First publ. in: Journal of Comparative Neurology 288 (1989), pp. 539-554

Evidence for the Stability of Positional Markers in the Goldfish Tectum

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ABSTRACT

Positional markers in the tectum, which are thought to guide growing axons to their target sites, have been proposed to be induced by axons, to be only transiently associated with the tectal cells, and then lost after long-term denervation periods (Schmidt: *J. Comp. Neurol. 177*:279–300, '78). To further investigate this concept, retinal axons were induced to regenerate into ipsilateral tecta which had been deprived of their retinal afferents for shorter (0–4 months) and longer periods (4–8 months). The paths of HRP-labeled regenerating axons of known retinal origin were traced and used as an operational test to decide whether the axons might navigate under the influence of positional markers. Two different kinds of experiments were performed:

1. The axons from a subpopulation of all ganglion cells in the retina were labeled by applying a small crystal of HRP at defined retinal regions. Independent of the denervation period of the tectum, the labeled regenerating axons traveled in abnormal but nonrandom routes. In early regeneration stages, axons exhibited signs of exploratory growth. They extended branches equipped with growth cones and filopodia into various regions of the tectum. In late regeneration stages, the axons lost these branches, exhibited U-turns and bends, and ended in terminal arbors in the retinotopic target region. These findings suggest that the axons travel under the influence of tectal positional markers and that these markers are not transient.

2. Axons from a surgically created temporal hemiretina were labeled by application of HRP to the optic nerve to test whether the temporal axons might expand into the caudal tectum in long-term-denervated tecta. The HRP-labeled axons coursed over rostral and midtectal regions. Instead of invading the caudal tectum they bent and terminated in the rostral tectal half. These results add further support for the conclusion that the path of regenerating retinal axons is governed by long-lasting positional markers.

Key words: path- and targetfinding, regenerating axons, retinotectal system, HRP

In the visual system of goldfish, the retinal axons terminate in the contralateral tectum in an orderly array, forming the retinotopic map. After injury of the optic nerve the axons regenerate and ultimately deploy their terminals at sites they had previously occupied (reviewed in Gaze, '70; and in Purves and Lichtman, '85).

One of the most widely accepted theories to explain the formation and reformation of orderly retinotectal projections is the chemoaffinity theory (Sperry, '63)—or its more recent versions, such as the gradient theory (Fraser, '80; Gierer, '87). Both postulate that tectal cells provide positional markers which are recognized by the ingrowing axons and assist them in target approach and target selection. These theories have gained substantial support from in vivo and in vitro experiments in both embryonic and regenerating systems (examples: Holt and Harris, '83; Harris, '86; O'Rourke and Fraser, '86; Thanos and Bonhoeffer, '86; Harris et al., '87; Walter et al., '87a,b; Stuermer, '88a; Vielmetter and Stuermer, '89).

The expression of positional markers by tectal cells has been questioned by size-disparity experiments in the regenerated retinotectal system (reviewed in Sharma and Romeskie, '84). For instance, regenerating retinal axons from a sur-

Accepted May 24, 1989.

TABLE 1. Survey of Experimental Tecta[1]

Regeneration period after T-rem (days)	Origin of labeled axons in retina	N of tecta of denervation periods (months)						
		0	1	2	3	4	5	8
20–55	DT	1	3	1	1	—	—	—
	VT	2	1	1	1	—	—	—
	DN	4	2	2	—	—	—	—
	VN	3	1	—	1	—	—	—
55–110	DT	5	1	2	1	1	—	—
	VT	3	1	—	2	1	2	1
	DN	2	1	1	—	1	1	—
	VN	3	—	—	1	1	1	—
110–280	DT	—	1	1	1	—	2	—
	VT	1	1	—	1	3	4	1
	DN	1	1	1	1	—	—	—
	VN	1	—	—	—	1	2	—
180	THR	—	—	—	—	—	4	2

[1]Survey of all experimental tecta used in this study. Abbreviations: DT, dorsotemporal; VT, ventrotemporal; DN, dorsonasal; VN, ventronasal; THR, temporal hemiretina.

gically created hemiretina first project to the appropriate rostral tectum. As regeneration progresses, the half-retinal projection expands over the rostrocaudal extent of the tectum (Schmidt et al., '78). Half-retinae immediately expand in tecta which have been deprived of retinal afferents for more than 4 months prior to the ingrowth of the regenerating axons (Schmidt, '78). To account both for the immediate expansion in long-term-denervated tecta and for the restricted projection in tecta without extended denervation periods two alternatives to the chemospecificity have been proposed. The first idea suggests that regenerating axons are not guided by markers on tectal cells but rather by degenerating axonal debris left by the previous projection (Murray, '76; Sharma and Romeskie, '77). The axons from a hemiretina are free to expand over the tectum when these remnants have been cleared away, e.g., in long-term-denervated tecta. Schmidt ('78) went further and proposed that axonal guidance might be mediated by markers which are not preexisting but imposed onto tectal cells by the retinal axons. These markers were thought to be preserved only in the presence of retinal axons and to disappear in tecta denervated for extended periods of time.

The above-cited studies assessed the organization of the axon terminals with electrophysiological recordings from the tectum and in selected cases by labeling the axons radioautographically. The resolution of these techniques is limited and they do not reveal the axonal pathways. As exemplified in a variety of studies, axonal trajectories give important information on the mode in which axons reach their termination sites (Fujisawa et al., '82; Thanos and Bonhoeffer, '86; Harris, '86) and they offer insights into their navigational strategies (Gierer, '87). The analysis of the paths of HRP-labeled regenerating retinal axons in the goldfish optic tectum showed that in contrast to earlier beliefs (Attardi and Sperry, '63; reviewed in Gaze, '70), regenerating axons do not follow normal pathways but instead travel in highly abnormal routes (Stuermer, '88b). In early regeneration stages, the axons branch widely over inappropriate regions of the tectum (Schmidt et al., '88; Stuermer, '88c) such that the tectal map is initially diffuse (Meyer et al., '85; Rankin and Cook, '86). However, axons in aberrant routes exhibit signs of course corrections, achieve target-directed routes from ectopic sites, and finally arborize in retinotopically correct regions. Early branch extensions, loss of ectopic branches, and the formation of target-directed routes are also observed when axons regenerate under continuous TTX-induced impulse blockade (Hartlieb and Stuermer, '89). Thus, these modes of growth are consistent with the concept that the axons navigate under the influence of positional cues on the tectum (Fujisawa et al., '82; Gierer, '87). These experiments do not of course rule out the possibility that positional cues originate from the axons of the earlier innervation and may be lost after long-term denervation.

The present study traced the pathways of HRP-labeled axons regenerating into tecta denervated for up to 8 months. The paths of the axons were used as an operational test to decide whether positional markers in tectum are transient and lost after long-term denervation or whether they are long-lasting and stable. Our results show that the axonal path is similar in tecta denervated for short and long periods and similar to that of axons regenerating into tectum shortly after optic nerve section (Stuermer, '88b,c; Humphrey and Stuermer, '88). They imply that axons are guided by long-lasting positional markers intrinsic to tectum.

These results have been published as abstracts (Busse and Stuermer, '87, '88).

MATERIALS AND METHODS

For all surgical manipulations, goldfish (40–60 mm in body length) were deeply anesthetized in 0.03% MS 222. To deprive the right tectum of retinal afferents, the left eye was removed. Axons from the remaining eye did not spontaneously form an ipsilateral projection to the denervated tectum (Sharma and Romeskie, '77; Meyer, '84). We confirmed this by applying HRP to the right optic nerve in seven fish after 15, 20, 25, 60, and 360 days. Labeled axons were only found in the contralateral left tectum. The remaining right eye was induced to regenerate to the denervated right tectum either immediately after the removal of the left eye or after delays of 1–8 months by removing the left tectum (tectal removal) (Table 1). The skull overlying the midbrain was opened with a scalpel blade and the left tectal lobe was removed by aspiration with a pipette connected to a low-pressure device. The bone was put back into place and fixed with super glue.

In 11 fish, the nasal half of the right retina was ablated concomitant with the removal of the left tectum. The retina was exposed by partially cutting away the cornea and removing the lens and an incision sparing the optic disk was made across the middle of the retina. The nasal half of the retina was removed by aspiration. The cornea and the lens were moved back into place. To determine whether the nasal half of the retina might have regenerated at the time the fish were killed the retinae were fixed, embedded in 2-hydroxyethylmethacrylate (Kulzer & Co. GmbH), sectioned at 5 μm, and stained with haematoxylin and eosine.

The regenerating axons were labeled with HRP at 20–280 days after tectal removal. To label regenerating axons of defined retinal origin small crystals of HRP were applied either temporally or nasally close to the optic disk in the dorsal or the ventral half of the retina. These punctate applications retrogradely labeled ganglion cells in a sector which extended from the HRP application site to the retinal periphery and anterogradely labeled their axons. The retinotopic destination of the labeled axons is a wedge-shaped region in the ventral or the dorsal half of the rostral and the caudal tectum, respectively (as illustrated for normal and regenerating axons in Stuermer, '88b). The size of the HRP crystal was small in fish whose tecta were denervated between 0 and 3 months and the sector of labeled ganglion

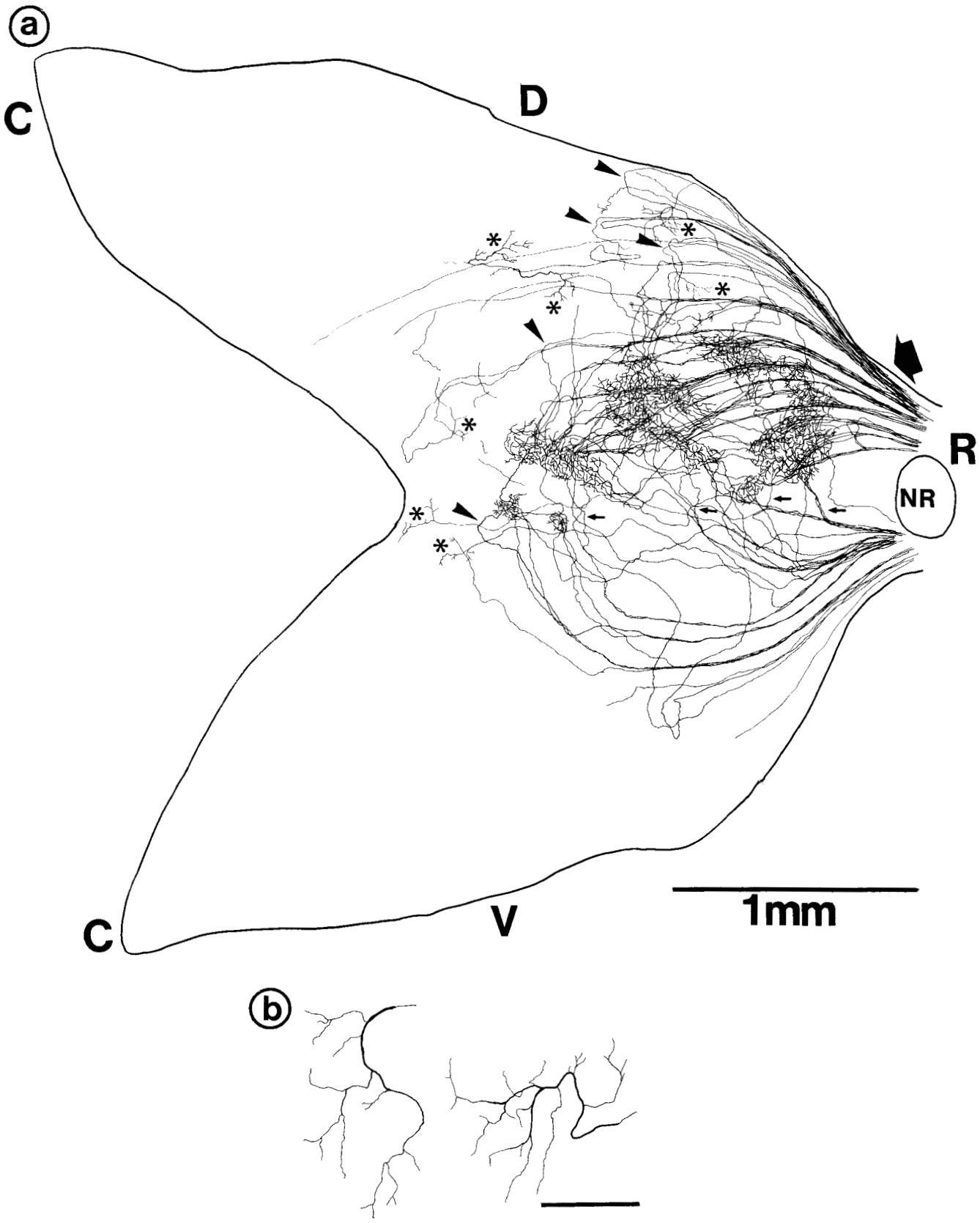

Figure 1

TABLE 2a. Labeled Axons in Denervated Tecta[1]

Regeneration period after T-rem (days)	Denervation period of right tectum (months)													
	0		1		2		3		4		5		8	
	A	GC	A	GC	A	GC	A	GC	A	GC	A	GC	A	GC
20–35	19	146	3	150	3	300	9	593	—	—	—	—	—	—
	19	760	—	—	—	—	1	516	—	—	—	—	—	—
	38	330	—	—	—	—	—	—	—	—	—	—	—	—
	9	346	—	—	—	—	—	—	—	—	—	—	—	—
35–70	61	339	14	350	28	700	3	228	—	—	—	—	—	—
	13	325	53	136	32	457	68	1,000	—	—	—	—	—	—
	21	382	—	—	—	—	—	—	—	—	—	—	—	—
	101	748	—	—	—	—	—	—	—	—	—	—	—	—
	67	291	—	—	—	—	—	—	—	—	—	—	—	—
	29	264	—	—	—	—	—	—	—	—	—	—	—	—
	57	588	—	—	—	—	—	—	—	—	—	—	—	—
70–160	137	173	22	440	59	347	14	288	17	474	10	457	40	1,232
	120	571	207	422	88	169	18	120	11	466	24	263	—	—
	42	117	140	583	52	743	47	160	—	—	2	322	—	—
	94	392	137	204	—	—	—	—	13	576	10	754	—	—
	—	—	56	339	—	—	—	—	53	661	8	276	—	—
	—	—	—	—	—	—	—	—	18	1,000	—	—	—	—

[1]Number of labeled ganglion cells in retina and number of labeled axons in the ipsilateral optic tract of long- and short-term-denervated tecta at increasing regeneration periods after removal of the right tectum (T-rem). A, labeled axons; GC, labeled ganglion cells.

TABLE 2b. Percentage of Labeled Axons in Denervated Tecta[2]

Regeneration period after T-rem (days)	Denervation period of right tectum (months)						
	0	1	2	3	4	5	8
20–35	7.4 (4)	2.0 (1)	1.0 (1)	0.8 (2)	n.d.	n.d.	n.d.
35–70	12.1 (7)	21.5 (2)	5.5 (2)	4.2 (2)	n.d.	n.d.	n.d.
70–160	40 (4)	32.2 (5)	25.3 (3)	16.3 (3)	3.6 (5)	3.2 (4)	3.3 (1)

[2]Relative number (in percent) of axons in the optic tract of long- and short-term-denervated ipsilateral tecta at increasing regeneration periods after removal of the right tectum (T-rem). Observed number in parentheses ( ). n.d., no data.

cells consisted of between 5 and 8% of the retinal area. In eyes connected to tecta which had been denervated for more than 3 months, larger HRP crystals were used to label a sufficient number of axons. This was necessary since only a small number of axons regenerated to long-term-denervated tecta (see Results). In these fish, the sector of labeled ganglion cells consisted of approximately 15% of the retinal area. Three to 4 days were allowed for the intraaxonal transport/diffusion of the HRP after intraretinal labeling.

To label all axons from the right retina crystalline HRP was applied to the stumps of the sectioned right optic nerve and 1–2 days were allowed for the anterograde transport/diffusion of the HRP.

Labeled axons were visualized in retinal and tectal whole mounts according to a protocol described by Stuermer ('88b). Briefly, the retina was removed from the dark-adapted right eye, fixed in 4% glutaraldehyde in PBS for 30 minutes, and incubated first in 0.7% cobalt chloride and then in o-dianisidine (OD, 1 mg/ml in PBS). The retina was flat mounted on a gelatinized slide, dehydrated through an ethanol series, and embedded in Permount. To prepare tectal whole mounts, the fish were perfused intracardially with 0.75% saline. The right tectum and the right optic nerve and tract were isolated, incubated unfixed in diaminobenzidine (DAB, 1 mg/ml in phosphate buffer), and fixed in 4% glutaraldehyde in PBS for 2 hours. The tecta were slit from the caudal pole, flat mounted onto gelatinized slides, dehydrated, and embedded in Permount.

HRP-labeled axons and their terminal structures were viewed with a Zeiss microscope under observation with oil immersion lenses (25×, 63×, and 100×). Since the axons run in various depths through the tectum it was not possible to photograph extended views of the labeled axons. The axonal trajectories were therefore traced through a drawing tube under observation with a 25× oil immersion lens resulting in drawings of 2 × 2 m. Axons coursing at various depths were drawn in one plane. To draw isolated axons, particularly their branches, the 63× and 100× oil immersion lenses were used. Selected axonal appendages were photographed with a Zeiss Axiophot equipped with Nomarski optics.

Quantitative measurements

For the quantification of axonal numbers, all labeled axons visible under inspection with a 63× oil immersion lens were drawn and counted. Axonal numbers were determined in the dorsal and the ventral brachium of the ipsilateral optic tract and in rostral, midtectal, and caudal regions in the denervated right tectum. The position of the three sample regions in tectum were as defined in Stuermer ('88b). On a camera lucida drawing of a tectum, the nucleus rotundus was connected to the caudal tectal ends by two rostrocaudal lines running through the middle of each hemitectum. Perpendicular to these lines, three parallel lines were drawn which ran from the dorsal to the ventral tectal edge through the rostral, middle, and caudal tectum. These lines intersected the rostrocaudal lines at three points which had equal distances from each other, from nucleus rotundus, and from

Fig. 1. **a:** Camera lucida tracing of all labeled ventrotemporal axons in a tectum denervated for 1 month (100 days after tectal removal). Axons end in terminal arbors, most of which are condensed into patches at the retinotopic target region which extends between the large arrow and the center of the tectum. Terminal arbors outside but in the vicinity of the target region are marked by asterisks. The axons travel over both the dorsal and the ventral hemitectum along the curved paths of the previous fascicles in the superficial fiber layer SO and through various routes in the deeper synaptic layer SFGS. Axons misrouted into the ventral hemitectum travel across the tectal equator into the dorsal hemitectum (small arrows). Arrowheads point to axons which bend before they deploy their terminal arbors. Abbreviations: R, rostral; C, caudal; D, dorsal; V, ventral; NR, nucleus rotundus. **b:** Two terminal arbors from the tectum in 1a. The arbors are similar in size and branching pattern to terminal arbors of regenerated axons in contralateral tecta (compare to Fig. 5 in Stuermer, '88b). Calibration bar: 100 μm.

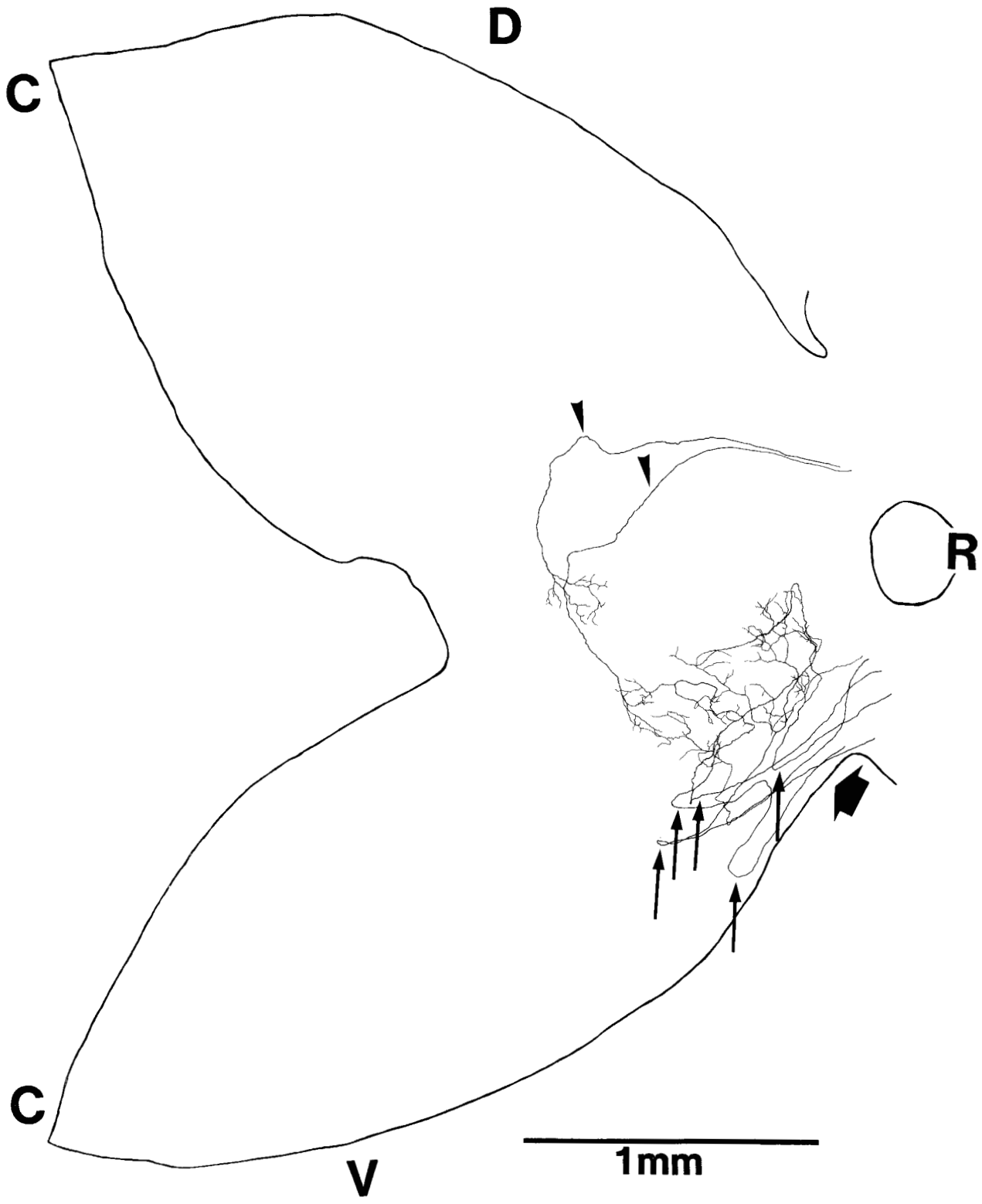

Figure 2

the caudal tectal ends. Labeled axons crossing the three parallel lines were counted.

A rough estimate on the number of ipsilaterally projecting axons, which differed considerably between long- and short-term-denervated tecta, was obtained as follows: In three arbitrarily chosen experimental whole-mounted retinae, in which HRP had been applied to bundles of axons as described above, the number of labeled ganglion cells was counted under observation with a 25× oil immersion lens. The labeled ganglion cells of these three retinae were N = 529, 673, and 658, respectively. The sectors in which the labeled ganglion cells resided were measured with a digitalizing tablet (Kontron), and were 0.890, 0.791, and 1.213 mm^2, respectively. The mean ganglion cell density, determined by dividing the number of labeled ganglion cells by the area of the sector, was 662 ganglion cells/mm^2. For all other experimental retinae, only the area of the sector in which the labeled ganglion cells resided was determined and multiplied by the mean ganglion cell density. The number of labeled axons coursing through the two brachia of the optic tract (before they entered into the ipsilateral tectum) was counted and expressed as a percentage of the number of labeled ganglion cells.

RESULTS

Consistent with previous studies (Easter et al., '78; Schmidt, '78; Lo and Levine, '80) only a small percentage of the labeled axons were found in the denervated ipsilateral tecta. We quantified the number of ipsilaterally projecting axons by comparing the number of axons counted in the ipsilateral optic tract to the estimated number of labeled ganglion cells in the retina as described in Materials and Methods (Table 2). The percentage of ipsilaterally projecting axons depended on the tectal denervation period. Tecta denervated for 4–8 months contained only 3–4% of retinal axons (Table 2b). Labeled axons which did not enter into the tectum via the optic tract but through alternate paths (Easter et al., '78; Lo and Levine, '80) were seen in five tecta, but the number was small. Only in one tectum (after application of HRP to the optic tract in fish with temporal hemiretinae, see last paragraph of Results) did as many as ten axons arrive through the posterior commissure. The remaining four tecta exhibited only one or two axons taking this route or other commissural paths.

Axonal paths in denervated tecta

We first consider axonal pathways at late regeneration stages, when the axons had formed terminal arbors. This was 60–280 days after tectal removal in tecta denervated for 0–3 months and 110–280 days after tectal removal in tecta denervated for 4–8 months. Figures 1a and 2 show camera lucida tracings of the paths of temporal axons in tecta denervated for 1 and 5 months, respectively. Figures 3 and 4 document the paths of nasal axons in tecta denervated for 1 and 5 months, respectively. Independent of the tectal denervation period, the axons had deployed terminal arbors of normal size and morphology (Fig. 1b) in regions retinotopic with respect to the sector of labeled ganglion cells in the retina. The terminal arbors of the temporal axons were confined to a region in the rostral tectum, as were those of nasal axons to a region in the caudal tectal half. Depending on whether the axons originated from the dorsal or ventral hemiretina, they terminated in either the ventral (Figs. 2, 3) or dorsal (Figs. 1a, 4) hemitectum. Only a few terminal arbors resided at ectopic locations (Figs. 1a, 4). In one fifth of the short-term-denervated tecta the terminal arbors were condensed into clusters instead of forming a continuous wedge (Fig. 1a). Similar termination patterns were formed in one third of the standard experimental condition, in which axons regenerate into the contralateral tectum following optic nerve section (Meyer et al., '85; Stuermer, '88b).

In both short- and long-term-denervated tecta the axons coursed in routes which were similar to those of regenerating axons in contralateral tecta after optic nerve section (Stuermer, '88b). Although these routes were aberrant when compared to those of normal axons (Stuermer and Easter, '84a,b; Stuermer, '86) they were strikingly target-oriented. Regenerating axons from the dorsal (Figs. 2, 3) or the ventral retina (Figs. 1a, 4) entered the tecta through both the appropriate and the inappropriate brachium of the ipsilateral optic tract. The percentage of axons in the inappropriate brachium was quantified in 20 tecta of different denervation periods. The mean percentage of misrouted axons was 20% ± 8.7% and varied little between tecta of short and long denervation periods. In the tectum most axons unspecifically followed the routes formerly occupied by the fascicles of normal axons in the superficial fiber layer stratum opticum (SO) (Stuermer and Easter, '84a,b; Stuermer, '86). Temporal axons predominantly used the rostrocentral fascicles ending in the rostral tectum (Stuermer and Easter, '84b). A few temporal axons traveled along the peripherocaudal fascicles, which ended in the caudal tectum (Stuermer and Easter, '84b) but left these routes in the rostral tectal half (Figs. 1a, 2). In contrast, nasal axons in peripherocaudal fascicles proceeded into the caudal tectum (Figs. 3, 4). The axons entered the synaptic layer stratum fibrosum et griseum superficiale (SFGS) either at the end of the fascicle routes close to the tectal equator (the boundary between dorsal and ventral hemitectum) or after their departure from the fascicle routes. Once in the synaptic layer, axons misrouted into ectopic tectal regions performed course corrections. Axons that grew past the level of their retinotopic target region looped back to it (Figs. 1a, 2, 4). Axons misrouted into the incorrect ventral or dorsal hemitectum curved, crossed the tectal equator, and grew towards their target in the opposite and correct hemitectum. Temporal axons crossed the tectal equator in rostral tectal regions (Figs. 1a, 2), as did nasal axons either immediately after their entrance into tectum (Fig. 3) or in caudal tectal regions (Figs. 3, 4).

The establishment of target-directed routes suggests that the axons must have had cues available to them to recognize their target. In tecta denervated for long periods (Figs. 2, 4) the axons performed similar course corrections as in tecta denervated for short periods (Figs. 1a, 3). These findings indicate that the position-encoding elements are present and preserved in long-term-denervated tecta.

To test whether in denervated tecta axons go through a phase of exploratory branching before they gain access to their target as in contralateral tecta after optic nerve section (Stuermer, '88c), we analyzed axonal trajectories at early

Fig. 2. Camera lucida tracing of all labeled dorsotemporal axons in a tectum denervated for 5 months (117 days after tectal removal). Terminal arbors reside in the retinotopic target region between the large arrow and the tectal center. Axons misrouted into the dorsal hemitectum curve (arrowheads) and cross from the inappropriate dorsal into the appropriate ventral hemitectum. Axons in the ventral hemitectum which have passed beyond the retinotopic target region perform U-turns (arrows) and return to the target region. Abbreviations as in Figure 1a. •

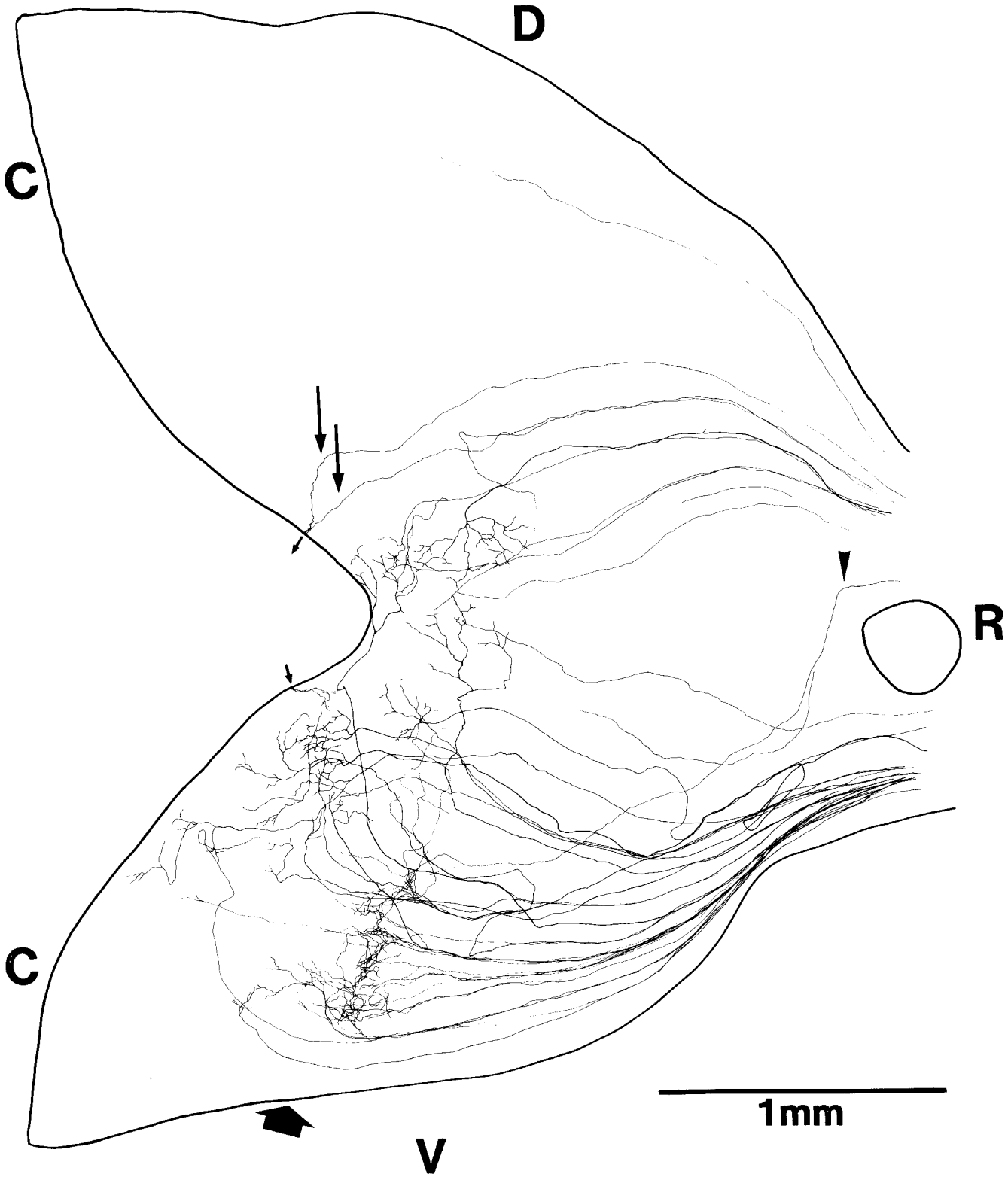

Fig. 3. Camera lucida tracing of all labeled dorsonasal axons in a tectum denervated for 1 month (118 days after tectal removal). Terminal arbors reside in the retinotopic target region in the caudal tectum (between large arrow and tectal center). Axons misrouted into the dorsal hemitectum cross into the ventral hemitectum either in very rostral (arrowhead) or in caudal regions of the tectum (medium-sized arrows). The two small arrows indicate axons interrupted by the caudal tectal incision. The axons either travel over the routes of the peripherocaudal or intermediate fascicles and then through SFGS towards their target. Abbreviations as in Figure 1a.

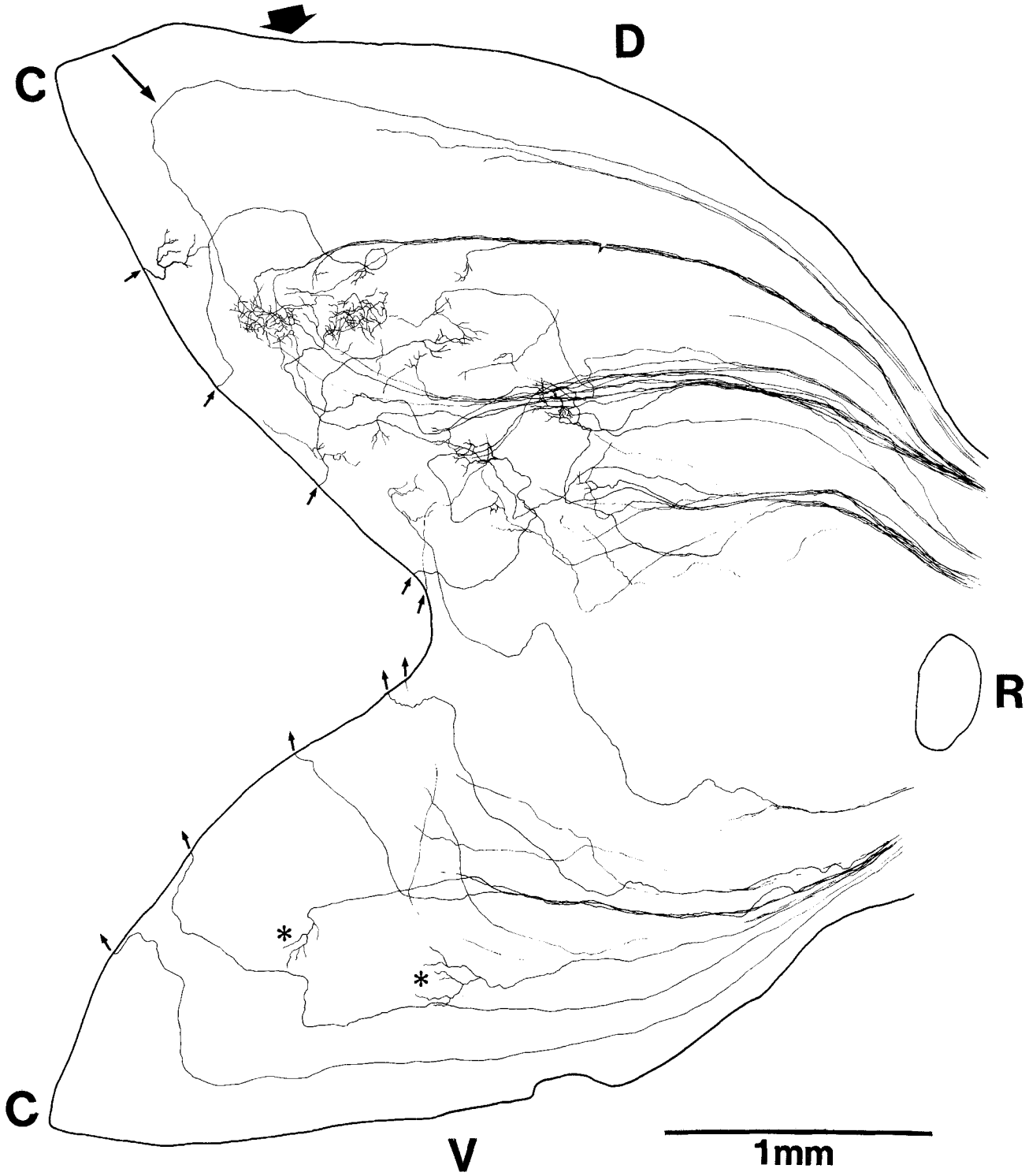

Fig. 4. Camera lucida tracing of all labeled ventronasal axons in a tectum denervated for 5 months (263 days after tectal removal). Most terminal arbors reside in the retinotopic target region in the caudal tectum (between large arrow and tectal center). Two ectopically located terminal arbors in the ventral hemitectum are marked by asterisks. Axons travel into caudal tectum over the fascicle routes in SO and through the synaptic layer SFGS. One axon passes beyond the target region but returns by bending rostrally (medium-sized arrow). Axons misrouted into the ventral hemitectum (interrupted by the caudal incision) cross into the dorsal hemitectum in caudal tectal regions (small arrows). Abbreviations as in Figure 1a.

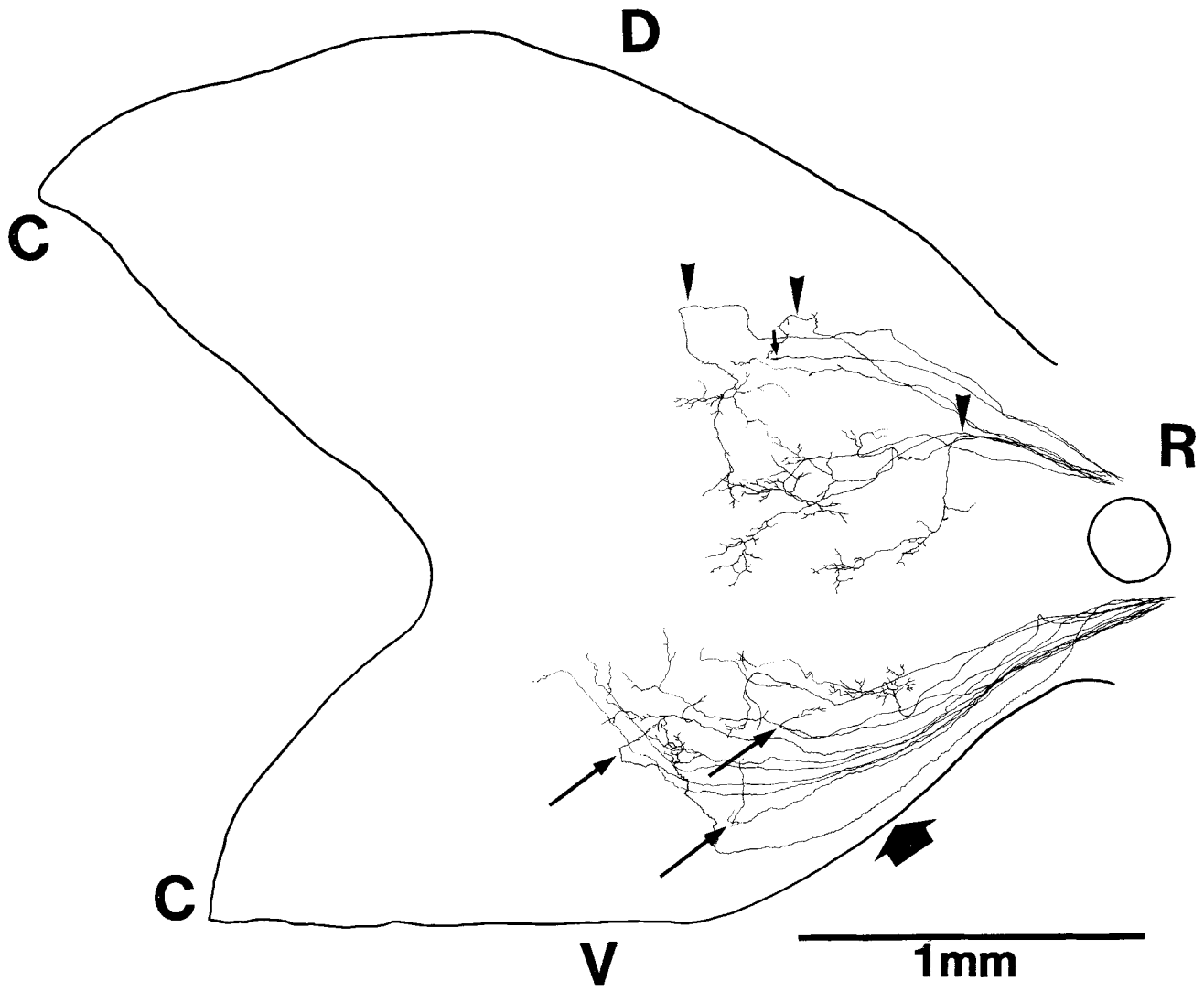

Fig. 5. Camera lucida tracing of all labeled dorsotemporal axons in a tectum denervated for 0 months (50 days after tectal removal). The retinotopic target region is indicated by the large arrow. Labeled axons course in abnormal routes through the rostral tectum. One axon following the route of a dorsal fascicle is unbranched and tipped with a leading growth cone (small arrow). The other axons have entered the synaptic layer and give rise to numerous side branches in the rostral tectum. Medium-sized arrows point to axons performing U-turns after their entrance into SFGS in the ventral hemitectum. Arrowheads indicate bends of misrouted axons in the dorsal hemitectum. Abbreviations as in Figure 1a.

regeneration stages. These are stages when axons have not yet reached their target regions and carry growth-related appendages such as growth cones and filopodia (20–60 days after tectal removal in tecta denervated for 0–3 months and 65–110 days after tectal removal in tecta denervated for 4–8 months).

Only 21% ± 9% of axons from the dorsal or the ventral retina passed through the incorrect brachium of the ipsilateral optic tract (16 fish) similar to axons at late-regeneration stages. This contrasts to axons regenerating into contralateral tecta which pass in equal numbers through both brachia at early stages after optic nerve section (Hartlieb and Stuermer, '87; Becker and Cook, '87). Axons had begun to establish routes which were similar to those described above for the late-regeneration stages. As long as the axons were in the superficial fiber layer they were unbranched and led by a single growth cone (Fig. 5). They traveled over the fascicle routes for various distances. Most temporal axons descended into the synaptic layer over the rostral tectum whereas most nasal axons followed the fascicles into the caudal tectum before they entered into the synaptic layer. Axons which had entered into the synaptic layer, however, emitted numerous side branches with growth cones and filopodia (Fig. 6b). These axonal ramifications covered up to 20% of the tectal surface and were found in retinotopic and ectopic tectal regions (Figs. 5, 6a). Branching, however, did not appear to be random since temporal axons preferentially branched in the rostral (Fig. 5) and nasal axons in the caudal tectum (Fig. 6a). Thus, widespread branching is typical for regenerating axons at early regeneration stages (Schmidt et al., '88; Stuermer, '88c). The branches and their growth cones might serve to explore the tectal territory

TABLE 3. Counts of Labeled Nasal and Temporal Axons in Denervated Tecta[1]

Denervation period of right tectum (months)	Nasal axons				Temporal axons			
	R	M	C	Σ	R	M	C	Σ
0	33	26	1	60	27	1	0	28
	41	30	5	114	20	12	0	32
	69	25	2	96	50	1	0	51
	121	58	0	179	63	1	0	64
1	52	67	16	135	34	13	1	48
	120	98	2	220	36	12	2	50
	10	26	9	45	9	4	0	13
	118	106	16	240	111	21	1	133
	21	41	10	72				
2	73	49	22	144	36	7	0	43
	50	44	3	97	38	4	0	42
	92	79	17	188	75	25	0	100
	74	63	1	138				
3	18	32	1	51	101	24	0	125
	64	44	0	108	91	12	0	103
4	20	15	3	38	12	3	0	15
	196	147	19	362	14	2	0	16
					22	7	0	29
5	74	39	7	120	115	13	0	128
	19	8	1	28	19	4	0	23
	35	26	7	68	130	7	0	137
8	55	17	3	75	117	21	0	138
					34	3	0	37
					53	18	0	71
THR					185	83	0	268
					44	12	0	56
					82	25	0	107
					286	131	0	417
					56	20	0	76
					28	8	0	36

[1]Distribution of labeled nasal and temporal axons in denervated tecta. R, rostral; M, midtectal; C, caudal; THR, temporal hemiretina (compare Figs. 7 and 11).

widely. The target-oriented routes seen at late regeneration stages may evolve from a preferential selection of appropriately oriented branches (Fujisawa et al., '82).

Quantitative evaluation of the distribution of regenerating axons over tectum

The camera lucida tracings (Figs. 1a–6) show that temporal and nasal axons differ in their distribution over tectum. We substantiated this by quantifying the distribution of temporal and nasal axons over rostral, midtectal, and caudal regions of tectum as described in Materials and Methods (Table 3). At both late- and early regeneration stages the axonal distribution was similar between tecta denervated for different periods, so the data were pooled.

At late-regeneration stages (Fig. 7a), the majority of temporal axons (81% ± 12.6%) coursed through the rostral tectum. Fewer axons (18.6% ± 11.7%) were found in midtectal and very few (0.4% ± 1.2%) in caudal regions. Nasal axons, in contrast, were more evenly distributed over rostral (58.8% ± 8.6%) and midtectal regions (34.5% ± 6.5%) and their number declined to 6.7% ± 2.8% in regions caudal to the retinotopic target site. The differential distribution of temporal and nasal axons was similar at early regeneration stages (Fig. 7b). Most temporal axons (83.6% ± 11.6%) were present in rostral regions; some (15.9% ± 11.2%) were in midtectal regions; and only 0.5% ± 1% were in caudal regions. Comparable numbers of nasal axons were found in rostral (49.8% ± 13.6%) and midtectal regions (43.9% ± 10%); fewer (6.3% ± 6.4%) were seen in regions caudal to the retinotopic target site.

These data suggest that both at late- and early regeneration stages temporal axons have a high preference for their retinotopically appropriate rostral tectum whereas nasal axons pass into caudal tectal domains. This preference was found in tecta denervated for short and long periods.

Pathways of regenerating axons from a temporal hemiretina in denervated tecta

The axonal pathways in long-term-denervated tecta illustrated in the foregoing sections support the view that the positional markers in tectum are long-lasting and survive despite the absence of retinal afferents. Electrophysiological recordings have shown, however, that axons from a temporal hemiretina immediately expand into inappropriate caudal regions of long-term-denervated tecta. This was interpreted to mean that positional markers were lost after long denervation periods (Schmidt, '78; reviewed in Sharma and Romeskie, '84). To decide whether axons from a temporal hemiretina would expand into caudal tectum and override the positional markers (possibly due to reduced competitive interactions with nasal axons) we traced the paths of axons from a temporal hemiretina in tecta denervated for 5 (nine fish) and 8 months (two fish). Between 100 and 180 days after tectal removal HRP was applied not to the retina but to the right optic nerve to label as many axons as possible. Inspections of serial sections revealed that the temporal half of the eye had a normally stratified retina whereas tissue in the nasal half appeared scrambled.

Four tecta denervated for 5 months and examined at 100–140 days after tectal removal did not contain labeled axons. Six of the remaining seven tecta (four tecta denervated for 5 months and two tecta denervated for 8 months) examined at 180 days after tectal removal contained small numbers of labeled axons (between 36 and 417) (Table 3, THR). Figure 8 illustrates a camera lucida tracings of labeled axons from a temporal half retina in a tectum denervated for 5 months. The labeled axons in this tectum as well as in the other five tecta (not shown) traveled through rostral and midtectal regions but did not grow into the caudal tectal half. Axons arriving at midtectal regions often curved and returned into rostral regions. Such course alterations were not only performed by single axons but often by fascicles of axons (Fig. 8). The routes of regenerating axons from temporal hemiretinae in long-term-denervated tecta were similar to those observed in earlier studies which investigated the routes of axons regenerating into the contralateral tectum shortly after optic nerve section (Humphrey and Stuermer, '88).

The absence of temporal axons from the caudal regions of the six tecta examined was substantiated by quantitative measurements of the relative number of labeled axons in rostral, midtectal, and caudal regions. The majority of labeled axons (74.1% ± 4%) were found in rostral regions; some axons (25.9% ± 4%) were found in midtectal regions; none were in caudal tectal regions (Fig. 9).

These data show that, even when regenerating in the absence of nasal axons, temporal axons exhibit a strong preference for the retinotopically appropriate rostral half in long-term-denervated tecta. We did not observe an expansion of temporal axons into caudal tectal regions. The striking rostrally directed bends of axons in midtectal regions suggest instead that the axons prefer to remain in the rostral tectal half.

DISCUSSION

Guidance cues or markers which are relevant for axonal pathfinding were suggested not to be permanently ex-

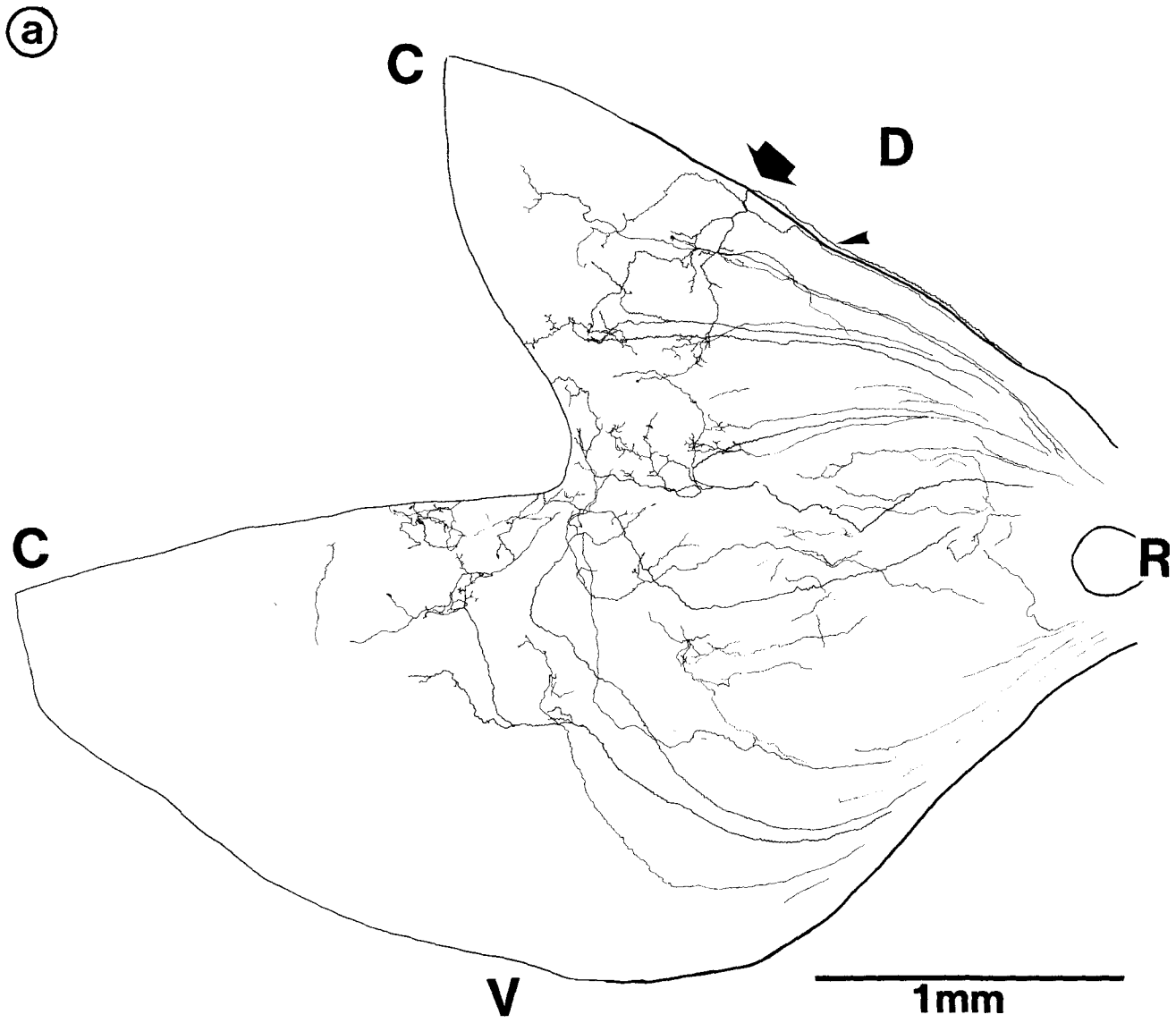

Fig. 6. **a:** Camera lucida tracing of all labeled ventronasal axons in a tectum denervated for 0 months (30 days after tectal removal). Nasal axons ramify preferentially in caudal tectal regions. Most axons grow over the routes of the previous fascicles in SO. Depending on whether they travel over more central or more peripheral fascicle paths, they enter the synaptic layer in more rostral or more caudal regions of the tectum and grow towards the retinotopic target region (between large arrow and tectal center). One axon (arrowhead) travels along the dorsal edge of the tectum. Its arborization is illustrated in b. Abbreviations as in Figure 1a. **b:** Axonal arborization from the tectum in a. Numerous side branches emerge from the axon. These branches carry growth cones (examples are marked by small arrows). The photomicrograph illustrates the growth cone at the tip of the longest axonal branch.

pressed by tectal cells but to be transiently associated with the tectum and lost after long tectal denervation periods (Schmidt, '78). To examine this concept we pursued the pathways of axons in ipsilateral tecta denervated for short (0–3 months) and long (4–8 months) periods. Correct targeting in both, long- and short-term-denervated tecta would implicate the presence and retention of guidance cues.

Retinal axons were induced to regenerate into ipsilateral tecta which had been denervated for short (0–3) and long (4–8 months) periods. The path of HRP-labeled axons from well-defined regions in the retina was analyzed to test whether the axons navigated under the influence of positional markers and whether these markers were lost after long denervation periods (Schmidt, '78) or whether they are intrinsic to tectum and stable (Meyer, '84). Much like axons regenerating into contralateral (nondenervated) tecta after optic nerve section, the axons in short- and long-term-denervated ipsilateral tecta traveled in abnormal routes. The routes, however, did not correspond to a random search; rather, they showed a strong bias for directions approximating the appropriate target position. There is much transient branching, but this appears to be followed by selection of target-oriented routes, finally ending in terminal arbors in retinotopically appropriate regions. The establishment of

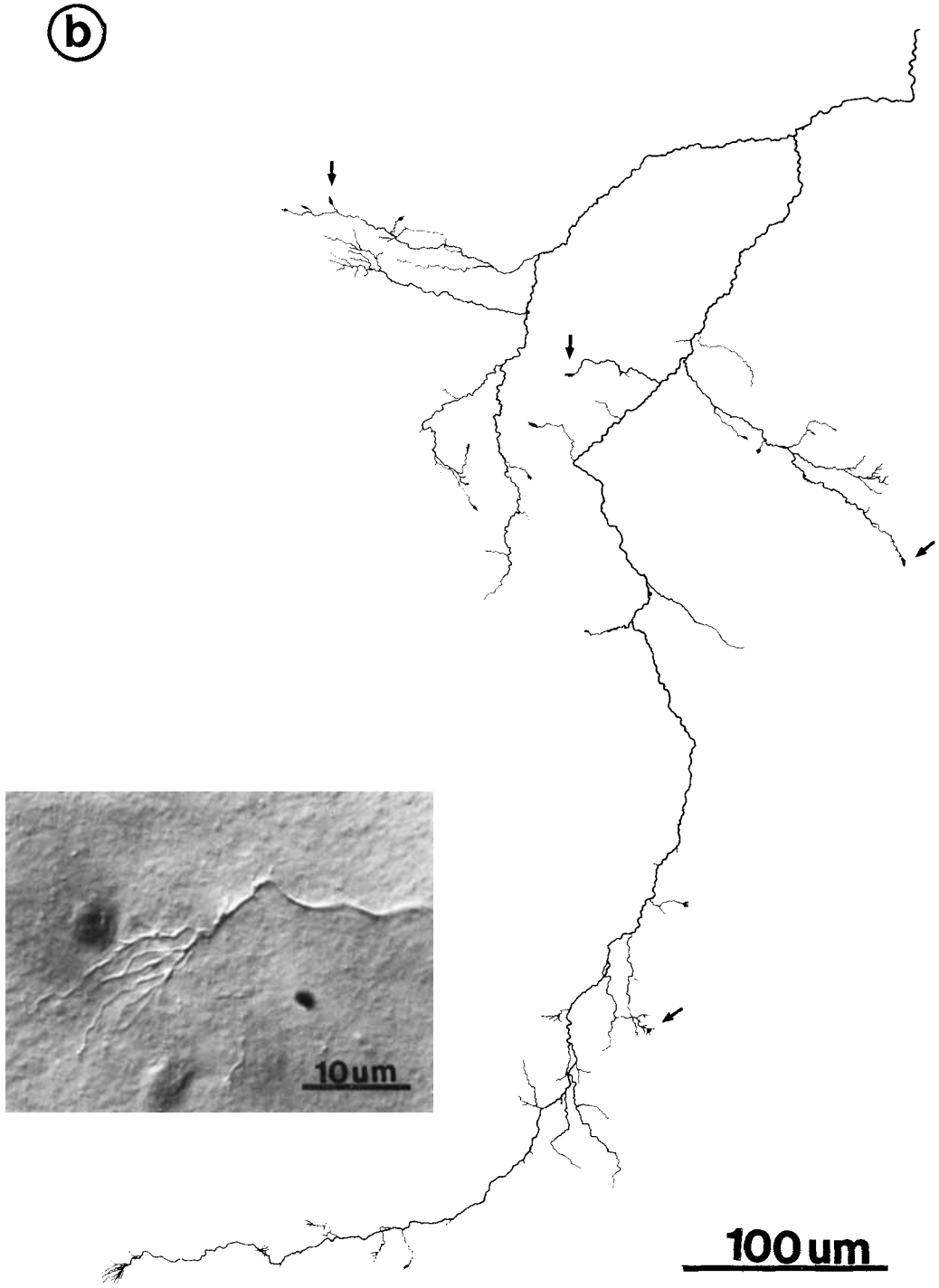

Figure 6b

target-directed trajectories and the termination in retinotopic regions of short- and long-term-denervated tecta indicates that the tecta provided and retained positional markers.

This conclusion contrasts with that of earlier studies (Sharma and Romeskie, '77; Schmidt, '78) which proposed that long-term-denervated tecta lose those elements which serve as guidance cues for regenerating axons. Possible guidance cues were proposed to be the degenerating debris of axons from the earlier retinotectal projection (Murray, '76; Sharma and Romeskie, '77; Schmidt, '78). However, unpublished data (Bastmeyer) indicate that debris had been cleared from long-term-denervated tecta by the time the axons grew in. This was shown by electron microscopic examinations of tecta denervated for 6 weeks and 4 months, respectively. After 6 weeks, degenerating retinal axons were still present in the superficial fiber layer but had disappeared from the synaptic layer. The retinorecipient layers

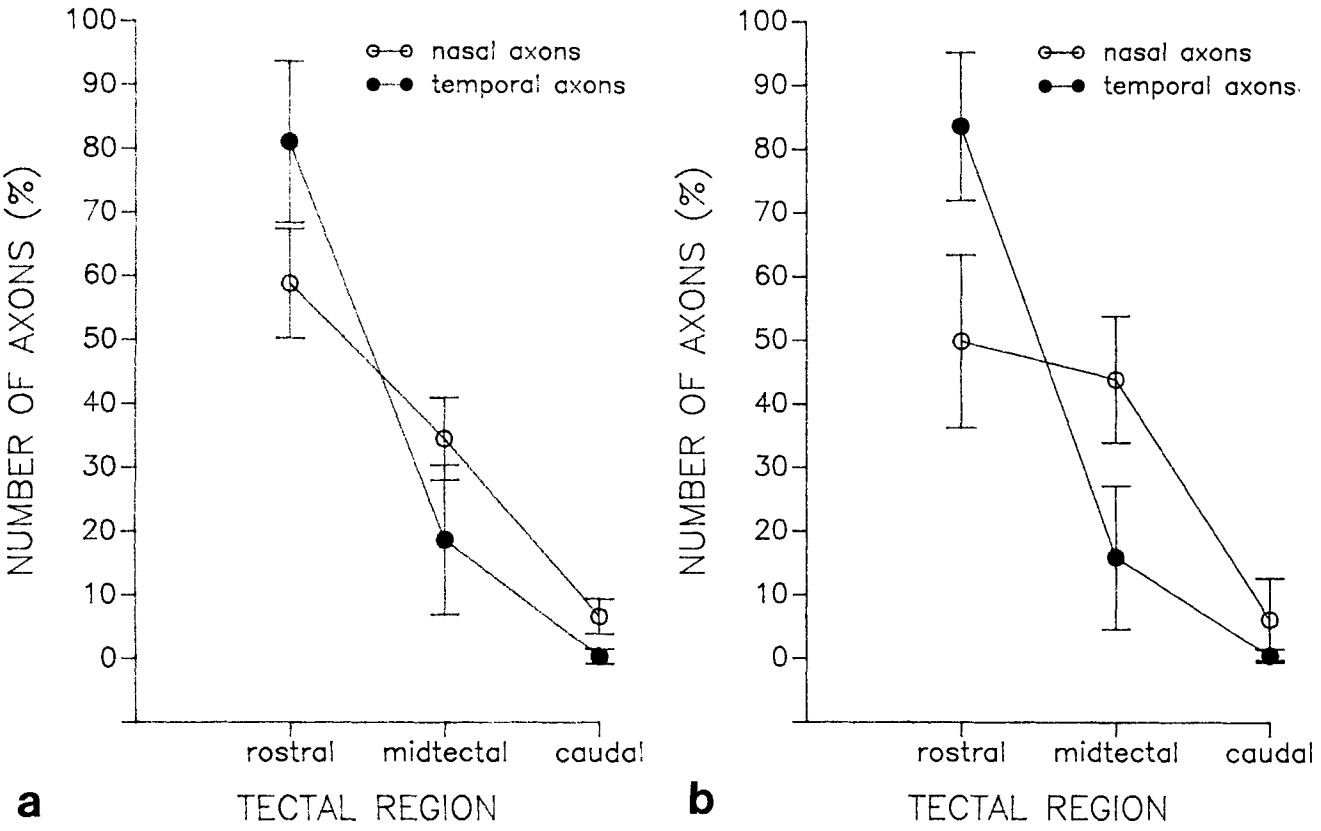

Fig. 7. Plots of the distribution of labeled temporal and nasal axons over rostral, midtectal, and caudal regions of denervated tecta at late (**a**)- and early regeneration stages (**b**). The number of temporal axons (closed circles) declines rapidly from rostral over midtectal and to caudal regions. Nasal axons (open circles) are distributed more evenly over rostral and midtectal regions and their number declines in caudal regions.

were devoid of axonal debris in tecta denervated for 4 months. Since in these tecta the routes established by the ingrowing axons were target-directed and nonrandom, axonal debris seems to be irrelevant for axonal path- and target-finding. In addition, the axonal debris is unlikely to serve a specific guidance function for regenerating axons because regenerating axons do not follow the pathways which had formerly been laid down by normal axons. The evidence that axons travel towards and terminate at retinotopic target regions in short- and long-term-denervated tecta argues against Schmidt's conclusion that positional markers are lost after long-term denervation.

Moreover, we found that fewer or none of the temporal axons erred into caudal regions of the long-term-denervated tecta whereas many more crossed through caudal territories in tecta without prolonged denervation periods (Stuermer, '88b,c). The reduction of these errors may be attributed to the fact that the number of axons in long-term-denervated tecta was smaller than in short-term-denervated tecta or in tecta reinnervated shortly after optic nerve section. In our earlier studies (Stuermer, '88b,c; Humphrey and Stuermer, '88) we suggested that regenerating temporal axons may be misled into caudal tectum since they grow in close association with and follow nasal axons. When temporal axons grow in the absence of nasal axons (Humphrey and Stuermer, '88) the number of errant temporal axons in caudal tectum is smaller. Thus, it is conceivable that regenerating axons are in a conflict between following axons unspecifically and responding to positional cues. This may be one of the reasons why most axons establish target-directed routes only after they had grown over considerable distance through the tectum. Another cause for axonal misroutings is the tendency of the regenerating axons to travel along the pathways which had been laid down by the fascicles of normal retinal axons. As proposed earlier, these pathways may be attractive to the regenerating axons either for mechanical reasons or because these pathways provide particular growth-permissive substances (Lo and Levine, '80; Stuermer, '88b,c). Once off these "highways" the axons appear to respond more readily to positional cues in that they establish routes directed to their targets.

To our knowledge there is only one concept that can account for the target-directed growth of normal and misrouted regenerating axons—namely, directional guidance by graded distributions of positional markers in the target tissue (Sperry, '63; Fraser, '80; Gierer, '87). This notion implies a specification of the position of the tectal cells by at least one gradient each for the two dimensions of the tectal field. When navigating in this system of gradients, the axons can perceive the appropriate direction of their target region from any ectopic site in the tectum even if they are still far away from the target itself. This directional feature is char-

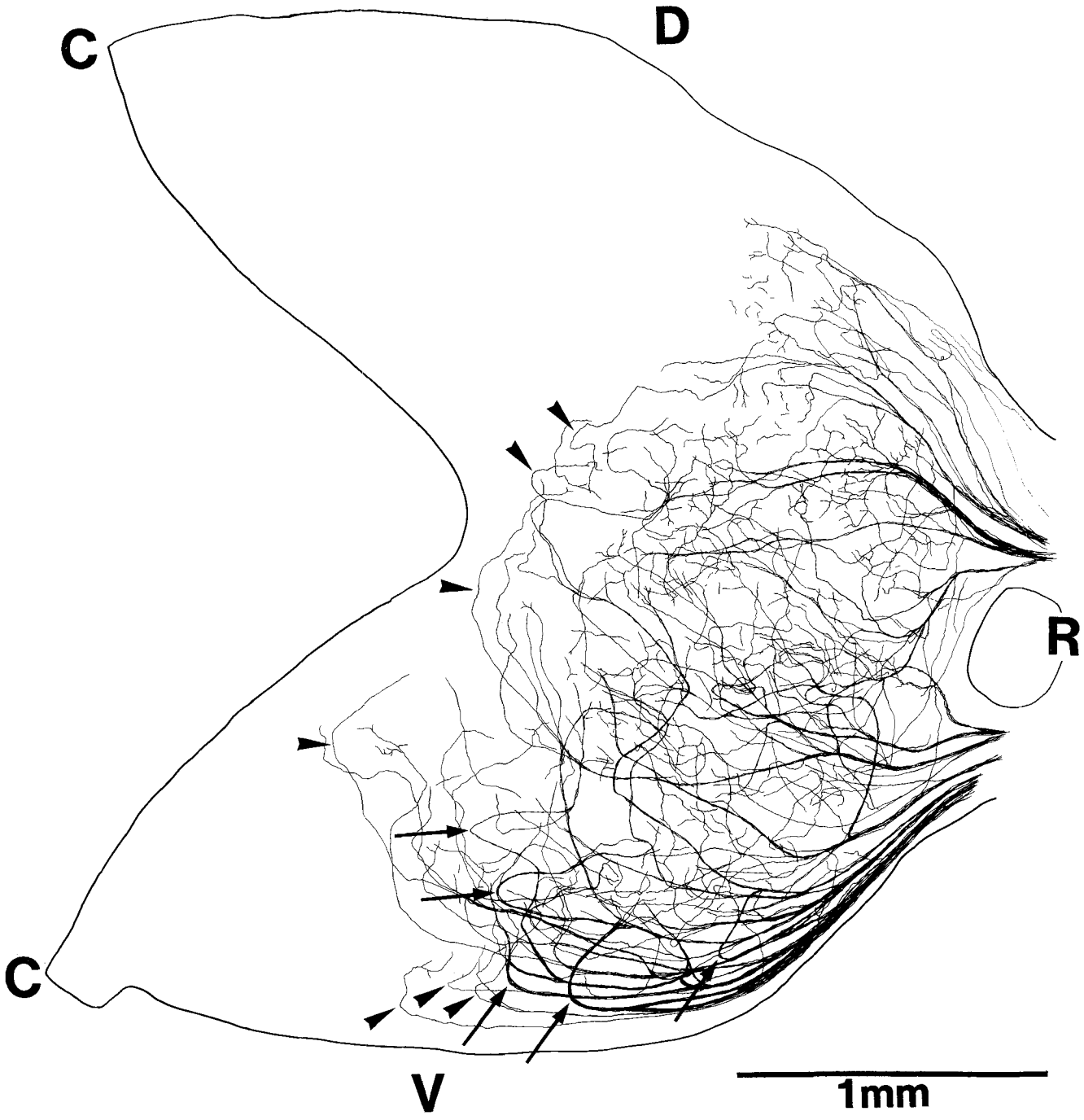

Fig. 8. Camera lucida tracing of labeled axons from a temporal half retina in a tectum denervated for 5 months (180 days after tectal removal). The axons travel through rostral and midtectal regions. In midtectal regions, single axons (arrowheads) and fascicles of axons (arrows) turn into rostral directions. Abbreviations as in Figure 1a.

acteristic for gradient models. Since numerous axons extended a large number of side branches with multiple growth cones we suggest that these axons might use these branches to seek out the direction of the target. Access to the target may be gained by the preferential selection and elongation of appropriately oriented branches and the withdrawal or elimination of the remaining ones (Fujisawa et al., '82; Stuermer, '88c). Whether all axons branched to find the location of the target is unclear.

Additional support for the presence and the retention of markers comes from in vitro experiments (Vielmetter and Stuermer, '89). When confronted with membranes from the rostral and caudal tectum temporal axons of fish accumulate on the membranes from the retinotopically appropriate rostral tectum. Temporal axons exhibited the same behavior when the membranes were obtained from tecta denervated for 3 and 10 months, respectively. We do not know whether the specific response of the temporal axons to sur-

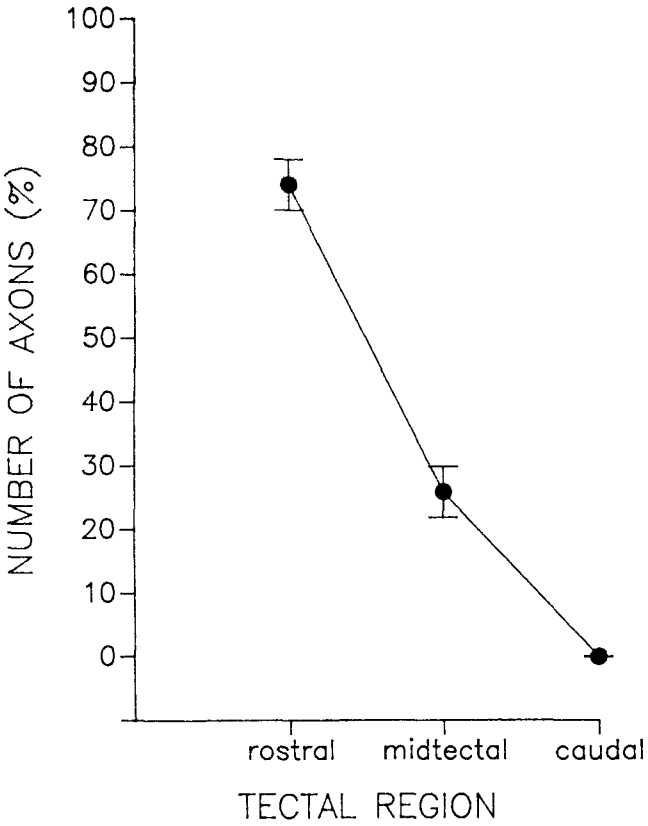

Fig. 9. Graph illustrating the distribution of axons from temporal hemiretinae over rostral, midtectal, and caudal regions of tecta denervated for 5 (N = 4) and 8 months (N = 2). Most temporal axons are found in rostral regions; some are in midtectal; and none are in caudal regions.

face components of tectal cells in vitro plays a role for the guidance of axons in vivo. Provided they do, then these experiments suggest that the relevant components are associated with the surfaces of the tectal cells and are long-lasting.

Half-retinal projections

The idea that tectal markers are lost after long-term deprivation was developed to account for the finding that a temporal hemiretina forms a projection restricted to the rostral hemitectum in short-term-denervated tecta and only expands after several months but immediately expands in tecta denervated for more than 4 months (Schmidt, '78). The projection was always topographically organized. Schmidt suggested that the formation of orderly projections is not inconsistent with a "marker-less" tectum since topographic ordering all along the pathway and a defined sequence of arrival of the regenerating axons would suffice for the establishment of terminal order (Singer et al., '79; Horder and Martin, '82; Springer and Mednick, '86; Rager et al., '86). Objections against this model come from the demonstration that the regenerating axons lose their normal relative order as they traverse the site of the nerve cut (Fawcett and Gaze, '81), arrive in an abnormal sequence in the tectum (Stuermer, '86), and travel through the tectum in highly aberrant routes (Stuermer, '88b,c). Therefore, the inference that the order of the axon terminals during regeneration can evolve passively from a preordering of axons and maintenance of neighborhood relationships cannot be held up. This consideration leads to the conclusion that even the formation of an expanded but topographically organized map requires the existence of positional cues on the tectum (Purves and Lichtman, '85).

Since our results support the existence of stable positional markers in the tectum we suggest that *if* expansion after longer regeneration periods occurred the retinal axons would have to override the positional markers.

When we traced the path of HRP-labeled axons from a temporal hemiretina, we did not discover an invasion of the temporal axons into the caudal regions of the long-term-denervated tecta, not even after 180 days. Not only were temporal axons absent from the caudal tectum—those at the transition zone between the rostral and caudal tectum exhibited striking bends as if avoiding to penetrate into the caudal tectum. Such routes may be interpreted to mean that the temporal axons either have a high affinity for the rostral tectum or might be repulsed due to an inhibitory component associated with the caudal tectum as demonstrated by recent in vitro experiments (Walter et al., '87b).

Technical considerations

The conclusions drawn from the results of this study are consistent with those of our earlier studies in which we applied similar techniques to analyze the path of regenerating retinal axons (Stuermer, '86, '88b,c; Humphrey and Stuermer, '88; Hartlieb and Stuermer, '89). Disquieting though is the fact that we failed to detect the expansion of the temporal retinal axons into the caudal tectum, a phenomenon which has been documented in several earlier studies (Schmidt et al., '78; Schmidt, '78; reviewed in Sharma and Romeskie, '84). The simplest explanation for the lack of expansion would be that the survival times were not long enough to allow the progression of the temporal axons into caudal tectum (Schmidt et al., '78). However, this argument does not find its full application here, since our fish survived for 6 months after nasal retinal ablation and separation of the axons from the contralateral tectum. Moreover, the clue of Schmidt's study was that temporal hemiretinae in long-term-denervated tecta form an expanded projection immediately (Schmidt, '78), i.e., after survival periods of 1–2 months, so long survival periods should not be required for expansion in these tecta.

Another explanation for the lack of expansion may be found in the low number of axons in our experimental tecta. The progression of axons into vacant regions of the tectum has commonly been attributed to the fact that axons compete with one another for tectal space (Fraser, '80) and an expansion into vacant territories releases the axons from the competitive pressure (Schmidt et al., '78; Easter, '85). We have noted that the number of axons is as small as 3–4% in tecta denervated for 4–8 months.

After removal of the tectum, Schmidt ('78) deflected the tract to bring it closer to the ipsilateral tectum and to facilitate tectal reinnervation. The fish used in Schmidt's study were three times as large as ours. This and the tract deflection may have contributed to a faster innervation of the tectum by a larger number of regenerating axons. Reduced axonal numbers probably decrease the competitive interaction of the axons. Thus, it is possible that the number of axons in tecta in our experiments was much smaller than in Schmidt's study, so that the axons had no tendency to expand.

Another possibility which may account for the incongruency of the electrophysiological data and our anatomical study is that HRP may not fill all axons or their branches. Thus it remains open whether HRP had failed to label those axons which the electrodes were able to detect. Based on our anatomical data, the arrival of axons in long-term-denervated tecta appeared to be considerably delayed and so was the phase of widespread branching and formation of target-directed routes and terminal arbors. However, the maps in long-term-denervated tecta were recorded at 30–40 days after the separation of the axons from the contralateral tectum (Sharma and Romeskie, '77; Schmidt, '78; Romeskie and Sharma, '80). Most long-term-denervated tecta in our study were devoid of axons at such an early time. Other experiments, however, have shown that the anatomical techniques are superior over electrophysiological recordings in demonstrating axonal processes. For instance, the earliest time at which visually evoked potentials can be recorded from regenerating axons in the fish tectum is at 30–40 days after optic nerve section (Schmidt and Edwards, '83). But HRP staining revealed that the axons arrive in the tectum already at 12 days and have formed large ramifications with numerous growth cones and filopodia all over the tectum shortly thereafter (Stuermer and Easter, '84a; Schmidt et al., '88; Stuermer, '88b,c). The electrodes fail to record from the axons which are in their exploratory growth phase. The map recorded at 30–40 days appears precise and topographically organized (Schmidt and Edwards, '83) although it has been shown to be diffuse when assessed with anatomical mapping techniques (Meyer et al., '85, Rankin and Cook, '86). Unfortunately these considerations do not provide a satisfying explanation which could account for the incongruency between the electrophysiological data and our anatomical study. It is perhaps the size of the fish, the surgery, or the technical differences in analyzing the maps which could account for the different results. To test this, electrophysiological recordings and anatomical axon-tracing techniques should be combined in the same experimental animals.

Paré and Levine ('82) have shown that as degenerated central pathways in the goldfish brain age, they become less permissive of axon growth. As a consequence Paré and Levine ('82) find fewer axons traveling over the tract of long-term-denervated tecta and more instead in the transverse commissure. Our finding that the number of axons reinnervating long-term-denervated tecta is drastically reduced as compared to short-term-denervated tecta could therefore be attributed to the loss of permissivity of the direct pathways leading to the tectum. However, in contrast to Paré and Levine ('82), we did not observe a compensatory increase in the number of axons taking alternate routes such as the transverse commissure, although we occasionally saw (and once as many as ten) axons in these paths. Paré and Levine ('82) used radioactive proline applied to the entire eye and evaluated the density of axons on sections with radioautography. We labeled subpopulations of the regenerating axons which were lesioned in the eye by using HRP and visualized them in whole mounts of the brain. These methodological differences in assessing the reinnervation of the ipsilateral tectum may account for the fact that the density of fibers in ipsilateral tecta appears more massive in the study by Lo and Levine ('80) than in ours and in Springer ('80). Evaluation of fiber densities by silver grains has its drawbacks and so has the HRP technique applied here. However, for the purpose of our study, which yielded at revealing the axonal trajectories in the tectum and testing of whether and how they find their targets, the HRP technique is superior to autoradiography and serves our purposes.

ACKNOWLEDGMENTS

We thank Drs. P. Raymond and T. Cox for reading the manuscript critically and A. Gierer for discussing with us the theoretical implications of the results. We also thank R. Groemke-Lutz for her skillful photographic reproductions.

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