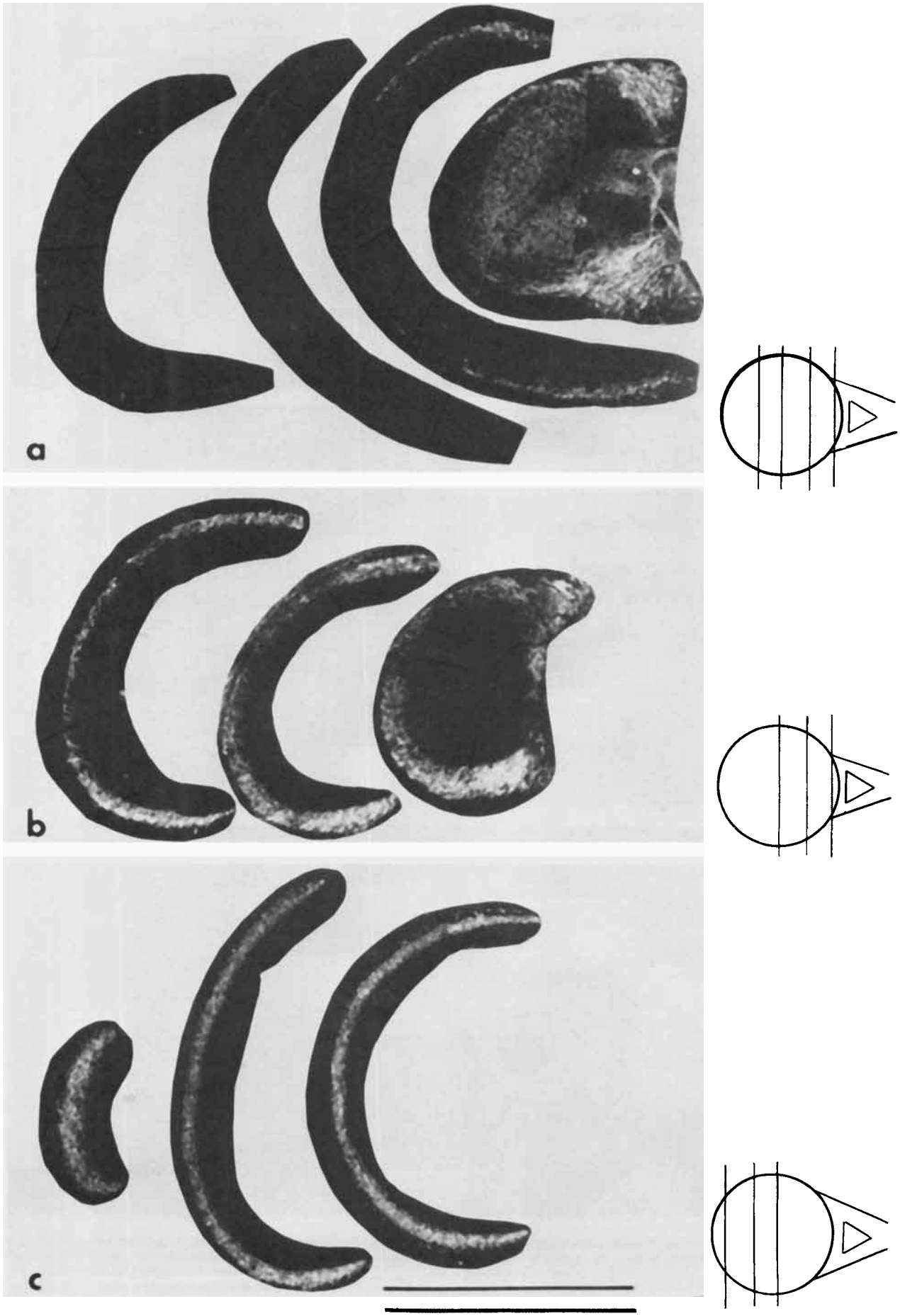

Figure 2

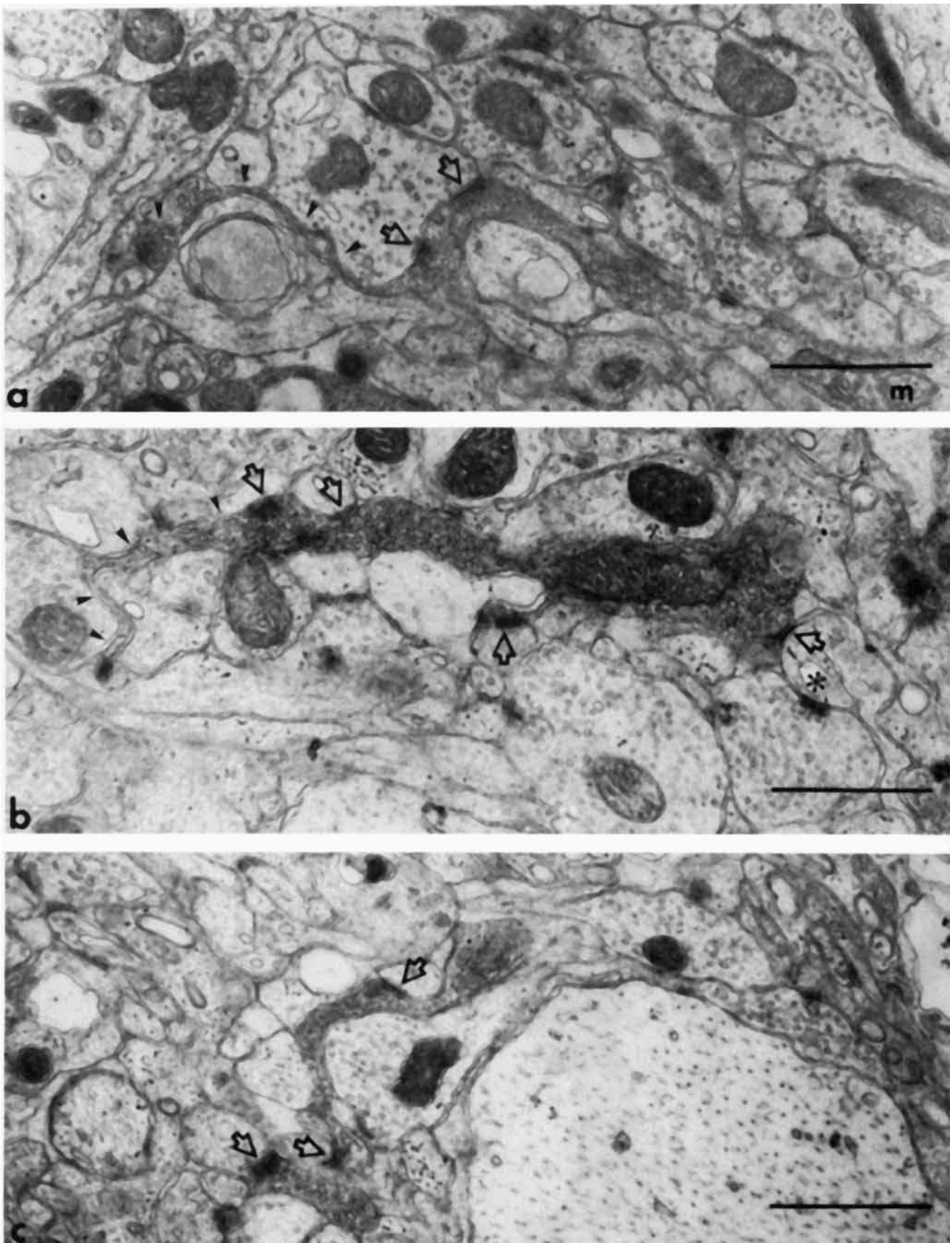

Fig. 3. Retinotectal terminals. From an ultrathin section adjacent to the semithin section/autoradiogram in Figure 5, from the region between the arrows labeled b and c in Figure 5. The micrographs in a–c of this figure show dark (degenerating) regenerated retinotectal terminals. They are small and narrow, and contain numerous ovoid vesicles and typical mitochondria (m). a. The terminal makes synapses (arrows) onto a vesicle-containing profile and exhibits a long extension (arrowheads). b. Another terminal with a long extension (arrowheads) and synapses onto dendrites (arrows). One dendrite (asterisk) is also contacted by a vesicle-containing profile not of retinal origin. c. Small terminal profiles with synaptic contacts onto small dendrites (arrows). Bar = 1 μm.

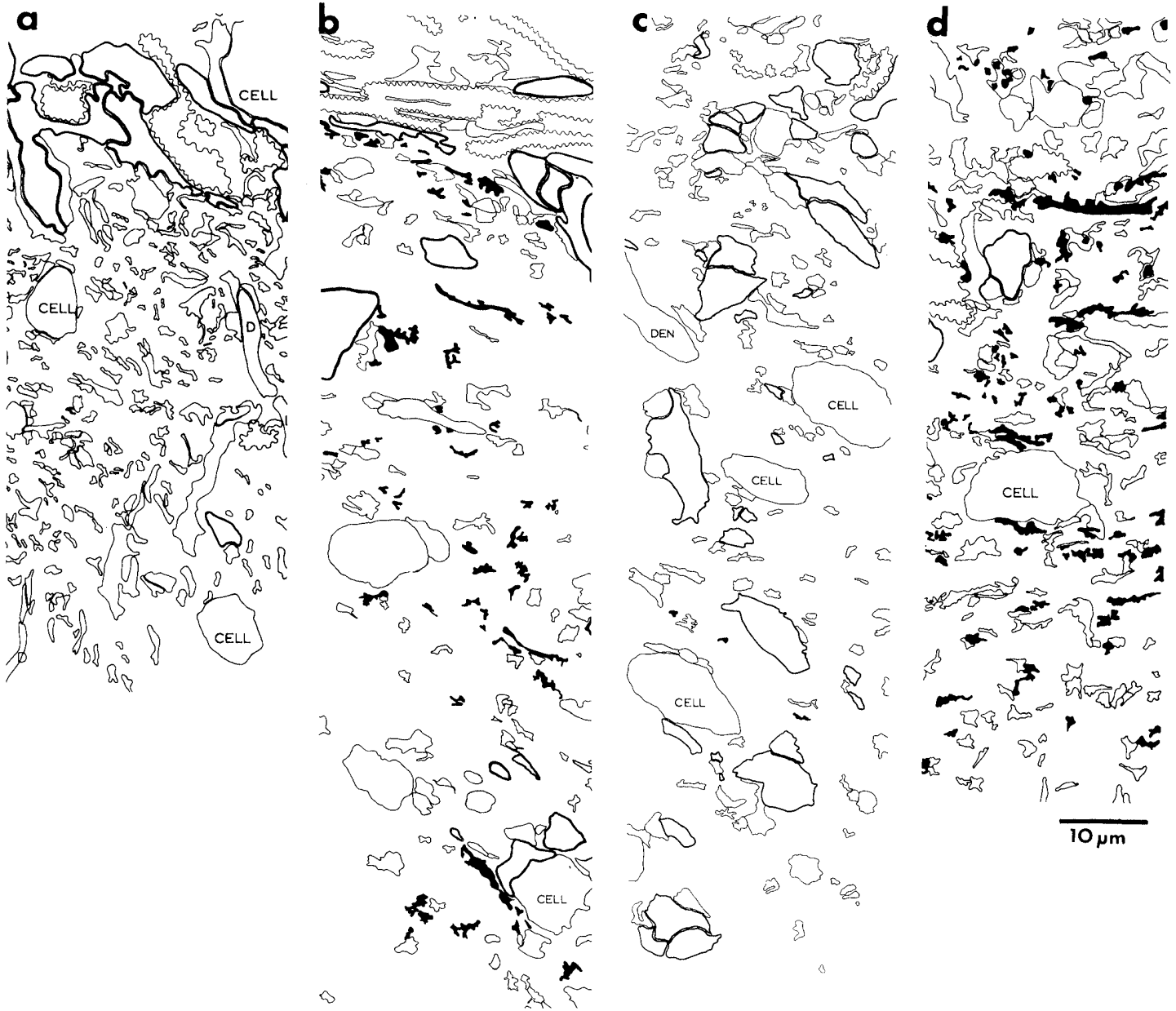

Fig. 4. Tracings from electron microscopic photomosaics of the S/SO/SFGS neuropil in rostral dorsomedial tectum. The filled black contours in b–d represent degenerating regenerated retinotectal terminals. The wavy lines enclose groups of myelinated fibers. Thin lines indicate pale glial processes, cell bodies (CELL), and large dendrites (DEN). Thick lines indicate dark glial processes, blood vessels, and pericytes. Calibration is the same in all panels. a. fourteen days after enucleation of the contralateral eye. b,c. Seventeen days after initial section of optic nerve, 1.5 days after second section: (b) peripheral, (c) central tectum. d. Sixty-six days after initial section, 2.5 days after second section.

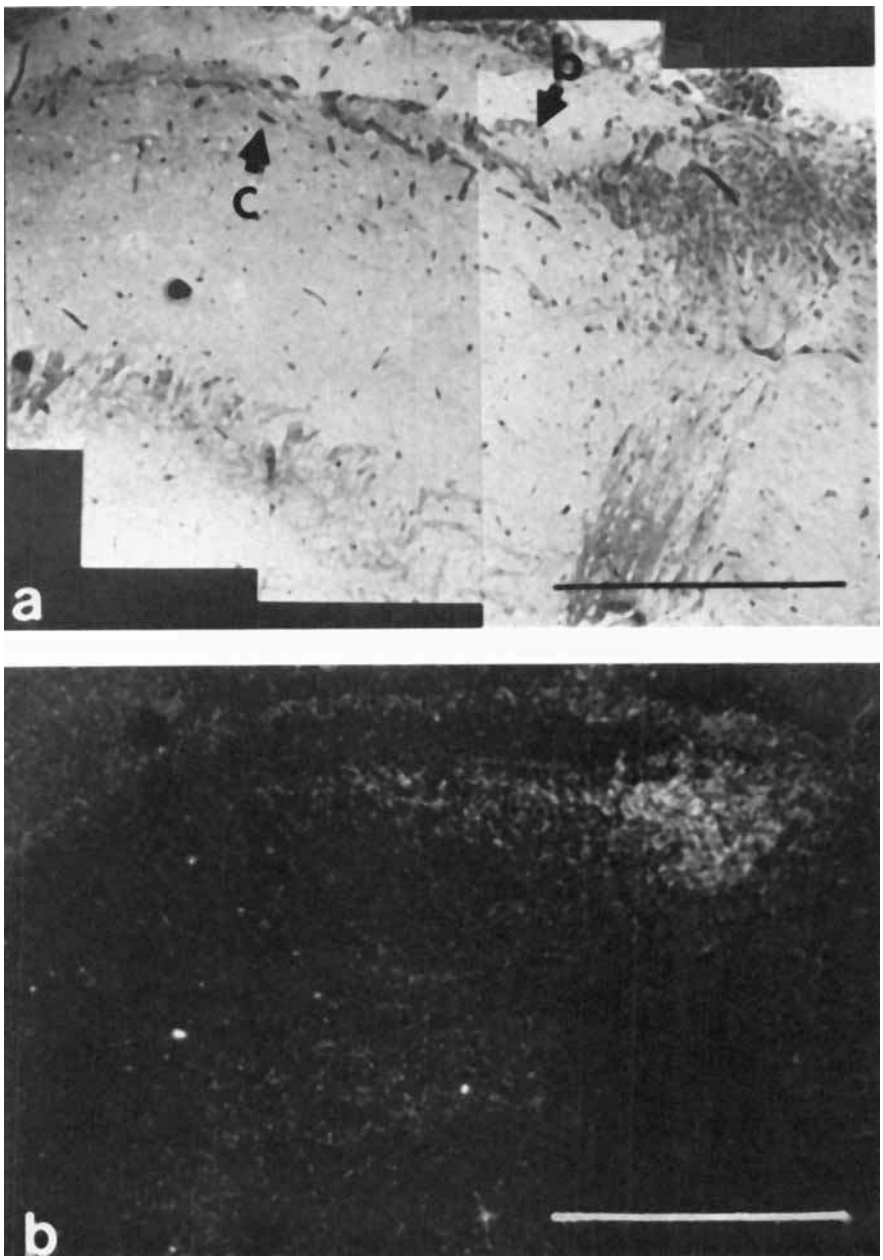

Fig. 5. Autoradiogram of a semithin section through dorsomedial rostral tectum. Seventeen days after initial nerve section, 2 days after proline injection, and 1 day after second nerve section: (a) brightfield and (b) darkfield illumination. The dorsomedial brachium of the optic tract is to the right, central tectum to the left. The silver grains (visible in b) over S, SO, and SFGS indicate the presence of regenerated retinal axons and terminals. The arrows point to the regions from which Figure 4b and c were traced.

In summary, the distribution of silver grains over semithin sections matched that of dark terminal profiles, retinal in origin, in neighboring ultrathin sections, at all regeneration stages tested here. This shows that synapse formation and axon regrowth were spatially and temporally linked; some of the ingrowing fibers must have formed synapses soon after they arrived at any region on the tectal hemisphere.

Synaptic connections. To this point, all identifications of the retinotectal terminal have depended upon recognizing it as degenerating, surely the most conservative approach. But when the degeneration time is very brief, the appearance of the degenerating terminal will be expected to have changed very little from normal. This allows one to infer which of the normal terminals are of retinal origin, and a number of workers have done so (Laufer and Vane-

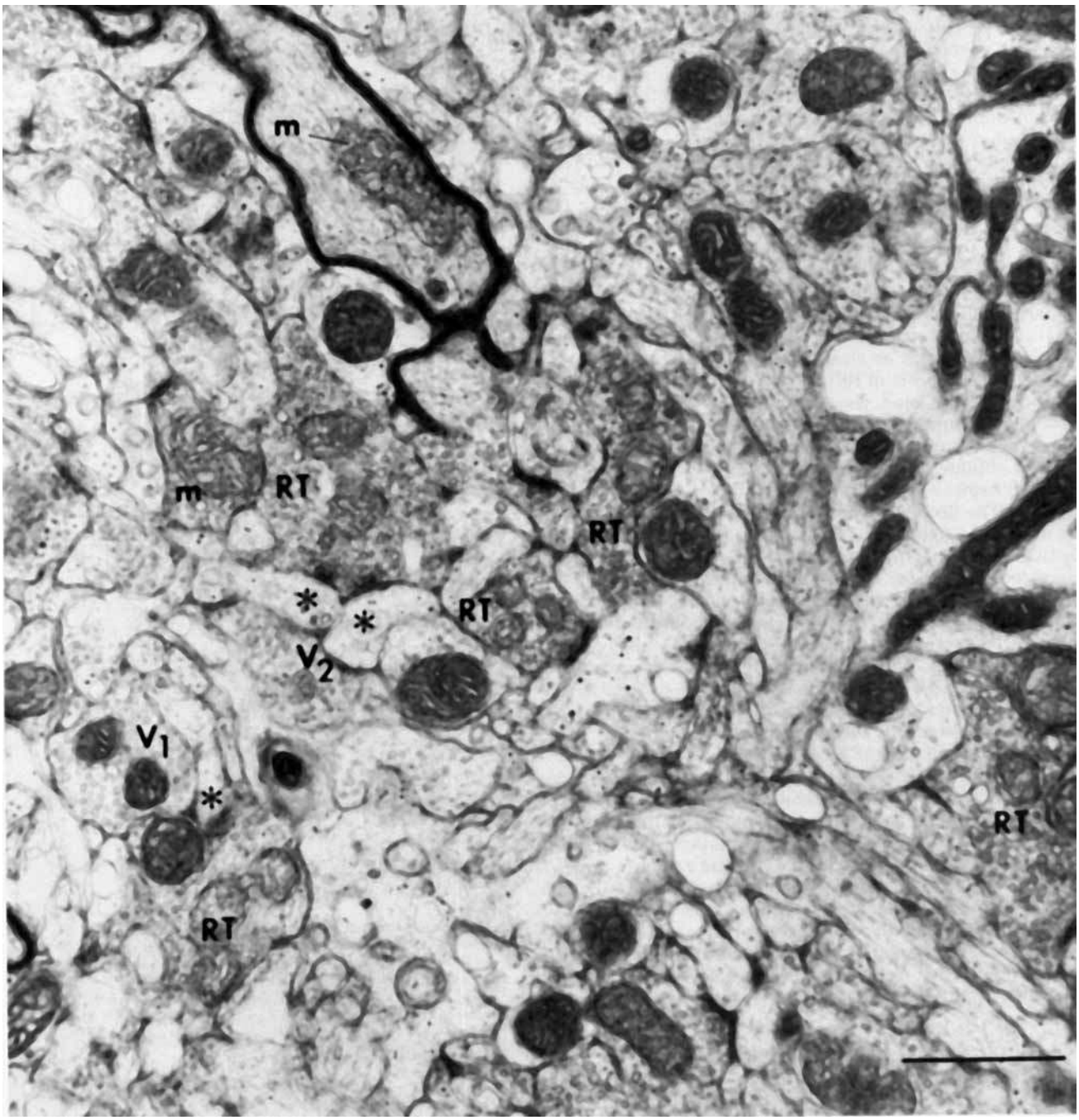

Fig. 6. Electron micrograph of SFGS in normal tectum showing several retinotectal terminal profiles (RT). They are electron dense and contain ovoid vesicles in high density and pale mitochondria (m) with irregular cristae and wide inter- and intracristal spaces. They synapse on clear dendritic profiles (asterisk). There are two other vesicle-containing profiles, V_1 and V_2. V_1 profiles contain round vesicles and dark mitochondria with regular cristae and narrow intracristal spaces. The V_2 profiles contain pleomorphic vesicles. Both V_1 and V_2 terminals synapse in common with RT onto dendrites (asterisks). Bar = 1 μm.

gas, '74a, b; Stuermer, '75, '78, '81a, b; Murray, '76; Airhart and Kriebel, '80; Ito et al., '80; Murray and Edwards, '82; Meek, '81a, b).

Most workers agree that the normal terminal can be recognized, and Figure 6 illustrates one. It is relatively dark. It contains a cluster of ovoid synaptic vesicles. When mitochondria are present, they are pale, with irregular cristae and wide inter- and intracristal spaces. There are three kinds of postsynaptic elements. One is clearly dendritic, and the other two contain vesicles, either round or

pleomorphic. These vesicle-containing profiles often contact the same dendrite as the retinotectal terminal. Such sets of synapses are often partially surrounded by glial processes, suggesting a glomerulus (Peters et al., '70).

The tracing in Figure 7 illustrates the stratification of such terminals. The letters to the left refer to the nomenclature used up to now, the ones on the right to the nomenclature of Grafstein ('67) and Landreth et al., ('75), modified by dividing F_3 into F_{3a} and F_{3b}. In S, the terminals were usually small, sometimes slightly elongated, and quite sparse. In SO, they only appeared between bundles of retinal fibers, and were therefore sparse. In the most superficial part of SFGS (F_{3a}), terminals with maximal dimensions of 5 μm length and 0.6–1.2 μm width were present, as well as large bulbous profiles and many small ones. Smaller profiles were often right next to bulbous ones and were arranged in groups or clusters, the glomeruli mentioned above. In the middle of SFGS (F_{3b}), the terminals did not form clusters. Instead, they appeared as either small profiles or as giant, long, isolated terminals, larger than the more superficial ones. These giant profiles ran parallel or slightly obliquely to the tectal surface (arrow, Fig. 7), and made synaptic contacts with small dendritic profiles en passant. The deepest layer of SFGS (F_4) was distinguished by its large, myelinated, retinal axons, comparable in size to the largest myelinated axons in SO. These deep axons were good candidates for the retinal afferents which enter directly into SFGS without having passed through SO (Springer and Gaffney, '81; Stuermer, unpublished observations). The retinal terminals were less numerous than in the upper layers, but the individual profiles were larger than most of those above and bulbous. Further, they were grouped together in "islands," with relatively large distances in between. One such island is encircled in Figure 7. This insular grouping of terminals could account for the patchy appearance of silver grains (Landreth et al., '75) and HRP label (Fig. 1) that was characteristic of this layer.

Following regeneration of the optic nerve, the retinotectal terminals were initially fewer and smaller than in the normal, as a comparison of Figures 4b and c with Figure 7 shows. With time, the numbers and sizes of the terminals increased (compare Fig. 4b, d). All three classes of postsynaptic profiles normally contacted were contacted in the regenerates. At 64 days, myelinated retinal axons were again visible and retinotectal terminals were detected emerging from myelinated axons (Fig. 8), as in normals (Fig. 6).

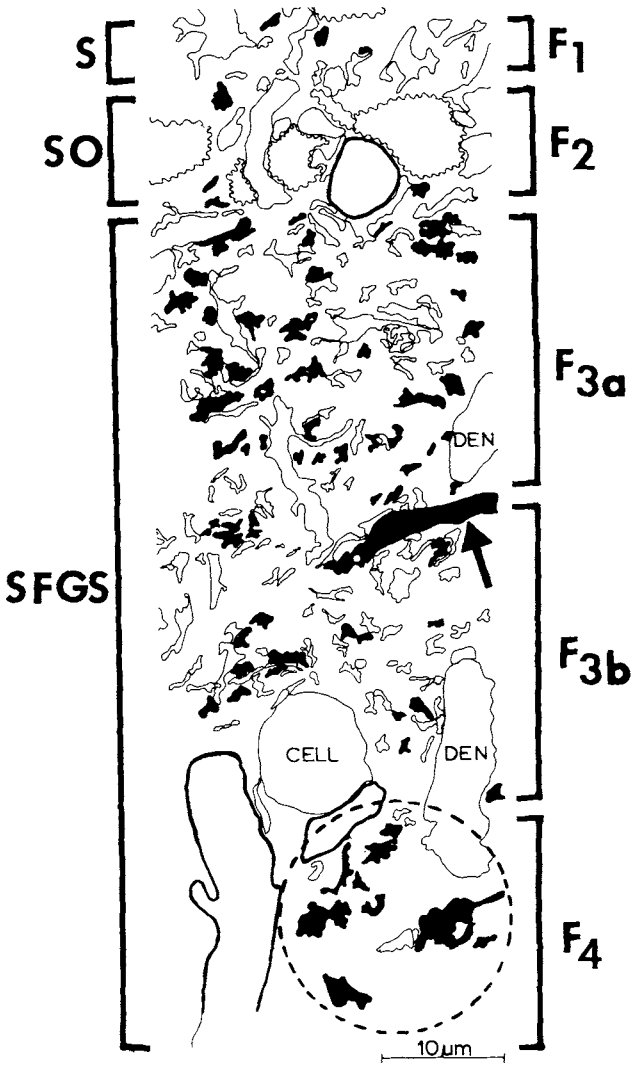

Fig. 7. Tracing from an electron microscopic photomosaic of the S/SO/SFGS neuropil of a normal tectum at a level similar to Figure 1. Blackened areas indicate intact retinotectal terminals. Other conventions are as in Figure 4. Brackets to the left indicate (from top to bottom) the three strata used above: S, SO, SFGS; brackets to the right indicate the layers after the nomenclature of Grafstein ('67) and Landreth et al. ('75). The dashed line encircles one island of terminals in F_4. The arrow points to a large horizontally oriented retinotectal terminal in F_{3b}.

Spatial order in the pathways

Optic tract and brachia. We labeled groups of retinal axons by applying HRP to a small region just dorsal or ventral to the optic disk. When the retinas were whole mounted, the labeled ganglion cells were found to be restricted to 10–20% of the retina, in a wedge-shaped region entirely contained in either the dorsal or ventral half. The labeled fibers were then traced in either whole mounts or sections through the tract and brachia.

Bunt ('82) has described the three-dimensional arrangement of fibers in the optic tract, and we have confirmed his observations. Figure 9a illustrates one view of an unlesioned tract, with the well-clustered fibers of ventral retinal origin. In Figure 10a, a more central section from the same animal, the labeled fibers have entered the dorsomedial brachium. The division was never absolutely perfect, for in all normal animals, a few labeled axons (three to ten) separated from the main group and entered the "inappropriate" brachium (Fig. 11). They were followed through their brachial path and found to penetrate into SO of the "inappropriate" hemitectum, but we were not able to determine whether they terminated there or crossed back to the other side. These misrouted axons, however, represented a small fraction of those fibers that traveled through the appropriate brachium. The estimate of how small a fraction is uncertain, because it is impossible to count all the fibers in the appropriate brachium. But if 10% of the retinal axons have been labeled, a plausible estimate based on the fraction of the retina labeled in this procedure, then they must

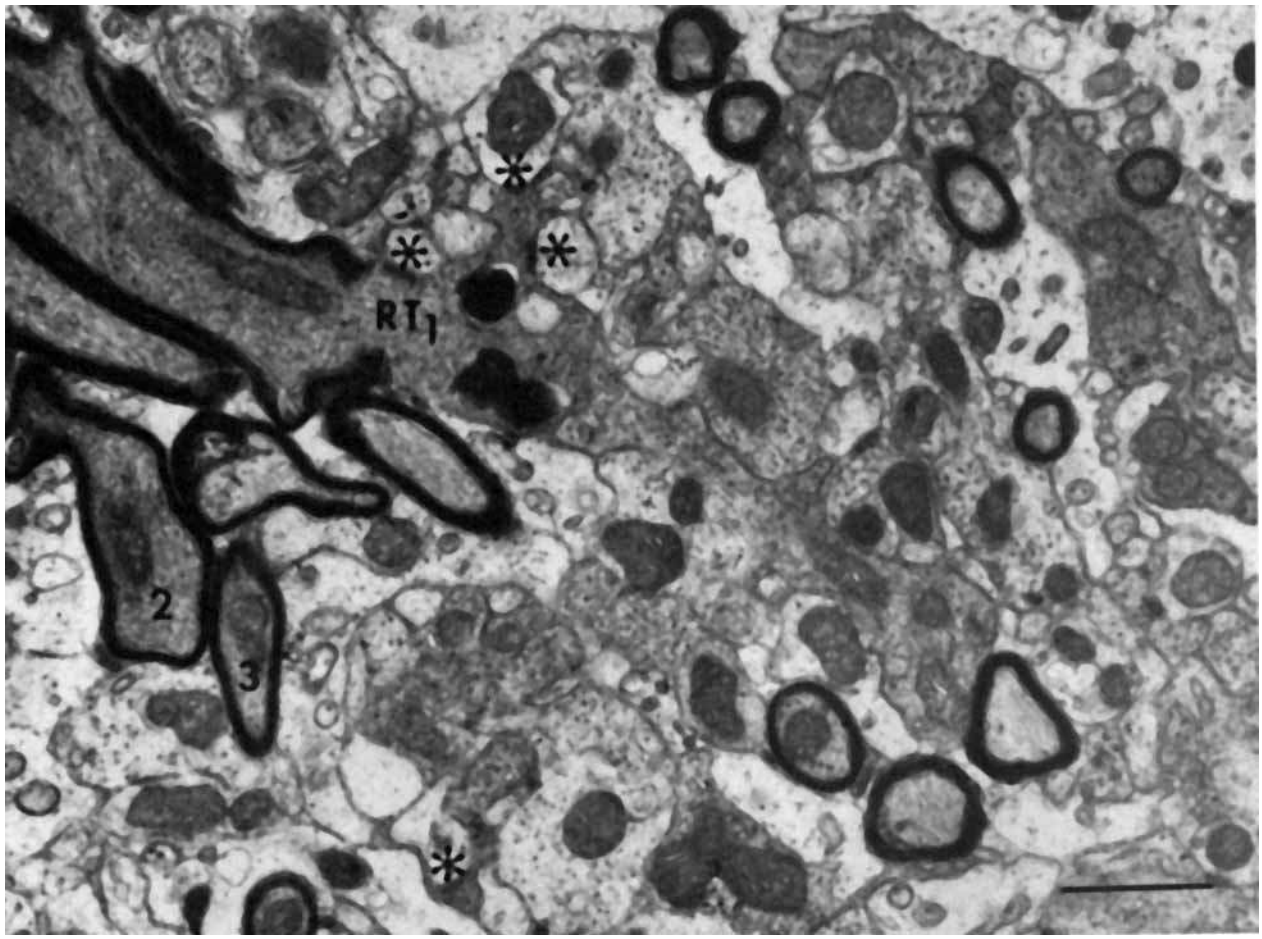

Fig. 8. Regenerated retinotectal terminal (RT_1) from an animal 64 days after nerve section and 3 days after eye enucleation. Note the synapses onto dendritic profiles (asterisks). Two myelinated axons with typical mitochondria (2, 3) are also degenerating. Bar = 1 μm.

number about 15,000 (Easter et al., '81). The three to ten errant axons would then be less than 0.1% of the total.

Five animals that had had one optic nerve sectioned earlier (18, 30, 60 (two fish), and 90 days) were labeled intraretinally. Both eyes were labeled either dorsally or ventrally. Most regenerated axons, especially those at early stages, stained weakly. They were much less well ordered than in the normal tracts, as they occupied the whole cross section (Figs. 9b, 10b) and deviated in various directions from the normal route. Fawcett and Gaze ('81) have made similar observations in the optic nerve and tract of *Xenopus*.

As Attardi and Sperry ('63) reported, most fibers entered the appropriate brachium (Fig. 10b). But the number of mistakes was much greater than on the normal side. Because many fewer fibers were labeled in the regenerates than in the normals, it was possible to count them in both brachia. The incorrect fibers ranged from 16 to 22% of the correct ones, more than 100 times greater than the normal figure of less than 0.1%.

Some additional observations are worth mentioning. First, there seemed to be two classes of regenerated fibers, large and small. The larger ones, which stained heavily, usually traveled in straight, direct routes through the tract and brachia into tectum. In contrast, the smaller and less densely labeled axons tended to travel in various directions in any given cross-section and often crossed or tangled with each other. Second, some trajectories suggested readjustments during regeneration. Some fibers bent through 180°. Others were sharply angled from one side of the tract to a new position on the other side, and entered the brachium on the opposite side from where their original path would have led them. Such changes occurred in both directions; that is, toward the appropriate and inappropriate brachia. The labeled fibers were followed into SO, where some turned to the other side of the tectal lobe, but most penetrated into the hemitectum on the same side as the brachium through which they had arrived. These observations suggest that a regenerating fiber may become misrouted, but also that it may alter its course in the tract.

Tectal fascicles. Retinal axons enter the tectal lobe at its rostroperipheral edges and create two fanlike radiations of fascicles over the dorsal and ventral hemitecta (Leghissa,

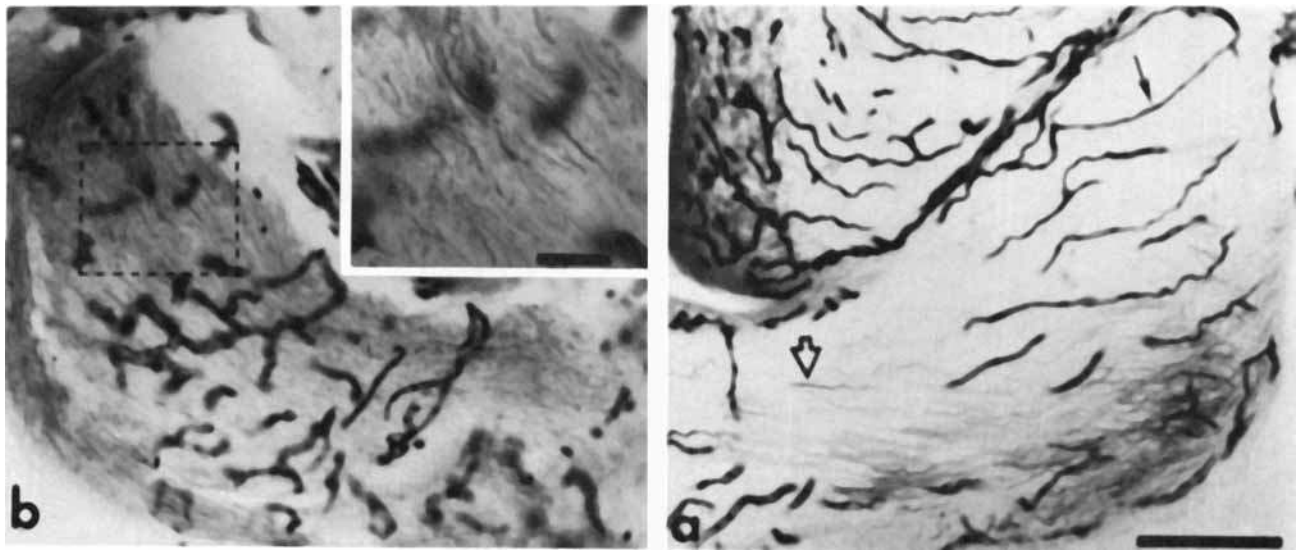

Fig. 9. Transverse sections through the optic tracts about 200 μm behind the chiasm, in an animal whose left optic nerve had been sectioned 64 days before. Crystals of HRP were inserted into corresponding ventral retinal regions 4 days before sacrifice. a. Normal tract. Labeled fibers are clustered and densely stained (open arrow). Blood vessels (thin arrow) stain heavily. b. Regenerated tract. Labeled fibers are faint and dispersed throughout the section. Inset: detail of region enclosed by dashed lines. Dorsal is up and right is to the left. Bar = 250 μm in main panels, 25 μm in inset.

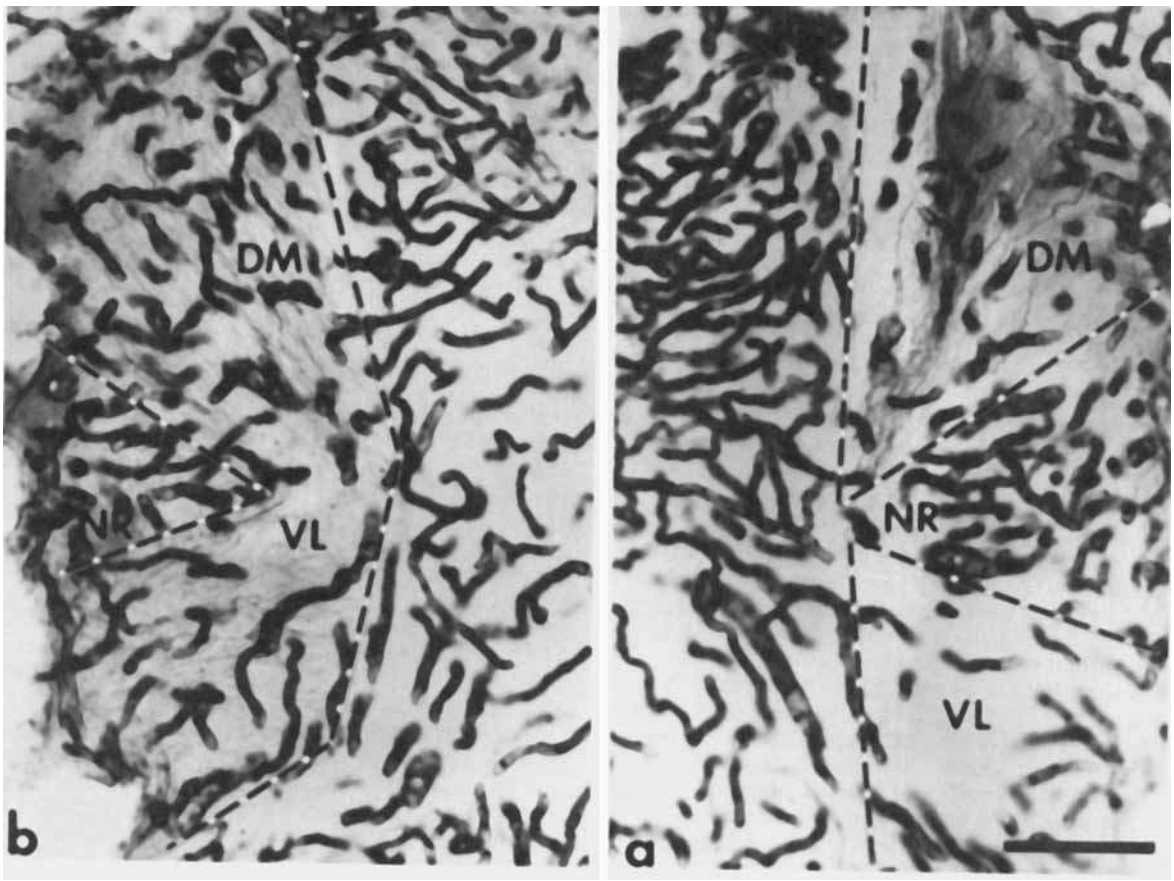

Fig. 10. Transverse sections of the same optic tracts as in Figure 9, with the same orientations. The sections were made at the level of the bifurcation of the tracts around nucleus rotundus (NR) into ventrolateral (VL) and dorsomedial (DM) brachia, bounded by the dashed lines. a. Normal side. Labeled (ventral) retinal fibers are confined to DM. b. Regenerated side. Labeled fibers are found in both DM and VL. Bar = 250 μm.

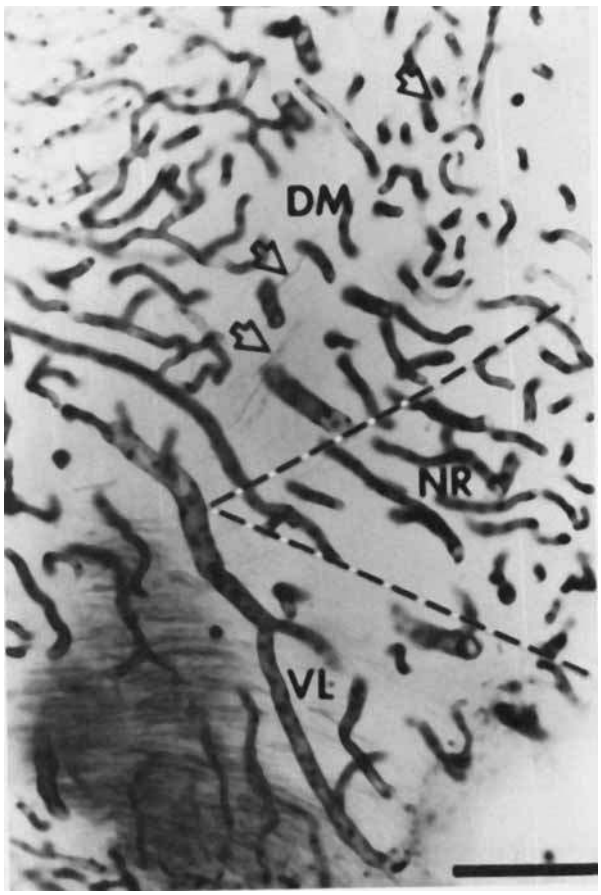

Fig. 11. Cross-section through the left (normal) optic tract at the brachial bifurcation. Dorsal retinal fibers were labeled with HRP. Most are in VL, the "correct" brachium, but a few (arrows) run in DM, the "incorrect" one. Same orientation as Figures 9, 10. Bar = 250 μm.

'55). A similar fanlike radiation of fascicles was noted after regeneration by Attardi and Sperry ('63) and Cook and Horder ('77), and was interpreted as evidence for some restoration of the normal fiber order. We have checked this observation using HRP, which is better suited to this task than the methods available to the earlier workers.

The cut central stump of normal and regenerated optic nerves (3 months postsection) was labeled with crystals of HRP, following which the contralateral tecta were reacted for HRP and whole mounted.

The arrangement in the normal animals was very orderly, confirming the earlier work. The fascicles diverged, fanlike, from the brachial entry point, and curved caudally and centrally, diminishing in thickness, until they disappeared at the equator. The fascicles seldom, and in many tecta, never, crossed one another. Figure 12a illustrates some of these features.

The arrangement of regenerated fascicles was much less orderly, as shown in Figure 12b. The fascicles were not so uniform in size as in the normals, were generally smaller, and crosssed one another frequently. Some of the rostral fascicles, directed toward the equator, where they would ordinarily disappear, crossed into the other hemitectum or turned caudally to cross many dorsoventrally oriented ones. Prominent rostrocaudally oriented fascicles were found in focal planes deep to SO (Fig. 13) where they are never seen in normals. Using other methods, earlier workers have observed aberrant fascicles in the SFGS of regenerates (Meyer, '80; Murray and Edwards, '82).

Thus, we have confirmed that regenerated retinal axons reassembled into fascicles in SO as claimed initially (Attardi and Sperry, '63; Cook and Horder, '77) and that these fascicles formed a fan. But many fascicles took aberrant routes, and the fan was very messy.

The retinal origins of the fascicles were demonstrated by labeling the fascicles with HRP. The normal pattern is shown in Figure 14a. Labeled ganglion cells occupied a part of a ventral half annulus, centered on the optic disk, and extending approximately 120° from the nasal boundary between dorsal and ventral hemiretina into ventrotemporal retina. This pattern has been reported earlier (Stuermer and Easter, '82a) and an analysis of its origin will be given elsewhere (Stuermer and Easter, '84). Here, we address the question of whether or not the regenerating axons reestablish the order implicit in this labeling pattern. When regenerated fascicles are labeled similarly, does the same retinal pattern result, or are the retinal origins of the regenerated fibers less well ordered?

Six fish (19, 60 (four fish), and 120 days postsection) had tectal fascicles labeled with HRP. The distribution of labeled ganglion cells varied. In two instances (both at 60 days) most of the labeled cells were in a nearly normal, appropriately placed, partial annulus (Fig. 14b,c). These results suggested that some, and perhaps all, of the axons that originally clustered together maintained or regained their association after regeneration. In another case (Fig. 14e), there was a hint of an arc, but most labeled cells lay elsewhere. The presence of labeled cells outside of the partial annulus indicated that some axons not previously associated with the group had joined it during regeneration.

In the other three cases, one of which is shown in Figure 14d, no annular pattern was evident. Most ganglion cells were within the ventral hemiretina but scattered at random (Fig. 14d). Many labeled cells were also found in the inappropriate dorsal hemiretina. These results indicated that either the HRP diffused more widely in the experimental tecta than in the normal ones or that the previous order was not regenerated. The labeled tecta were reacted for HRP, and the label site was no larger in the experimentals. Therefore we conclude that regenerating axons may, but do not always, course with many of their original neighbors, and that they always course with abnormal neighbors.

Tectal SFGS. Earlier work has shown that the regenerated fibers in tectum did not occupy the same pathways as originally, but these reports did not distinguish between disorder in strata of fibers and neuropil. The experiment of Figure 14 probed the order in the fiber layer, SO. We investigated the order of axons in the synaptic layer, SFGS, in another experiment.

In this case, HRP was inserted deeply into the rostral dorsomedial tectum, which produced stereotyped patterns of labeled ganglion cells. These could then be compared to the patterns after labeling an experimental tectum. If the two patterns differed, then that implies that axons and perhaps terminals in the SFGS were not in the same sites as in normals. If the regenerated pathway showed less order (a more chaotic distribution of labeled ganglion cells)

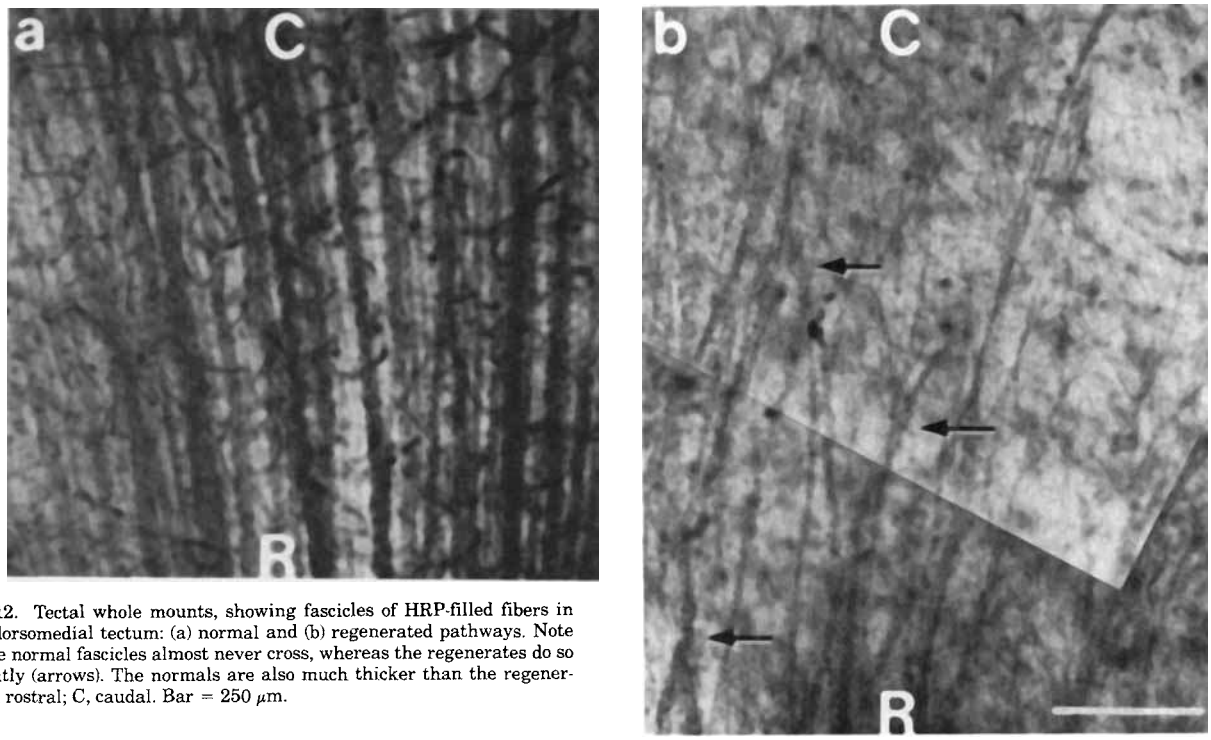

Fig. 12. Tectal whole mounts, showing fascicles of HRP-filled fibers in SO of dorsomedial tectum: (a) normal and (b) regenerated pathways. Note that the normal fascicles almost never cross, whereas the regenerates do so frequently (arrows). The normals are also much thicker than the regenerates. R, rostral; C, caudal. Bar = 250 μm.

following deep tectal labeling than after fascicular labeling (Fig. 14), then that would imply that regenerating fibers must have made additional errors in the SFGS.

In retinas with unsectioned optic nerves (N = 13), the labeled ganglion cells were always confined to the ventral half, in a sickle-shaped zone (Stuermer and Easter, '82a). The blade of the sickle was a partial half annulus (PHA in Fig. 15a) centered on the optic disk. These cells were probably labeled through the fascicle in SO which coursed through the label site. The handle of the sickle had two parts. One was a cluster of labeled cells (CL in Fig. 15a), always in ventrotemporal retina, at a site topographically related to the label site in tectum. These cells were probably labeled through their terminals. The cluster and the partial half annulus were linked by a linear connector (LC in Fig. 15a), which we interpret as those cells with axons which passed through the label site at a level deep to SO. This interpretation has been developed elsewhere (Easter and Stuermer, '82; '83; '84), but even without our interpretation, the pattern itself is a sign of order, and we use it as such.

The tecta contralateral to sectioned optic nerves were labeled similarly after a range of survival times (7 (two), 9 (two), 10 (three), 11 (four), 14 (three), 16, 17, 18, 19, 25, 26, 34, 65, 75, 100 (four), and 110 days (two)). At the two earliest times, 7 and 9 days, and in two cases at 10 days, no ganglion cells were labeled. In one 10-day animal, a few cells were labeled. In all fish that had survived longer than 10 days, labeled ganglion cells were scattered over the entire retinal surface, both dorsal and ventral (Fig. 15b,c). Many retinas had more labeled cells in ventrotemporal retina, the tectotopically "appropriate" site (Fig. 15c), but this slight bias did not seem to improve with longer survivals. Earlier work has shown that the retinotopia of the terminals improves with time, following optic nerve section (Stuermer, '78, '81a; Meyer, '80). The fact that we noted no such improvement reinforces our belief that this procedure labels both axons and terminals. The existence of labeled cells all over the retina implied that axons that would normally not pass through the label site were there after regeneration. We interpret this early and prolonged disorder as consistent with the results (in Amphibia) of Fujisawa ('81) and Fujisawa et al. ('82), who showed that individual axons ranged widely over tectum before terminating.

The degree of disorder in SFGS can be compared to that of the optic tract and SO. Recall that the majority (about 80%) of the regenerating axons in the tract chose the correct brachium and therefore grew into the correct hemitectum. The fascicle labeling confirmed this pattern, as most cells labeled through dorsal tectum were in ventral retina. But the deep tectal labeling showed generally less bias toward ventral retina. We infer that some axons must have entered the incorrect hemitectum after having entered the correct one through SO. The axonal paths in SFGS are probably the least constrained in the regenerated retinotectal system, as Figure 13 suggested.

DISCUSSION

Our results show that regenerating axons arrive first in rostroperipheral tectum and progress along a curved path that takes them both caudally and centrally. This course resembles the normal trajectory of the fascicles. At any tectal site, the axons invade SO and SFGS at about the same time. Wherever grains were found in the light microscopic autoradiograms, retinal afferent terminals were found electron microscopically. They were in the correct

Fig. 13. Tectal whole mount, showing fascicles of HRP-labeled fibers in the synaptic layer, SFGS, deep to SO, in a tectum innervated by a regenerated optic nerve. R, rostral; C, caudal. Bar = 250 μm.

stratum (SFGS), they had many of the same morphological characteristics as the normal ones, and they contacted the same classes of postsynaptic elements. The regenerated terminals were smaller and sparser initially, but their number and size increased with time. These observations reinforce the impression gained from earlier work that the regenerated retinotectal projection is quite normal.

A rather different impression emerged from the studies of small groups of regenerated axons. Their spatial order was assessed and compared with the normal at four levels: the optic tract, the brachia, SO, and SFGS. In none of these did regenerating axons reestablish their normal order, but neither were they randomly located. In the optic tract, they were more dispersed than normally (Fig. 9). At the brachial bifurcation, roughly 20% entered the incorrect brachium vs. less than 1% in normals (Fig. 10). In SO, the axons reformed fascicles in a fanlike array, but the fascicles often crossed one another, which practically never occurred in normals (Fig. 12). Further, the axons contributing to individual fascicles came from ganglion cells that were more widely scattered in retina than in normals, although most of them still derived from the appropriate retinal half, and in some cases even from annular regions as in normals (Fig. 14). In SFGS, the normal order was almost entirely missing (Figs. 13, 15), which suggests that axons wandered far from the fascicle through which they had entered tectum before terminating.

These results are inconsistent with parts of Attardi and Sperry ('63), but the techniques that we have used are quite different from theirs. Before commenting on the differences, it is useful to review the relevant literature, drawn from both amphibians and fish.

Survey of earlier work

Attardi and Sperry ('63) used a modified Bodian silver stain to evaluate the order of regenerated axons and terminals in goldfish tectum. They concluded that axons "regenerating from retinal remnants preferentially select appropriate afferent routes through the optic tract and across the tectum to terminal tectal sites" (quoted from Meyer and Sperry, '76). Later studies with electrophysiology determined only the positions of the terminals in tectum and did not treat the subject of order in the pathway (Gaze and Jacobson, '63). (Little was known about spatial order in the pathways at that time. The conventional wisdom, drawn from experiments on monkey (Hubel and Wiesel, '60) and frog (Lettvin et al., '59), was that the nerves were scrambled.) The electrophysiologically determined retinotectal map was coarsely ordered at first, and more finely so later, but it never reached the same precision as the normal (Gaze and Keating, '70). Autoradiographic mapping techniques confirmed this sequence of events (Stuermer, '78, '81a,b; Meyer, '80). The conclusion that axons did not immediately deploy their terminals at the correct tectal sites was secure. The routes taken by the afferent axons were shown in other experiments in which part of the pathway (retinal or tectal fascicles) was cut and the denervated tectal zone was then determined either anatomically or electrophysiologically (Horder, '74; Stuermer, '78, '81a,b; Udin, '78; Meyer, '80). In normals, such lesions completely denervated certain predictable regions. Following these lesions in regenerates, the zone which would normally have been completely denervated was incompletely so; this suggested that axons must have grown through abnormal routes into the spared zone. Therefore, the original view—that regeneration was an orderly reacquisition of the original pathway—had to be modified.

More recently, axons have been labeled selectively with HRP. The spatial order is quite striking in normals (particularly in fish) and therefore provides a good basis for comparision with the regenerates (Scholes, '79; Rusoff and Easter, '80; Easter et al., '81; Bunt, '82). Fawcett and Gaze ('81) and Dawnay ('81) labeled some regenerated axons with HRP in the optic nerves of *Xenopus* and goldfish and found that they were less well ordered than in normals. It was suggested that the axons might reorder more centrally, but Fujisawa and his collaborators (Fujisawa, '81; Fujisawa et al., '81, '82) traced optic axons through tectum to their sites of termination and found that the regenerates followed very abnormal routes, consistent with the idea that they wandered until finding the correct site.

We have approached the problem somewhat differently, by inserting HRP into tectum and then discovering which ganglion cells were represented there. Our results (this report; and Stuermer and Easter, '82a) are similar to those of Cook ('83). The labeled retinal ganglion cells were far more widely scattered than in normals, which implies one or more of the following three possibilities: (1) The HRP may have diffused more widely in the regenerated tecta

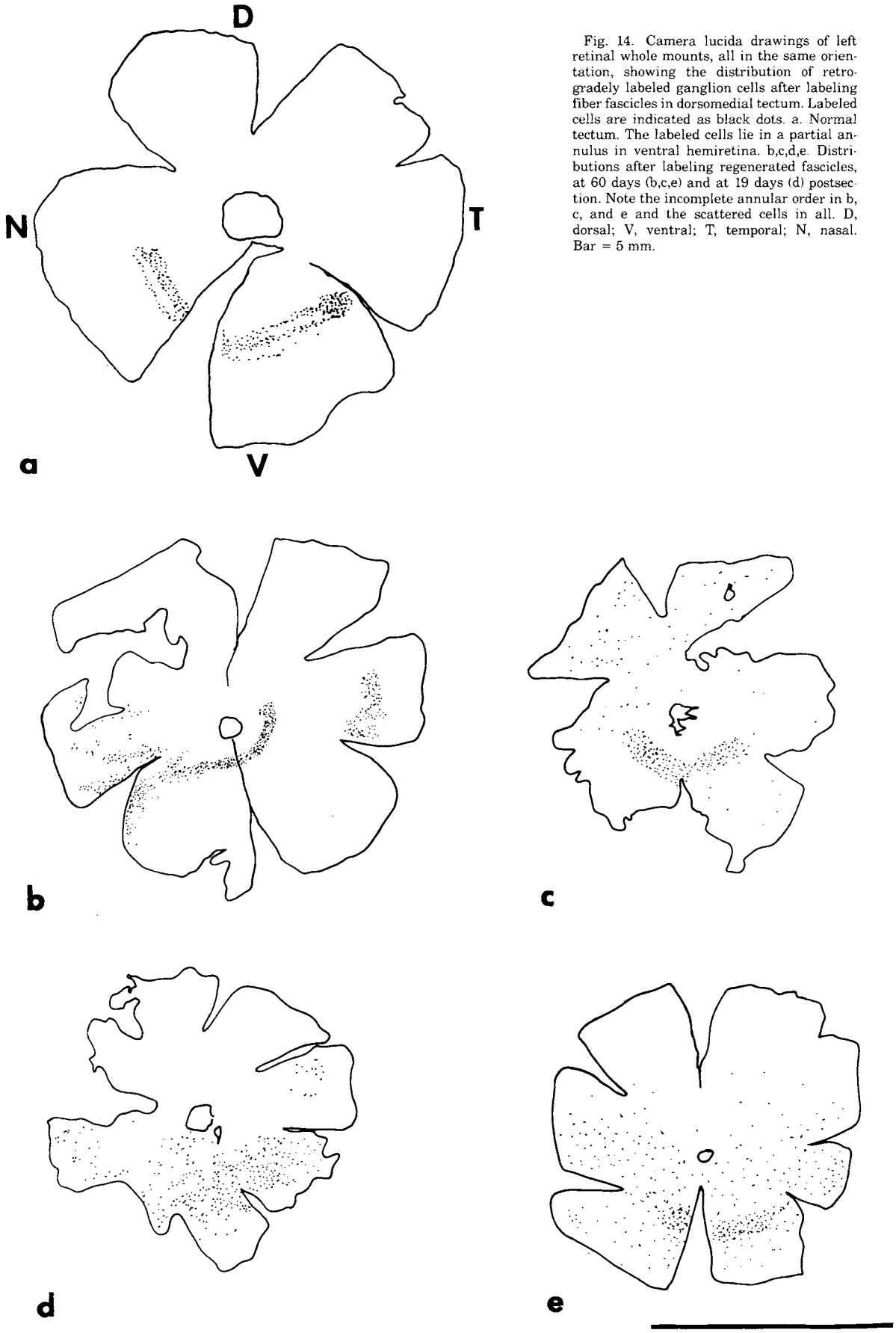

Fig. 14. Camera lucida drawings of left retinal whole mounts, all in the same orientation, showing the distribution of retrogradely labeled ganglion cells after labeling fiber fascicles in dorsomedial tectum. Labeled cells are indicated as black dots. a. Normal tectum. The labeled cells lie in a partial annulus in ventral hemiretina. b,c,d,e. Distributions after labeling regenerated fascicles, at 60 days (b,c,e) and at 19 days (d) postsection. Note the incomplete annular order in b, c, and e and the scattered cells in all. D, dorsal; V, ventral; T, temporal; N, nasal. Bar = 5 mm.

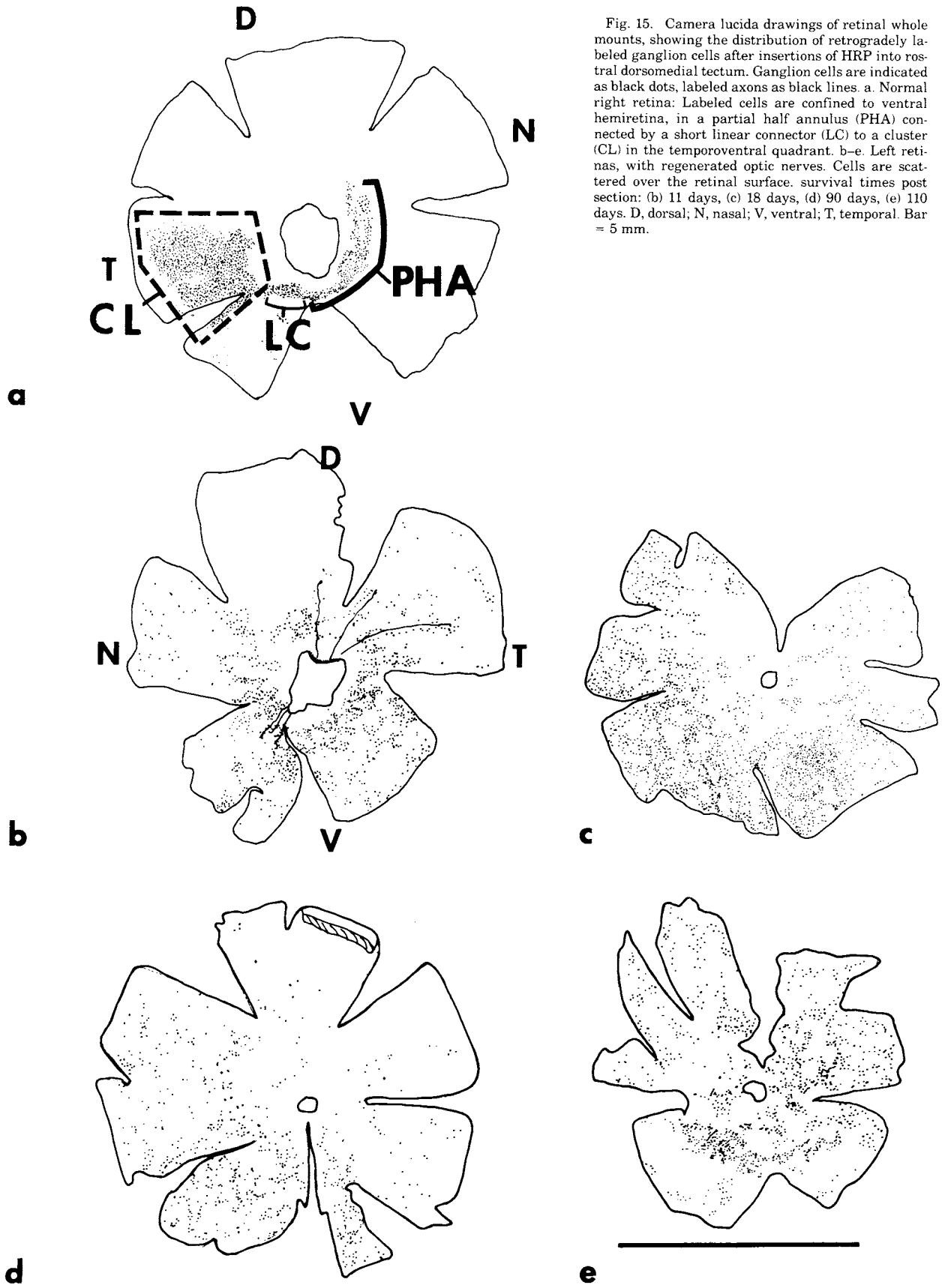

Fig. 15. Camera lucida drawings of retinal whole mounts, showing the distribution of retrogradely labeled ganglion cells after insertions of HRP into rostral dorsomedial tectum. Ganglion cells are indicated as black dots, labeled axons as black lines. a. Normal right retina: Labeled cells are confined to ventral hemiretina, in a partial half annulus (PHA) connected by a short linear connector (LC) to a cluster (CL) in the temporoventral quadrant. b–e. Left retinas, with regenerated optic nerves. Cells are scattered over the retinal surface. survival times post section: (b) 11 days, (c) 18 days, (d) 90 days, (e) 110 days. D, dorsal; N, nasal; V, ventral; T, temporal. Bar = 5 mm.

than in the normals. (2) The regenerated fibers may pick up HRP more avidly than normals, so that they would label in the presence of very dilute extracellular HRP. (3) The regenerated fibers occupied different paths in the regenerates than in the normals. We have described a control experiment which caused us to doubt the first of these. The second is a logical possibility, but if the regenerates occupied the same pathways as the normals, then the retinal whole mount ought to show similar, but larger, fields of labeled cells. For instance, following fascicular label (Fig. 14) there ought to be a thicker partial annulus, and after label in SFGS (Fig. 15), all three portions of the retinal pattern (partial half annulus, linear connector, and cluster) ought to be abnormally large. Such was not the case; the labeled cells lay far from one another, often in patches that could not be explained by diffusion to contiguous regions (e.g., Figs. 14b, 15b–e). Therefore, we favor the third alternative, that regenerating axons did not restore the order of the original tectal pathway, but instead wandered through tectum without any clear order. However, despite their tortuous courses, they ultimately reached their retinotopic termination sites (Stuermer, '78, '81a; Meyer, '80; Fujisawa et al., '82).

Our results confirm the conclusions of the earlier studies and extend them by showing where the mistakes were made and to what degree.

A possible origin of coarse retinotopia

The predominantly rostrocaudal orientation of the aberrant fascicles in SFGS was a striking feature. This merits some comment because it suggests a conservative explanation of how the initial coarse retinotopia might be established in spite of errors in the selection of pathways. In normals, axons exit from a fascicle into SFGS in a predictable order: temporal first, nasal last (Stuermer and Easter, '82b; '84). If axons grow into the "wrong" fascicles but then exit in the normal order then all of the axons from temporal retina would have exited their fascicles in rostral tectum, no matter which fascicles they were in. Therefore, these axons would be in the "appropriate" half tectum and would have to search a relatively short distance before finding their retinotopic termination sites. Axons from nasal retina would be expected to exit over the caudal half of their fascicles. But many of these fascicles would be restricted to the rostral half tectum; therefore a substantial number of axons retinotopically related to caudal half tectum would have to go there through extrafascicular pathways. We suggest that these are the axons which fasciculate in SFGS and course caudally.

The rule, "temporal first, nasal last" could account for the coarse retinotopia that Meyer ('80) has described early in regeneration. According to Meyer, axons initially terminate in the proper hemitectum (rostral vs. caudal) but show no more refined order. This is consistent with our suggestion: All temporal axons and half of the nasal ones would be in rostral hemitectum initially; about half of the nasal axons and none of the temporal ones would be caudal. The subsequent refinement of the map would reflect homing by individual axons.

Time course of regenerative synaptogenesis

In an earlier ultrastructional study, Murray ('76) first observed regenerated synapses in goldfish tectum 28 days after the nerve was cut, 10 days later than we have found (this report and Schmidt et al., '83). The difference is probably attributable to the fact that Murray examined only midtectum, whereas we have found that the first synapses were rostral.

Evaluation of current hypotheses

The evidence that axons from nonneighboring ganglion cells fasciculate together, take abnormal routes, and finally terminate retinotopically weakens the hypothesis that a selective affinity between axons of neighboring ganglion cells would provide a selective reordering among them and determine their retinotopic termination (Cook and Horder, '77).

The idea that regenerating axons may be led passively to the tectum by mechanical guidance (Horder and Martin, '78) can also be excluded. Those axons that have taken incorrect paths terminate retinotopically (Horder, '74; Easter and Schmidt, '77; Easter et al., '78; Udin, '78; Fujisawa, '81; Fujisawa et al., '82). Moreover, axons showed signs of having actively changed their positions in order to enter their correct brachial path.

Our results relating to axonal pathfinding are compatible with the idea of local chemotactic growth suggested by Sperry ('63). Our results are consistent with those of Fawcett and Gaze ('82) and Gaze and Fawcett ('83), which support the idea that growing axons have an affinity for particular pathways or parts of pathways. The regenerated pathways described in this report were not totally disordered, only much less precisely ordered than the original ones. It is possible that the regenerating axons followed the same rules as new ones, but were more disordered because there were so many more of them and so much more space available for them to invade than during de novo development. Although they did not recapture the original paths perfectly, they came rather close until they arrived in SFGS. At this level, pathfinding seemed to break down into a random search, not a guided one. This is contrary to the prediction of the hill-climbing model for retinotectal projections of Gierer ('83). Ultimately, despite all the freedom to err, the majority of regenerated retinotectal terminals were positioned correctly in tectum, as other work has shown. The success of this final search is explicable most easily in terms of long-lived tectal markers that regenerating axons recognize: the original chemospecificity hypothesis (Sperry, '63).

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