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EFFECT OF TEMPERATURE ON RATE OF GOLDFISH OPTIC NERVE REGENERATION: A RADIOAUTOGRAPHIC AND BEHAVIORAL STUDY

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SUMMARY

Optic nerve regeneration was examined in goldfish radioautographically, as well as by a variety of behavioral measures of visual function. The rate of regeneration was markedly enhanced by increasing temperature above the ambient level (20 °C). The earliest functional indication of recovery was the appearance of an autonomic response to a change in illumination, then the ability to localize food visually and at a still later time, an optomotor response. Even the early behavioral indications of return of function appeared some time after there was radioautographic evidence that the tectum had been reinnervated.

INTRODUCTION

The optic nerve of the goldfish has proved useful for studying regeneration, in great part because the central nervous system of fish and amphibia regenerates in a highly ordered fashion with apparent full recovery of function[14,15]. Previous studies have focused primarily on the nature of anatomical and electrophysiological indices of recovery. Morphological correlates of reestablishment of functional connections in the tectum do not, however, permit one to make conclusions about function, and electrophysiological studies usually measure only presynaptic events in the tectum, with little direct indication of return of function. Since regenerating axons may transport qualitatively and quantitatively altered macromolecular components[4], and since altered transport may have synaptic consequences[12], it was of considerable interest to establish the nature of the return of vision following nerve section.

In the present study we have utilized several methods to determine return of vision and to establish the role of temperature upon the rate of recovery. We have also examined the degree of variability in the observed time of return of function.

MATERIALS AND METHODS

Surgical procedures

Goldfish *(Carassius auratus)*, 6–7 cm in body length and weighing 8.5–11 g, were anesthetized with tricaine methanesulfonate (250 mg/l). The conjunctival membrane surrounding the left eye was cut and the optic nerve was crushed with a pair of curved forceps. The right eye was removed, thereby rendering the fish blind until the remaining optic nerve regenerated its central nervous system connections. Completeness of separation of the nerve was established visually under a dissecting microscope, and fish were discarded if the sheath surrounding the nerve or adjacent artery was severed. Sham nerve crushes involved the same procedures, including positioning the forcep tips around the exposed optic nerve. Fish were allowed to recover at room temperature and groups of 5–15 fish were then placed in 3.8 liter tanks which were maintained at various temperatures, as stated below. Fish to be maintained at 35 °C, were first placed in a 30 °C tank and the temperature was progressively raised to 35 °C over a 10 day period followed by surgery. All tanks were aerated and filtered and the fish were maintained in continuous light and were fed daily for the duration of the experiment. Identification of individual fish within groups was made possible by initial selection of subjects with unique body pigment patterns.

Visual testing

In initial experiments the whole body pursuit (optomotor, OPM) response was used as an index of visual function. Fish were tested in an optomotor drum twice weekly. The apparatus and testing procedures are described in detail in the preceding paper[16].

A second test involved dropping small pellets of food into the tanks and noting whether fish either caught a food pellet as it was sinking, or made non-random movements in locating the food after it had dropped to the bottom. Although somewhat difficult to quantify, the method was of interest since it had been used previously as a measure of regeneration[10] and could thus be used to relate the present experiment to previous studies. In subsequent experiments, the possible contribution of olfactory cues in food localization was eliminated by using food pellets embedded in clear vinyl plastic[16].

A third method involved respiratory deceleration to a light stimulus. A fish was considered to see if significant (10%) deceleration to the stimulus was detected; details are presented in the preceding paper[16].

In addition, limited tests of optokinetic nystagmus were made using the eyestalk method[2]. Fish were clamped in a foam restrainer, placed in the optomotor drum and eye movements were observed. Repeated saccades in the direction opposite the drum rotation were scored as optokinetic nystagmus.

Radioautography

Fish were blinded by removal of the right eye and crush of the left optic nerve as described above, and were maintained at 30 °C. The remaining eye was injected

with 25 μCi of L-[2,3-^{3}H]proline (30–40 Ci/mole) in 5 μl by means of a 30-gauge needle, either 1, 3, 6, 11, 14 or 19 days following surgery (2–3 fish per time point). After 24 h, the fish were decapitated and the heads were fixed in Bouin's solution, dehydrated with butyl alcohol and embedded in paraffin. Sections (8 μm thick) were dipped in Kodak NTB2 emulsion, were exposed for 7 days, then developed and stained with hematoxylin–eosin.

RESULTS

Return of the optomotor response (OPM)

Following bilateral enucleation, fish maintained at 30 °C, 25 °C or 20 °C failed to meet an OPM test criterion of 60 sec of total tracking time during the 180 sec test period (Fig. 1). Control fish in which the right eye had been enucleated, but with a sham nerve crush of the left optic nerve, demonstrated consistently high tracking times at all temperatures studied (Fig. 1). The experiment showed that the surgical procedure itself and the temperature of storage or testing of the fish per se had little effect on performance.

Recovery of vision in fish whose left optic nerve had been crushed and right eye enucleated following maintenance at 30 °C, 25 °C or 20 °C is shown in Fig. 2A. An accelerated return of vision at 30 °C in comparison with the 20 °C group was apparent. In view of this result, an attempt was made to determine whether fish maintained at a still higher temperature (35 °C) would regenerate even more rapidly, but this was not the case (Fig. 2B).

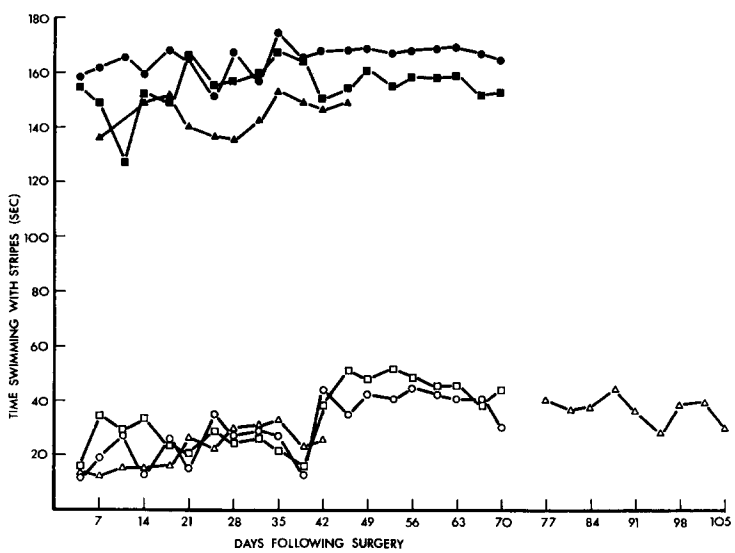

Fig. 1. Mean time swimming with stripes in optomotor drum after surgery. Sham groups (right eye removed and sham nerve crush on left eye): ●—●, 30 °C (n = 5); ■—■, 25 °C (n = 5), ▲—▲, 20 °C (n = 9). Blind fish (both eyes enucleated): ○—○, 30 °C (n = 5); □—□, 25 °C (n = 5); △—△, 20 °C (n = 5).

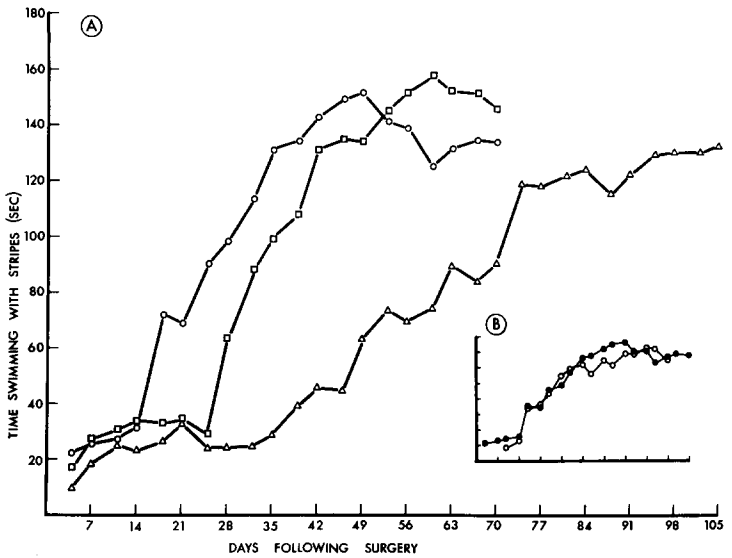

Fig. 2. A: mean time swimming with stripes in optomotor drum following surgery. Fish had right eye removed and left optic nerve crushed. ○—○, 30 °C; □—□, 25 °C; △—△, 20 °C (n = 15 per group). B: comparison of rate of regeneration in fish maintained at 35 °C (○—○, n = 8) vs. 30 °C (●—●, n = 15). Same scale as in A.

While the data in Fig. 2A might appear to reflect a gradual return of vision, it should be borne in mind that each curve represents the mean tracking time of a group of fish and does not accurately reflect the time course of improvement for an individual fish. In fact, individual plots show a rapid phase of recovery, but at a different time after crush for each fish, probably because of variability in the precise site of each nerve crush and differences in intrinsic regenerative capacities among individual fish. In order to eliminate the variation in the rate of return of function during the active phase of recovery, the data were examined using a criterion about which each fish's optomotor curve could be superimposed. The least between- and within-group variance resulted when we used a criterion of two consecutive tests in which 100 sec or greater of tracking was seen. When this normalization is made (Fig. 3), the change in tracking just prior to and following achievement of the criterion can be examined. Mean days calculated to achieve the 100 sec criterion are shown in Fig. 5. Student *t*-tests showed that each of the groups differed from one another ($P < 0.05$). Recalculation with a criterion of 60 sec had little effect on the computed result (Fig. 5). The apparent rapid phase of recovery of optomotor responding (Fig. 3) was re-examined in a small number of fish at 30 °C that were tested daily, and the period of rapid recovery was found to extend over 5 days.

Food localization

As with the OPM test, temperature affected the rate of return of ability to localize food (Fig. 4). Blind fish made random movements in locating food, while sham-operated animals responded within several seconds of food presentation and

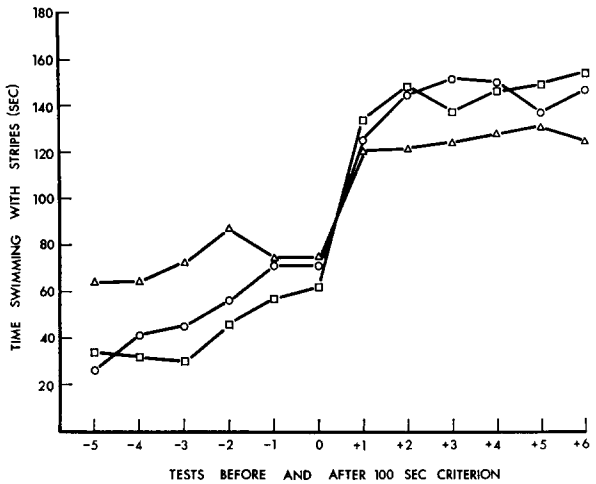

Fig. 3. Mean time swimming with stripes in optomotor drum in regenerating fish prior to and following achievement of a criterion of two consecutive optomotor tests of at least 100 sec. Points +1 and +2 indicate first time the criterion was achieved. Interval between points on abscissa is 3–4 days. ○—○, 30 °C; □—□, 25 °C; △—△, 20 °C.

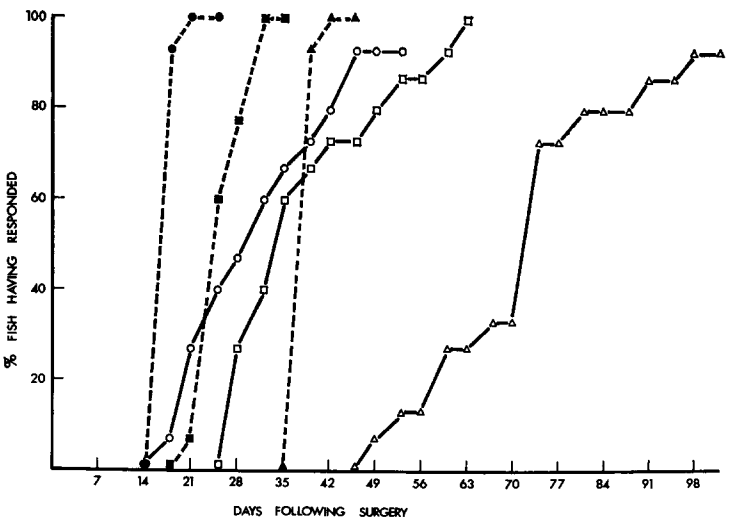

Fig. 4. Percent regenerating fish that were identified as localizing food or having achieved two consecutive tests of 100 sec in the optomotor drum as a function of time after surgery. Fish were examined daily for food localization and twice weekly for the optomotor response. Both behaviors were examined in the same fish. Sham and blind groups are omitted since shams responded and continued to respond immediately while blind fish never responded. Unplotted points reflect absence of responding fish. Food localization: ●---●, 30 °C; ■---■, 25 °C; ▲---▲, 20 °C. Optomotor: ○—○, 30 °C; □—□, 25 °C; △—△, 20 °C.

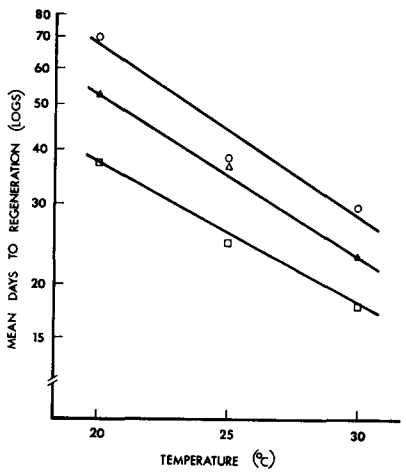

Fig. 5. Mean days to regeneration (on log scale) as a function of temperature. Food localization ($Q_{10} = 2.06$) □—□; optomotor, 60 sec criterion ($Q_{10} = 2.30$) △—△; optomotor, 100 sec criterion ($Q_{10} = 2.35$) ○—○. Standard error of means for food localization measure ± 0.12–0.87 days and for optomotor response ± 1.83–4.25 days. Lines were drawn on the basis of a linear regression analysis.

always made direct movements in obtaining food. Mean days of recovery are seen in Fig. 5. All groups differed significantly from one another ($P < 0.0001$).

In order to facilitate comparison of the data for the OPM and food localization indices, the data in Fig. 2A are replotted in Fig. 4 in terms of percent fish that had a score of at least 100 sec of tracking with the stripes for two consecutive tests. Comparison of the two measures (Figs. 4 and 5) reveals that mean days to locate food at each temperature is less than the mean days to achieve the 100 sec criterion for OPM. Recalculation using an optomotor criterion of 60 sec does not eliminate the different onset times of the two responses but reduces the disparities at each temperature (Fig. 5).

In all cases, the time disparities between the food and OPM measures were significant ($P < 0.01$). Thus it appears that during the course of regeneration, fish are able to locate food much before they can track the striped drum. This result was somewhat unexpected since we considered the ability to locate food required a greater degree of visuomotor coordination than was necessary for OPM. The possibility remained that olfaction interacted with minimal vision to enable food localization while it did not facilitate OPM. In order to ascertain whether the disparity between onset of the food localization and optomotor responses was actually contributed to by olfactory cues, a separate group of regenerating fish was tested for food localization using food pellets that could not provide olfactory cues (see Methods). These fish were also tested for OPM twice weekly as described above, as well as for their response to changes in illumination 10, 15, 17 and 20 days following surgery. A fourth test of vision in the same fish, the optokinetic response, was performed 18 days following surgery.

The results obtained for these fish are presented in Fig. 6. Vision as assessed

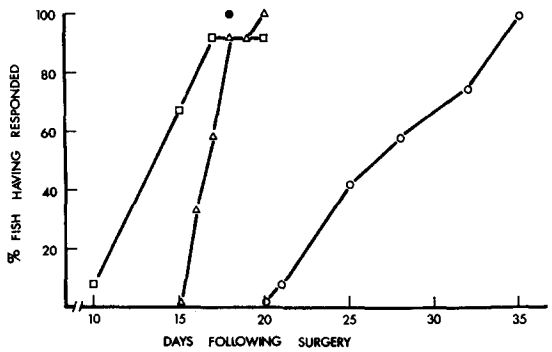

Fig. 6. Percent regenerating fish that were identified as seeing using shadow, food, optomotor and optokinetic tests as a function of time after surgery. All 4 tests were made in same fish. A fish that showed a respiratory deceleration to the shadow (10%), snapped at the plastic coated pellets or scored at least 100 sec of swimming with the stripes for two consecutive tests was considered to see. Fish were maintained at 30 °C. Shadow, □—□; food, △—△; optomotor, ○—○; optokinetic, ●.

by the respiratory response to the shadow was observed in 8 fish on day 15. Food localization was not observed until day 16 (4 fish) (mean = 17.3 days) and OPM appeared at considerably later times (mean = 28.8 days with a 100 sec OPM criterion and 21.9 days with a 60 sec criterion). Since olfactory cues were ruled out, the present data confirm that vision as measured by food localization is restored sooner than it is by OPM. Optokinetic nystagmus was elicited in all 12 fish on day 18, while none of the fish yet displayed an optomotor response, demonstrating that the pursuit response by eyes and body are experimentally separable.

Radioautography

The efficacy of the nerve crush procedure was substantiated by radioautographs of fish that were sacrificed 2 or 4 days following surgery. Labeled optic nerve fibers were absent in the tectum of all 6 fish examined at these time points (Fig. 7a and b), although the regenerating optic nerve could be seen nearing the chiasma in the day 4 fish. Of the 3 fish sacrificed on day 7, all showed evidence that the optic nerve had reached the tectum. The dorsolateral and ventrolateral regions of the rostral tectum and the entire caudal tectum had not yet been reinnervated (Fig. 7c and d). By day 12 the tectum appeared to be completely reinnervated by retinal fibers (Fig. 7e and f), since radioautographs of these tecta did not appear to be significantly different from those of day 15 (Fig. 7g) or day 19 fish (Fig. 7h). In addition, retinal efferents to all of the various tectal lamina which normally receive retinal input[11] could be seen as early as day 12. Considerable innervation of the ipsilateral tectum by regenerating optic fibers was observed in fish sacrificed after day 7, and is reported elsewhere[17].

DISCUSSION

While the rate of regeneration in the anuran peripheral nervous system is known to be temperature dependent[9], the present studies indicate this to be true for the teleost

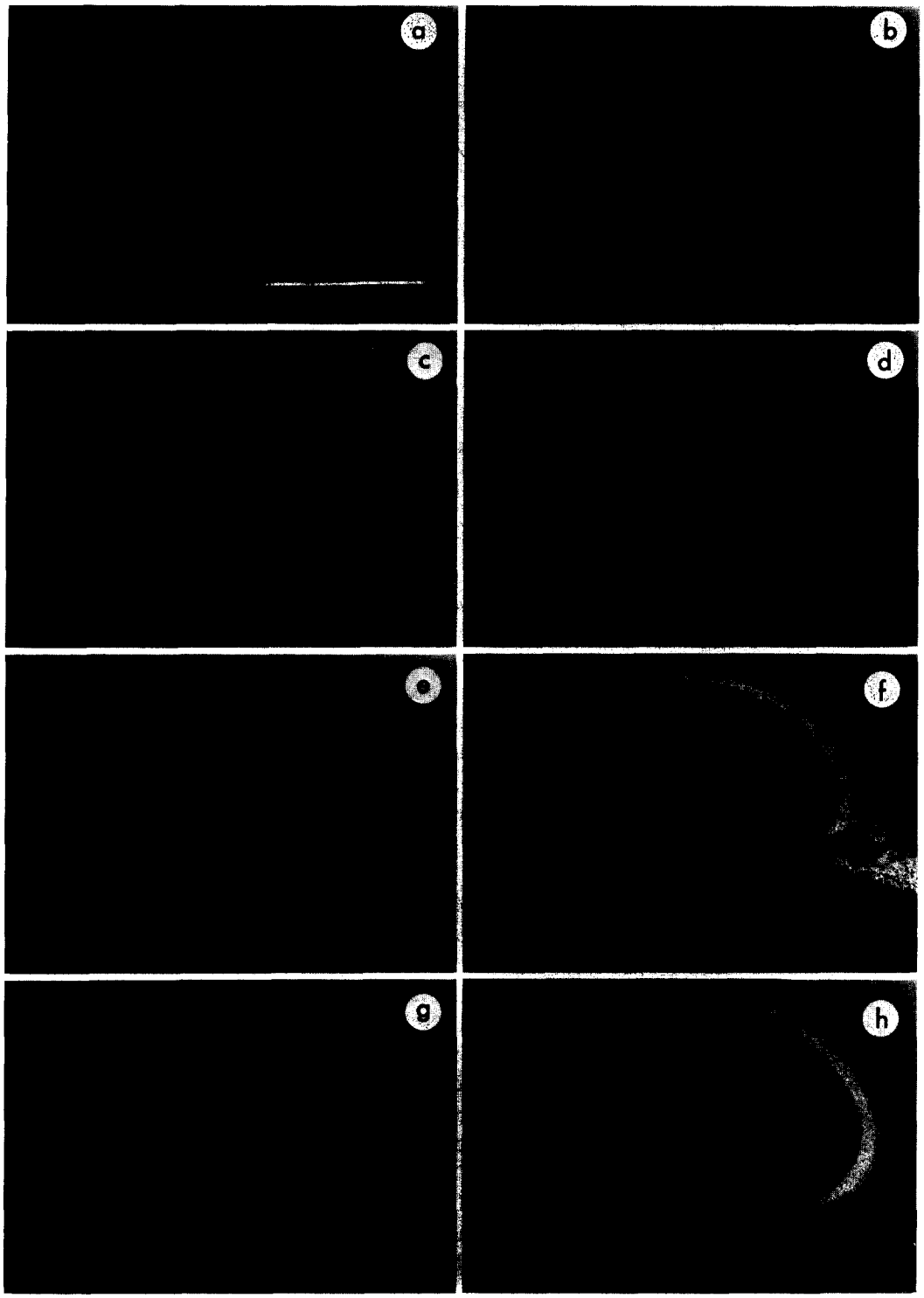

Fig. 7. Time course of tectal reinnervation by optic fibers in fish maintained at 30 °C and which had right eye removed and left optic nerve crushed. Photomicrographs were taken with both transmitted and incident light to illustrate tissue section and emulsion grains (scale, 1 mm; only contralateral optic tectum is presented). Absence of grains in cross (a) and parasagittal (b) section of fish sacrificed 4 days after nerve crush. Partially innervated tectum of fish sacrificed at 7 days in cross (c) and parasagittal section (d). Completely innervated tectum of fish sacrificed at 12 days in cross (e) and parasagittal section (f). Cross-section of fish sacrificed at 15 (g) or 19 days (h) following nerve crush.

central nervous system as well. In anurans, the rate of regeneration appears to be affected by two processes. Between 9 and 22 °C, temperature has a marked effect on the latency of regenerating fibers to cross the site of crush but the rate of elongation beyond the crush is minimally affected; between 22 and 26 °C there are no apparent differences in the latent period. In the present study, there is no measure of the latent period, but the time course of regeneration by the various behavioral criteria appears to be linear between 20 and 30 °C (Fig. 5). It would therefore appear that either the putative latent period in the goldfish is non-existent or brief relative to the elongation process, or that there is a latent period, but it has the same Q_{10} as the elongation process. In the anuran system, the rate of nerve elongation is unaltered between 9 and 22 °C but is elevated at 26 °C. The present data suggest that the rate of elongation of the goldfish optic nerve is also unaffected at a high temperature since regeneration at 35 °C does not lead to faster return of function than at 30 °C.

It has previously been observed that recovery of vision as evidenced by return of food-localizing behavior in regenerating goldfish corresponded to the observed time for arrival of slowly transported protein from the eye to the tectum in unoperated fish at 21 °C, leading to the suggestion that slow axonal transport mediates the regeneration process[8]. It has also been reported that the rate of slow axonal transport in the goldfish visual system is essentially unaltered between 9 and 20.5 °C[7], although a striking temperature dependence of rapid flow has been observed[3]. If indeed slow axonal transport is not temperature-dependent within, as well as above the stated limits, the observation would argue that slow transport is causally unrelated to regeneration since the present data clearly indicate the temperature-dependent nature of the recovery process. The present studies also indicate that temperature must be considered as an important factor in comparing biochemical and behavioral regeneration data between and within studies.

The various behavioral measures reported here add to the previously demonstrated neurophysiological and anatomical correlates of regeneration[5]. Behavioral techniques are particularly useful in that they provide a direct measure of functional synaptic reconnection in contrast with electrophysiological studies in goldfish which typically measure only presynaptic impulses of the retinal ganglion cells at tectal locations. Also, they are non-invasive, thus permitting multiple measurements over time in the same subjects. The present studies indicate that vision continues to improve for considerable periods after the first indications of reconnection are demonstrable. It remains possible that the regenerated visual system is in some way deficient relative to the normal eye at the time we judge vision to be restored, since morphological evidence of remyelination along the optic tract is not complete for several months following disruption[10]. There is as yet little additional information concerning return of vision in fish other than the observation that recovery of visual acuity[18] and color vision[1] are complete in *Astronatus ocellatus* as early as 40 days after optic nerve section.

Recovery of function as measured by the various behavioral responses follows an orderly progression at the temperatures tested (Fig. 6) and in each case, the recovered function eventually appeared complete. Autonomic measures of vision are

detected first, and the optokinetic eye movements are seen at the same time, followed by food localization and finally by return of OPM. Examination of mean days to recovery for food localization and OPM indicated temperature curves of similar slopes (Fig. 5). This result suggests that the neural elements participating in the recovery are qualitatively similar for each behavior, and militates in favor of the possibility that a single process mediates the times to recovery of each behavior. For example, the total number of optic nerve fibers that synapse in the tectum may be the determinant of each observed behavior, and elicitation of food localization may require fewer functional synapses than are required for appearance of OPM.

It is of interest that radioautographic evidence of regeneration of tectal projections at 30 °C appeared grossly complete before any of the various behavioral responses had been restored. It should also be noted that some of the behaviors tested may not be directly subserved by the tectum. For example, optokinetic nystagmus is mediated by extratectal nuclei in the goldfish[16] and there is evidence that sharks can discriminate black from white patterns without the tectum[6]. In ablation experiments, we found that the shadow, food localization and OPM responses are lost following tectal ablation[16] and the loss of the shadow response is consistent with a previous study which demonstrated that scotomas result following tectal ablations in goldfish[13]. The possibility that non-tectal nuclei are also necessary for these behaviors is not precluded, since tectal ablation might interfere with some aspect of the behavior subsequent to primary reception elsewhere.

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