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Normal Numbers of Retinotectal Synapses During the Activity-Sensitive Period of Optic Regeneration in Goldfish: HRP-EM Evidence Implicating Synapse Rearrangement and Collateral Elimination During Map Refinement

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Optic and nonoptic fibers and synapses were counted in the primary optic innervation layer (S-SO-SFGS) in anteromedial tectum in normal goldfish and in fish 30, 60, and 240 d after the optic nerve was crushed. A newly developed "cold-fill" HRP-labeling protocol was used to label optic afferents for electron microscopy, and counts were then made on EM photomontages of columns through the HRP-labeled S-SO-SFGS.

Normal numbers of retinotectal synapses were present at 30 d regeneration, at a time when activity-dependent refinement of the optic projection is incomplete. Normal numbers were also found at 60 and 240 d, when refinement is largely completed. In contrast to this constancy in optic synapse numbers, there was nearly 10 times the normal number of optic fibers in the SFGS at 30 d, and these were reduced by 50% at 60 d, remaining over 4 times normal at 240 d. These findings imply extensive rearrangement of optic synapses during map refinement. They also indicate that synapse rearrangement is associated with the elimination of optic collaterals.

How growing nerve fibers navigate along specific pathways in the brain and ultimately choose appropriate target cells with which to form synapses remains a fundamental problem in neurobiology. There is long-standing evidence, particularly from work in lower vertebrates, that synapse formation is regulated by intrinsic cellular markers. Optic fibers can regrow to their appropriate retinotopic position in tectum following surgical diversion to abnormal starting positions or can relocate their appropriate piece of tectum when it has been transplanted to an inappropriate region of tectum (Sperry, 1963; Meyer, 1980, 1984; Gaze and Hope, 1983). More recent work, however, suggests that the formation of connections may be more flexible than implied by a rigid cell marker system (Schmidt et al., 1978; Cook, 1979; Horder and Martin, 1982; reviewed by Cowan and Hunt, 1985; Fraser, 1985). In particular, the establishment of specific synapses may be regulated by impulse activity. In goldfish, regenerating optic fibers initially form a diffuse projection that is roughly retinotopic and that becomes more spatially restricted or refined (Meyer, 1980; Meyer et al., 1985; Rankin and Cook, 1986). This refinement is apparently mediated by impulse activity since it can be inhibited by impulse blockade (Meyer, 1983; Schmidt and Edwards, 1983) or abnormal visual experience (Cook and Rankin, 1986). A similar transformation is seen when fibers from 2 eyes are made to regenerate into one tectum. Initially both groups of fibers overlap and then subsequently segregate. This segregation also is prevented by impulse blockade (Meyer, 1982; see Reh and Constantine-Paton, 1985, for data on frogs). Similar findings have been reported for the developing visual system in mammals (Dubin et al., 1986; Stryker and Harris, 1986; reviewed by Cowan et al., 1984; Easter et al., 1985).

Although these findings suggest that functional synapses play a role in the formation of refined retinotectal maps (as postulated by Prestige and Willshaw, 1975; Changeux and Danchin, 1976; Willshaw and von der Malsburg, 1976; Whitelaw and Cowan, 1981), it is not clear what the synaptic correlates of these refinements are. Three distinct synaptic scenarios can be envisioned. The first is *selective synapse formation* in which only position-specific synapses are formed. Optic synapses would gradually accumulate at the appropriate retinotopic position (presumably modulated in an activity-dependent fashion), so that the establishment of normal numbers of synapses is tightly correlated with the refinement of neural topography. A previous electron microscopic study has suggested this is the case for goldfish optic regeneration (Murray and Edwards, 1982). The second is *synapse reduction* in which growing axons would make an excess number of synapses prior to map refinement and eliminate the incorrect ones. Although there is evidence for synapse elimination in other systems (reviewed by Purves and Lichtman, 1985), there is little direct evidence for transient inappropriate synapses during regeneration in goldfish (Murray and Edwards, 1982; Airhart and Norden, 1985; but see Hayes and Meyer, 1984, 1988a). Third is *synapse rearrangement* whereby growing axons also make synapses but in which these are numerically constrained. Fibers would then remove and reform synapses until all their connections are located at the

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correct tectal position. There is no clear evidence for it in this system and only indirect evidence for other CNS systems (reviewed by Cotman and Nieto-Sampedro, 1984).

The goldfish retinotectal projection offers a number of attractive features to test these possibilities. The large majority of optic fibers projects to one target, the tectum, and primarily to one layer in that target, the S-SO-SFGS (Meyer, 1980; Springer and Gaffney, 1981; Murray and Edwards, 1982; Meyer et al., 1985). During regeneration there are no substantial exuberant projections to other nuclei after 30 d (Springer, 1981), and there is no apparent neuronal death or birth of afferent or target cells (Murray et al., 1982; Meyer et al., 1985). Also, two phases of the topographic refinement are distinguishable: one at 30 d, in which all fibers have invaded the entire tectum but project with poor retinotopic order, and a later one at 60 d, in which fibers have become spatially restricted (Meyer, 1980; Meyer et al., 1985; Rankin and Cook, 1986).

Materials and Methods

A detailed description of the HRP cold-fill labeling protocol has been presented elsewhere (Hayes and Meyer, 1988a, b). What follows is a summary of the surgical, labeling, and histochemical procedures and a description of the methods used in the laminar analysis of fiber and synapse numbers.

Surgery and HRP labeling

Goldfish (4.5–6.0 cm) were anesthetized in a tricaine (Finquel) solution. The left optic nerve was crushed intraorbitally. After rearing for 30, 60, and 240 d at 19–21°C, fish were labeled with HRP (see Table 1). Briefly, fish were anesthetized as above and placed in an ice bath for 3 min. The left eye was then enucleated, and the optic nerve stump was sucked up into a 4–6 mm segment of polyethylene tubing (1.14 mm i.d.) that was sealed at one end with tacky wax and contained a 25–30% solution of HRP (Type II, Sigma). The tube was glued into place with cyanoacrylate adhesive and the orbit filled with vaseline. Fish were maintained for 48–72 hr at 5–7°C.

Tissue preparation and HRP histochemistry

Anesthetized fish were perfused transcardially using, first, a tricaine in fish Ringer's solution followed by 2 cold aldehyde fixative solutions (see Hayes and Meyer, 1988b, for details). The entire fish was postfixed at 5–7°C for 45–60 min, and the brain was removed and postfixed cold for 2–3 hr.

Brains were sectioned coronally at 100 μm in PBS maintained at 9–11°C with a vibratome. Individual sections were stored in proper sequence at 5–7°C for 2–12 hr. Sections were processed for HRP histochemistry using a modification of the Adams (1981) diaminobenzidine-cobalt (DAB-Co) protocol.

Electron microscopy

Tectal sections were osmicated and embedded using propylene oxide and Epon–Araldite. In one fish (HRETC16), sections were dehydrated in ethanolic–phosphotungstic acid (E-PTA) (see Table 1). Ultrathin and semithin sections were cut from blocks parasagittally, perpendicular to the tectal surface. Semithin sections for light microscopy were stained using sodium borate-buffered toludine blue. Ultrathin sections were floated onto 1.0-mm-hole Formvar grids and stained on grid using methanolic uranyl acetate and then lead citrate. Each section spanned the entire primary optic layer consisting of the superficial optic fiber lamina (S), the intermediate optic fiber lamina (SOi), the deep optic fiber lamina (SO), and the superficial fiber and gray lamina (SFGS) and the adjacent overlying marginal fiber lamina (SM) and underlying central gray lamina (SGC) (for laminar nomenclature see Fig. 1, and Vanegas et al., 1974; Meek, 1981).

Controls

The possible effects of the HRP cold-fill labeling procedure on retinotectal terminal degeneration and the specificity of optic afferent labeling were examined at the electron microscopic level in tectal material (1) not reacted with DAB-Co (or other chromogen) but labeled using HRP and (2) labeled with fish Ringer's instead of HRP and then reacted using DAB-Co. Using cellular landmarks to identify the borders of the unlabeled S-SO-SFGS (see below), this region was examined and photographed at the ultrastructural level in three fish (Table 1).

Table 1. Fish used for the HRP-EM laminar analysis

Fish	Days after optic nerve crush	Sample columns analyzed (*n*)	Total synapses counted (*n*)	Total fibers counted (*n*)
HRETC14[a]	–	–	–	–
HRETC16[b]	–	1	384	85
HRETC18[c]	–	5	2268	481
HRETC19[d]	–	1	478	224
HCREG17[d]	30	3	1380	1833
HCREG18[d]	30	1	569	828
HCREG19[d]	30	1	602	766
HCREG5	60	1	433	548
HCREG6	60	1	519	541
HCREG7	60	3	1342	919
HCREG13	240	1	586	379
HCREG14	240	3	1337	1331
Total		21	9898	7935

[a] Tube with fish Ringer's instead of HRP (cold-fill control).

[b] Osmicated material dehydrated in E-PTA.

[c] Two columms from unreacted sections (cold-fill control) and 3 columns from DAB-reacted material.

[d] Unreacted control sections (for light microscopy only).

Laminar analysis of synapse and fiber numbers

Electron micrographs for the sample columns were taken from equivalent anteromedial tectal regions in all animals. The amount of DAB product formed a gradient from the cut surface to the central region of each vibratome section. Typically, the middle of the section was lightly labeled, and the edges were so heavily labeled that the ultrastructure was not optimal. Both the middle of the section and the flanking regions showing good ultrastructure were used for the electron micrographs.

Montages. Contiguous electron micrograph montages representing 14 × 160 μm columns were made that spanned the S-SO-SFGS. Individual electron micrographs were taken at a magnification of 6000 or 6300. Using a carbon calibration grid, micrographs were calibrated and printed at ×18,000; additional survey micrographs of labeled profiles not included in the montages were taken at ×2500–30,000. The tangential laminar boundaries of the S-SO-SFGS, the radial orientation of the prominent Type 1 neuron (Meek, 1981) apical dendrites, and the tectal surface were used to align the column of micrographs along a radial path that was sometimes stepped laterally to minimize the presence of capillaries and cell bodies. Columns extended at least 6 μm into the unlabeled flanking SM and SGC.

Counting procedure. The montages of sample columns through the S-SO-SFGS were divided into 30–40 rectangular bins, 14 μm wide and 3 μm deep. Under magnification, each of the following profiles was marked on the micrograph and tabulated by bin: labeled synapses, labeled unmyelinated fibers, labeled myelinated fibers, unlabeled synapses, and unlabeled myelinated fibers.

Laminar boundaries. For both the thickness measurements and synapse and fiber counts (Tables 2–4), laminar boundaries in labeled sections were defined as follows: the upper boundary of the S-SO-SFGS, the SM-S boundary, was placed immediately above the bin containing the labeled S lamina. The SO-SFGS boundary was placed immediately below the bin containing large labeled, transversely sectioned fascicles of fibers. The lower SFGS-SGC boundary was placed just below the deepest bin containing labeled synapses. The SOi was defined as an unlabeled lamina between the S and SO.

The upper and lower boundaries of the S-SO-SFGS could be roughly estimated in unlabeled tectal material using the following previously

Table 2. Summary of mean synapse numbers per sample column

	Labeled synapses (LSYN)	Unlabeled synapses (USYN)	Total synapses (TSYN)	(LSYN/TSYN × 100) %
Normal development (n = 5, 3 fish)	174 ± 15	238 ± 42	411 ± 33	42.6 ± 6.0
30 d regeneration (n = 5, 3 fish)	167 ± 20	283 ± 48	449 ± 64	37.3 ± 2.4
60 d regeneration (n = 5, 3 fish)	162 ± 14	251 ± 13	413 ± 19	39.4 ± 2.2
240 d regeneration (n = 4, 2 fish)	172 ± 16	246 ± 42	418 ± 46	41.4 ± 4.4

described neurocytological landmarks (Murray and Edwards, 1982). The superficial SM just above the S lamina consisted of a dense network of transversely cut unmyelinated fibers. The deep SFGS-SGC border, although more difficult to resolve with precision in unlabeled material (Murray and Edwards, 1982), was just below the sparsely distributed Type 1 (Meek, 1981) neuronal cell bodies.

Synaptic criteria. The following criteria were followed when identifying synapses. Each synaptic contact that was counted contained a single postsynaptic density with synaptic vesicles in proximity to the presynaptic membrane facing an identifiable postsynaptic process. Labeled synapses were counted on terminals containing DAB-type product and on the few darkened terminals showing degenerating pale mitochondria. Labeled terminals were sometimes so heavily filled with DAB product that synaptic vesicles in apposition to postsynaptic densities were difficult to resolve. In these cases, a synapse was counted if the DAB product was confined within the terminal membrane and if synaptic vesicles were seen near a density spanning the membrane facing a potential postsynaptic profile. Labeled terminals often contained more than one synapse and these were counted separately.

Labeled synapses in sample columns were recounted by a second person using more conservative criteria in which a synapse was defined as above but also had to show a clearly defined synaptic cleft with cleft material. Numbers of labeled synapses counted this way were significantly less than the value determined using the preceding criteria but were a consistent fraction of that number in all fish. The former criteria, however, appears to give the more accurate estimate of synapses because when labeled synapses were counted the first way and added to unlabeled synapses, we found that the total synapses per column were essentially the same as the total synapses counted from columns of unreacted HRP-labeled sections in the same animal, where synaptic contacts could often be more easily resolved (see Results).

Axonal criteria. Each clearly separated fiber ensheathed by myelin was counted as a myelinated fiber. Unmyelinated labeled fibers were identified by the presence of DAB product in profiles containing oriented microtubules and lacking synapticlike vesicles. Both fasciculated and individual axonal profiles were counted. Fascicles and the fibers per fascicle were also counted. When counting fibers within large fascicles, fiber labeling was not uniform and not all fibers were always clearly labeled. Since large fascicles seen in the SFGS near the heavily DAB-labeled lateral edges of the section often had all their (as many as 120) fibers labeled and since fascicles encountered away from the edges showed progressively fewer labeled fibers, it was assumed for counting purposes that most or all of the fasciculated fibers within labeled fascicles are optic. This assumption is also supported by previous ultrastructural studies (Murray, 1976; Murray and Edwards, 1982) and by our observation that in fascicles sectioned longitudinally labeling was sometimes discontinuous along the length of a single optic fiber.

Measurements and statistics. Mean labeled, unlabeled, and total synapse numbers were derived by summing the total numbers of synapses from the counting bins within the S-SO-SFGS and pooling data from the sample columns. Mean fiber numbers, however, were the sum of the numbers of fibers counted only in bins in the SFGS. Mean fiber numbers per column were made from the SFGS because fascicles were not always seen in the S and SO. For computing the means, the sample columns from the same fish were considered the same as columns from different fish because their numbers were just as variable (data not shown). However, to be conservative in the statistical tests of significance, only the first column that was analyzed in each individual fish was used; that is, one fish was considered one observation and only the columns from different fish in each experimental group were pooled.

The mean depth profiles of fibers and synapses for normal fish and for fish at 30, 60, and 240 d regeneration were made by aligning the individual sample columns at the SO-SFGS border and averaging the numbers of fibers and synapses in the aligned bins. The thicknesses of the S-SO, SFGS, and S-SO-SFGS were measured directly from the electron micrograph montages. The statistical significance of any possible differences in mean numbers of profiles or in laminar thickness was determined using both a 1-way analysis of variance test (ANOVA) and a 1-way multivariate analysis of variance test (MANOVA) and several nonparametric tests of significance.

Results

Light microscopy of retinotectal fibers

This HRP method labeled the entire anterior–posterior and medial–lateral extent of the primary retinotectal innervation layer (S-SO-SFGS) in normal fish and fish after 30–240 d optic regeneration in a dense and uniform fashion (data shown in Hayes and Meyer, 1988b). Semithin plastic sections from the anteromedial tectum in different fish showed that the laminar distribution of labeled optic fibers in fish as early as 30 d regeneration was similar to that seen by others in normal fish using autoradiographic (Meyer, 1980), HRP (Stürmer and Easter, 1984; Meyer et al., 1985), and cobalt (Springer and Gaffney, 1981) methods. The SOi lamina between the labeled S and SO sub-

Table 3. Summary of mean fiber numbers per sample column

	Labeled fibers (LFIB)	Unlabeled fibers (UFIB)	Myelinated fibers	% Myelinated fibers	LFIB in fascicles	% LFIB in fascicles	Labeled fascicles	Mean LFIB/fascicles
Normal development (n = 5, 3 fish)	51 ± 8	24 ± 8	47 ± 2	92.1 ± 3.5	28 ± 6	52.9 ± 9.2	10.2 ± 3.3	2.9 ± 0.5
30 d regeneration (n = 5, 3 fish)	460 ± 93	27 ± 3	1 ± 3	0.3 ± 0.6	400 ± 94	86.4 ± 4.0	23.6 ± 6.0	17.8 ± 6.9
60 d regeneration (n = 5, 3 fish)	231 ± 39	25 ± 10	14 ± 9	5.9 ± 3.8	200 ± 37	86.6 ± 4.3	25.6 ± 6.7	8.0 ± 1.2
240 d regeneration (n = 4, 2 fish)	223 ± 70	33 ± 4	147 ± 11	65.8 ± 5.0	192 ± 67	85.4 ± 4.4	22.8 ± 5.1	8.5 ± 3.1

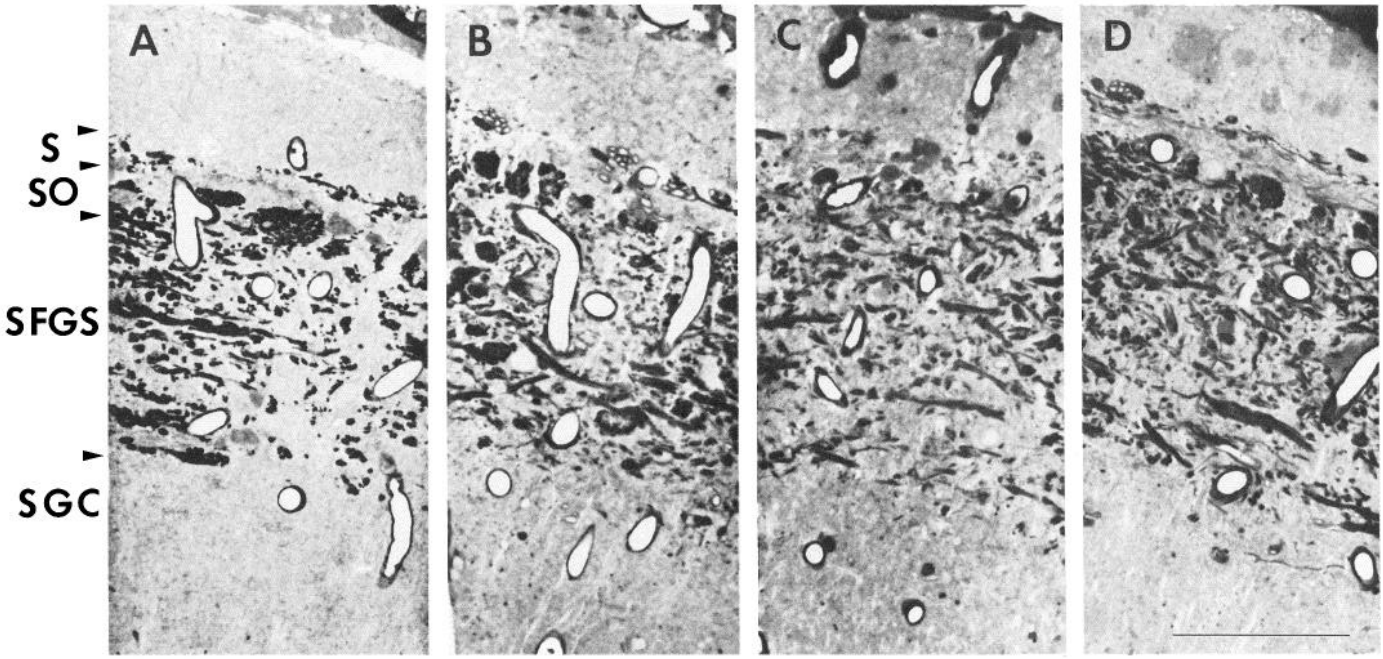

Figure 1. Light micrographs of 1 μm semithin sections cut parasagittally through the primary optic innervation laminae of anteromedial tectum of goldfish. Optic afferents labeled with HRP using DAB-Co as chromogen. The S, SO, and SFGS are denoted by lines at the left of each micrograph. *A,* Normal unoperated fish (HRETC19); *B,* 30 d regeneration (HCREG19); *C,* 60 d regeneration (HCREG7); *D,* 240 d regeneration (HCREG14). Scale bar, 50 μm.

laminae showed little or no label in normal and regenerating fish (Fig. 1, *A–D*). The deeper optic laminae in the SGC and SAC-SPV were also selectively reinnervated. These deeper projections were always sparse and were not studied at the electron microscopic level. Control material not labeled with HRP or not reacted histochemically showed no product (Table 1).

Electron microscopy of optic fibers and synapses

Optic fibers and synaptic terminals in the S-SO-SFGS were densely filled with flocculent DAB product in the S, SO, and SFGS of normal fish. HRP-labeled retinal terminals were, as reported by others (Murray and Edwards, 1982; Airhart and Kriebel, 1984), typically large, and filled with synaptic vesicles, and they often made multiple asymmetric synaptic contacts onto dendritic spines and vesicle-containing postsynaptic profiles (Figs. 2*A*, 3). In addition, labeled optic terminals making axosomatic and axodendritic contacts onto Type 1 neurons in the deep SFGS and onto their proximal dendrites in the mid-SFGS were seen less frequently (Hayes and Meyer, 1988b). Fiber label in normal fish showed, as reported previously (Murray, 1976; Murray and Edwards, 1982), that myelinated optic fibers formed large fascicles in the SO, and these often contained over 50 fibers. Labeled fibers in the SO were invariably sectioned transversely in our parasagittally oriented material, indicating they traveled mediolaterally in midtectum. Optic fibers in the S and SFGS were either not fasciculated or formed smaller fascicles containing 2–8 fibers. These fibers were often sectioned obliquely, indicating they coursed in all possible directions (Fig. 2*A*). From the label it was evident that optic terminals in normal fish were often grouped in clusters that were near or in contact with labeled axonal profiles in the S and SFGS (Fig. 3; Hayes and Meyer, 1988a, b).

There was no evidence of DAB-like product in any of the

Table 4. Summary of changes in S-SO-SFGS thickness

	S-SO-SFGS depth (μm)	% change from normal	SFGS depth (μm)	% change from normal	S-SO depth (μm)	% change from normal
Normal development (n = 5, 3 fish)	87 ± 4	—	65 ± 5	—	22 ± 3	—
30 d regeneration (n = 5, 3 fish)	101 ± 3	+16.1	77 ± 5	+18.5	24 ± 4	+9.1
60 d regeneration (n = 5, 3 fish)	109 ± 11	+25.3	83 ± 14	+27.7	27 ± 9	+22.7
240 d regeneration (n = 4, 2 fish)	103 ± 2	+18.3	77 ± 6	+18.5	26 ± 5	+18.2

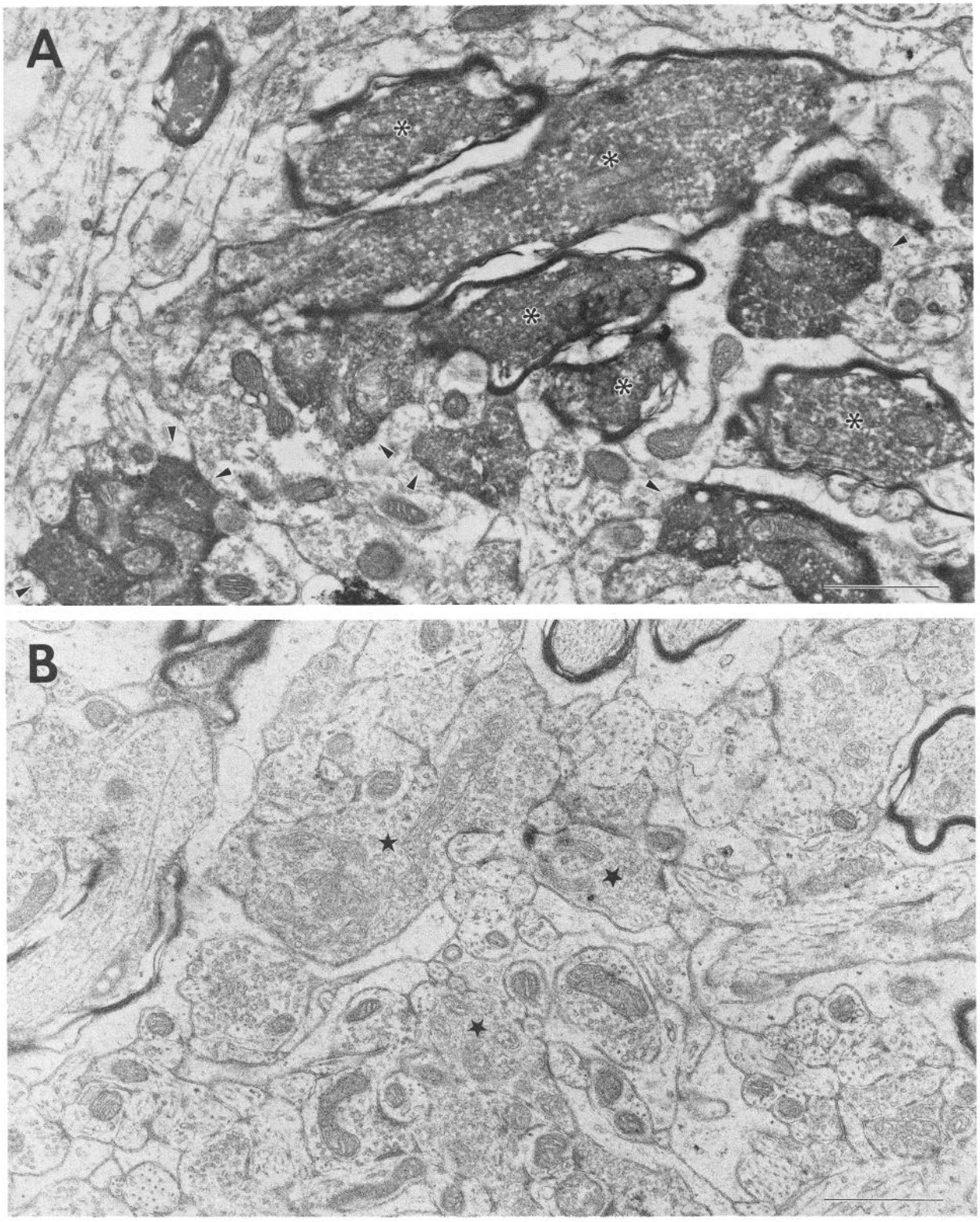

Figure 2. Electron micrographs of the SFGS in a normal fish (HRETC18) labeled using the HRP cold-fill method. *A,* DAB-Co reacted material showing high concentration of labeled optic fibers and synapses. Note myelinated optic fibers (*asterisks*) are often fasciculated, and optic terminals making synaptic contacts (*arrowheads*) form clusters near or in contact with fascicles. *B,* Nonreacted material showing absence of label or overt fiber or terminal degeneration. Note presumptive optic terminals (*stars*) with pale mitochondria make many normal synaptic contacts. Scale bars, 1 μm.

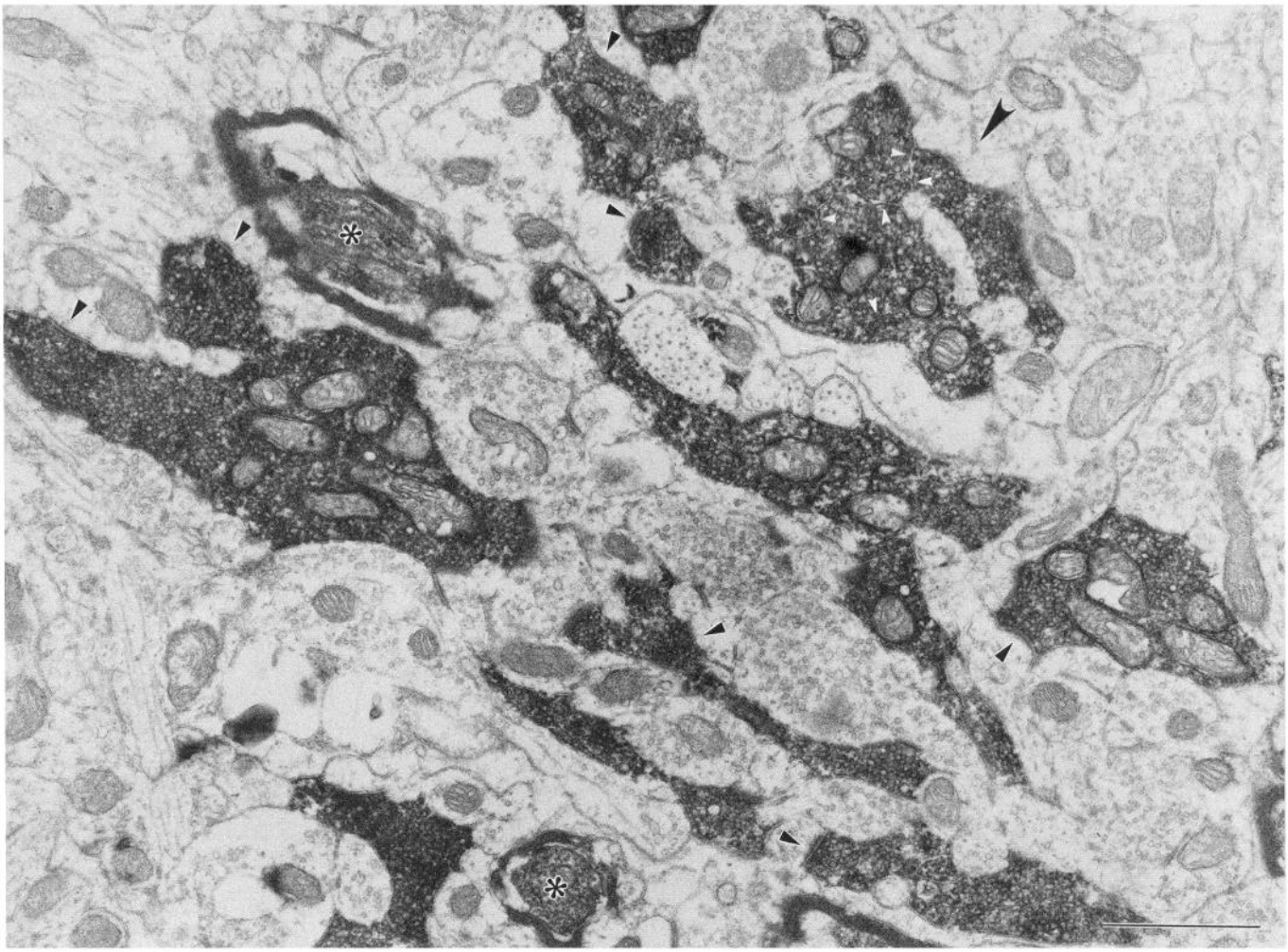

Figure 3. Electron micrograph of labeled optic terminal clusters forming many synapses (*small arrowheads*) in the SFGS of normal fish (HRETC18). Note the terminal cluster in the upper right corner (*large arrowhead*) contains many distinct profiles in direct contact with each other (*white arrowheads*). Scale bar, 1 μm.

sections that were not HRP-labeled or not reacted with chromogen (Fig. 2*B*; Hayes and Meyer, 1988b). However, some putative optic terminals identified by the presence of large pale mitochondria (Airhart and Kriebel, 1984; Stürmer and Easter, 1984; but see Murray and Edwards, 1982) showed disrupted and dilated membranes. These profiles were seen making many morphologically normal synapses (Fig. 2*B*). Unidentifiable degenerating profiles were only very rarely seen in unlabeled and labeled normal fish or fish with regenerating optic nerves.

At 30, 60, and 240 d regeneration, large fascicles, previously seen in unlabeled material (Murray, 1976; Murray and Edwards, 1982) were heavily labeled. These fascicles contained many more fibers than that seen in the SFGS of normal fish (Fig. 4). During regeneration, labeled synaptic terminals were also observed making many normal types of synaptic contacts (axospinous, axodendritic, and axosomatic), as well as atypical, possibly nascent synapses in the S, SO, and SFGS but not in the SOi (Figs. 5, 6; see Hayes and Meyer, 1988b, for a detailed description of the synaptology of regenerating optic fibers at 14–21 d regeneration). As noted in normal fish, labeled synaptic terminals were typically grouped in clusters that were in contact with or in close proximity to labeled fibers (Figs. 3, 5, 6).

Quantitative spatiotemporal analysis of retinotectal regeneration

The depth profile analysis was carried out in normal fish and fish regenerating for 30, 60, and 240 d (see Table 1).

Normal. The mean depth profile of synapses (Fig. 7*A*) showed that optic (labeled) synapses were restricted to the previously described optic innervation laminae: S, deep part of the SO, and SFGS. The boundaries for these laminae were sharply defined. Optic synapses were observed in the 4–6 μm S lamina and deep part of the 18–20 μm SO lamina and in the 63–65 μm SFGS. In the SFGS, optic synapses were inhomogeneously distributed, with the majority in the upper two-thirds of the SFGS. Unlabeled synapses in the S-SO-SFGS tended to be inversely distributed to optic synapses.

The mean depth profile for fibers (Fig. 7*B*) showed that there were many optic fibers in the SO. While there were comparatively few fibers in the S lamina, significant numbers of individual optic fibers and optic fascicles were observed in the SFGS, where they are inhomogeneously distributed.

Regeneration. Mean depth profiles for fish at 30 d (Fig. 7, *C, D*), 60 d (Fig. 7, *E, F*) and 240 d (data not shown) regeneration

Figure 4. Low-magnification electron micrograph showing large numbers of fasciculated optic fibers (*large arrrowheads*) and nearby synapse-bearing optic terminals (*small arrowheads*) in the SFGS at 30 d regeneration (HCREG18). Scale bar, 2 μm.

showed that the distributions of regenerating (labeled) and unlabeled fibers and synapses were similar to those observed in normal fish. Briefly, labeled profiles were always confined to optic laminae of the S-SO-SFGS and were not observed in the SM, SOi, and upper SGC (Hayes and Meyer, 1988a, b). The same inverse relationship in the number of labeled versus unlabeled synapses seen in normal fish is restored as early as 30 d regeneration (Fig. 7, *A, C, E*). Also as in normal fish, regenerating optic fibers obeyed laminar boundaries while distributing themselves throughout the depth of the SFGS.

Changes in the S-SO-SFGS thickness during regeneration

The laminar borders, defined by the distribution of labeled profiles in the depth profiles (see Materials and Methods), were used to measure the thicknesses of the S-SO and SFGS in each of the sample columns in normal and regenerating fish (Table 1). There was a 16% increase over normal in the S-SO-SFGS thickness at 30 d, and laminar hypertrophy was seen for at least 240 d after nerve crush. These differences in the S-SO-SFGS thickness were found to be highly significant ($p < 0.005$). This overall increase in the thickness of the S-SO-SFGS was the result of similar increases in both the S-SO and SFGS laminae during regeneration (Table 4).

Changes in fiber and synapse numbers during regeneration

Synapses. As a control for the labeling procedure, total synapses were counted in the S-SO-SFGS (borders defined in Materials and Methods) in 2 sample columns from one normal HRP-labeled fish (HRETC18) in sections that were not processed histochemically, and 3 additional columns were counted in this same fish from alternate sections that were processed histochemically (Table 1). The counts of total synapses in the S-SO-SFGS of the 2 columns not processed histochemically were similar to the total (labeled plus unlabeled) synapses counted in DAB-processed material from the same fish and from 2 other normal fish.

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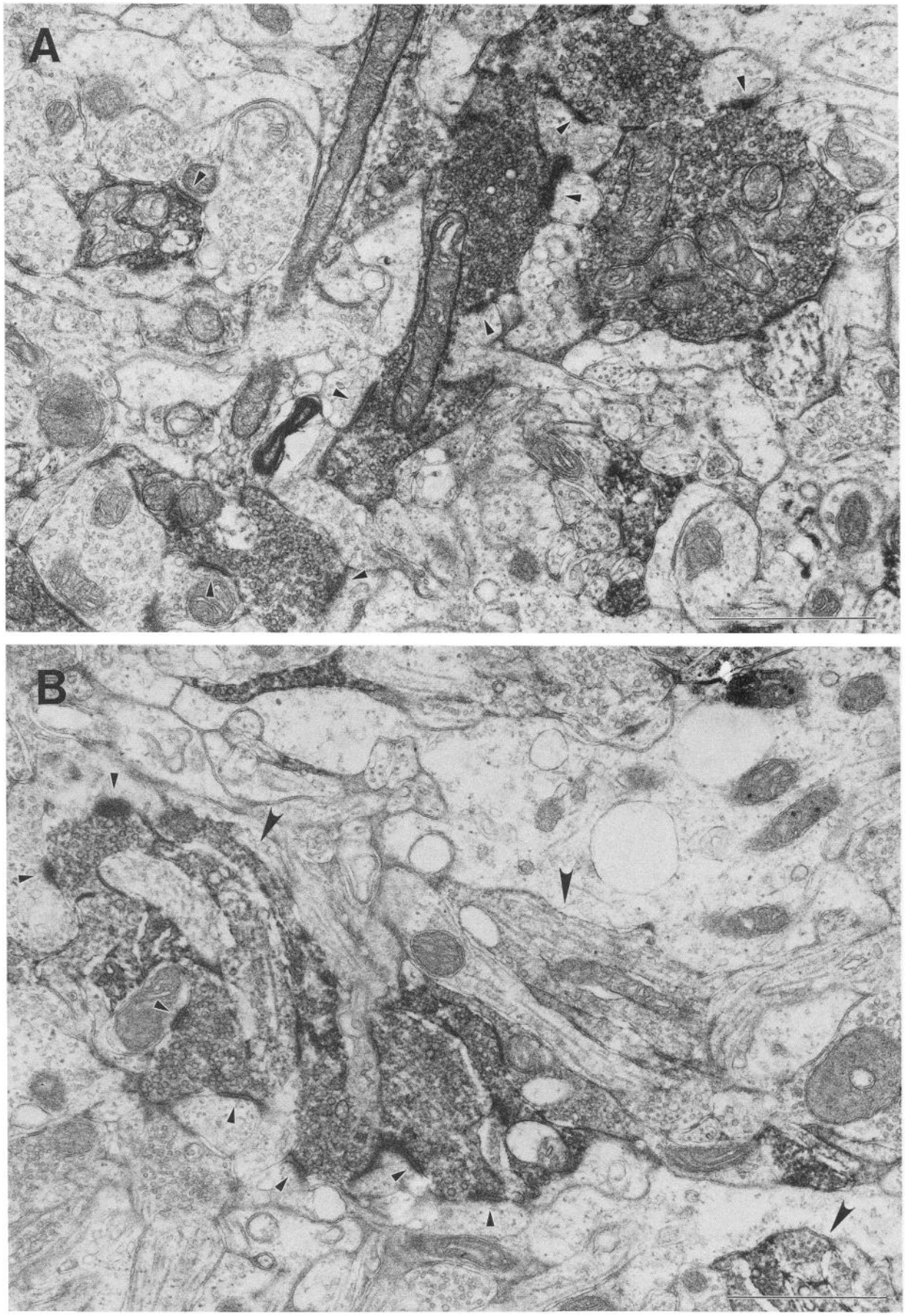
Figure 5. High-magnification electron micrographs of optic terminals in the SFGS at 30 d regeneration (HCREG19). *A,* Normal terminals filled with synaptic vesicles making numerous synapses (*small arrowheads*). *B,* Terminals associated with fasciculated optic fibers (*large arrowheads*) making many synapses (*small arrowheads*). Note that distinct terminal profiles are in intimate contact with each other. Scale bars , 1 μm.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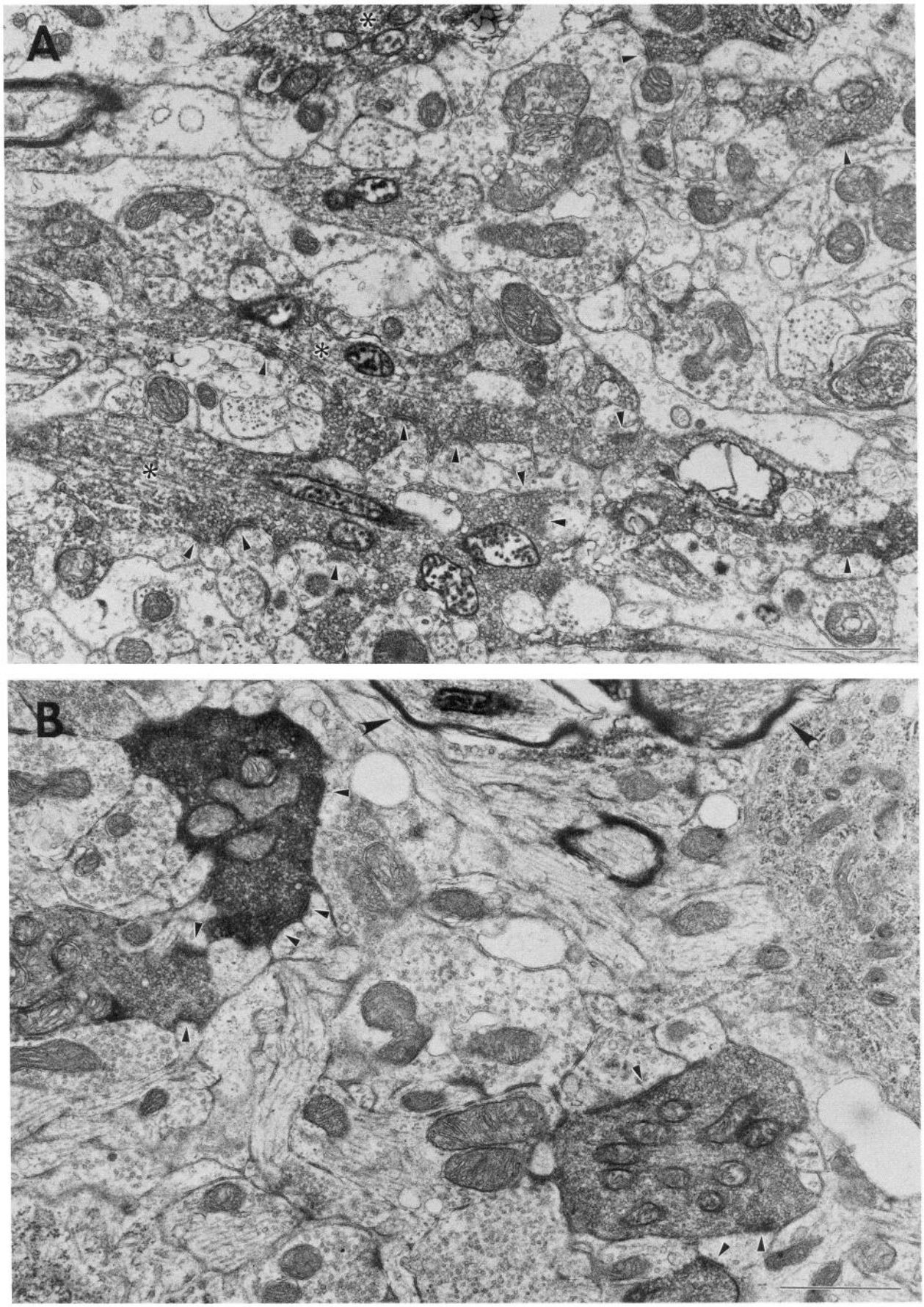

Figure 6. High-magnification electron micrographs showing optic synapses in the SFGS at 60 and 240 d regeneration. *A*, 60 d (HCREG7): Numerous labeled synapses (*arrowheads*) made by optic fibers (*asterisks*). *B*, 240 d (HCREG14): Numerous synapses (*arrowheads*) made by optic terminals typically seen in normal fish. Note nearby fasciculated optic fibers (*large arrowheads*). Scale bars, 1 μm.

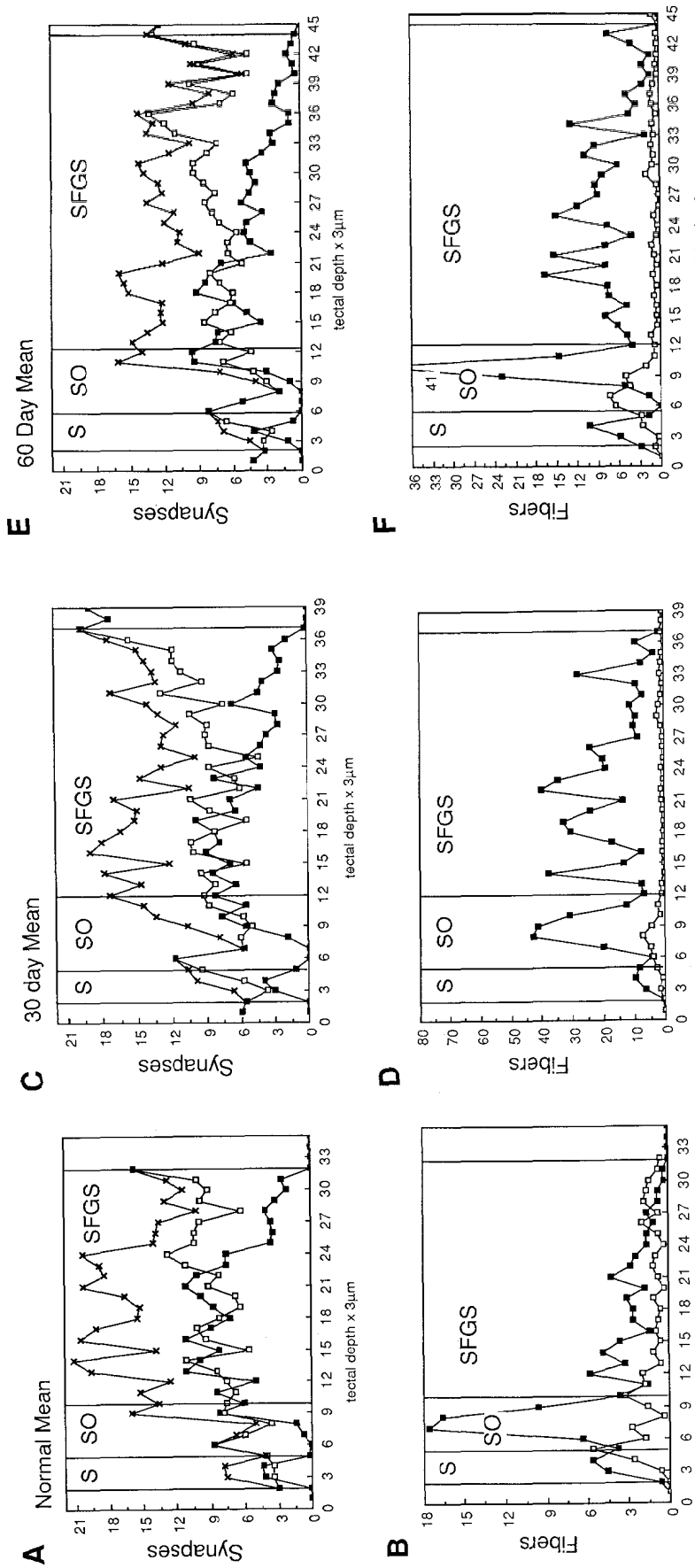

Figure 7. Mean depth profiles of optic and nonoptic fiber and synapse numbers in the S-SO-SFGS of normal fish and fish with optic nerve crush, made by superimposing data from 5 sample columns representing 3 fish (see Materials and Methods). *A* and *B*, Normal fish; *C* and *D*, 30 d regeneration; *E* and *F*, 60 d regeneration. Symbols: *A*, *C*, and *E*, labeled (*filled squares*), unlabeled (*open squares*), and total (*crosses*) synapses; *B*, *D*, and *F*, labeled (*filled squares*) and unlabeled (*open squares*) fibers. Note the range of optic fiber numbers in *B* is 0–18; *D*, 0–80; and *F*, 0–36.

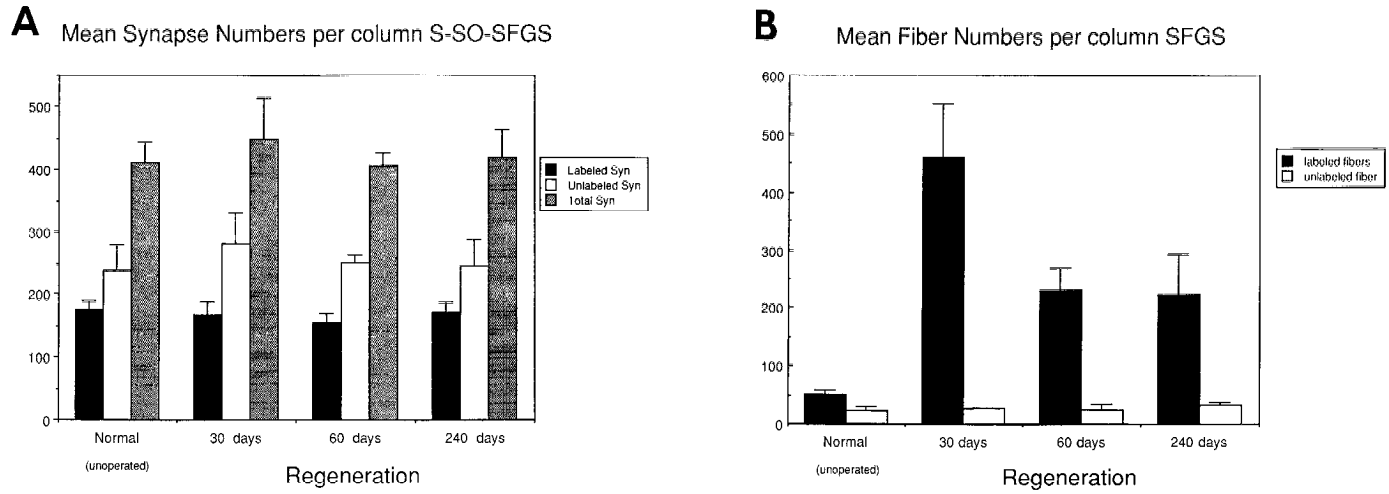

Figure 8. Histograms of mean numbers of total optic and nonoptic fibers and synapses in normal and regenerating fish (see Tables 2 and 3 for data, and the number of columns and fish). *A,* Synapse numbers per column S-SO-SFGS: labeled (*solid bar*), unlabeled (*open bar*), and total, i.e., labeled plus unlabeled (*shaded bar*). *B,* Fiber numbers per column SFGS (labels as in *A*).

The numbers of labeled ($p > 0.2$), unlabeled ($p > 0.25$), and total ($p > 0.3$) synapses in the S-SO-SFGS were roughly normal at all times examined during regeneration (Table 2, Fig. 8*A*). In addition, the differences in the mean percentage of labeled synapses relative to total synapses in the S-SO-SFGS (see Table 2) were small and not statistically significant ($p = 0.08$).

Fiber numbers. Counts of labeled and unlabeled fiber numbers in the SFGS showed that during regeneration there was a 9-fold increase in the normal number of labeled fibers at 30 d regeneration. Between 30 and 60 d, the number of labeled fibers was halved, but over 4 times the normal number remained in the SFGS at 60 and 240 d (Table 3, Fig. 8*B*). These differences in optic fiber numbers were found to be highly significant ($p < 0.005$). No significant differences in unlabeled fiber numbers were found ($p > 0.25$).

Optic fiber myelination. In the SFGS of normal fish, over 90% of the optic fibers were myelinated, whereas during 30, 60, and 240 d regeneration, <2, <10, and <70%, respectively, of the optic fibers were myelinated. At 30 and 60 d, this means that less than the normal numbers of labeled myelinated fibers were observed in the SFGS. However, there was more than 3 times the normal number of myelinated optic fibers at 240 d (Table 3). Thus, the number of myelinated optic fibers increases slowly, in agreement with findings of Murray (1976). This indicates that remyelination is not related to map refinement and that tectal oligodendroglia can myelinate many more than the normal number of fibers.

Optic fiber fasciculation. In agreement with previous qualitative studies (Murray, 1976; Murray and Edwards, 1982; Hayes and Meyer, 1988a, b), many of the optic fibers were found to be contained in fascicles. To quantify this in the present study, the following profiles in each of the sample columns from normal fish and fish with regenerating nerves were counted (Table 3, Fig. 9, *A, B*): (1) fibers in labeled fascicles; (2) labeled fascicles; and (3) labeled fascicles with >80, >40, >20, >10, >5, and <5 fibers per fascicle.

In normal fish, 53% of the optic fibers were included in fascicles. There were, on average, 10.2 fascicles per column SFGS and each fascicle contained an average of 2.9 fibers. At 30 d, 86% of the optic fibers were fasciculated. Each sample column contained over twice the normal number of fascicles, and each fascicle had 6 times the normal number of fibers. At 60 and 240 d, the percentage of fibers contained in fascicles and the number of labeled fascicles in the SFGS were unchanged from 30 d (Fig. 9*A*). Although the number of fascicles per column SFGS remained over 2 times normal, the number of fibers per fascicle decreased by half at 60 d and was unchanged at 240 d.

The relative numbers of labeled fascicles per SFGS column with >80, >40, >20, >10, >5, and <5 fibers also changed greatly in normal fish and fish with regenerating nerves (Fig. 9*B*). An abnormal population of fascicles containing between 10 to over 80 fibers was present in fish at 30 d. Between 30 and 60 d, the numbers of labeled fascicles with more than 10 fibers were reduced, and those with more than 40 fibers had all but disappeared, while the total number of fascicles remained the same.

Discussion

The principal findings of this study are that normal numbers of optic synapses were regenerated before the period of activity-dependent map refinement. In contrast, nearly 10 times the normal numbers of optic fibers were present before map refinement, and these were halved thereafter. We will discuss synapses and fibers in turn, and then we will argue for synapse rearrangement by fiber remodeling during optic regeneration.

Optic synapse numbers in normal fish and after optic nerve regeneration

The estimate in the present study that 43% of synapses in the SFGS are optic is comparable to the 37% estimated by Murray and Edwards (1982) but somewhat higher than the 27% estimated by Airhart and Kriebel (1984) and the 15% by Meek (1981). In general, these differences can be explained by whether optic synapses were labeled and how well they were labeled. Meek (1981) used no label. Airhart and Kriebel (1984) used HRP but had to use a correction factor for incomplete labeling. Murray and Edwards (1982) filled a high percentage of terminals with HRP in normal fish, but the filling method was not as efficient as ours since it did not label regenerating fibers earlier than 12 weeks after nerve crush.

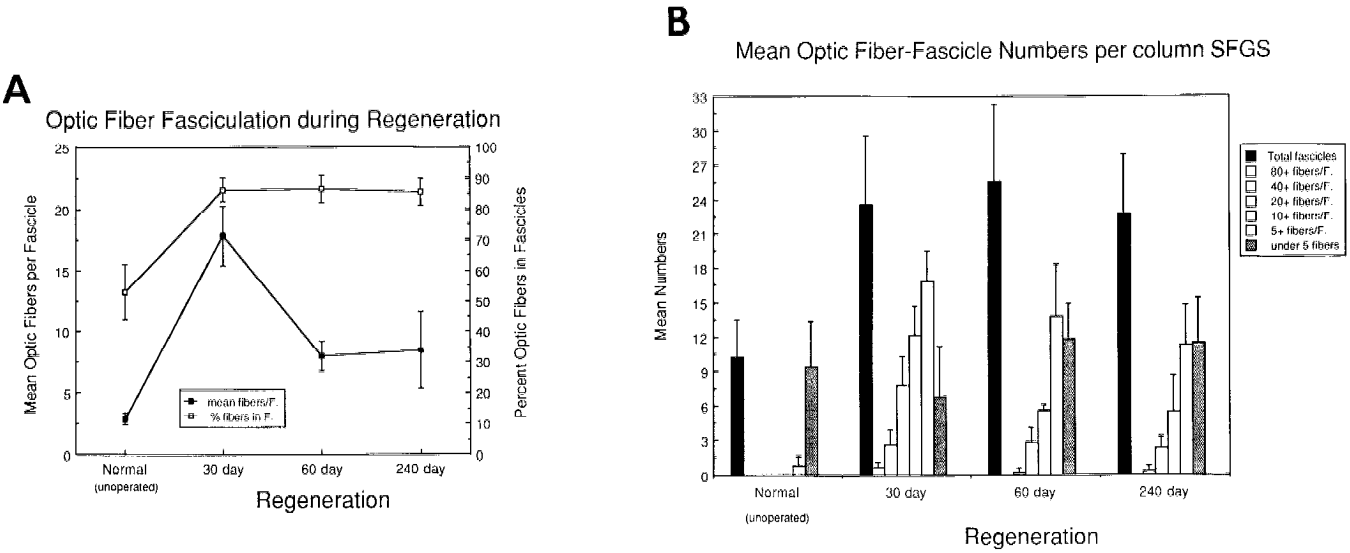

Figure 9. Graphs of optic fiber fasciculation during regeneration. *A,* Mean number (*filled squares*) and mean percentage (*open squares*) of labeled fibers fasciculated per sample column SFGS (see Table 3 for data, and the number of columns and fish). *B,* Mean number of fascicles per column SFGS: total (*filled bar*), fascicles with >80 fibers (*first open bar* to the left of filled bar), >40 fibers (*second open bar*), >20 fibers (*third open bar*), >10 fibers (*fourth open bar*), with >5 fibers (*fifth open bar*), and <5 fibers (*shaded bar*). The number of sample columns and fish used is as in *A*.

The synaptic counts during regeneration indicated that normal numbers of synapses were reformed by 30 d, prior to activity-dependent map refinement (Meyer, 1983; Schmidt and Edwards, 1983). The present findings appear to contradict a previous quantitative EM study of regeneration in goldfish (Murray and Edwards, 1982), in which it was argued that fewer than the normal number of synapses was present until 12 weeks regeneration. However, their measures were much less direct. Terminals, rather than synapses, were counted, and the number of synapses was computed based on the assumption of the number of synapses per terminal about which there is some disagreement (Murray and Edwards, 1982; Airhart and Kriebel, 1984). The computation also required an estimate of SFGS thickness, which changes during regeneration and which is difficult to estimate using histological criteria. In fact, they found the SFGS returned to normal by 42–77 d, in contrast to the permanent abnormal thickening we saw in labeled material. Finally, the differences between these data were comparatively small and easily explained by measurement errors. They reported that total synapse numbers were 87% of normal at 21–35 d and 82% of normal at 42–77 d (these percentages were calculated by us from Table 4 of Murray and Edwards, 1982). On the other hand, it might be argued that our counts are too high because of a change in the size of synapses during regeneration. Previous measurements of regenerating synapses, however, show them to be of normal size (Murray and Edwards, 1982; Hayes and Meyer, 1988a).

Proliferation and elimination of fasciculated optic fibers during regeneration

The main conclusion is that optic collateral sprouting on tectum dramatically increases the number of optic fibers by 30 d, and that by 60 d these are sharply reduced by branch elimination. Nevertheless, as late as 240 d, many more optic fibers are present in the SFGS than normal, as also was reported by Murray and Edwards (1982). Regenerating fibers have been shown to branch extensively in the optic nerve (Murray, 1982) and in fascicles on tectum (Murray, 1976). It seems likely that some and perhaps most of this branch reduction originates in tectum, although reduction from within the optic nerve may also play a role (Murray, 1982). This reduction cannot be attributed to cell death, which is absent (Murray et al., 1982; Meyer et al., 1985).

With respect to fiber organization during regeneration, over 80% of the observed optic fibers were found to be contained in fascicles in the SFGS. The present finding that the number of optic fascicles remained about the same during regeneration when the number of optic fibers per fascicle was halved indicates that optic collaterals are eliminated from within fascicles.

Evidence for inappropriate synapse formation and synapse rearrangement in goldfish optic regeneration

Of the 3 mechanisms posed in our introductory remarks to explain how position-specific synapses are redeployed in this system, only *synapse rearrangement* is supported by our data. This follows directly from the finding that there were normal numbers of optic synapses before and after the period of map refinement.

Before map refinement at 30 d, the extent of retinotopic disorder is substantial. Tracing studies using WGA-HRP indicate that retinal ganglion cells which normally project to about one-tenth of tectum are projecting across about one-third of tectum (Meyer et al., 1985; Rankin and Cook, 1986; see also Meyer, 1980; Cook, 1983). Thus, few optic fibers and synapses would be expected to be appropriately positioned during the period of gross retinotopography. This is consistent with previous EM observations that optic fibers at 14–30 d regeneration often make synapses from within fascicles as they grow through tectum (Hayes and Meyer, 1988a, b) and that inappropriate synapses can form (Hayes and Meyer, 1984, 1988a), as well as electrophysiological evidence for functional synapses at an early stage of regeneration and for time-dependent changes in receptive field size (Northmore and Masino, 1984; Adamson et al., 1984, in frog). In this context, the present evidence implies that optic fibers initially make retinotopically inappropriate synaps-

es, that fibers extensively rearrange their synaptic connections during regeneration and that synaptic number is regulated independently of retinotopy.

It is likely that activity regulates the pattern but not the number of synapses during this refinement. Impulse blockade using intraocular TTX does not affect the numbers of optic synapses formed by regenerating fibers (Hayes and Meyer, 1986, 1989). In contrast, when fibers are made to regenerate onto a surgically produced half tectum forming a retinotopically inappropriate compressed projection, the number of optic synapses per column was found to be normal (Hayes and Meyer, 1988c; see also Murray et al., 1982); that is, each fiber makes half its normal number of synapses. Thus, the number of optic synapses appears to be set by the target cells. We have proposed elsewhere that this limit promotes competitive interactions important for map refinement (Hayes and Meyer, 1988a).

In conclusion, present findings in goldfish optic regeneration support a mechanism for neural mapping in which functional synapses are rearranged through an activity-dependent process, possibly in the way proposed by Hebb (1949) and Stent (1973), though the present data would also be compatible with a variety of other models (Prestige and Willshaw, 1975; Changeux and Danchin, 1976; Willshaw and von der Malsburg, 1976; Whitelaw and Cowan, 1981).

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