Published in final edited form as:

Exp Neurol. 2008 April ; 210(2): 592–601.

Growth Dynamics and Morphology of Regenerating Optic Fibers in Tectum are Altered by Injury Conditions: An In Vivo Imaging Study in Goldfish

Amy J. Dawson^{*} and Ronald L. Meyer

Department of Development and Cell Biology, University of California at Irvine, Irvine, CA 92697-2305

Abstract

The dynamic behavior of axons in systems that normally regenerate may provide clues for promoting regeneration in humans. When the optic nerve is severed in adult goldfish, all axons regenerate back to the tectum to reestablish accurate connections. In adult mammals, regeneration can be induced in optic and other axons but typically few fibers regrow and only for short distances. These conditions were mimicked in the adult goldfish by surgically deflecting 10–20% of optic fibers from one tectum into the opposite tectum which was denervated of all other optic fibers by removing its corresponding eye. At 21–63 days, DiI was microinjected into retina to label a few fibers and the fibers were visualized in the living fish for up to 5–7 hours. The dynamic behavior and morphology of these regenerating deflected fibers were analyzed and compared to those regenerating following optic nerve crush. At 3–4 weeks, deflected fibers were found to form more branches and to maintain many more branches than crushed fibers. Although both deflected and crushed fibers exhibited stochastic growth and retraction, deflected fibers spent more time growing but grew for less distance. At 2 months, both deflected and crushed fibers became much more stable. These results show the morphology and behavior of fibers regenerating into the same target tissue can be substantially altered by the injury conditions, that is, they show state dependent plasticity. The morphology and behavior of the deflected fibers suggests they were impaired in their capacity to grow to their correct targets.

Keywords

Regeneration; Topography; Axon; Retina; Tectum; Plasticity

Introduction

Many forms of nerve injury in humans, such as spinal cord injury, stroke and head trauma, involve the disruption of axons. An ideal way to repair these injuries would be to induce these axons to regenerate past the injury, grow to the correct part of the nervous system and reestablish functional connections with the appropriate target neurons.

Insight into what is necessary for orderly regeneration can be gained from studying animals that can spontaneously regenerate such as lower vertebrates. Particularly notable in the

Correspondence Author: Ronald L. Meyer, Department of Development and Cell Biology, University of California at Irvine, 4101 Natural Sciences 2, ZOT 2305, Irvine CA 92697, Email: rlmeyer@uci.edu; Telephone: 949.824.5565; FAX: 949.824.5565.

^{*}Current Address for AJD: Arts and Sciences, New River Community College, 5251 College Drive, Dublin, VA 24084

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

completeness and fidelity of regeneration is the retinotectal system of the adult goldfish and other teleosts (22). When the optic nerve is severed in the goldfish, all retinal ganglion cells survive and after about a week begin to regenerate past the injury and being to invade tectum. By 3–4 weeks, regenerating fibers encompass the entire tectum. In contrast to development where fibers follow orderly paths to their targets, regenerating fibers are highly disorganized. About 40% of fibers enter the wrong brachial division of the optic pathway and invade the incorrect mediolateral half of tectum (5,6,12,40,41). Fibers that should terminate in the posterior half of tectum initially form synapses in the anterior half (23,27). These targeting errors are corrected and by 3 months fibers form a retinotopic map roughly comparable to the normal projection. Good lamination and normal numbers of synapses are restored. And perhaps most importantly, fish recover excellent pattern and color vision (1,17,28,29,33,37).

How regenerating fibers correct these initial errors to generate orderly connections is not well understood. Gradients of Eph ligands and receptors persist in the adult and are thought to guide axons to produce a rough topographic projection. Activity is then utilized to sharpen the projection to generate fine retinotopy. Just how Eph and other cues such as activity directs and regulates the growth of regenerating axons is unclear. There are reasons to believe that the regulation of axonal growth is more complicated than a simple response to local cues. One reason for thinking this is the plasticity of the mapping (13,26,36,39,42,44). When half of tectum is removed, fibers will form a compressed retinotopic projection onto the remaining half of tectum. When half of retina is removed, fibers can form an expanded retinotopic projection across the entire tectum. Thus, the position to which a fiber terminates is effected by the presence of other fibers. These findings imply that regenerating axons interact with each other as well as with tectum. How they interact with each other is poorly understood.

To explore this issue, we asked how regenerating fibers would behave if only a small number of them grew into a denervated tectum. Under these conditions, interaction between fibers should be greatly diminished. To obtain direct information on the dynamic behavior of axons we used in vivo imaging to visualize regenerating axons in the adult goldfish. We found that both the behavior and morphology of axons in a low density projection was greatly altered compared to axons in a normal density projection following optic nerve crush. In the low density projection axons branched earlier, and although they were very active in extending and retracting processes, their net growth was less. This may explain why fibers in a low-density projection have trouble forming a topographic projection (25,43).

Materials and methods

Animals

Adult common goldfish (*Carassius auratus*), 5–7 cm in body length, were maintained in 10-gallon aquaria at 19°C–21°C under a 12-hour light/dark diurnal illumination regimen. Animal care and use conformed to NIH standards and procedures were approved by the animal use and care committee at University of California at Irvine.

Surgery

Goldfish were anesthetized with 0.05% tricaine methanesulfonate (Sigma Chemical Co., St. Louis, MO). Under stereomicroscope, one eye was removed by cutting the optic nerve, ocular muscles and any other tissue holding the eye in place with fine iridectomy scissors. A flap was made in the cranium overlying the midbrain region and held back in place with a tungsten hook to expose the tecta. Any fat deposits and blood clots were aspirated away with a curved glass pipette. A fine tungsten needle was used to excise a small strip of innervated tectum at the medial edge by cutting longitudinally along the anterior-posterior axis while keeping the anterior end intact. Another cut perpendicular to the anterior-posterior axis was then made at

the posteriormost level of the longitudinal incision. The incisions went down to the level of the optic ventricle so the layers of the tectal strip were intact. An incision was made perpendicularly to the anterior end of the denervated tectum and the excised innervated tectal strip was deflected into the incision of the denervated tectum, posterior-end first (25). These deflected fibers correspond to ventronasal retina. Deflecting other fibers from tectum is considerably more difficult because their axonal path is shorter; they are further from the host tectum; and, lateral regions curve ventrally to become inaccessible. The cranial flap was tucked into place and the fish resuscitated and allowed to recover for 21–63 days under normal housing.

DiI injection

Fish were anesthetized as above and injections with 0.25% solution of DiI (Molecular Probes, Inc., Eugene, OR) in 100% ethanol were given in the retina. A small piece of sclera was removed with a fine tungsten hook and a DiI-filled glass needle (15–20 μm in diameter) mounted on a microliter syringe was inserted through the underlying choroid. The needle was advanced into the retina by 100 μm intervals and 1.6 nl of DiI was injected at each interval up to a depth of 500–600 μm (total of 5–6 injections). Injections were given in the ventronasal quadrant of retina, which corresponds to the deflected fibers' origins. Two injections were given at neighboring locations to ensure a greater probability of labeling deflected fibers. Fish were resuscitated and left to recover until the time of imaging, four to six days after injections.

DiI visualization

At 21–63 days after the enucleation and deflection surgery, fish were anesthetized with 0.05% tricaine methanesulfonate and given an injection of d-Tubocurarine (2 $\mu\text{g/g}$ fish, Sigma Chemical Co., St. Louis, MO) into the epaxial (dorsal) musculature to prohibit movement during the imaging period. The fish were wrapped in moist gauze and under stereomicroscope the cranial flap was removed to expose the dorsal half of tecta. Any overlying fat deposits and blood clots were aspirated. Fish were moved to a holder apparatus similar to those used in electrophysiological studies (24) to further prevent movement. Fish were respired by a glass tube placed in the mouth through which oxygenated water was pumped over the gills. Labeled fibers were visualized with a post-mounted Olympus fluorescence microscope (Melville, NY) equipped with rhodamine optics and a 20X water immersion objective (N. A. 0.4; Nikon Corporation, Melville, NY). A cooled CCD camera (Star 1, Photometrics, Ltd., Tucson, AZ) was used to collect digitized images of the fibers. These images encompassed an area of about 180 X 200 μm . Images were stored on optical disks as 12-bit files. A series of images in the Z-axis at 4 μm intervals was obtained approximately once every hour for a 5–7 hour period. The Z-series included all focal planes in which the fibers were in focus as well as focal planes below and above the planes of focus to make certain every piece of the fibers was visualized. The tecta were illuminated only during the Z-series acquisition time and kept moist throughout the entire imaging period with teleost Ringers. Blood flow in the tecta was checked before and after each Z-series. The experiment was terminated if blood flow slowed or stopped. Fish were sacrificed at the end of experiments by pithing through the open cranium.

Data analysis

The 12-bit image files (4094 gray scale values) from each Z-series time point were scaled to 8 bits (255 gray scale values) and converted to TIFF files with image processing software, ImagePro Plus (Media Cybernetics, Silver Spring, MD). The useable gray scale of the 12-bit image was always less than 8 bits in depth so it was possible to map the 12 bits into 8-bit space without the loss of information. The image file that contained most of the field of view in focus was used as a template to create a composite with all parts of the fibers in focus. This was accomplished by selecting pieces from each image (equals one focal plane) that were in focus

and pasting them over the template to create an image that had all relevant fibers in focus in a montage fashion. Z-series montages were done for each time point and the position of the end of each fiber was measured to determine changes in fiber length and in direction of growth by comparing each time point with the first time point of the experiment. This comparison was achieved by putting the montage of the first time point in register with each subsequent montage and combining the two images as different color channels in one color image. Any differences in fiber length were traced, measured, and converted to microns following calibration with a stage micrometer. Changes in fiber trajectories and varicosity movements were observed qualitatively. All statistical analyses were done using SigmaStat (SPSS Corporation, Chicago, IL) and rounded to the nearest hundredths.

Results

The fibers surgically diverted into the ipsilateral denervated tectum will be referred to as the “deflected fibers”. Their behavior will be described and compared to fibers regenerating into tectum after an intraorbital nerve crush. The data for these “nerve crushed fibers” come from a previous study (8). The deflected fibers arise from ventronasal retina (see surgery in Materials and Methods) and were therefore labeled by making spot injections of DiI into ventronasal retina. In the previous nerve crush study, spot injections were made into different regions of retina including ventronasal retina. Only ventronasal fibers from the previous nerve crush study will be considered so that fibers from the same part of retina can be compared. Only the dorsal half of tectum can be directly visualized. In early regeneration, the growing ends of fibers were located in an area of dorsal tectum that was considerably larger than their normal topographic position. This was true of both deflected and crush fibers most of which have not yet reached their target area. Fibers were found beginning at about 1/3 the distance along the anterior to posterior dimension and were within the most medial 2/3 of the dorsal half of tectum. In late regeneration, crushed fibers were typically within a few hundred micrometers of each other in posterior tectum near the medial edge, their appropriate tectal position. Deflected fibers, however, remained nearly as dispersed as in early regeneration. The methodology for labeling and imaging optic axons in the adult goldfish and the corresponding technical issues has been discussed previously (7,8,20).

Deflected optic fibers: early regeneration

Fifteen adult goldfish were used to observe the dynamics of deflected fibers at 21–31 days after surgery herein referred to as “early regeneration”. A total of 92 endings were visualized and used for analysis. The labeled optic fibers originated from the ventronasal quadrant of the retina. Fiber endings were typically found in the middle third or posterior third of the host tectum but a few were observed in the anterior third of the host tectum. Fibers were also in the main optic lamina, SFGS, as judged by depth from the tectal surface. A salient feature of these fibers was their morphological complexity. They were typically branched. Of the 92 endings followed, 62 (67%) of those were branches (see Table 1). Most fish (13/15) had at least one branch in the field of view and many of the fish (7/15) had four or more branches in the field (see Figures 2, 3, 4). In addition, 21 branches formed during the imaging period. This observation contrasts with the simple unbranched fibers seen in the crushed fibers (see below). It should be noted that axon was much longer (typically 750–1500 μm) than the imaging field (about 180 X 200 μm). The number of branches is simply a measure from the sampled area, not an absolute measure of the number of branches per axon.

Axonal behavior was scored for every 60-minute interval as growth, retraction, or stability (no detectable change in length). Fibers typically alternated between different behaviors during the imaging period. Nearly a third of the fibers (32%) displayed growth, retraction and stability during the 5–7 h imaging session. Over half (52%) of the fibers alternated between two

dynamics: growth and retraction, growth and stability, or retraction and stability. Only 16% of the fibers consistently displayed one behavior the entire imaging period. Of these, 11 fibers grew, 2 retracted, and 2 were stable for the entire imaging period.

Behaviors for each of the 60-minute epochs were pooled and analyzed to look for difference in the time spent in each behavior. As a group, fibers had episodes of growth 41% of the time, which was significantly more frequent than epochs of retraction (30%; $p = 0.003$, Z-test). The frequency of growth episodes (41%) was also significantly greater than episodes of stability (29%; $p = 0.001$, Z-test). There was no significant difference between the frequency of retraction and stability epochs (30% versus 29%; $p = 0.83$, Z-test).

The net translocation of each fiber (distance moved, see methods) was also analyzed. Net translocation of individual fibers was calculated by adding the total distance extended and then subtracting the total distance retracted during the whole imaging period, giving a measurement of positive, negative, or zero net translocation. There were 59 fibers (64%) with positive net translocation (growth), averaging $+20\ \mu\text{m}$, and 26 fibers (28%) with negative net translocation (retraction), with an average distance of $-17\ \mu\text{m}$. Only 8% of the optic fibers (7/92) displayed zero net translocation, which included fibers that showed no detectable change in length for the entire imaging period as well as those that extended and retracted equal distances so as to finish within $5\ \mu\text{m}$ of their initial position. There was no significant difference between net growth ($+20\ \mu\text{m}$) and net retraction ($-17\ \mu\text{m}$) distance averages as shown by the Mann-Whitney rank sum test, a non-parametric test, ($p = 0.26$). Averaging together all positive, negative, and zero net translocation values of the entire group gave a measurement of overall net translocation, which was $+8\ \mu\text{m}$. A measurement of “gross movement” (motility) was also calculated by summing the absolute value of distance traveled at every 60-minute interval, that is, without regard to whether it was growth or retraction. The gross movement average for these fibers was $17\ \mu\text{m}$ (see Table 2).

Comparison to nerve crushed fibers

The morphological features of nerve crushed fibers ($n = 40$) were simple in nature. Fibers had club like endings and many displayed straight or slightly serpentine trajectories as they invaded the tectum (Fig. 1). They also showed appropriate ordering along the anterior-posterior tectal axis (8). Notably, there was little or no branching. Only 7 fibers in 5 of 22 fish imaged had single short branches about $15\text{--}100\ \mu\text{m}$ long. These were typically terminal bifurcations. Substantial branching was only seen in late regeneration (20).

The most striking and surprising difference between the nerve crushed and deflected fibers was the high degree of branching in the deflected fibers in early regeneration. There were a total of 62 branches observed in the deflected fibers (in 15 fish) compared to only 7 branches seen in the nerve crushed fibers (in 22 fish). This difference was highly significant (Z-test, $p = <0.001$). Deflected fibers also had branches forming during the 5- to 7-hour imaging period, for a total of 21 new branches. In nerve crushed fibers, no new branches were observed (Table 1). There were obvious qualitative differences as well. In the nerve crushed fibers, the few branches that were seen were typically terminal bifurcations with short symmetrical branches. Deflected fibers were much more complex. Branches were sometimes long and highly variable in length. They originated at various positions along the shaft. In some cases, second order branching was observed (Fig. 3, 4).

The deflected fibers also differed from nerve crushed fibers in their dynamic behavior. Deflected fibers spent more time growing. They had a significantly higher number of growth epochs (41%) compared to crushed fibers (28%; Z-test, $p = 0.004$). They also had a lower number of stable epochs (29%) compared to crushed fibers (41%; Z-test, $p = 0.01$). The time

spent retracting was similar (30% versus 31%; Z-test, $p = 0.89$). Thus, the deflected fibers were more active and most of this activity was growth rather than retraction.

Surprisingly, this increased time in growing did not translate into more net growth. In net growth, deflected fibers grew significantly less distance (20 μm) compared to crushed fibers (69 μm , Mann-Whitney rank sum test, $p = 0.002$). The other growth measures showed trends in the same direction. The average overall net translocation was +8 μm for deflected fibers compared to +18 μm for crushed fibers (Mann-Whitney rank sum test, $p = 0.25$) and average gross movement for deflected fibers was 17 μm compared to 49 μm for crushed fibers (Mann-Whitney rank sum test, $p = 0.12$). Since these growth measures were overall averages, the apparent lack of a difference in overall growth might result if some deflected fibers grew more and other deflected fibers retracted more than crushed fibers. However, this was not the case. Comparison of the groups showing net retraction (-17 μm versus -19 μm) or net stability (7 versus 8) revealed no significant differences. Thus, although deflected fibers spent more time growing, they apparently grew shorter distances than crushed fibers.

Deflected optic fibers: late regeneration

Since the behavior and morphological features of deflected optic fibers were very different from nerve crushed optic fibers during the first month of regeneration, this raised the question of how stable this difference might be. To determine this, 9 additional fish with deflection surgery were imaged at 34 to 64 days herein referred to as “late regeneration”. A total of 40 endings were observed. The morphological features of the late regenerating deflected fibers were similar to the early regenerating deflected fibers (compare Fig. 5 to Figs. 2, 3, 4) with many branches and bifurcating fibers found in nearly every fish (8/9). The only morphological difference between early deflected fibers and late deflected fibers was that early regenerating fibers formed new branches ($n = 21$) significantly more than the late fibers ($n = 3$; Z-test, $p = 0.04$), but the proportion of the endings that were branches did not differ between the two groups (see Table 1; Z-test, $p = 0.89$). Also, unlike a small proportion of early regenerating deflected fibers (20%), all of the late regenerating deflected fibers were found in middle to posterior third of tectum. The translocation behaviors of early regenerating and late regenerating deflected fibers differed in only three areas. Early regenerating deflected fibers retracted by larger distances on average than late regenerating deflected fibers (-17 μm versus -7 μm ; Mann-Whitney rank sum test, $p = 0.01$. See Table 2). Late regenerating deflected fibers had fewer epochs of growth (30 % versus 41%; Z-test, $p = 0.02$) and a larger number of stable epochs compared to early regenerating deflected fibers (44% versus 29%; Z-test, $p = 0.002$). Thus, while late deflected fibers were morphologically similar to early deflected fibers, they were not as mobile and retracted by smaller distances. Late phase deflected fibers appeared to be very comparable to those previously described for late phase crushed fibers. (This earlier study (20) was done by a different group. Because of possible methodological differences in the measurements, a quantitative comparison was not made.)

Discussion

The major findings of this study are that optic fibers regenerating into tectum were substantially affected by the type of surgical insult. In early regeneration, deflected fibers which constituted a fraction of fibers redirected across the midline into a denervated tectum, differed from those regenerating after optic nerve crush in that they: 1) spent more time growing 2) grew less distance, and 3) formed many more branches. The growth dynamics and morphology of deflected fibers will first be discussed in comparison to nerve crushed fibers and then possible explanations and implications will be addressed.

Dynamics

Regenerating deflected fibers typically exhibited stochastic behavior alternating between growth, retraction and stability. In this respect, they were closely comparable to fibers regenerating after an optic nerve crush (8). The major difference was that deflected fibers spent more time growing (41% versus 28% of the time) and exhibited fewer episodes of stability (29% versus 41%) compared to nerve crushed fibers. They spent more time growing: however, they grew for shorter distances. Net growth of deflected fibers was 20 μ m compared to 69 μ m for nerve crushed fibers. These differences were only seen in early regeneration at about 20–30 days. At later times (34–63 days), deflected fibers became more stable spending less time growing or retracting. Their behavior was indistinguishable from those following nerve crush during the second month of regeneration (20).

Morphology

The most salient difference between the deflected and nerve crushed fibers was in the formation of branches in early regeneration. The large majority (67%) of deflected fibers had branches and branch initiation was frequently observed in real time (23% of the time). In contrast, axons regenerating following nerve crush were typically (89%) unbranched processes and branch formation during imaging was never observed. During the second month, this difference disappeared. Nerve crushed fibers began to form branches and elaborated a terminal arbor by 60 days (8,38). This corresponds to the time when good retinotopic order is reestablished (28,34). At the same time, deflected fibers showed no further increase in the incidence of branch formation. Therefore, deflected fibers can be thought of as forming branches prematurely.

Interrelationship between dynamics and morphology

It is not unreasonable to think that the early branch formation by deflected fibers altered the growth dynamics. Deflected axons would be distributing their capacity for extending axonal processes into numerous small branches. If growth capacity is limited, then growth might be seen at many branches while the magnitude would be reduced in proportion to the number of branches into which the growth capacity is divided. Since nerve crushed fibers have few if any branches, all of the growth potential could be expressed at the tip of the one fiber. Thus, while one might see more instances of growing fibers in the deflected condition, the amount of elongation would be greater in the nerve crushed fibers.

Plasticity of axonal behavior

Developmental studies of growing axons have shown that the local environment at the tip of the growing axon dictates its shape and the behavior. This has been particularly well studied in the optic pathway of lower vertebrates and mammals (21). Optic axons in the optic nerve have a simple spike-like ending and grow relatively rapidly. In animals with bifurcating optic projections, axons abruptly slow upon reaching the chiasm which is a choice point in most animals. They then become cone shaped and display multiple filopodia that extend and retract searching their environment. After choosing to grow ipsilaterally or contralaterally, they enter the optic pathway reverting to a spike-like rapidly growing process. Upon entering colliculus or tectum, they again slow and begin to branch. These behaviors have been shown to be regulated by diverse guidance cues that are differentially distributed along the pathway (21).

The present study on regenerating axons is a departure from this simple picture. Here we show that the same axons (optic axons from ventral nasal retina) that are in the same tissue (dorsal posterior tectum) and that are at the same stage of regeneration can behave very differently depending on the circumstances. Therefore axonal growth behavior is not strictly dictated by some fixed property of the tectal environment. Rather it is plastic and depends on the state of the system.

What accounts for state-dependent axonal growth plasticity?

The intent of the present study was to test the effect on reducing the number of regenerating fibers. Although this is the most salient difference, there are a number of other differences between the nerve crush and deflection condition that could, strictly speaking, account for the effect on axonal behavior. These include the distance of the axotomy to the cell body, damage to the tectum and proximity to the lesion site. There is little prior data in this system clearly implicating any of these of these alternative possibilities. In fact, there are reasons for thinking they were not relevant. An earlier study on goldfish used different sized eye lesions to vary the number regenerating fibers (43). Usually the projection was orderly but when the number of fibers was about 10–15% of normal (comparable to the present study), fibers failed to form an orderly projection. In this case, distance grown and distance to cell body was the same and tectum was not lesioned.

Although axotomy close to the cell body can produce a more robust (2,10,19,45) or faster (45) regenerative response, this is doubtful in the present study. The distance to the cell body is not as different between the two conditions as it might first seem. The nerve crush was actually some distance from the cell body. The crush was positioned 1–2 mm behind the eye and since the labeled ganglion cells were at the periphery of retina, this adds an additional 1–2 mm of intraretinal axon. The deflected fibers were probably only about 2 mm longer. As for tectal damage, the fibers were imaged at 500–2000 μ m from the site of tectal injury. Previous light and electron microscopy analysis of the tectum following deflection revealed no effect of surgery on tectal structure except in the immediate vicinity of the insertion point of the deflected fibers (15,25). For proximity to the lesion, there is indirect evidence that optic nerve crush causes axons to sprout near the axotomy (30). However, in the present study and in ongoing studies of deflected fibers, we have not seen evidence for sprouting at the injury site. Although these alternative possibilities remain as caveats, they do not seem particularly likely.

Number of fibers and competition

There is good evidence that optic fibers interact with each other to generate retinotopic order (18,42). When half of tectum is removed and the optic nerve crushed, fibers from the entire retina become squeezed into the remaining half tectum to form a compressed retinotopic projection from the entire retina (13,44). Similarly, when half of retina is removed and the nerve crushed, regenerating fibers from the remaining half of retina can expand across the entire tectum (36). Since the general result is that all fibers become accommodated and all tectum becomes innervated, it has been suggested that fibers compete for tectal space (18,26,39,42).

What fibers compete for has not been clearly proven. It could be for synaptic sites. During regeneration, fibers reform the normal number of synaptic connections by 1 month. This number remains constant thereafter even though fibers are changing positions to refine retinotopy (17,31). If half of tectum is removed to produce a compressed projection, the number of optic synapses per unit projection area of tectum is the same as in the normal tectum (14,32). In other words, on average, each fiber forms half its normal number of synapses. Thus, there seems to be a fixed number of synaptic sites in tectum that fibers compete for. In a previous ultrastructure study (16) of the same deflected “low density” projection as was used here, the number of synaptic connections per unit area in the densest part of the projection was less than half the normal number. Although each fiber is presumably forming more synapses than normal, there are limits as to how many synapses a fiber can form. As a result, synaptic sites are unfilled.

Fibers might also compete for a trophic factor such as BDNF, which could conceivably be the molecular basis of competition for synaptic sites. Optic axons in lower vertebrates express the

cognate BDNF receptor, trkB, and tectum produces limited BDNF (4). Exogenous application of BDNF directly to axons during development promotes branching (3,4) and similar results have been obtained for regenerating fibers (Dawson and Meyer, unpublished).

Since the number of fibers in the deflected condition is only 5–10% of normal, it would be reasonable to think that in the deflected projection, interfiber interaction and, in particular, competition for synaptic sites or BDNF would be far less than with nerve crush. This condition could favor branching and alter dynamic behavior. For example, the amount of BDNF per fiber could be far greater for deflected fibers and could promote branching similarly to that seen with exogenous BDNF.

Implications for successful regeneration

The findings here that deflected fibers branch earlier and grow more slowly than after nerve crush suggest that their ability to grow to their appropriate place in tectum to form a retinotopic projection may be diminished. There is some earlier evidence that this is the case. An electrophysiological mapping study on fish with the same deflection surgery as used here showed that topographic organization was quite poor (25). A similar result was also obtained using large retina lesions (43). Topography was also analyzed using small “spot” injections of WGA-HRP into retina (35). Following nerve crush, labeled fibers typically converge into one small region of tectum (28,33). In the deflected animals, some fibers did converge into a small tectal region, but there was also labeling dispersed over an area 4 fold greater than in the normal density projection indicating poor topography. In previous mammalian studies, limited regeneration has been obtained following injury to optic fibers or spinal cord, but there has been no clear evidence that these fibers form an orderly projection onto their proper targets. For example, in a study where regeneration of a few optic fibers into the superior colliculus was obtained and topography analyzed, there was an apparent lack of any topographic order (11).

These findings suggest that to achieve successful regeneration in mammals, it may be necessary to have a critical number of fibers or to counteract effects of injuries located close to the site of innervation. Consistent with this idea is a recent report in young hamsters that when axons were cut in the optic tract and treated with a bridging agent that promoted rapid healing, large numbers of optic axons were able to regenerate into colliculus (9). These animals could localize objects indicating that topography was restored.

Acknowledgements

This work was supported by the National Institutes of Health Grant EY014200 to R.L.M.

Abbreviations

BDNF

brain-derived neurotrophic factor

WGA-HRP

wheat germ agglutinin - horseradish peroxidase

References

1. Arora HL, Sperry RW. Color discrimination after optic nerve regeneration in the fish *Astronotus Ocellatus*. *Dev Biol* 1963;7:234–243.
2. Cancalon PF. Survival and subsequent regeneration of olfactory neurons after a distal axonal lesion. *J Neuocytol* 1987;16:829–841.

3. Cohen-Cory S, Fraser SE. BDNF in the development of the visual system of *Xenopus*. *Neuron* 1994;12:747–761. [PubMed: 8068082]
4. Cohen-Cory S, Lom B. Neurotrophic regulation of retinal ganglion cell synaptic connectivity: from axons and dendrites to synapses. *Int J Dev Biol* 2004;48:947–956. [PubMed: 15558485]
5. Cook JE. Errant optic axons in the normal goldfish retina reach retinotopic tectal sites. *Brain Res* 1982;250:154. [PubMed: 6182948]
6. Cook JE. Tectal paths of regenerated optic axons in the goldfish: evidence from retrograde labeling with horseradish peroxidase. *Exp Brain Res* 1983;51:433–442.
7. Danks AM, Kim P, Wang Z, Meyer RL. Imaging of individual normal and regenerating optic fibers in the brain of living adult goldfish. *J Comp Neurol* 1994;345:253–266. [PubMed: 7929900]
8. Dawson A, Meyer R. Regenerating optic fibers correct large-scale errors by random growth: Evidence from in vivo imaging. *J Comp Neurol* 2001;434:40–55. [PubMed: 11329128]
9. Ellis-Behnke RG, Liang YX, You SW, Tay DK, Zhang S, So KF, Schneider GE. Nano neuro knitting: peptide nanofiber scaffold for brain repair and axon regeneration with functional return of vision. *Proc Natl Acad Sci U S A* 2006;103:5054–5059. [PubMed: 16549776]
10. Fernandes KJ, Fan DP, Tsui BJ, Cassar SL, Tetzlaff W. Influence of the axotomy to cell body distance in rat rubrospinal and spinal motoneurons: differential regulation of GAP-43, tubulins, and neurofilament-M. *J Comp Neurol* 1999;414:495–510. [PubMed: 10531542]
11. Fischer D, Heiduschka P, Thanos S. Lens-injury-stimulated axonal regeneration throughout the optic pathway of adult rats. *Exp Neurol* 2001;172:257–272. [PubMed: 11716551]
12. Fujisawa H. Retinotopic analysis of fiber pathways in the regenerating retinotectal system of the adult newt *Cynops pyrrhogaster*. *Brain Res* 1981;206:27–37. [PubMed: 6162521]
13. Gaze RM, Sharma SC. Axial differences in the reinnervation of the goldfish optic tectum by regenerating optic nerve fibers. *Exp Brain Res* 1970;10:171–181. [PubMed: 5434497]
14. Hayes WP, Meyer RL. Optic synapse number but not density is maintained during regeneration onto surgically-halved tectum in goldfish: HRP-EM evidence that optic fibers compete for fixed numbers of postsynaptic sites on tectum. *J Comp Neurol* 1988;274:539–559. [PubMed: 2464623]
15. Hayes WP, Meyer RL. Retinotopically inappropriate synapses of subnormal density formed by misdirected optic fibers in goldfish tectum. *Dev Brain Res* 1988;38:304–312.
16. Hayes WP, Meyer RL. Retinotopically inappropriate synapses of subnormal density formed by surgically misdirected optic fibers in goldfish tectum. *Dev Brain Res* 1988;38:304–312.
17. Hayes WP, Meyer RL. Normal numbers of retinotectal synapses during the activity sensitive period of optic regeneration in goldfish: EM-HRP evidence implicating synapse rearrangement and collateral elimination during map refinement. *J Neurosci* 1989;9:1400–1413. [PubMed: 2703883]
18. Honda H. Competitive interactions between retinal ganglion axons for tectal targets explain plasticity of retinotectal projection in the servomechanism model of retinotectal mapping. *Dev Growth Differ* 2004;46:43–65.
19. Hull M, Bahr M. Differential regulation of c-JUN expression in rat retinal ganglion cells after proximal and distal optic nerve transection. *Neurosci Lett* 1994;178:39–42. [PubMed: 7816335]
20. Johnson F, Dawson A, Meyer R. Activity-dependent refinement in the goldfish retinotectal system is mediated by the dynamic regulation of process withdrawal: an in vivo imaging study. *J Comp Neurol* 1999;406:548–562. [PubMed: 10205027]
21. Mann F, Harris WA, Holt CE. New views on retinal axon development: a navigation guide. *Int J Dev Biol* 2004;48:957–964. [PubMed: 15558486]
22. Matsukawa T, Arai K, Koriyama Y, Liu Z, Kato S. Axonal regeneration of fish optic nerve after injury. *Biol Pharm Bull* 2004;27:445–451. [PubMed: 15056844]
23. Matsumoto N, Kometani M, Nagano K. Regenerating retinal fibers of the goldfish make temporary and unspecific but functional synapses before forming the final retinotopic projection. *Neurosci* 1987;22:1103–1110.
24. Meyer RL. Eye-in-water electrophysiological mapping of goldfish with and without tectal lesions. *Exp Neurol* 1977;56:23–41. [PubMed: 558902]
25. Meyer RL. Deflection of selected optic fibers into a denervated tectum in goldfish. *Brain Research* 1978;155:213–227. [PubMed: 688017]

26. Meyer RL. "Extra" optic fibers exclude normal fibers from tectal regions in goldfish. *J Comp Neurol* 1979;183:883–902. [PubMed: 762275]
27. Meyer RL, Kageyama GH. Large scale synaptic errors during map formation by regenerating optic axons in the goldfish. *J Comp Neurol* 1999;409:299–312. [PubMed: 10379922]
28. Meyer RL, Sakurai K, Schauwecker E. Topography of regenerating optic fibers in goldfish traced with local wheat germ injections into retina: Evidence for discontinuous microtopography in the retinotectal projection. *J Comp Neurol* 1985;239:27–43. [PubMed: 4044930]
29. Murray M. A quantitative study of regenerative sprouting by optic axons in goldfish. *J Comp Neurol* 1982;209:352–362. [PubMed: 7130462]
30. Murray M. A quantitative study of regenerative sprouting by optic axons in goldfish. *J Comp Neurol* 1982;209:352–362. [PubMed: 7130462]
31. Murray M, Edwards M. A quantitative study of the reinnervation of the goldfish optic tectum following optic nerve crush. *J Comp Neurol* 1982;209:363–373. [PubMed: 7130463]
32. Murray M, Sharma S, Edwards M. Target regulation of synaptic number in the compressed retinotectal projection of goldfish. *J Comp Neurol* 1982;209:374–385. [PubMed: 7130464]
33. Olson M, Meyer R. The effect of TTX-activity blockade and total darkness on the formation of retinotopy in the goldfish retinotectal projection. *J Comp Neurol* 1991;303:412–423. [PubMed: 2007657]
34. Olson MD, Meyer RL. The effect of TTX-activity blockade and total darkness on the formation of retinotopy in the goldfish retinotectal projection. *J Comp Neurol* 1991;303:412–423. [PubMed: 2007657]
35. Olson MD, Meyer RL. Activity- dependent retinotopic refinement in a low density retinotectal projection in the goldfish: evidence favoring synaptic cooperation over competition. *J Neurosci* 1993;14:208–218. [PubMed: 7506763]
36. Schmidt JT, Cicerone CM, Easter SS. Expansion of the half retinal projection to the tectum in goldfish: an electrophysiological and anatomical study. *J Comp Neurol* 1978;177:257–278. [PubMed: 621291]
37. Schmidt JT, Edwards DL. Activity sharpens the map during the regeneration of the retinotectal projection in goldfish. *Brain Res* 1983;269:29–39. [PubMed: 6307483]
38. Schmidt JT, Turcotte JC, Buzzard M, Tieman DG. Staining of regenerated optic arbors in goldfish tectum: progressive changes in immature arbors and a comparison of mature regenerated arbors with normal arbors. *J Comp Neurol* 1988;269:565–591. [PubMed: 3372728]
39. Schneider GE. Competition for terminal space in formation of abnormal retinotectal connections, and a functional consequence. *Anat Rec* 1971;169:420.
40. Stuermer CA, Easter SS. A comparison of the normal and regenerated retinotectal pathways of goldfish. *J Comp Neurol* 1984;223:57–76. [PubMed: 6200514]
41. Udin SB. Permanent disorganization of the regenerating optic tract in the frog. *Exp Neurol* 1978;58:455–470. [PubMed: 304414]
42. Udin SB, Fawcett JW. Formation of topographic maps. *Annu Rev Neurosci* 1988;11:289–327. [PubMed: 3284443]
43. Udin SB, Gaze RM. Expansion and retinotopic order in the goldfish retinotectal map after large retinal lesions. *Exp Brain Res* 1983;50:347–352. [PubMed: 6641867]
44. Yoon M. Reorganization of retinotectal projection following surgical operations on the optic tectum in goldfish. *Exp Neurol* 1971;33:395–411. [PubMed: 5124957]
45. Zagrebelsky M, Buffo A, Skerra A, Schwab ME, Strata P, Rossi F. Retrograde regulation of growth-associated gene expression in adult rat Purkinje cells by myelin-associated neurite growth inhibitory proteins. *J Neurosci* 1998;18:7912–7929. [PubMed: 9742159]

Figure 1.

Retinotopic optic fibers following optic nerve crush. The fibers originated from ventronasal retina and were at 31 days after crush. The arrow indicates the ending of the fiber followed for the entire five hours of imaging. Notice its simple structure. Anterior is to the top of each panel and medial is to the right of each panel. Time inset is in hours and minutes. Scale bar represents 20 μm .

Figure 2.

Deflected optic fibers at 24 days after surgery. There is one obvious branch point in the image and the left-hand fiber of the bifurcation is labeled with an arrowhead. The right-hand fiber of the bifurcation is labeled by a solid arrow. The left-hand fiber grew by 42 μm and the right-hand fiber grew by 110 μm . The thin arrow in the 3:55 panel indicates a branch that had formed, but it totally retracted by 6:00. Anterior is to the top and medial is to the right of each panel. Scale bar represents 20 μm .

Figure 3. Deflected fibers at 22 days after surgery. Two arrowheads label 2 branches that were forming and growing during the imaging period. The arrow indicates a fiber that had a bifurcation, both sides of which grew. Anterior is to the top and medial is to the right of each panel. Scale bar represents 20 μm .

Figure 4.

Deflected optic fibers at 29 days after surgery. Notice how complex the branching pattern is in this particular field. The arrow indicates a fiber that bifurcated at its ending by the 3:05 time point. The arrowhead labels a branch that extended for a total of 13 μm during the imaging period. Anterior is to the top and medial is to the right of each panel. Scale bar represents 20 μm .

Figure 5. Deflected optic fibers at 52 days after surgery. The arrow and arrowhead both indicate branches within the field. Notice again the complexity of the branching. Little growth and retraction was observed at this time. Anterior is to the top and medial to the right of each panel. Scale bar represents 20 μ m.

Table 1

Summary of Branching in Early and Late Regenerating Low-Density Projections and Early Regenerating Normal-Density Projections¹

	Normal-Density Fibers: ER*	Low-Density Fibers: ER	Low-Density Fibers: LR
Total Number of Endings	40	92	40
Number of Endings That Were Branches	7	62	28
Number of Branches Formed During Imaging Period	0	21	3

¹ A higher percentage of the endings observed in early regenerating (**ER**) low-density projections were branches compared to normal-density projections (Z-test, $p = <0.001$). The number of branches observed in early regenerating and late regenerating (**LR**) low-density projections were statistically similar (Z-test, $p = 0.89$), however, there was a significant difference in the number of new branches formed during the imaging period in the early low-density group compared to the late low-density group (Z-test, $p = 0.04$). The early regenerating low-density projections also had significantly more new branches form during the imaging period than normal-density projections (Z-test, $p = <0.001$).

* The data for the Normal-Density Fibers column is taken from Dawson and Meyer, 2001.

Table 2

Summary of Translocation Measurements for Low-Density Optic Fibers during Early and Late Regeneration and Normal-Density Optic Fibers during Early Regeneration¹

	Normal-Density: Early Regen *	Low-Density: Early Regen	Normal-Density: Late Regen **	Low-Density: Late Regen
Net Growth Average	+69 μm ; n = 15	+20 μm ; n = 59	+17 μm ; n = 3	+17 μm ; n = 22
Net Retraction Average	-19 μm ; n = 17	-17 μm ; n = 26	-11 μm ; n = 8	-7 μm ; n = 11
Net Stability	0 μm ; n = 8	0 μm ; n = 7	0 μm ; n = 16	0 μm ; n = 7
Overall Net Translocation Average	+18 μm ; n = 40	+8 μm ; n = 92	-2 μm ; n = 27	+7 μm ; n = 40
Gross Movement Average	49 μm ; n = 40	17 μm ; n = 92	Not Done	25 μm ; n = 40

¹ For low-density and normal-density optic fibers, distance in microns for the different translocation behaviors and the numbers of fibers displaying the different growth behaviors are shown. Low-density optic fibers in early regeneration (**ER**) retracted by larger distances than late regenerating (**Late Regen**) low-density fibers (Mann Whitney Rank Sum test, $p = 0.01$). There were no other statistically significant differences between those two groups' translocation behaviors. Normal-density optic fibers in early regeneration grew by larger distances than low-density fibers in early regeneration (Mann Whitney rank sum test, $p = 0.002$), but there were no other significant differences in the translocation behaviors of normal-density and low-density optic fibers during early regeneration. See text for explanations and calculations used to derive the translocation behavior averages.

* The data for "Normal-Density: Early Regen" is taken from Dawson and Meyer, 2001.

** Data for "Normal-Density: Late Regen" is taken from Johnson et al., 1999.