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BILATERAL TECTAL INNERVATION BY REGENERATING OPTIC NERVE FIBERS IN GOLDFISH: A RADIOAUTOGRAPHIC, ELECTROPHYSIOLOGICAL AND BEHAVIORAL STUDY

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SUMMARY

Following unilateral enucleation and optic nerve crush in goldfish, the remaining nerve regenerates and innervates both optic tecta. Approximately 5% of the nerve fibers reach the ipsilateral optic tectum (IOT) via the ipsilateral tract at the chiasma. Comparable debris in both tracts was not sufficient to result in an IOT projection since when both nerves were crushed simultaneously the usual pattern was seen, i.e., each nerve innervated a contralateral optic tectum (COT). When the arrival of one nerve at the chiasma was delayed by staggering the nerve crushes, the nerve that first arrived at the chiasma partially innervated the IOT. In most instances the entire IOT was innervated, however, the stratigraphic distribution of fibers in the various tectal lamina was atypical.

Electrophysiological analysis indicated that fibers from each area of the retina innervated the IOT visuotopically. The COT was ablated in order to determine whether the IOT projection could mediate behavior. All fish failed to respond to changes in illumination as measured by respiration and failed to swim with or against the stripes in an optomotor drum. Thus, the IOT input, possibly because of its sparseness, could not be shown to be behaviorally functional.

INTRODUCTION

Following [^{3}H]proline injection into the eye of the goldfish, rapidly transported protein appears in the contralateral optic tectum (COT), with very little background labeling as evidenced by radioactivity in the ipsilateral optic tectum (IOT). Biochemical and radioautographic analyses reveal that the background labeling is due to systemic radioactivity that labels the IOT in situ[3]. [^{3}H]Proline has proved useful in the study of regenerating goldfish optic nerve in analytic, as well as radioautographic studies of the optic nerve and tectum[13].

In a previous study that examined the effect of temperature on the rate of optic nerve regeneration[20], the [^{3}H]proline method detected the regeneration of an anomalous ipsilateral retinotectal projection in fish subjected to left optic nerve crush and right eye enucleation. This report contains biochemical, radioautographic, electrophysiological and behavioral studies of this ipsilateral innervation.

MATERIALS AND METHODS

Surgical procedures

Under tricaine methanesulfonate anesthesia, one eye of each fish was removed and the optic nerve of the other eye was crushed intraorbitally. A complete description of the fish and this surgical procedure has been described previously[20]. For tectal ablations, access to the brain was gained by hinging a flap of cranium, which was replaced after aspiration of the tectum. The cranial flap reattaches within 2 weeks leaving a faint scar. After surgery fish were housed in 3.8 liter tanks at 30 ± 1 °C unless otherwise noted.

Biochemical and radioautographic analyses

[^{3}H]Proline was injected intraocularly (see preceding paper for details) and the fish were kept at 30 ± 1 °C throughout the incorporation period. Unoperated fish, normally maintained at 20 ± 1 °C, were kept at 30 °C for at least 24 h prior to administration of labeled proline. At 24 or 48 h after intraocular (IO) injection of labeled proline, tecta were removed and homogenized separately in cold distilled water. Acid-insoluble radioactivity was determined by a filter paper method[11]. Protein concentration was determined spectrophotometrically[10]. Radioautographic methods were as previously described[20].

Electrophysiology

One to 5 months postoperatively, the projection of the left eye was mapped onto both tecta using standard electrophysiological mapping techniques[6]; details of the present method are given elsewhere[16]. The anesthetized fish was positioned in air with water perfused over the gills and with its eye at the center of a large hemisphere. The cranium was opened and a microelectrode was inserted to record from several retinal fiber terminals in the tectum. A small spot of light was used to map those areas on the hemisphere which excited the fiber terminals recorded. The position of the eye was monitored by projecting the position of the optic disc onto the hemisphere and the maps were then corrected for any shift of eye position. After mapping, several fish were revived, injected with [^{3}H]proline and processed for radioautography.

Behavioral assays

Fish were tested for vision in a striped rotating drum that reliably elicits an optomotor response[21]. After placement in the drum for 2 min of adaptation, time swimming with the stripes in a 3 min test period was recorded. Normal fish spend at least 140 sec and enucleated fish less than 50 sec swimming with the stripes[21]. A second test of vision consisted of placing the fish in a foam restrainer and slowly passing a black piece of plastic over the fish[21]. This procedure results in a respiratory deceleration in normal fish and provides a measure of whether the fish are capable of seeing and responding to changes in illumination. Respiration was monitored with a thermistor placed near the mouth of the fish.

RESULTS

Biochemical analysis

The arrival of axonally transported protein in the optic tectum after IO injection of [^{3}H]proline was examined in fish 17 days following optic nerve crush and unilateral enucleation. The specific radioactivity of COT and IOT protein was determined and compared with that of unoperated control fish (Table I). It is apparent that the regenerating optic nerve has reached the COT by 17–19 days. In agreement with the results of Grafstein and Murray[4], comparison of the COT labeling in operated fish with that in control fish indicates that optic nerve regeneration is accompanied by an increase in the amount of labeled transported protein. Unexpectedly, the specific radioactivity of IOT protein in operated fish was also found to be significantly elevated over that in control fish. Labeling in the IOT should result only from local incorporation supplied by systemic circulation.

Radiography

Since the increased IOT radioactivity could represent an ipsilateral retinotectal projection, the nature of the IOT labeling was examined radioautographically. Thirteen fish were injected IO with 30 or 60 μCi of [^{3}H]proline at 12–50 days after optic nerve crush and unilateral enucleation, and were killed 24 h following injection.

All fish showed heavy radioautographic grain density in the retinal ganglion cell terminal layers of the COT which indicated that the optic nerve had regenerated

TABLE I

Accumulation of transported protein in optic tectum in goldfish following optic nerve crush and unilateral enucleation

Operated fish (n = 10) were injected intraocularly with 27 μCi [^{3}H]proline 17 days after removal of the right eye and crushing the left optic nerve. Unoperated control fish (n = 8) received the same dose of [^{3}H]proline. Acid-insoluble radioactivity in individual tecta was determined for both groups at 48 h. The results are expressed as mean ± S.E.M.

	Specific radioactivity (disint./min/µg protein)	
	Operated	*Control*
Contralateral	242.3 ± 16.9	42.8 ± 3.9
Ipsilateral	9.8 ± 1.6	1.3 ± 0.1

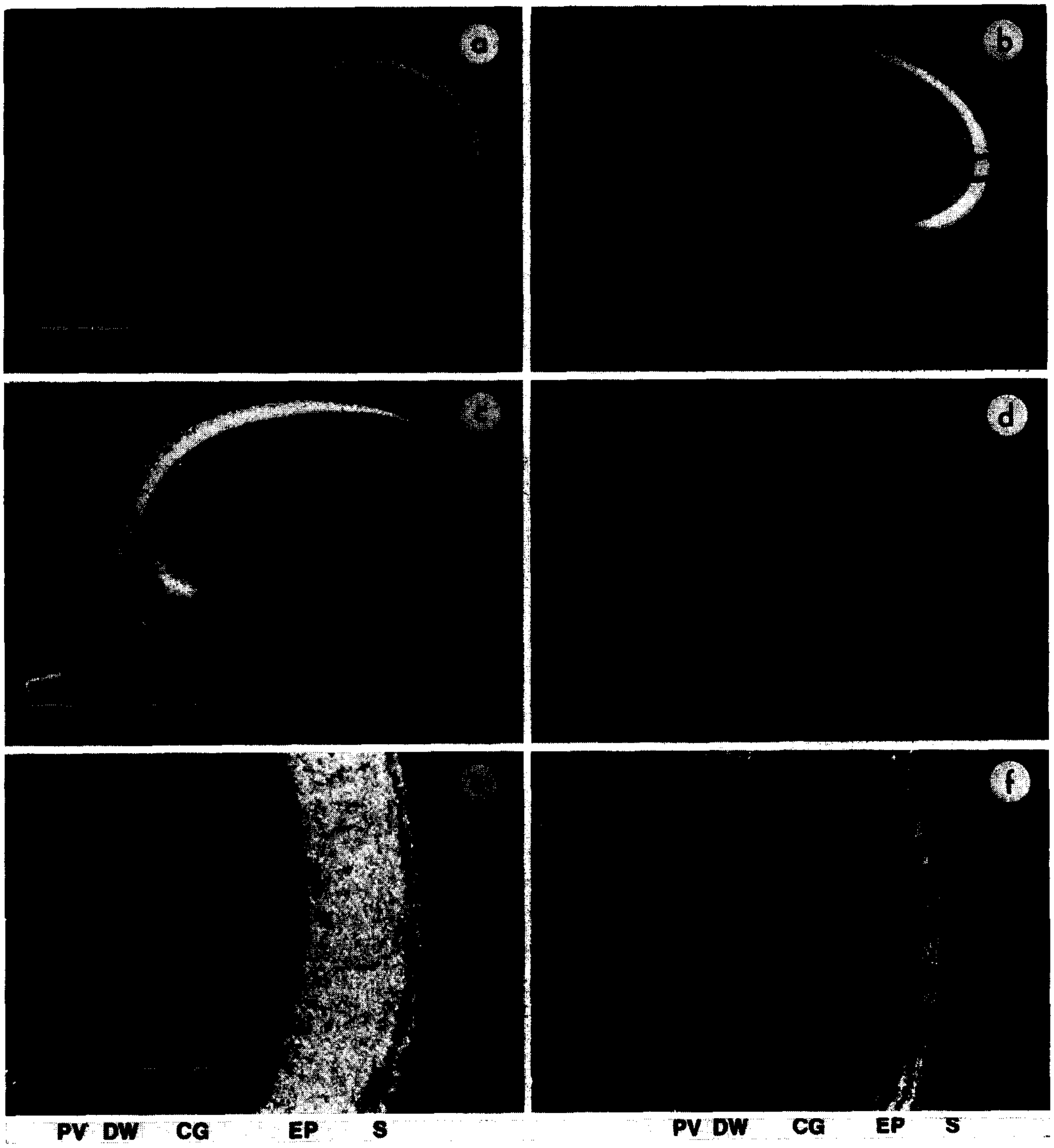

Fig. 1. a: cross-section of a normal goldfish brain after IO injection of [^{3}H]proline into one eye. Microphotographs were taken with both transmitted and incident light to illustrate both the tissue section and the emulsion grains. Grains are localized in the optic tectum contralateral to the injected eye and are not evident in the IOT. Scale 1 mm. b: cross-section of the brain of a goldfish in which one eye had been removed and the optic nerve of the other eye crushed. The fish then received an IO [^{3}H]proline injection and were processed for radioautography. Grains are present in the IOT following regeneration. c: parasagittal section of the brain of a fish described in "b" showing IOT labeling following regeneration. d: COT of fish described in c. e: stratigraphic distribution of grains in the COT following regeneration of the fish described in "b". Grains are above background in the synaptic lamina (S), external plexiform lamina (EP), central gray (CG), deep white (DW) and periventricular region (PV). Scale 0.2 mm. f: stratigraphic grain distribution in the IOT of the fish described in "b". Grains are predominantly localized in the external plexiform lamina (EP).

(Fig. 1b, c) and the labeling pattern was the same as that of the COT of unoperated fish (Fig. 1a). All regenerating fish also evidenced appreciable grain densities in the IOT (Fig. 1b). There was a significant concentration of grains, considerably above background, in the external plexiform layer, indicating that some regenerating optic fibers had innervated the IOT. In most fish the entire IOT was labeled (Fig. 1b, d) while in others the rostral portion of the IOT or medial and lateral or only lateral areas of the IOT were labeled. In all instances the grain distributions appeared continuous and patchy innervation of the IOT was not observed. In addition, the density of the IOT projection did not appear to be related to the interval between surgery and sacrifice. Microdensitometric analysis of the IOT and COT of unstained sections suggested that 3–10% of the optic fibers destined for the COT had entered the IOT and this estimate is consistent with the previous biochemical data.

Optic fibers terminate in specific tectal lamina and the COT of the regenerating fish appeared to have a normal stratigraphic distribution (Fig. 1c, e). However, the distribution of fibers in the IOT (Fig. 1d, f) of all fish was not comparable to that in the COT. Silver grains in the IOT were restricted to the superficial portion of the usual location of the external plexiform stratum while grains were absent in all instances in the synaptic stratum at the boundary of the marginal fiber stratum. The ipsilateral tract was examined along its extent in several fish to assess innervation of other retinal target nuclei. In general, it appeared that nuclei which were labeled contralaterally were also labeled ipsilaterally, but less densely. In particular, grains were observed over nucleus preopticus pars magnocellularis, thalamic nuclei (nucleus dorsolateralis, medialis and lateralis), pretectal nuclei (rotundus, geniculatus and also over the basal optic nucleus of the mesencephalon[17]) (Fig. 2a). In some fish innervation of these regions was not seen, presumably because ipsilateral labeling in these areas was too sparse for a conclusive assessment. The optic nerves in goldfish are reported to be completely crossed in that direct projections to the ipsilateral side of the brain have not been found[18]. However, we have recently found that primary projections to ipsilateral non-tectal nuclei do exist[22]. In view of this recent finding, the question arises of whether any part of the ipsilateral non-tectal projections observed in the present study following regeneration is anomalous. By comparison of ipsilateral and contralateral radioactivities in unoperated (Fig. 2b) and experimental fish brains, we conclude that there are anomalous ipsilateral projections to these nuclei. Although several routes to the IOT are available[19], the fibers consistently reached the IOT via the ipsilateral tract at the chiasma (Fig. 2c).

To determine whether the high maintenance temperature employed (30 °C) was essential for the appearance of the ipsilateral projection, 3 fish were prepared as above and were maintained at 23 °C. Radioautographs revealed an ipsilateral projection in these fish as well. Since optic nerve fibers are reported to follow channels in degenerating debris[12], the possibility that the debris in the ipsilateral tract causes fibers to enter the ipsilateral tract was examined. The right eye of 3 fish was removed two weeks prior to crushing the left optic nerve. This procedure should result in debris of a different age in the ipsilateral tract relative to that in the contralateral tract at the time when the nerve reaches the chiasma. Fish were injected with [^{3}H]proline 19

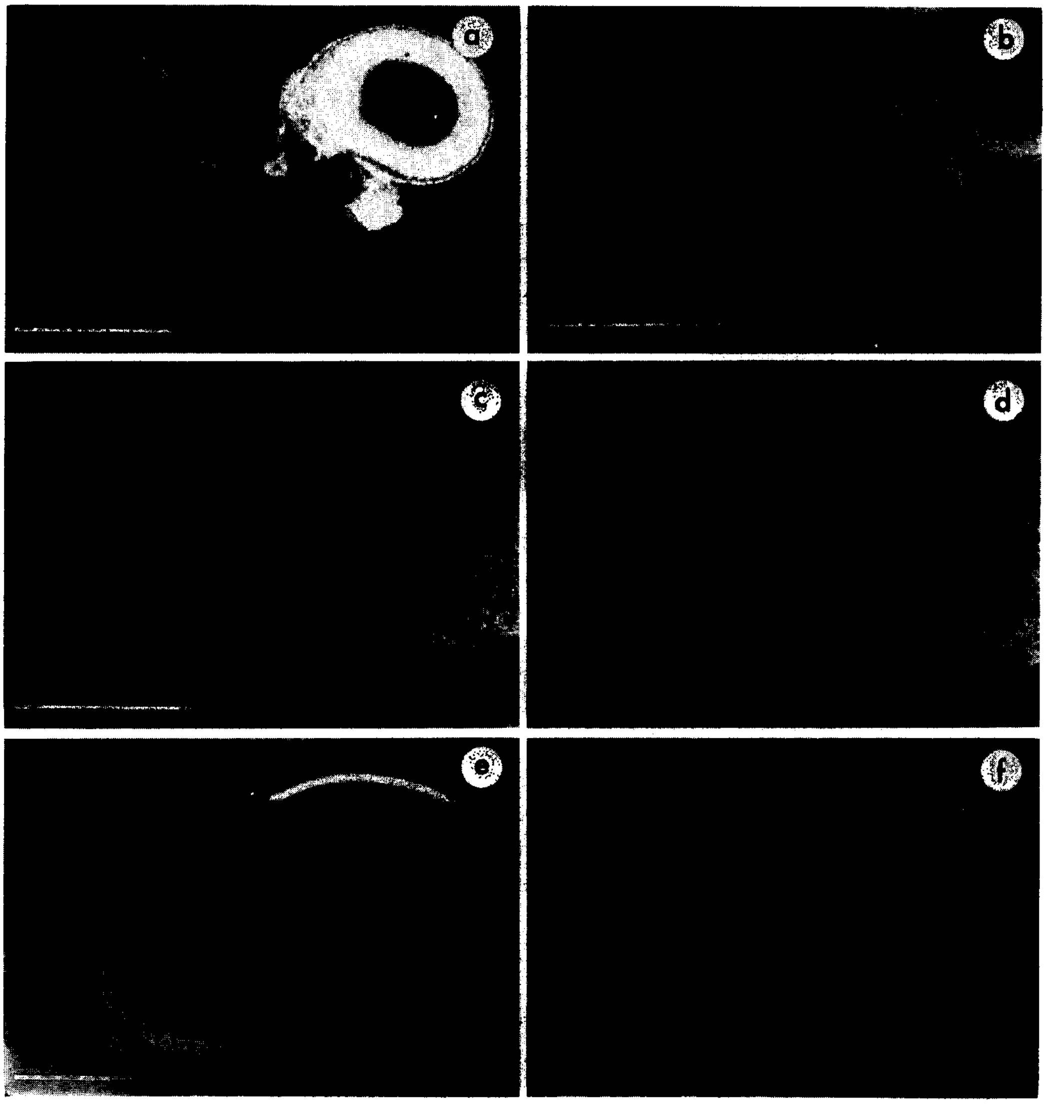

Fig. 2. a: radioautograph of the brain of a fish that had one eye removed and optic nerve of the other eye crushed (cross-section). Following regeneration, grains are found in both the COT and IOT. In addition, grains are localized in non-tectal sites on both the ipsilateral and contralateral sides of the brain. Scale 1 mm. b: cross-section of the brain of a normal fish showing grains only in the COT. Grains are also seen at non-tectal sites contralaterally, as well as ipsilaterally (arrow). The grain densities are heaviest on the contralateral side. Scale 1 mm. c: optic chiasma of a fish described in "a", demonstrating fascicles of optic nerve fibers turning ipsilaterally. Scale 0.5 mm. d: when an eye is removed 2 weeks prior to crushing the nerve of the other eye, the regenerating nerve does not turn ipsilaterally; grains are not observed in the ipsilateral optic tract or in the IOT (e). Scale 1 mm. f: following simultaneous crush of both optic nerves each nerve regenerates back to a COT. Grains are found in the COT, but not in the IOT.

days following nerve crush. Radioautographs revealed that the ipsilateral projection was absent in 2 of 3 fish, both at the chiasma (Fig. 2d) and IOT (Fig. 2e). Since debris is reported to be present as long as 3 months following enucleation[12], this result suggests that the quality of the debris in the degenerating tract may be a factor in eliciting the ipsilateral projection.

To further examine the role of the degenerating tract, both optic nerves of 3 fish were crushed simultaneously. In this preparation, the state of degeneration of both optic tracts should be comparable when the regenerating nerves reach the chiasma and an ipsilateral projection was predicted. Radioautographs of the tectum following regeneration did not however reveal an ipsilateral projection in any of the fish (Fig. 2f).

Since simultaneously crushing both optic nerves did not result in an ipsilateral projection, we entertained the possibility that enucleation produces a more marked degeneration response in the remaining optic nerve stump than after crushing the nerve. Since the crush leaves the endoneurium intact, we examined the effect of a surgical cut of the other optic nerve. We might expect simultaneous crush of one optic nerve and cut of the other nerve to result in the appearance of an ipsilateral projection of the crushed nerve such as that seen after enucleation. This was found to be the case, but the difference appears not to be between crushing and cutting per se but in the time lag in regeneration of the cut nerve compared to the crushed nerve. Although simultaneously crushed nerves do not show ipsilateral innervation, when the optic nerves are crushed two days apart, the nerve which is crushed first develops an ipsilateral projection. These observations suggest that some of the regenerating fibers will invade the ipsilateral tract if the ipsilateral tract is unoccupied when the growing fibers reach the chiasma.

Electrophysiology

Eight fish which had been subjected to left nerve crush and right eye enucleation

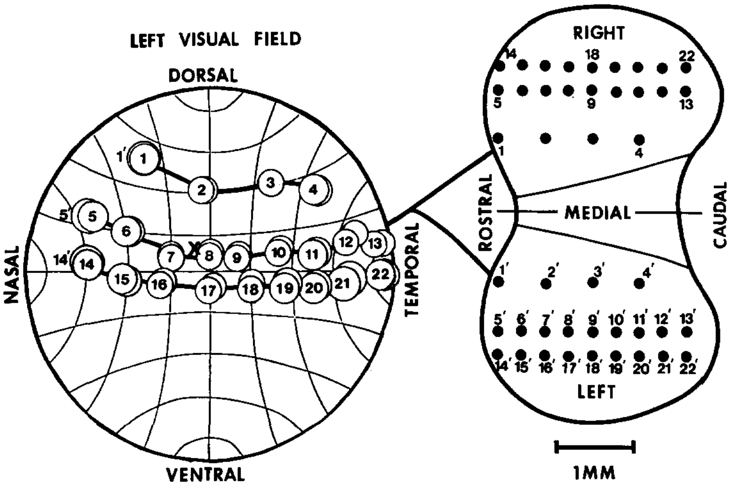

Fig. 3. Projections from the left eye mapped onto the left and right tectum 27 days after optic nerve crush and removal of the right eye. " × " marks the projection of the optic disk onto the hemisphere.

were mapped and all showed regeneration of a normal projection to the COT. In all fish, light-driven activity was also recorded in the IOT. The extent of the IOT projection recorded was highly variable, ranging from a single point in one fish to a complete and orderly projection in several fish (Fig. 3). The projection to the IOT is normally organized (dorsal field projects medially and nasal field rostrally) resulting in a mirror image projection. For ease of comparison, electrode penetrations were made at positions mirror symmetric across the midline. The receptive field of units from the IOT overlap extensively with those from the corresponding positions on the COT in every case, and even superimpose in some instances. This overlap was also apparent in those fish where only a few points could be mapped ipsilaterally.

Four of the fish were successfully revived for radioautography. In two fish with only a few recordable points, the projection was very light, although the extent of the projection was somewhat wider than would be indicated by the electrophysiology. In the two fish with extensive ipsilateral maps, the distribution of grains was more extensive and the density greater, although still much less than contralaterally. All 4 fish showed heaviest tectal labeling in the lateral-ventral region of the ipsilateral tectum.

Behavior

Since the electrophysiological data indicated that the fibers were arranged visuotopically in the IOT and in many cases represented most of the visual field, the visual function of the ipsilateral projection was examined. Thirty-six days following right eye enucleation and left optic nerve crush 12 fish were tested in the optomotor drum to verify that regeneration had occurred. With the stripes moving clockwise, the fish had a mean time of swimming with the stripes of 145 ± 8.4 sec (± S.E.M.), indicating recovery of vision[20]. To determine whether the ipsilateral projection was behaviorally functional, the COT was then ablated, leaving the fish with the eye projecting to the IOT. Since the route of the IOT fibers is through the chiasma, ablation of the COT should not disrupt the IOT projection. Eight days following COT ablation the fish were placed in the optomotor drum and time swimming with and against the clockwise moving stripes was recorded. Since a reversed visuotectal map is present on the IOT and since the tectum cannot distinguish between which eye is innervating it, it was expected that the fish would swim against the stripes. Mean time swimming with the stripes was 40.2 ± 8.2 sec and time swimming against the stripes was 31.7 ± 11.7 sec, scores that are indistinguishable from those of blind fish[20]. In addition, as is evident in the scores, fish were free of circling movements within the 10 day test period, although a few fish began to evidence circling movements several weeks later.

Although the fish appeared blind in the optomotor test, it remained possible that they could detect changes in illumination. The results of the shadow test, however, also indicated that the fish were blind, since they showed an insignificant mean respiratory deceleration (2.3 ± 1.5%). Three days after behavior testing, 6 of the fish were injected with [^{3}H]proline and were allowed to survive 24 h. Radioautographs revealed an ipsilateral projection in all of the fish, suggesting their presence at the time of behavioral testing.

To examine the possibility that the combined surgical trauma of having an eye as well as a tectum removed results in fish that can see but that do not respond in these tests, a group of 12 fish had the right eye and left tectum removed. When tested 7 days after surgery, their mean time swimming with the clockwise moving stripes was 135.2 ± 8.8 sec and against the stripes 2.2 ± 1.6 sec. In addition, their mean respiratory deceleration to the shadow was 40.3 ± 4.4%. Thus, there is no evidence of surgical trauma when fish are tested soon after surgery. These results taken together indicate that the ipsilateral projection is not behaviorally functional by these tests. While we saw no indication of vision, it remains possible that psychophysical testing (e.g., use of more intense light) may reveal that some vision is mediated by the IOT.

DISCUSSION

Investigations of optic nerve regeneration in both fish and anurans indicate that regenerating optic nerve fibers maintain their original spatial configurations in the optic nerve[5] and return to their original tracts following deflection[1]. While these results are obtained when both optic tecta are left intact, the optic nerve is reported to retinotopically innervate the IOT via a number of different routes when the COT has been ablated[19]. Thus, rostrocaudal tectal locus specificity is maintained while right–left tectal specificity and the usual course of the fibers are not. In contrast to the previous studies, the present experiments indicate that right–left specificity, as well as specificity in the course of fibers, is partially vitiated in the presence of an intact tectum when both the ipsilateral and contralateral tracts are available to a regenerating nerve. Of possible relevance is the report of bilateral innervation of the frog tectum when an optic nerve is damaged at an early embryonic stage[7].

The predominance of reconnection to the COT indicates that the COT and IOT are not equally attractive to the regenerating fibers. The outgrowing ganglion cell axons may have a greater affinity for the contralateral tract and tectum, or may be channeled by mechanical constraints at the chiasma. An obligatory role for the IOT in attracting fibers can be excluded, since we have found in the present study that some fibers turn ipsilaterally at the chiasma even if the IOT is absent.

A possible explanation for the anomalous ipsilateral projection is that the regenerating nerve fibers are attracted to the debris of the degenerating tract distal to the nerve section (either the crush or enucleation). This hypothesis would seem to be supported by the present finding that an optic nerve fiber will follow a tract that has recently degenerated but not one in which degeneration began several weeks earlier. The debris hypothesis cannot alone account for the formation of the ipsilateral projection, since when two nerves are crushed simultaneously each nerve stays within its appropriate tract even though the debris in both tracts are identical. It seems therefore likely that each nerve by its presence prevents the other nerve from entering its tract. When the presence of one optic nerve at the chiasma is eliminated by enucleation or is delayed by staggering the nerve crushes, its occlusive properties are eliminated and a portion of the other nerve enters the unoccupied ipsilateral tract.

Thus, the seemingly cohesive forces between fibers of each nerve that are in effect when the nerve reaches the chiasma may in fact be partially explained by the presence of the other nerve at the chiasma.

A question arises as to how the rather sparse anomalous ipsilateral projection can mediate the remarkably representative projection on the IOT that was observed electrophysiologically. A process that randomly selects fibers from the entire nerve could result in the selection of a representative population. In explaining how the selection might happen, one must take into account the observation that the goldfish optic nerve is itself normally retinotopically organized. Should fascicles nearest the degenerating ipsilateral tract be deflected, they might be expected to represent a rather limited region of the retina. Roth[15], however, notes that while there is retinotopic organization within the nerve, the orientation rotates about the nerve axis and in addition central fibers move to the perimeter, so that a large portion of the fibers from the retina would during their course come into contact with the degenerating ipsilateral tract.

A small but representative population of axons could be furnished by the participation of lead fibers[9]. If the goldfish retina were subdivided into domains, each with central ganglion cells that furnish advancing lead fibers, and if a number of such fibers were attracted into the degenerating ipsilateral tract, the same principles of selective adhesivity that lead to the retinotopic organization of the normal optic nerve could organize the anomalous projection. Another possibility is that ipsilateral fibers arise by sprouting, most likely rostral to the chiasma. If so, a single ganglion cell would project to both sides of the tectum. Such collateral sprouting has been demonstrated in neonatal rats following unilateral enucleation[2].

Ipsilateral retinotectal projections following ablation of one tectum are reported to have a normal stratigraphic distribution following regeneration[19], although in some instances the entire tectum is not reinnervated[8]. In the present experiments, the ipsilateral projection usually encompasses the entire tectum. However, fibers fail to innervate the synaptic lamina at the border of the marginal fiber stratum in the IOT. It is of possible relevance that in the longnose garfish each optic nerve normally projects to both tecta and the most superficial band is also missing in the IOT[14].

The ipsilateral projection seen in the present study appears to be electrophysiologically intact but behaviorally non-functional. Following COT ablation, experimental fish are left with only an ipsilateral projection. Behavioral tests which examined responses to the optomotor drum or to a change in illumination failed to demonstrate any evidence of visual function. This may simply reflect the small number of fibers rather than a failure to form functional synapses. The more extensive ipsilateral tectal projections that are observed when only one tectum is ablated[19] are currently being examined for their ability to mediate vision.

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