STUDIES ON SELECTIVE NEUROREGENERATION IN LOWER VERTEBRATES

Thesis by

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In Partial Fulfillment of the Requirements
for the Degree of
Doctor of Philosophy

California Institute of Technology
Pasadena, California

1977

(Submitted December 6, 1976)

ACKNOWLEDGEMENTS

I am grateful to Professor Roger Sperry for his patience, support and guidance. I appreciate his attempt to provide an atmosphere which encouraged independent thinking and the pursuit of one's own ideas.

I thank Chuck Hamilton for many helpful suggestions and stimulating discussions. Special thanks to Ron Meyer, who collaborated in part of this work, for unselfishly sharing his knowledge, and being a good friend.

I wish to thank my husband, Ron, and children, Lori and Jeff for the many sacrifices and continual support throughout the long course of my education.

The financial support received from the California Institute of Technology, National Institues of Health, Evelyn Sharp, ARCS, and Phi Kappa Phi are gratefully acknowledged.

ABSTRACT

The specificity with which terminal axons of the peripheral and central nervous systems functionally innervate foreign fields was studied in frog and goldfish.

The effects of competitive reinnervation by left and right cutaneous nerves were carried out in frog in an effort to test for the existence of left-right biochemical differentiation of neural tissue. The dorsal cutaneous nerves were cut, transposed and allowed to regenerate under different conditions of denervation and competition and the resultant reinnervation and reflex patterns were determined by behavioral and electrophysiological mapping techniques. There was no indication that growth patterns and functional reconnections were influenced by the specific laterality of the fibers.

In goldfish, the course of functional recovery of vision during the compression of the retinotectal projection that follows hemitectal ablations was investigated with fish maintained postsurgically in diurnal and continuous-light environments. Behavioral and electrophysiological mapping methods indicated that vision was restored throughout the temporal half-field, originally blinded by the tectal lesion regardless of the postoperative lighting conditions. The scotoma diminished in an orderly anterior-posterior fashion and color discriminability was concommitant with visual recovery. Functional topographic reorganization of the retinotectal projection implies that locus-specific affinities between retinal and tectal neurons, which may play a prominent role in the direction of nerve growth and formation of synapses under more normal circumstances, are not permanently fixed even in mature goldfish.

The results of the present investigations indicate that factors such as availability of terminal sites and the tendency of fibers to seek terminal connections seem to override any qualifications imposed by the existence of lateral or locus specificity on the formation of terminal connections. The return of color vision in goldfish, however, indicates prespecification of retinal and tectal cells for color, and selective reconnection of neurons according to their specificities which survived the compensatory developmental pressures created by the retinotectal size disparity.

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I. INTRODUCTION

Definitive studies in the 1940s showing selective reinnervation of the midbrain tectum by regenerated optic fibers (Sperry, 1943, 1944, 1945, 1948), were instrumental in reversing the skepticism of previous decades towards the concept of functional and anatomical specificity. The observation that severed optic axons grew back to the tectum with great precision to form functional connections at their original terminal sites regardless of the maladaptive behavioral effects resulting from 180° rotation or transplantation of the disconnected eye led Sperry to propose a theory of neuronal specificity based on embryonic cytochemical differentiation of individual neurons in the retinal and tectal fields with locus-specific affinities between the corresponding primary and secondary neurons (1943, 1944, 1951, 1965). A radical departure at its inception, the chemoaffinity theory received wide support from behavioral, anatomical and physiological studies, after regeneration of the optic nerve in mature organisms (Gaze, 1959; Maturana, et al., 1959; Attardi & Sperry, 1963; & Gaze & Jacobson, 1963), and in developing embryos in which locus specificities between retinal and tectal cells have been demonstrated to exist even before the initial outgrowth of optic axons from the retina (DeLong & Coulombre, 1965; Jacobson, 1968; Hunt & Jacobson, 1972a;

1972b; 1973; 1974; & Crossland et al., 1974).

Along other dimensions, it was noted that many aspects of adaptive behavior required refined patterns of connections and it was suggested that the notion of cytochemical differentiation must be extended to levels beyond locus specificity to include cytospecific affinities for functional cell types (Sperry, 1951; 1965). The prediction is largely borne out by results showing selective reconnection of specialized cell types after regeneration of the optic nerve in lower vertebrates (Maturana et al., 1959; Arora & Sperry, 1963; & Keating & Gaze, 1970).

Integrated behavior is dependent upon the orderly response relationship of peripheral organs and complex neural networks of the central nervous system. Somehow, all sensory and motor systems must form orderly associations both centrally and peripherally. Evidence that cytodifferentiation of extra-neural tissue (Sperry, 1951), similar to that proposed for the nervous system comes from studies showing preferential affinity of motoneurons for their own muscles (Sperry & Arora, 1965; Marlotte & Mark, 1970; & Grimm, 1971), and the orderly reestablishment of cutaneous reflex patterns after regeneration (Sperry, 1951).

The following work deals with some rather divergent aspects of specificity in nerve growth. Because of the above indications that specificity can be manifested in extremely subtle and diverse forms, the question of whether the existence of cytochemical specification of nerves from the left and right sides of the body could account for the complex patterning of anatomical and functional structures on either side of the midline was investigated in the first experiment. The other two studies deal mainly with the functional aspects of the exceptional plasticity indicated in the retinotectal system which has recently created renewed controversy over the developmental mechanisms involved in the formation of patterned connections. Each experiment is preceded by its own introduction.

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II. TESTS FOR LEFT-RIGHT CHEMOSPECIFICITY IN FROG CUTANEOUS NERVES*

Whether the cytochemical differentiation of the nervous system involved in regulating the developmental patterning of nerve fiber pathways and connections includes lateral chemical specification of bisymmetric structures on left and right sides has yet to be determined. The possible existence of some form of left-right biochemical specificity is suggested by the discriminative precision with which fibers of the mammalian optic nerve divide at the chiasm, and many other patterns of fiber decussation in central nerve tracts; the innate cerebral asymmetry in man (Geschwind & Levitski, 1968; Witelson & Pallie, 1973), the lateralization of song control in avian species (Nottebohm, 1971) and certain other anatomical asymmetries like that of the habenular nucleus in anura (Braitenberg & Kemali, 1970; Morgan _et al_., 1973, Scott, 1973).

On the other hand, many studies on nerve development and regeneration involving left-right interchanges have failed to disclose evidence in support of such specificity (Sperry, 1945, 1951; Sperry & Miner, 1949; Hibbard, 1965; Gaze, 1970, Jacobson, 1970). In particular, the readiness

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*This work has been published by M. Y. Scott and R. W. Sperry (1975) in _Brain, Behavior and Evolution_, 11:60-72.

with which surgically transposed right and left nerves have been found to connect to mirror structures on the wrong side, both in the centers and periphery has seemed to point to the absence of lateral specification. Such specification is not required to account for the aforementioned anatomical asymmetries of the brain which can conceivably be explained in other terms, as can also the selective decussation at the midline (Sperry, 1963).

In view of the foregoing, the report of Franzisket (1959) describing competitive cutaneous reinnervation experiments in frogs with nerves from left and right sides is of outstanding interest in that it has been taken to present a definite demonstration of lateral specificity. Ipsilateral and contralateral nerves, after being cut and surgically deflected into the same cutaneous field, were observed to display differential growth effects depending on the laterality of the competing fibers. In other instances, fiber outgrowth was found to be severely retarded when inserted into neural tissue of the opposite side. The present study was an attempt to confirm Franzisket's findings. Additional related observations on the growth behavior of nerves deflected into foreign cutaneous environments are also included. In general, the present findings on Rana pipiens fail to confirm the earlier results of Franzisket (1959) on the closely related Rana esculenta. A brief presentation of the findings was made earlier

(Scott, 1972).

General Procedures

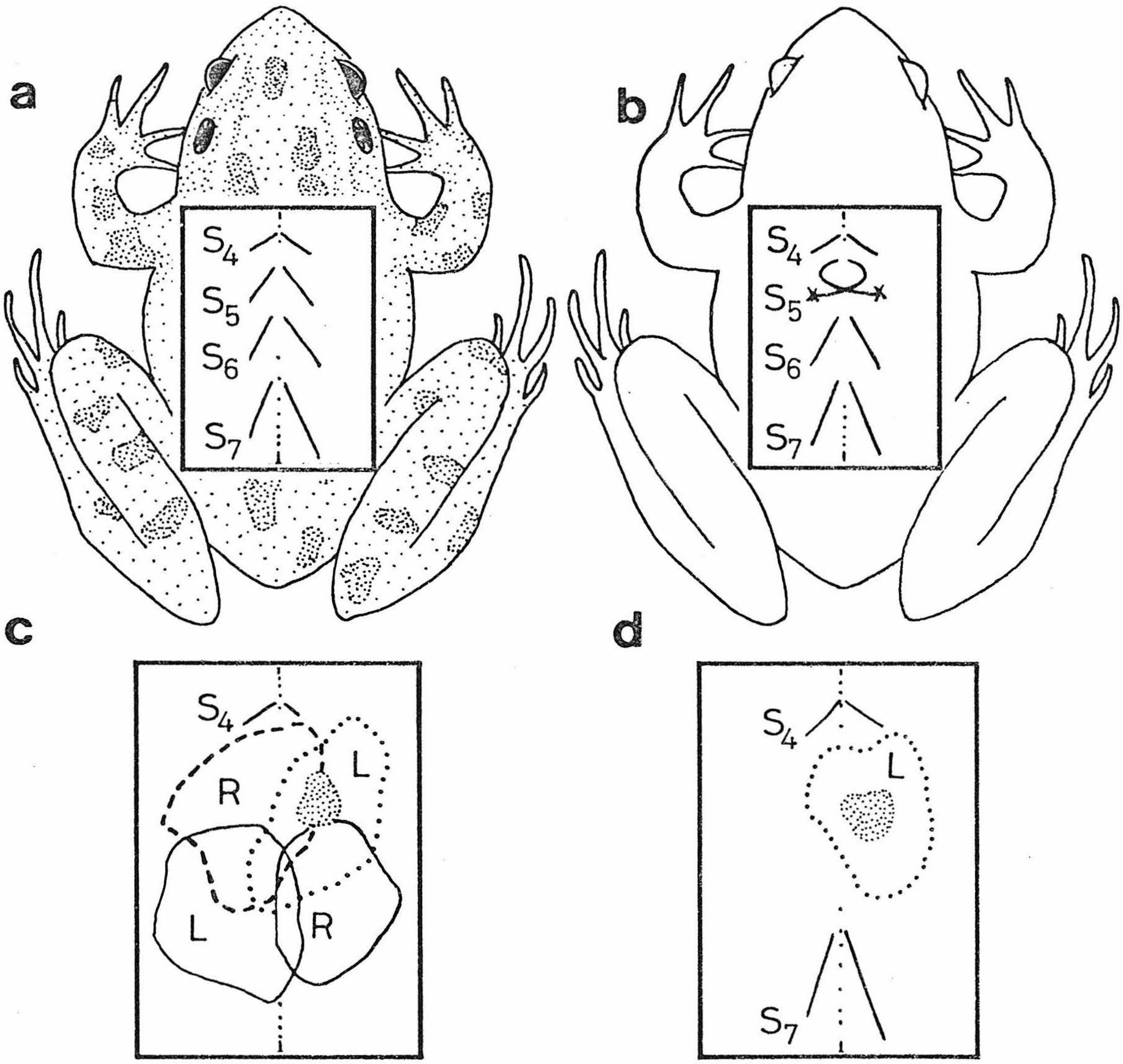
Adult *Rana pipiens* 7.5 - 10 cm in length were used throughout. The medial rami of the dorsal cutaneous nerves of this species grow out of the fascia close to the midline and lie rather loosely in the dorsal lymphatic sac (fig. 1a), a feature facilitating lateral crossing of cutaneous nerves. There was no apparent abnormal stretch on the nerves as a result of their being deflected into contralateral cutaneous fields. Adrian *et al*. (1931) estimated that each of these nerve branches is comprised of about 50 sensory fibers. When describing surgical and electrophysiological recording procedures in the present investigation, we refer to whole nerve bundles and not to individual fibers.

Surgery was performed with the aid of a low-powered binocular dissecting-scope. Each animal was anesthetized in a glass container with ether vapor prior to surgery. After operation, the animals were housed three to four in 5-gallon terraria and maintained on a diet of live meal worms offered three times a week.

Drawings traced from enlarged photographs of each specimen showing cuticular pigmentation, lateral ridges and other distinguishing features were marked with stimulation points. Wiping reflexes were elicited by touching or local stroking of the skin with thin bristles exerting 1 - 4 gm pressure. Behavioral responses were recorded two to three times a week after the onset of the first response signalling contralateral innervation of the skin and were continued until the time of electrophysiological recording.

Compound action potentials were recorded from the cutaneous nerves monopolarly with platinum electrodes during tactual stimulation. Each nerve was lifted out of the skin through a small transverse slit at the midline and mounted on the recording electrode. The reference electrode was attached to the forelimb of the animal. Signals were amplified and displayed on an oscilloscope with simultaneous audiomonitoring. During recording sessions the animals were anesthetized with ether and also paralyzed with an intramuscular injection of 50 μ of <u>d</u>-tubocurarine. The animals were reanesthetized at the first indication of arousal.

Fig. 1. (a) Dorsal cutaneous rami of spinal nerves (4-7) shown in cutout as they appear after emergence from dorsal musculature. (b) Reciprocal cross attachment at S5. (c) Detail showing typical regenerated fields of crossed nerves (dashed & dotted lines) and adjacent S6 normal nerves as mapped electrically. Stippled area represents behaviorally responsive region of left (contralateral) hindlimb prior to electrical mapping. Analogous region for right nerve has been omitted for simplicity. (d) Behaviorally responsive region of crossed left nerve from same frog before (central stippled area) and after elimination of overlapping innervation.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Experiment 1: Reciprocal Cross of Dorsal Cutaneous Nerves

Surgery

The dorsal cutaneous nerves of the midtrunk were exposed bilaterally through a longitudinal midline incision. The dorsomedial rami of left and right spinal nerve V were then sectioned distally where they enter the integument and the freed ends were crossed to a symmetrical region on the opposite side of the back (fig. 1b). In one group of five animals, the cut ends of the nerves were sewn to the subcutaneous fascia with surgical silk (60 μ diameter). In another group of six, the nerves were anchored with a tungsten needle in the intended position while the deflected skin was carefully replaced, after which the needle was withdrawn. A similar surgical procedure was performed in a third group of 10 animals except that the freed tips of the crossed nerves in these cases were inserted intradermally with a fine tungsten needle into the inner layer containing the nerve plexus and blood vessels.

Results

No difference in reinnerative efficacy was noted for the two nerve-cross procedures used in the first two groups. In both, the misdirected wipe responses began to appear among the residual ipsilateral responses in the reinnervation area on about the 15th postoperative day. The area yielding crossed responses enlarged gradually to cover an average area of 0.18 cm^2 where it stabilized toward the end of the fourth week in the eight cases that survived, four from each group. In the third group of animals, the crossed responses were obtained earlier, the first ones appearing as early as 1 week after operation. Also, by the end of the fourth week, in all eight cases that survived, the region evoking crossed responses had spread over an area slightly larger than in the previous two groups averaging 0.28 cm^2. Ipsilateral responses were interspersed among the contralateral responses throughout the reinnervated regions.

The dimension of the sensory fields for the crossed and neighboring uncrossed dorsomedial rami were compared by electrical mapping in eight (four total from groups 1 and 2 and four from group 3) of the 16 survivors 5 - 7 weeks after surgery. The extent of the crossed fields mapped electrophysiologically were found in six of these eight frogs not to differ significantly from those of adjacent rostral and caudal ipsilateral dorsomedial rami (fig. 1c). In the remaining two cases, the regenerated cutaneous fields were approximately one-half to three-fourths as large as the adjacent ipsilateral nerves. Although contralateral reflexogenous zones of group 3 animals were noticeably larger than those of groups 1 and 2, reinnervated regions did not differ in size between the groups when measured electrophysiologically. Contrary to the conclusion from previous studies that fibers of opposite laterality exhibit mutual repulsion in growth, extensive overlap of cutaneous fields of indigenous and deflected contralateral nerve bundles was evident in all the present cases.

As can be seen in figure 1c, the area innervated by the transposed nerve mapped electrically was considerably larger than the same area mapped by behavioral reactions. This discrepancy can conceivably be explained on the basis that reinnervation was insufficient or somehow substandard in outer areas for effective spatial and/or temporal summation in the firing of central neurons; or that some form of central inhibition (activated, perhaps, by simultaneous stimulation of ipsilateral fibers) suppressed the latent behavioral response.

Isolation of Reinnervated Zones

The following procedure was undertaken in an effort to test some of these possible alternatives. All innervation save that from the crossed nerve was eliminated from a large section of reinnervated skin in each of the remaining eight frogs, by making an incision through the skin surrounding the area of the test nerve and transecting the adjacent ipsilateral nerves. Behaviorally responsive areas determined in this manner with overlapping ipsilateral innervation eliminated were much expanded and were comparable to those mapped electrophysiologically. Figure 1d shows reflexogenous zones mapped from the same animal just before and a day after surgical isolation of the crossed nerve. The results imply that the full extent of the reinnervated region as determined electrically was potentially functional also for behavioral responses. Apparently the intermixed ipsilateral fibers in the periphery of the reinnervated area were responsible for preventing behavioral manifestation by some mechanism of reciprocal inhibition.

Spinal Section

The competitive reinnervative effects described by Franzisket (1959) were obtained in spinal animals. It seemed unlikely, but not impossible that the disparity between his results and those reported here were attributable to the effects in his animals of the spinal transection. Accordingly, the spinal cord was severed in two additional animals at the level of spinal nerve I, between the first and second vertebrae, on the 30th day after nerve crossing. Mechanical stimulation and application of 10% AcH to the skin 0.5, 4, 24, 48 and 72 hours after spinal surgery failed to elicit satisfactory localizing responses although the simpler extensor thrust was well executed. Cutaneous stimulation also failed to produce good localizing wipe responses in six normal animals after spinal section.

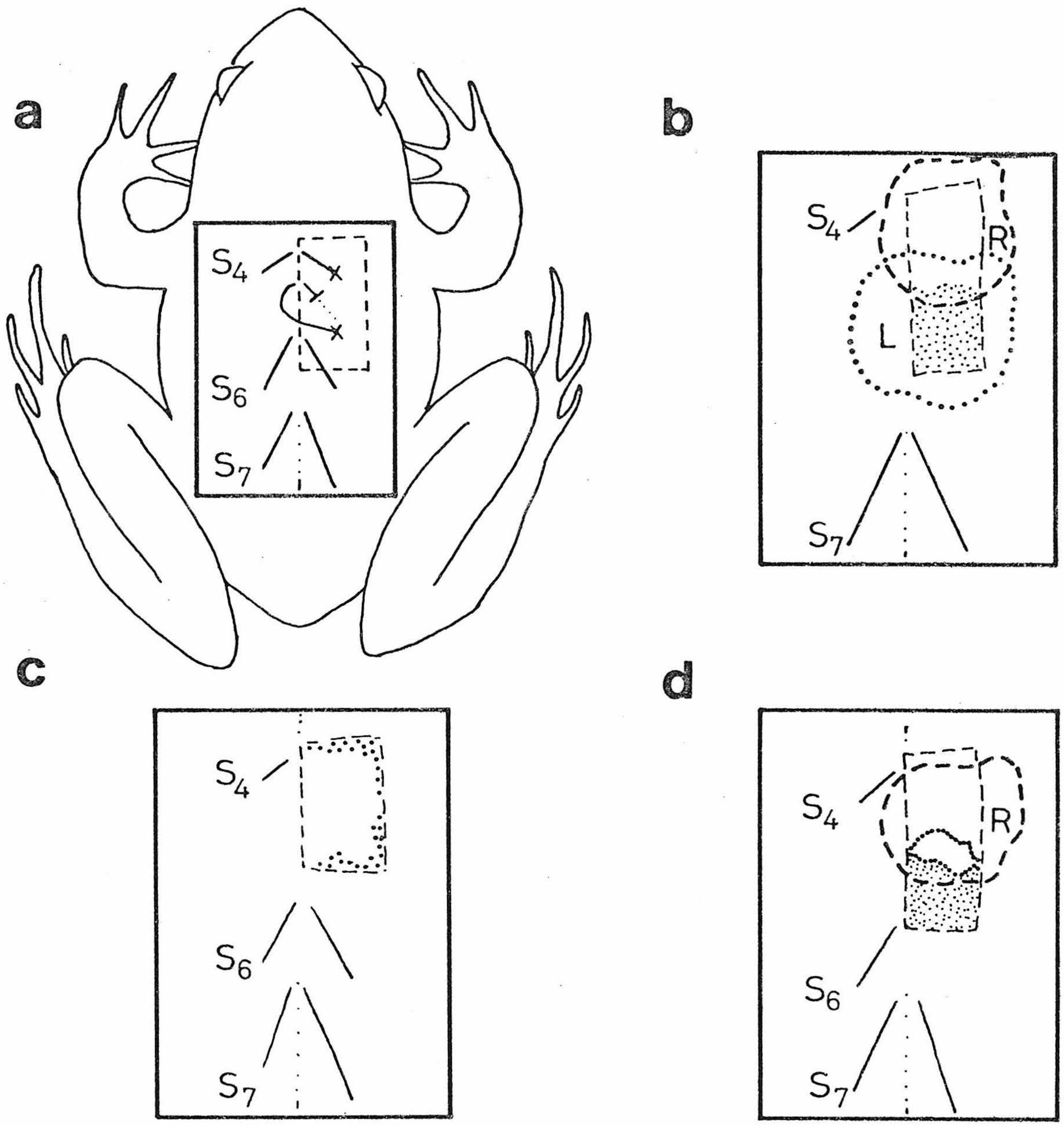
On the third day after spinal surgery, electrophysiological mapping of the transposed and adjacent nerves showed cutaneous receptive fields of these frogs to be essentially identical to those obtained in animals without spinal section. The condition of the spinal animals progressively deteriorated and precluded further testing and the animals were given a lethal dose of ether on the third day.

Experiment 2: Competitive Reinnervation of Denervated Skin by Nerves of Opposite Laterality

Surgery

An incision through the skin was made unilaterally in seven frogs and extended with scissors to isolate a rectangular area of skin approximately 1.1 x 2.0 cm covering the dorsolateral trunk between midline and lateral ridge and including the insertion points of the fourth and fifth spinal nerves. The incised skin was elevated and the two attached cutaneous nerves were transected where they enter the skin. The anterior nerve was rejoined to the center of the rostral half of the denervated patch. Instead of reconnecting the posterior nerve, the corresponding contralateral nerve trunk was freed through the same incision, sectioned distally and crossed over to the caudal half of the denervated rectangle where the cut end was inserted intradermally near the middle of the caudal half. The incised skin patch was then carefully replaced in its original position (fig. 2a). The frogs were kept on a moist pad until the reimplant seemed secure before being returned to the home terraria. Two frogs had to be discarded because the patches failed to adhere. Of the remaining five, the reimplants healed without complication. The aim of the surgical procedure was to determine the effect on reinnervation of having a contiguous competitive

Fig. 2. (a) Initial operation: section and reattachment of right nerve S4 to anterior half of circumsected skin patch (dashed line), removal of right cutaneous nerve S5 and its replacement by contralateral S5. Different features of resultant reinnervation from three separate animals are shown in b, c, & d. (b) Electrophysiological maps for right (dashed line) and left (dotted line) regenerated nerves. Stippled area represents region of contralateral behavioral responses prior to electrical mapping. (c) Peripheral stippling indicates residual ipsilateral responses after section of S4 & S5. (d) Dashed line circumscribes region reinnervated by ipsilateral S4 as mapped electrically. Stippling shows reflexogenous zone of regenerated contralateral S5 prior to section of ipsilateral S4. Dotted line indicates expansion of reflexogenous zone after section of ipsilateral S4.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cutaneous field supplied with regenerating sensory fibers of opposite laterality.

Results

Behavioral testing was carried out daily until the onset of the first responses, then on every second - fourth day until the time of electrophysiological mapping. Sensitivity to tactile stimulation within the isolated rectangle began to return in all cases 11 - 12 days after surgery. Normal and misdirected responses appeared at about the same time from rostral and caudal zones respectively, and the sensitive area spread to fill the incised area by the 30th day. Contralateral reflexes remained confined to caudal portions of the patch, and normal reflexes were generally restricted to the rostral portion (fig. 2b) except for occasional points along the caudal and caudolateral incision lines that presumably resulted from ingrowth of fibers from outside the incision boundary. Contralateral and ipsilateral behavioral responses were not intermixed within the incised skin patch except for the above mentioned peripheral regions. Contralateral responses were occasionally elicited from points caudal to the incision line in one case.

Electrophysiological mapping was carried out on two of the surviving four frogs 5 weeks after surgery. The results showed that the two test nerves had each partially invaded the territory innervated by the other to form a common overlapping field (fig. 2b). As can be seen in figure 2b, both ipsilateral and contralateral nerves were found to extend beyond the incision scars to reinnervate regions occupied by adjacent nerves. After the recording sessions were completed, both nerves were cut and behavioral mapping was repeated the next day for residual responses. The stippled area in figure 2c represents the area from which wipe reflexes were obtained--all with the ipsilateral limb. The results confirm the assumption that ipsilateral fibers from adjacent tissue had grown across the incision line. Although the terminal fields of the two regenerated nerves, one from each side of the body, overlapped extensively by electrical mapping, the behaviorally reactive zones for ipsilateral and contralateral limbs had been clearly demarcated, excepting for the above mentioned regions immediately adjacent to the incision lines. Unlike the results reported in the first experiment, ipsilateral responses were not interspersed within the main body of contralateral responses.

The remaining two frogs were used to map the extent of the regenerated field of the anterior nerve electrophysiologically. After this was completed, the rectangular patch was reincised and the ipsilateral nerve was ablated leaving intact the surgically crossed posterior nerve as the sole innervation to the incised area. Testing of localizing responses the next day revealed that terminal fibers of the contralateral nerve extending into the overlap territory formerly occupied also by the ipsilateral anterior nerve were sufficient to trigger wiping reflexes (fig. 2d). Figure 2d also shows the regions from which these misdirected responses were elicited from the same animal immediately prior to the electrophysiological recording session.

Experiment 3: 180° Rotation of Skin Patch

Surgery

A rectangular piece of skin 2.0 x 2.2 cm extending the width between the lateral ridges and encompassing the cutaneous fields of the dorsomedial rami of the fourth and fifth spinal nerves was cut free, rotated 180° and replaced leaving the two pairs of attached nerves intact.

Results

Mechanical stimulation of the rotated patch elicited contralateral wiping reflexes on the day following rotation. The reversed reflexes were obtained consistently from the patch until the attached nerves were sectioned 1 - 3 months later. From the 26th day onward, normal reflexes were occasionally elicited along border regions presumably a result of fiber ingrowth across the incision scars. Following section of the attached nerves at either 32 (two frogs) or 96 (two frogs) days after operation, appropriate ipsilateral responses were readily elicited 2 - 3 mm inside all borders of the rectangle. Again it was evident that ipsilateral cutaneous fibers had invaded and functionally innervated cutaneous regions of opposite laterality that already were predominantly supplied by their original contralateral fibers.

After section of all medial nerve branches suppling the rotated skin reactive areas were found to persist along the lateral regions of the patch and were similar to those found in normal animals after elimination of the same medial cutaneous nerves. These medial branches of the dorsal cutaneous rami constitute the main nerve supply of the dorsal integument. However, the dorsolateral branch, another division of the dorsal cutaneous ramus, innervating the adjacent side skin around the lateral ridge, extends medially to overlap somewhat with the dorsal medial nerves. It was evident that even fibers of the dorsolateral branches had reinnervated the foreign contralateral patch with what appeared to be normal terminal connections.

There was no indication that regenerating neurites of either dorsomedial or dorsolateral branches of the dorsal cutaneous rami were inhibited in their growth as they advanced across the incision into contralateral skin to make functional connections in areas already innervated by neural tissue of the opposite side.

Experiment 4: Unilateral Denervation of the Dorsal Trunk

It is conceivable that nerve rami originating on one side of the midline restrict their innervation fields mainly to the same side because of some growth limitations, e.g., a maximum length particular fibers can attain. To test this hypothesis, a major portion of the dorsum was unilaterally denervated by sectioning the innervating dorsocutaneous nerves to see if contralateral fibers would be stimulated to cross the midline and extend abnormal distances into the opposite side.

Surgery

A longitudinal incision about three dermatomes long was made along the right lateral ridge in eight frogs. Transverse cuts to the midline at both ends of the rostrocaudal incision produced a rectangular skin flap which was folded back to expose the medial dorsal cutaneous nerves. All nerves connecting with the flap as far distally and proximally as possible were sectioned and removed after which the denervated flap was replaced.

Results

Behavioral mapping carried out several hours after operation showed that the reflexogenous fields of the left dorsal cutaneous rami extended slightly beyond the midline and were similar to those previously determined electrophysiologically in normal animals. Between 21 and 25 days after unilateral denervation it was possible to elicit contralaterally misdirected cutaneous localizing reflexes with the left limb from most of the previously denervated flap on the right side. The exception was a 2 mm strip along the lateral ridge where ipsilateral responses predominated and some remaining insensitive loci were occasionally encountered. Recutting around the flap and redividing any nerves from the right side which were found to have regenerated eliminated all ipsilateral responses. Unresponsive regions were again observed along the lateral margin after this second operation.

After another period of 3 - 4 weeks, electrophysiological recording from the left midline cutaneous nerves indicated that two of every three nerves from which recordings were made had expanded their terminal fields across the midline all the way to the opposite lateral ridge to cover the entire dorsum. Behaviorally unresponsive zones remained along the lateral ridge even though electrophysiology showed that some of these regions were indeed innervated.

Discussion

No evidence was obtained in the foregoing that would demonstrate the existence of lateral cytochemical specificity. In all cases regenerating fibers of left and right cutaneous nerves appeared to grow with equal readiness into overlapping fields of neighboring nerves regardless of fiber laterality. The differential inhibition of growth exerted by fibers of one laterality on fibers from the opposite side as described earlier by Franzisket (1959) under very similar experimental conditions was not found in this investigation.

The disparity between Franzisket's results and those obtained here remains to be explained. As far as can be ascertained, surgical and behavioral testing methods employed in the two studies were similar. Franzisket, however, used chronic spinal animals while the spinal animals used in the present investigation were acute preparations. Perhaps this factor was critical though it seems unlikely as does the possibility that such closely related species as R. esculenta and R. pipiens would have developed such a difference for the patterning of peripheral connections.

The appropriate pattern of cutaneous innervation at the midline can conceivably be accomplished during development without reciprocal lateral repression of fiber outgrowth and in fact may argue against such repression. In normal frogs there is considerable right-left overlap of cutaneous fibers at the midline although this midline overlap is by no means of the magnitude found between adjacent dermatomes. One might ask how cutaneous fibers normally are restricted from growing more extensively across the midline since Experiment 4 has shown that fibers from one side do have the potential for innervating the entire dorsum. The answer remains to be determined but may conceivably be found in the interplay of such factors as timing of fiber outgrowth, the tendency of growing fibers to fill nearest areas first, limitation on number of active terminals per fiber, maximum density of terminal sites per unit area, and the like, without need to invoke lateral chemospecificity.

The present results are consistent with numerous surgical rearrangement experiments that have indicated an equal interchangeability of neural structures from one side to the other under many conditions. None of these experiments, however, including the present, firmly exclude the possible existence of a left-right specificity that may yet be revealed by different kinds or more refined tests. Recent findings on neonatal rats and hamsters by Lund and Lund (1973) and Schneider (1973) during the course of the present investigations seem suggestive but not definitive and one can only conclude at this point that the question still remains open.

Since left and right cutaneous nerves normally overlap somewhat across the midline the observed absence of misdirected reflexes along the midline and their presence after ipsilateral denervations requires some explanation. The function of those fibers that have crossed the midline must be suppressed or inhibited in some way, presumably through concomitant stimulation of overlapping ipsilateral fibers. Differential density of fiber terminals from the two sides in conjunction with central reciprocal inhibition of the weaker of the two conflicting resppnse tendencies offers a plausible basis by which the spinal centers determine ipsilateral vs. contralateral responses.

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III. BEHAVIORAL TESTS OF COMPRESSION OF RETINOTECTAL PROJECTION AFTER PARTIAL TECTAL ABLATION IN GOLDFISH*

Recent studies indicate an exceptional plasticity in the retinotectal projection in goldfish (Cook & Horder, 1974; Gaze & Sharma, 1970; Sharma, 1972a; Sharma, 1972b; Yoon, 1971; & Yoon, 1972). After removal of the caudal half tectum, the normal topographic projection from the retina, which is regularly restored on the tectum after section and regeneration of the optic nerve (Attardi & Sperry, 1963; Gaze, 1970; Hunt & Jacobson, 1974; Jacobson, 1970; Sperry, 1944; & Sperry, 1951), was found to be reinstated in complete but compressed form on the residual rostral half tectum. This orderly compression of the retinal projection onto half tectum in goldfish has since been confirmed by many studies and with a number of surgical variations. To achieve the compressed map, considerable readjustment of original connections seem necessary. The underlying mechanism by which the plastic remapping occurs and the extent to which the projection may be

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*Scott, M. Y., <u>Experimental Neurology</u> (1976). In Press.

functional through tectum and efferent system remains to be determined.

Evidence for the compression comes primarily from electrophysiological studies in which elaborate arguments have been presented that the mapped potentials are from optic axon terminals rather than from tectal cells or axons coursing across the tectum (Gaze & Sharma, 1970). It has been assumed almost without question that synapsis occurs although possible postsynaptic recordings have been reported by only one investigator (Yoon, 1975). Despite absence of direct physiological evidence for synapse formation, evoked tectal potentials, in the context of retinotectal plasticity, have generally come to be translated into "presynaptic terminals" (Hunt & Jacobson, 1974).

The aim of the present investigation was to determine whether the compressed projection is behaviorally functional. A visually mediated conditioned respiratory response was utilized as an assay for function at various intervals following removal of the caudal half tectum, with optic nerve left intact. The results show a gradual behavioral recovery of the conditioned response from over the entire visual field. A preliminary report has been published (Scott, 1975).

Materials and Methods

Surgery. Goldfish (*Carassius auratus*) 6 to 7.5 cm in body length were housed in glass aquaria under a 12-hr light: 12-hr dark schedule. For surgery, fish were anesthetized in a .05% solution of tricaine methanesulfonate (Finquel, Ayerst) and the tectum exposed through a reflected cranial bone flap. The caudal half tectum was separated from the rostral half by an incision, down to the ventricle, made with an electrolytically sharpened tungsten knife. The caudal half was then lifted out by aspiration and teased away from the brain and the cranial flap wedged back into position. The optic nerve was left intact in all cases. Operated fish were quickly revived by infusion of water over the gills and recovered without apparent motor deficits. Fish were anesthetized and the extraocular muscles torn away from the globe with jewelers forceps prior to mapping sessions.

Apparatus. Fish were lightly anesthetized then immobilized, by securing an elasticized strap wrapped around the body, to a clay block mounted on a mechanical manipulator in a water-filled tank 20 x 40 cm adjacent to a 46 cm water-filled hemispheric dome (Meyer, 1974; Schwassmann & Kruger, 1965). In an effort to reduce reflective images, the interior surface of the hemisphere was sandblasted except for a 3 cm circular region at the apex which was left clear. The interior of the tank was lined with flat-black closed-cell neoprene except for a 1.2 cm round peephole left uncovered, to expose the test eye.

The transilluminated optic disc was visualized through the peephole and aligned along the central axis of a perimeter circumventing the hemisphere at the equator. Differential recording electrodes were positioned behind the gills contralateral to the test eye, and just in front of the mouth, with the ground embedded in the clay block behind the fish. Respiration was visually displayed on a Beckman Dynograph Recorder with simultaneous audiomonitoring.

Training and Testing Procedure. A visual discrimination task was selected in these mapping experiments to determine whether the compressed projection, if functional, were transmitting useful information. A conditioned respiratory response technique was developed as a behavioral measure because the procedure readily allows sequential mappings from the same animal, without undue damage, and at the same time enables sufficient immobilization of the fish to accurately map the visual field.

Differential suppression of respiration to the 5 sec presentation of a red or green flashing light was accomplished by pairing one of the colors (CS+) with an electroshock to the tail. The shock, delivered through stainless steel electrodes positioned on either side of the tail, was adjusted to an effective value for each fish individually, and ranged from 15-50 microamps for 200-500 ms. In order that the stimuli be presented to the same point in space, a Y-shape fiber optics light pipe was used. The stem of the optical conduit was terminated at a mount on the perimeter, forming a light source of 1.5° visual angle. The mount then could be slid through 180° of the perimeter. The lights were equated for brightness according to Yager (1967).

Fish were habituated in preconditioning sessions by presenting the stimuli (20 trials each) without shock. Most fish habituated within the first five presentations and some did not respond to either light. It was observed that fish not giving an initial startle response were more difficult to train later.

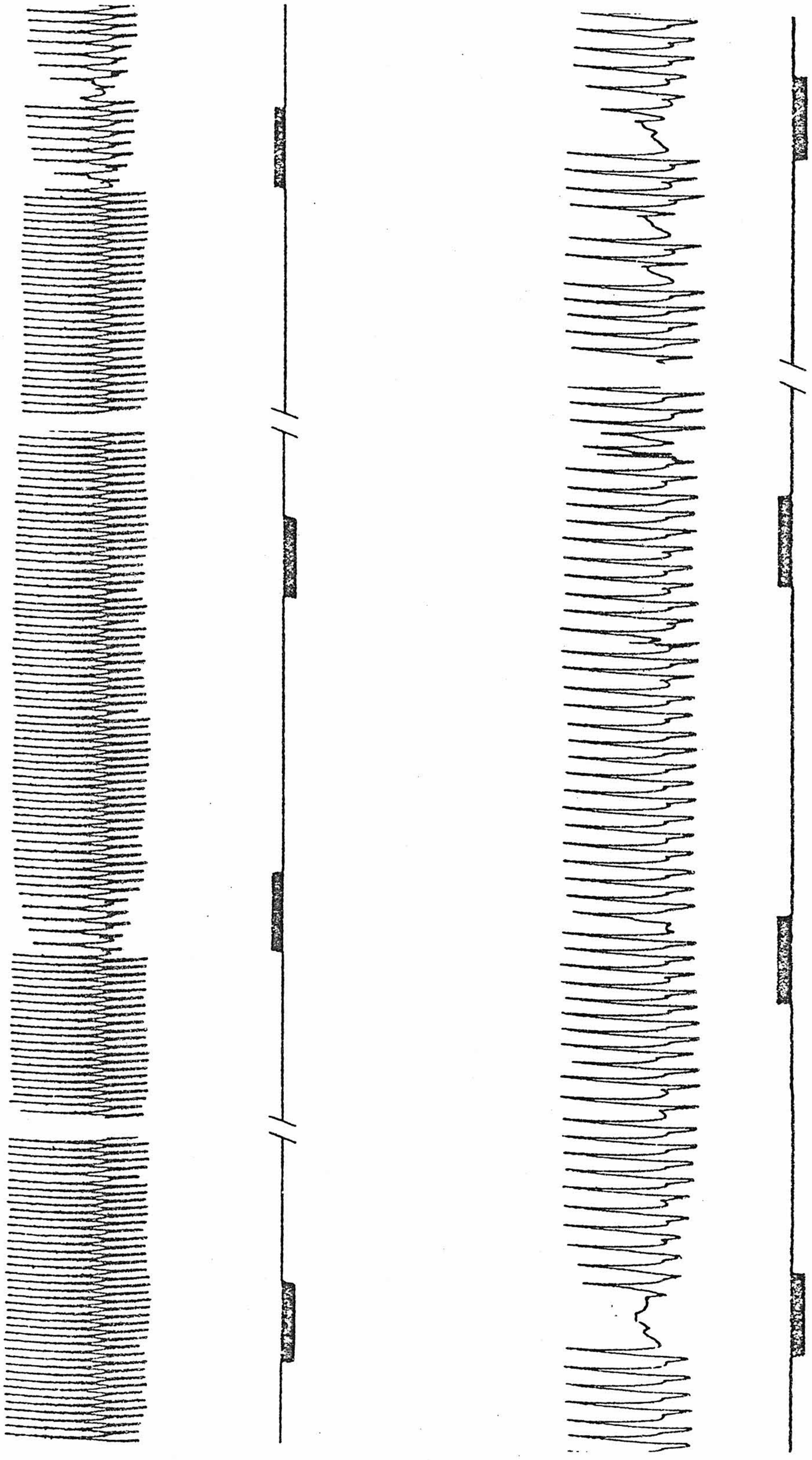
In conditioning sessions, stimuli were presented at 40° in the anterior field. After a shock reinforced trial, a 120 sec recovery period was allowed before the next presentation, and at least 10 min of quiet after each series of five reinforced trials. Following the first block of five continuously reinforced trials, shock was administered intermittently until 20 consecutive responses to the CS+ and less than 20% to the neutral stimulus (CS-) was obtained. As fish generalized throughout the field even when reinforced at only one position, it was not necessary to train at different angles. Some fish initially gave weak responses at extreme posterior positions, but responded vigorously to later presentations at those angles when weak responses were immediately followed by a light-shock trial at the 40° training position. A few early fish were reinforced at several positions in the anterior field during training.

In general, it was more difficult to obtain differential responding when the green light was paired with shock. Although readily reacting to the green light, it appeared difficult to inhibit generalization of the "fear" response to red light (CS-) presentation, which may indicate an innate aversion to the color red. Six fish were discarded because adequate differential responding could not be obtained.

During mapping sessions, discriminanda were presented pseudorandomly at variable intervals ranging from 20-90 sec. The visual field was mapped along the equator by alternating the test angle between anterior and posterior fields. All angles were tested at least twice with 3-5 CS+ each time to give a total of 10 or more trials per angle tested. Test angles ranged from 60° in the anterior field ($+60^{\circ}$) to 80° in the posterior field (-80°) with the optic disc falling on 0°.

As can be seen in figure 3, dramatic suppression of respiration to the onset and sometimes the duration of the positive stimulus makes the conditioned response clearly differentiable from normal prestimulus respiration, whereas, respiration to the neutral stimulus remains unchanged. Responses to reinforced and neutral stimuli were tallied for each angle. Trials were deleted if prestimulus noise interferred with unambiguous scoring of responses.

Fig. 3. Respiratory potentials showing differential suppression to presentation of 5 sec red and green lights in 2 goldfish. Bars above and below baseline denote onset and duration of the two colors.

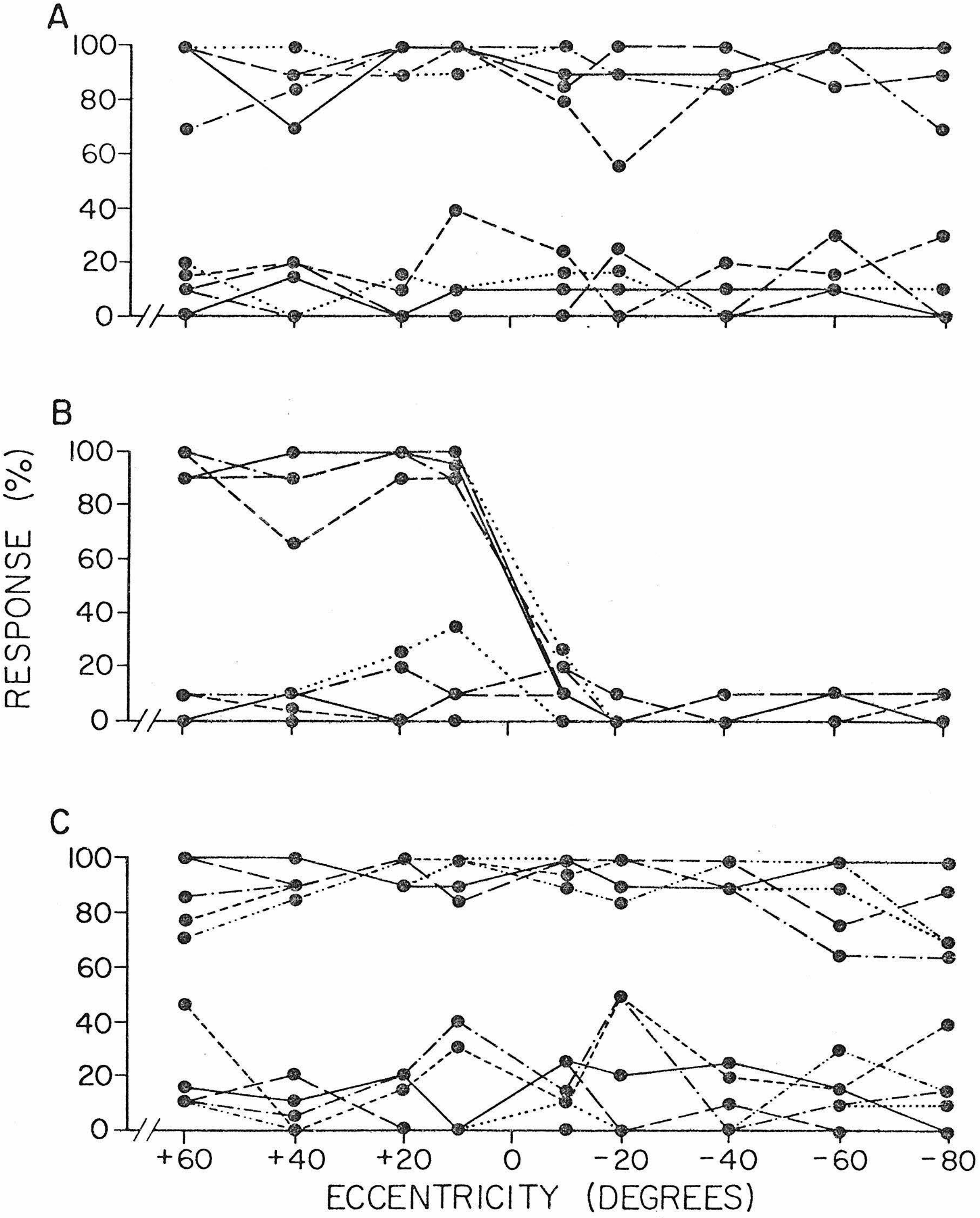


Check for Hue vs. Brightness Discrimination. Following the final training session, two fish (one green CS+ and one red CS+) were given additional trials under varying stimulus brightness conditions. The intensity of the CS+ was decremented in steps of one-half by inserting one or more Kodak neutral density filters (N.D. 0.3) into the light path. Both fish continued responding normally to the CS+ until the intensity was decreased to one-eighth of the original value, where responding ceased altogether. Neither fish switched response preference to the CS- at any of the decreased CS+ intensities. Responding resumed when the CS+ was increased by one-half so that light intensity was brought up to one-fourth of the original value. Varying the brightness of the CS- had no effect on response pattern.

Results

Individual visual field maps of five fish before and one week following excision of the caudal half tectum are shown in figures 4A and B. Differential responding obtained at all angles prior to tectal surgery, shown by separation of the CS+ and CS- curves in figure 4A, indicates good discrimination throughout the field. Following tectal ablation, responding was normal in the anterior field, but fell off to both stimuli at the vertical meridian, indicating a scotoma in the posterior half field (fig. 4B).

Fig. 4. Visual field maps of the left eye obtained behaviorally. The upper set of curves in each graph show CS+ responses, and the lower show CS- responses. $(+)^{\circ}$ = anterior visual field; $(-)^{\circ}$ posterior visual field. (A) Normal fish. (B) Same fish as in (A) 1 wk after lesion of the caudal half of the contralateral optic tectum, showing scotoma in the posterior half field. (C) A different group of fish showing complete restoration of the visual field 8 - 10 mos after removal of contralateral caudal half tectum.

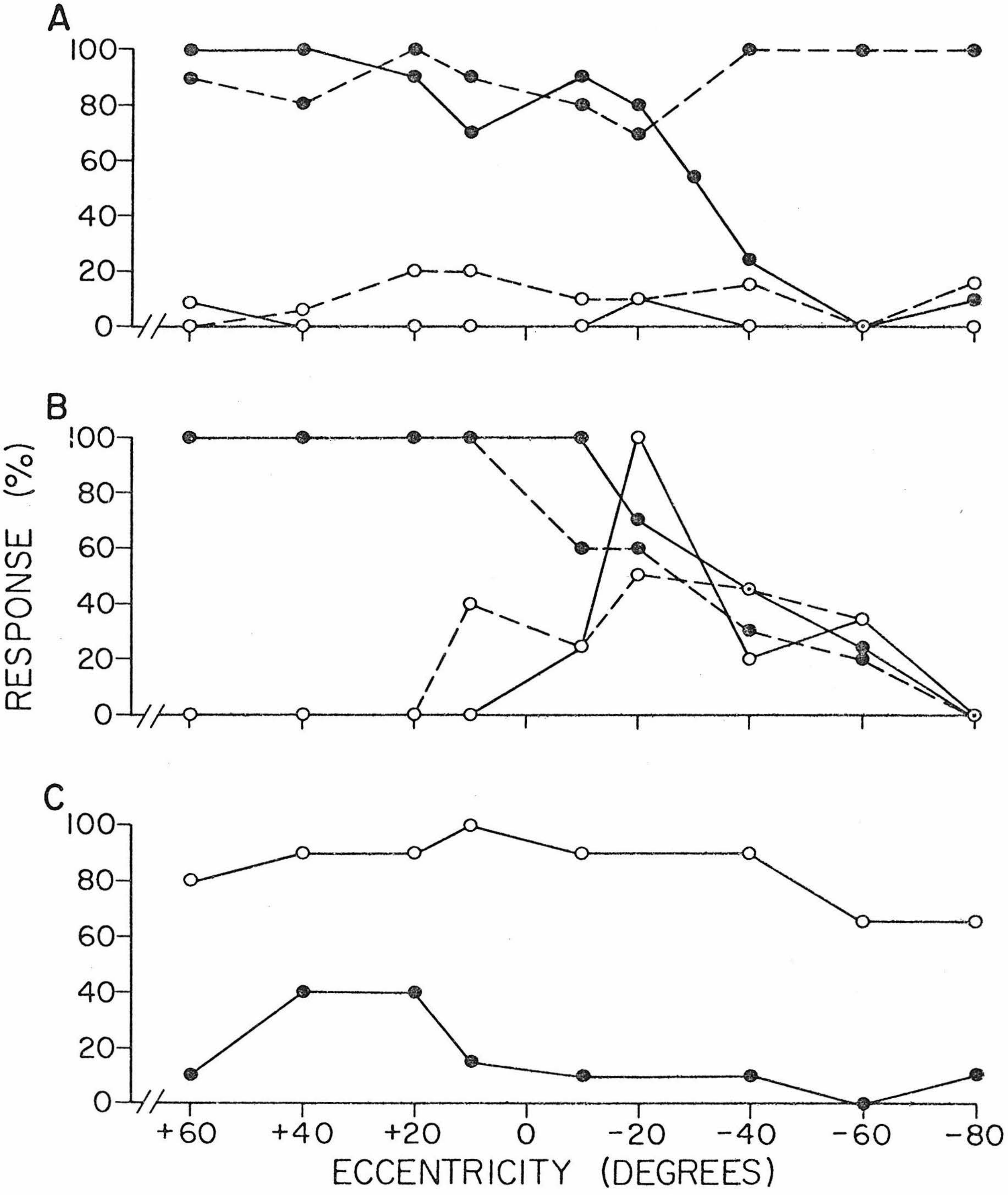

A
B
C
100
80
60
40
20
0
RESPONSE (%)
+60
+40
+20
0
-20
-40
-60
-80
ECCENTRICITY (DEGREES)

Recovery Following Tectal Ablation. Mapping 8 - 18 months after surgery showed recovery of vision in the blind field created by tectal lesion in all eight fish in this series. Recovery maps from six fish (four red and two green CS+) are plotted in figure 4C. Differential responding is evident throughout, and the curves show remarkable similarity to the visual field maps of normal fish in figure 4A. Red-green discrimination does not appear to be impaired after tectal lesion and reorganization of the retinotectal projection onto remaining half tectum.

Two fish, not included in figure 4C, are treated in figure 5. Fish 5A (red CS+) did not show full recovery on the first testing at 532 days with a scotoma remaining in the posterior 40^{o} of the visual field. Retesting the following day, however, showed full visual field recovery. Responses to the CS- were significantly depressed in the first, compared to the second testing, and to those obtained from fish in figure 4C. The diminished responses to the CS- on the first day may have reflected an attention/motivational factor which differentially effected performance in the recovered posterior field, suggesting a possible threshold or other difference in the reconnection between temporal and nasal fibers in the reorganized projection.

Fish 5B (green CS+) showed deficits in the posterior

Fig. 5. (A) Response curves from a fish showing partial (solid line at 532 days postop) and full recovery of the visual field (dashed line at 533 days postop). Red-positive stimulus. (B) Response maps from another fish, 312 (solid) and 320 (dashed) days after surgery showing incomplete recovery of the visual field in both testings. Green-positive stimulus. (C) Same fish as in (B) following reversal training to red as the positive stimulus showing a fully recovered field. See text for discussion.


A
B
C
100
80
60
40
20
0
RESPONSE (%)
+60
+40
+20
0
-20
-40
-60
-80
ECCENTRICITY (DEGREES)

visual field when tested on two occasions, eight days apart (fig. 5B). Vision appeared to be marginal, at best, beyond -20^{o}, with the complete disappearance of differential responding. The unusual increase in responding to the CS- in the posterior field was suspicious, and the fish was subsequently trained on a reverse discrimination task, which he readily acquired. The resultant map, after reversal, is shown in figure 5C and shows full-field recovery, at least for red light. Whether this fish could see green light in the posterior field is unclear, but the results of the two other green-positive fish, included in figure 4C, indicate recovery of vision for green light does occur. Reversal training was attempted on other fish originally conditioned to red to see if a similar scotoma could be produced in the posterior field. These attempts were unsuccessful, however, as differential responding could not be obtained following commencement of shocks to green light.

Time Course of Recovery. In this series, four fish were trained and mapped prior to surgery. Seven days after ablation of the caudal half tectum tests established the extent of the blind field and served as a reference for comparison with subsequent intermittent mappings from the same fish. All fish were found to be blind posterior to the vertical meridian in the initial postoperative test. After three weeks, response maps showed no change in the blind field, but during the fifth weeks, a noticeable expansion of vision over 20-30° had occurred. From the fifth week onward, variability of recovery between fish increased with restoration of vision across the entire field ranging from 84 to 112 days.

Time sequences of recovery for the four fish are shown in figure 6. Responses to the neutral stimulus have been omitted for clarity. Fish 6A, with an initial scotoma in the entire posterior field, gradually recovered vision in the blind field in an orderly anterior-posterior fashion as seen in the sequential mappings at the indicated post-surgical intervals. Response maps from the other three fish (fig. 6B-D) again show an orderly reestablishment of connections from retina to tectum, with the central edge of the scotoma extending gradually into the temporal field. These curves clearly illustrate the regularity of recovery, with expansion always proceeding from center to periphery; no indication of vision was found in the posterior field before restoration in central regions.

Another observation from these data was that color vision returned concommitantly with the first indication of vision. In figure 7, the difference in percent responses to the positive and neutral stimuli, averaged over four fish, are plotted for each indicated recovery interval. These curves are unambiguous in showing immediate discrimination between red and green lights at all angles where vision was first demonstrable. Comparison of figures 6 and

Fig. 6. Sequential response maps from 4 fish (A-D) showing visual field expansion in an orderly center-periphery fashion. Recovery intervals in days. Responses to the CS- have been omitted for clarity.

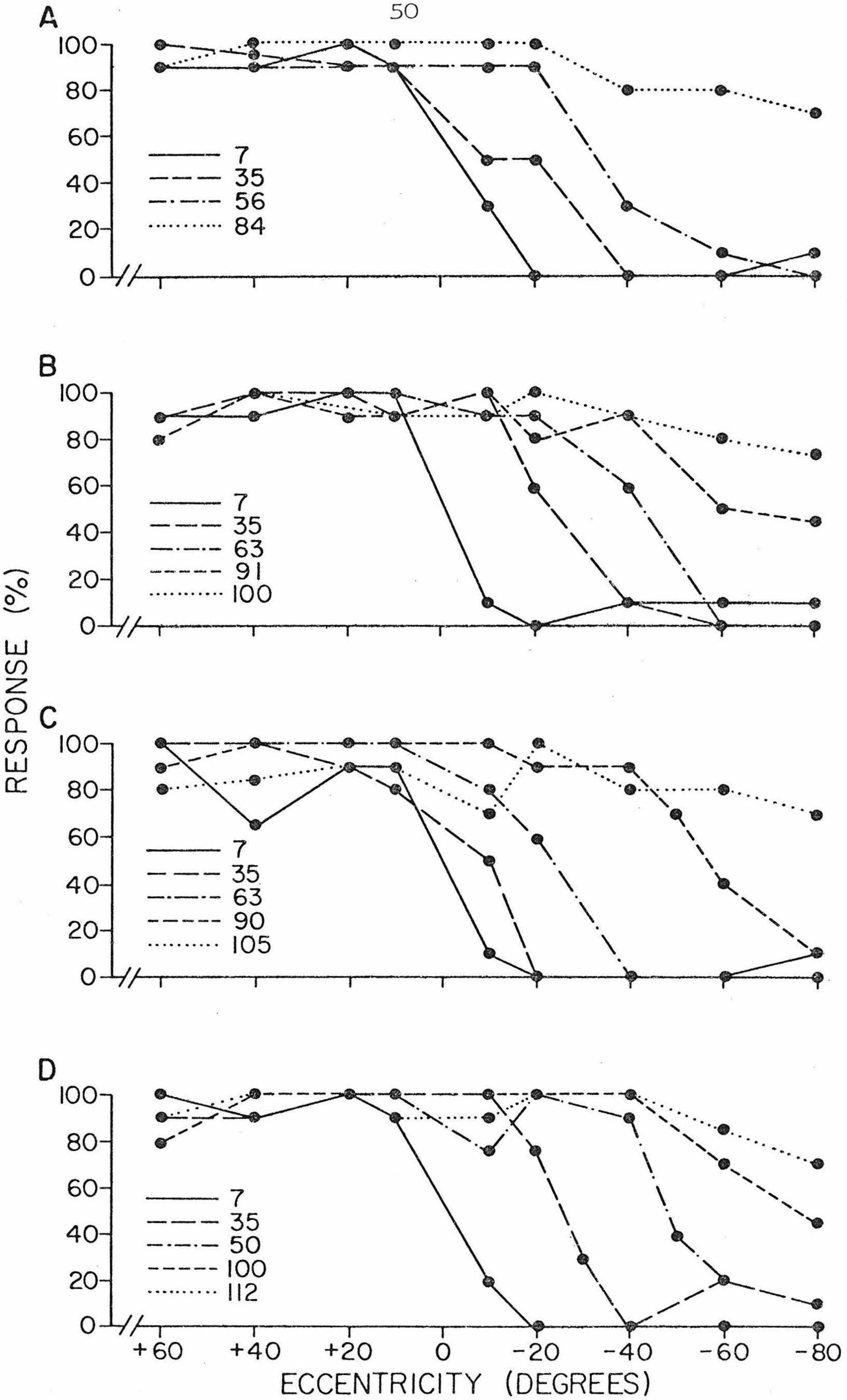
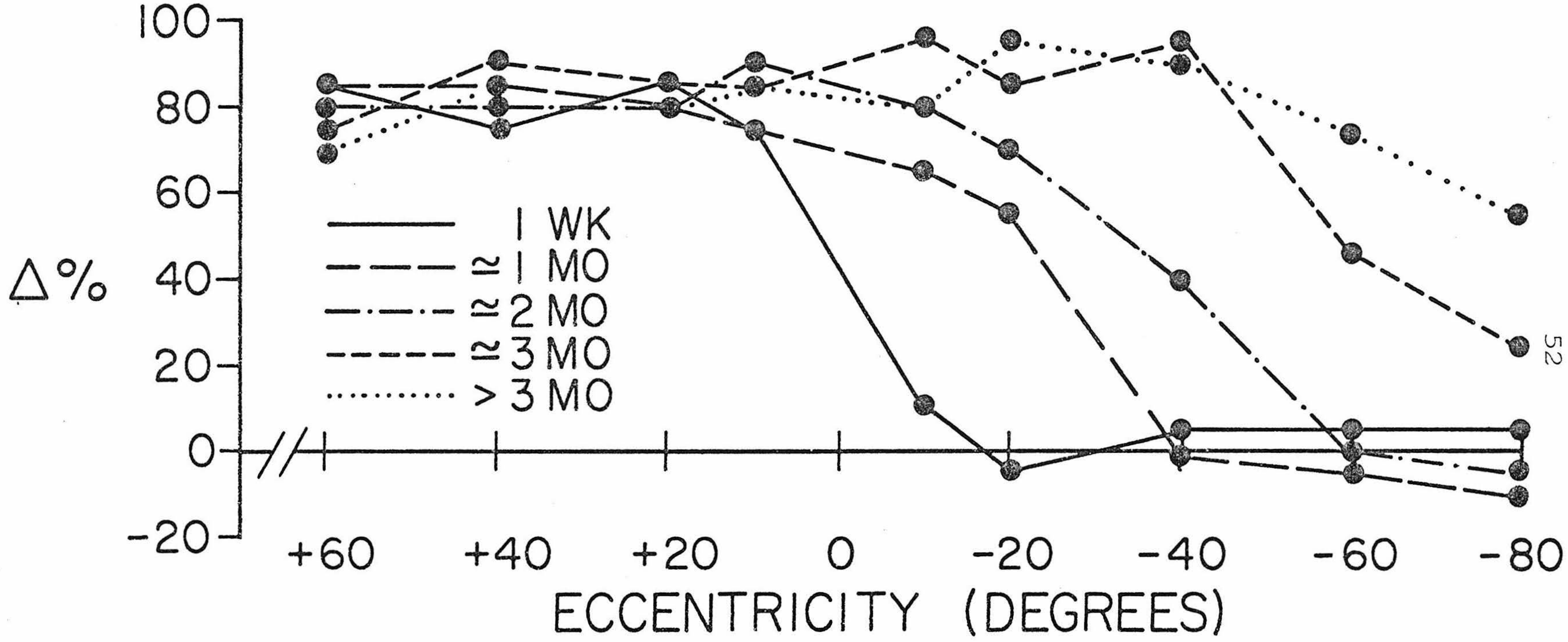

A
B
C
D
RESPONSE (%)
0
20
40
60
80
100
7
35
56
84
7
35
63
91
100
7
35
63
90
105
7
35
50
100
112
+60
+40
+20
0
-20
-40
-60
-80
ECCENTRICITY (DEGREES)

Fig. 7. Differential response curves (percentage response-CS+ minus percentage response-CS-) showing sequential recovery of color vision at the indicated recovery intervals. Each point is the average of the 4 fish in Fig. 4.


Δ%
100
80
60
40
20
0
-20
+60
+40
+20
0
-20
-40
-60
-80
ECCENTRICITY (DEGREES)
1 WK
≃ 1 MO
≃ 2 MO
≃ 3 MO
> 3 MO

7 show striking similarities in recovery curves determined by percent responses to the positive stimulus alone and by percentage differential responses to the positive and neutral stimuli.

Discussion

Behavioral mapping of the visual field, employing a conditioned respiratory response, indicates a recovery of vision over the entire formerly blind field created by excision of the caudal half tectum. Differential suppression of respiration to red and green lights throughout indicates, furthermore, the restoration of color vision to the recovered field. The present results demonstrate that the compressed retinotectal projection mapped by electrical methods is behaviorally functional through tectum and efferent system.

Sequential recovery maps show a gradual orderly expansion of the visual field, caudalward from the central region that extends to the posterior pole and reaches a plateau within 84-112 days after surgery. The observed behavioral recovery is consistent with the time course for compression determined by electrophysiology with the optic nerve left intact (Yoon, 1971). The expansion, starting from the central edge of the scotoma, extended 20-30^{o} every several weeks. Biweekly mappings sometimes failed to show a noticeable change in the size of the field from a previous testing; for example, at three weeks, the field map was essentially unchanged from the one week postoperative reference map. When field expansion did occur, it was always found to be orderly and continuous with the previously recovered field with no islands of recovery restored sporadically within the scotoma.

Although differential responding was always obtained, the conditioned responses at newly recovered points were sometimes weaker (shorter in duration) than elsewhere. The differences in response characteristics disappeared by the time the full field was recovered, however, perhaps as the result of maturation of the new synaptic connections, or the more effective driving of tectal cells through concentration of synaptic contacts by addition of new synapses, or spatial localization of existing terminals. There was some indication that the reestablished representation of the posterior visual field was not always equivalent to a normal projection, even after long recovery intervals, but these cases were exceptional and recovered fish on the whole performed in a manner indistinguishable from normals in their ability to detect and differentiate the 1.5° stimuli.

Functional rejoining of fibers from the whole eye to the remnant half tectum like the results of electrical mapping of compression, may appear at first to violate the principles of the neurospecificity model of Sperry which explains the formation of orderly connections in terms of cytochemical differentiation and specific labeling of individual neurons in the retinal and tectal fields with selective matching of pre- and postsynaptic elements according to the prescribed specificities (Sperry, 1944; Sperry, 1965). However, the orderly graded progression of recovery in the foregoing does suggest rather selective reconnection in the sense that the nasal fibers, deprived of their terminal connections after excision of the caudal tectum, formed synapses in the order of their original proximity to the remaining tectum. Without cell-unique specification for position and positional preferences of some kind, a random recovery throughout the posterior field would be expected since all axons coursing beyond the rostrocaudal midline of the tectum were interrupted concurrently, and presumably had equal opportunity to make connections on a purely mechanical nonselective basis.

Respecification of synaptic affinities has been proposed (Meyer & Sperry, 1976; Sperry, 1975; Yoon, 1971, 1972, 1975) as one means by which the half tectum could accommodate the influx of foreign axon terminals. Other investigators (Gaze & Hope, 1975; Gaze & Keating, 1972) deny the necessity for affinities between retinal and tectal neurons for the formation of ordered maps, and suggest that retinal fibers merely align themselves according to nearest neighbors on the available tectal space. The pattern of visual recovery found here does not distinguish the above two divergent positions. Another aspect of the results, however, is suggestive of the existence of predetermined affinities. The majority of operated fish after recovery performed throughout as well as normal fish on the red-green discrimination task, indicating a return of color vision to the restored temporal field. Differential responding in the time course study was immediately apparent with the first clear indication of vision in the expanding field. Great precision and selectivity in matching up of color cells of the retina and tectum would seem a logical necessity. These findings concur with those of Arora and Sperry (1963) in supporting the notion of cytospecific differentiation of neurons in the retinal and tectal fields along the color dimension (Sperry, 1951). Observations from compression studies in goldfish in which the optic nerve was divided and allowed to regenerate (Cook & Horder, 1974; Sharma, 1972a; Yoon, 1975) indicate that only appropriate retinotectal connections are formed initially. Reorganization appears to occur secondarily with time, suggesting that the influence of the position-dependent affinities may be diminished in light of the mismatch situation.

The present results show that the reorganized retinotectal projection induced by extensive damage to the optic tectum in goldfish, is behaviorally functional. The full functional capacity of the compressed projection is yet to be determined. Visual acuity tests, for example, were not administered, but acuity is presumably diminished by the greater convergence of optic axons required in reconnecting the whole eye to a half tectum. Perceptual distortions may also be introduced by the compression and the accuracy of visual localization remains to be determined. Another unknown factor concerns the extent to which the plastic changes extend to deeper centers of visuomotor coordination.

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IV. FAILURE OF CONTINUOUS LIGHT TO INHIBIT COMPRESSION OF RETINOTECTAL PROJECTION IN GOLDFISH*

According to a recent report by Yoon (1975), goldfish maintained in a continuously illuminated environment fail to show the usual plastic compression of the retinotectal projection following a partial lesion to the optic tectum. Under normal diurnal lighting conditions, excision of the posterior half of the tectum regularly induces a gradual formation of a compressed retinotopic projection of the entire visual field onto the anterior tectal remnant in about three months (Gaze & Sharma, 1970; Meyer, 1974; 1976; Sharma, 1972; & Yoon, 1971; 1972). The plastic remapping occurs regardless of whether the original projection to the residual anterior half tectum is left intact, or whether the optic nerve is cut and allowed to regenerate. In an environment of continuous light, however, Yoon (1975) found that the compression was suppressed provided the optic nerve was not crushed at the time of surgery. Visual field maps obtained by postsynaptic recordings from tectal cells indicated that the projection to the remaining anterior half tectum originated exclusively from the appropriate anterior half of the visual field even after recovery periods

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*This paper co-authored with R. L. Meyer has been submitted for publication in Exp. Brain Res.

of up to 253 days. The suppression was apparently not a simple retinal pathology which prevented optic fiber regeneration, because after optic nerve crush, compression was seen to occur in the usual fashion. Furthermore, when compression was inhibited by continuous light, subsequent transference of the fish to a diurnal light cycle resulted in compression within three weeks.

In the present experiments, the effect of continuous light on retinotectal compression was further investigated by mapping with presynaptic recording methods and by assaying for visual function with behavioral testing. The animals, surgical techniques, and environmental conditions were, as far as could be determined, the same as those used by Yoon. A preliminary report of the results have been published (Scott & Meyer, 1976).

Materials and Methods

Common goldfish (Carassius auratus) 5 - 7 cm in body length sustained a lesion of the posterior half of the tectum while under tricaine methanesulfonate anesthesia, and were subsequently transferred to transparent 10 gal glass aquaria with water temperature between 22-25°C. Three separate experimental runs were distributed over an 18 month period. The ambient illumination, provided by flourescent lighting, was measured at the walls of the aquaria and ranged from 0.86-1.16 log footlamberts for one set of fish and 1.30-2.21 log footlamberts for the other two groups. For the last two groups, a battery powered back-up system was installed to provide light in the event of a general power failure. The emergency system was triggered only once, near the termination of the experiments, when all but one fish had already been tested for the final recovery.

For both electrophysiology and behavioral mapping, the eye was immobilized prior to testing by resection of the extraocular muscles and strapping the fish to a rigid holder which was immersed in an aquarium. One wall of the aquarium was formed by the flat side of a water-filled hemisphere 46 or 62 cm in diameter (Schwassman & Kruger, 1965; Meyer, 1974; 1976; & Scott, 1976). The eye was positioned at the center of this wall, and the fish rotated until the optic disc projected through the vertex of the hemisphere. Visual stimuli were presented at the hemisphere surface through nearly 180° of the visual field. This system eliminated the refractive errors associated with the more conventional methods of mapping with the fish in air.

Electrophysiological mapping. For electrophysiology, fish were additionally curarized and a cranial opening was made through which Pt Ir glass insulated electrodes, 100K-5M ohms impedance, were systematically lowered into the superficial 50-200 μ of the tectum. Single and multiple units with 3-5° receptive fields were mapped with a 2°

black disc against a white background (see Meyer, 1974; 1976). The source of these kinds of units is generally thought to be terminal optic axon arborizations (see Gaze & Sharma, 1970).

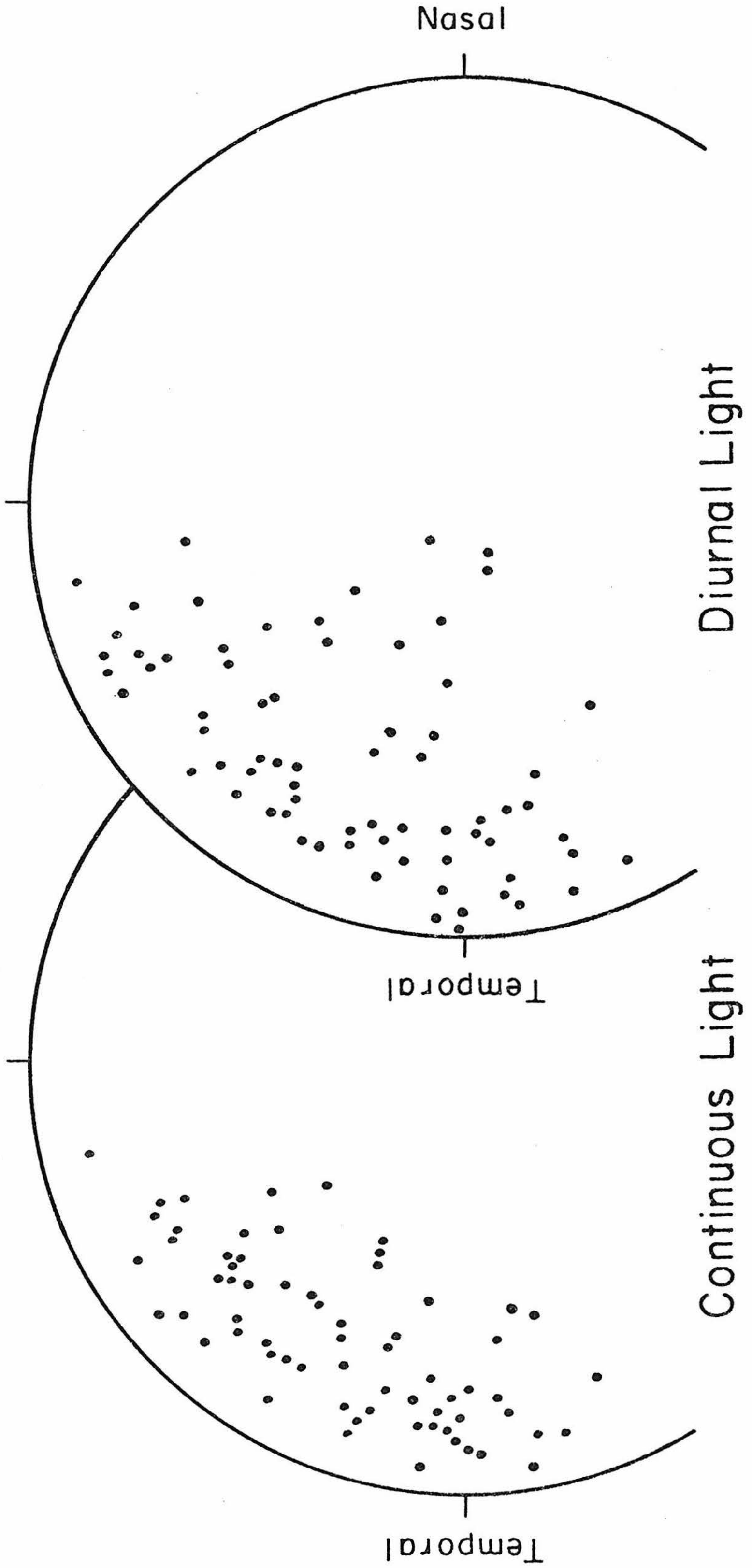
Behavioral mapping. A conditioned respiratory response to a flashing red spot of light of 5 sec duration was employed as a behavioral measure (see Scott, 1976). Respiratory suppression was achieved by pairing the onset of the stimulus with an electroshock administered across the tail through two stainless steel electrodes. The 1.5° light was 1.7° mw/cm^2 at the source and was presented around the "equator" of the hemisphere against an average ambient test room illumination of 0.56 log footlamberts. The intervals at which fish were first tested for recovery and the frequency of the tests were varied (see below) to control against possible effects of the brief (3-4 hr) exposure to the diminished lighting conditions of the test room on recovery. For some fish mapping began two weeks postoperatively and was repeated several times. In others, initial testing was delayed for up to 24 weeks after surgery and was repeated only if full recovery were not indicated.

Results

Electrophysiology. Ten fish were mapped at 45 days to 9 months following surgery and two of these were successfully revived for a second later mapping. The extent and rate of recovery was essentially the same as 13 similarly operated controls maintained under 12 hr light-12 hr dark (Fig. 8). The acute effect of this surgery is a scotoma of the entire temporal half of the visual field. By 2 months, this field deficit had been reduced to the temporalmost quarter of the visual field. At 3 - 4 months usually most of the field had become represented on the anterior remnant as an inhomogenous retinotopic compression in which the temporal half field was squeezed onto the posterior third of the remnant and nasal half field onto the anterior two-thirds. At longer periods this inhomogeneity in the projection decreased but never disappeared, and a small (10-20°) scotoma often persisted in temporalmost superior field. While this is somewhat at odds with the complete and homogenous compression previously reported using eye-in-air recording techniques, it is the typical result using eye-in-water recording. In addition, small regions of temporal field occasionally came to project too far rostrally in violation of the strict nasotemporal retinotopography though still in accordance along the inferior-superior topography. Though this anomalous pattern has not been reported by Yoon, we and others (Gaze & Sharma, 1970) typically find it when the optic nerve is left intact.

Behavior. Five fish were mapped at two weeks after surgery to determine the extent of the typical scotoma produced by tectal lesion. Vision appeared to be normal in

Fig. 8. A scatter plot of the temporalmost receptive fields obtained by electrophysiological recording. Each dot represents the receptive field position corresponding to a mediolateral row of 6 - 9 recording points obtained in the posteriormost regions of the tectal remnant. All ten experimental fish are included in the continuous light plot. For the diurnal control, the data are from ten other fish with comparable surgery and postoperative survival times.


Nasal
Diurnal Light
Temporal
Continuous Light
Temporal

the anterior half field and to 20° or so into the posterior field. Sequential mapping thereafter showed an orderly anterior-posterior expansion of the visual field with full recovery being achieved within 10 - 14 weeks. The pattern of visual recovery was similar to that for fish maintained postoperatively under a 12 hr light-12 hr dark cycle (Scott, 1976). A summary of results for all fish is shown in Table 1. Twelve of the 13 fish kept in continuous light following tectal ablation eventually recovered vision over the entire posterior field tested. The 13th fish showed a field expansion to 70° in the posterior field when tested at 10 weeks, but died prior to the next mapping.

Six of the above fish were also mapped electrophysiologically 1 - 3 days after behavioral testing. Expansion of the field along the equator was usually 10 and sometimes 20° less than that seen behaviorally at early stages of recovery, but both showed a graded recovery over time with representation out to 80° in the posterior field by 10 - 14 weeks.

Discussion

In these experiments, compression of the retinotectal projection proceded in continuous light with essentially the same pattern and time course as under diurnal lighting. Electrophysiological mapping indicated that fibers from the posterior field formed terminal arborizations on the foreign

TABLE 1

VISUAL FIELD RECOVERY[a]

	2 wks	6 wks	10 wks	14 wks	24 wks
CL1[b]	20	40	60	80	
CL2	30	60	80		
CL3	20		70[c]		
CL4	20		80		
CL5	20			80	
CL6		50	80		
CL7		60	80		
CL8			80		
CL9			70	80	
CL10			50	80	
CL11					80
CL12					80
CL13					80
DL[d]	0(1 wk)	20(5 wk)	40 (9 wk)	80 (14 wk)	80

[a] In degrees posterior to vertical meridian

[b] CL = Fish kept in continuous light environment

[c] Died prior to final testing

[d] DL = Typical fish kept in diurnal light environment

anterior tectal remnant displacing the original presynaptic fibers in the process, and the behavioral results show that these terminals formed functional connections with tectal cells. The reason for the apparent discrepancy with Yoon's finding is not clear. The only obvious difference in experimental procedures was the manner in which fish were tested for compression. Our findings do not preclude the possibility that the formation of functional synapses was inhibited in a select class of cells which were preferentially sampled by Yoon's techniques, but it seems more likely that the suppression of compression involves additional genetic or environmental factors which were not duplicated under our conditions.

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