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Intraocular BDNF Promotes Ectopic Branching, Alters Motility and Stimulates Abnormal Collaterals in Regenerating Optic Fibers

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Abstract

A great deal of effort has been invested in using trophic factors and other bioactive molecules to promote cell survival and axonal regeneration in the adult central nervous system. Far less attention has been paid to investigating potential effects that trophic factors may have that might interfere with recovery. In the visual system, BDNF has been previously reported to prevent regeneration. To test if BDNF is inherently incompatible with regeneration, BDNF was given intraocularly during optic nerve regeneration in the adult goldfish. In vivo imaging and anatomical analysis of selectively labeled axons were used as a sensitive assay for effects on regeneration within the tectum. BDNF had no detectable inhibitory effect on the ability of axons to regenerate. Normal numbers of axons regenerated into the tectum, exhibited dynamic growth and retractions similar to controls, and were able to navigate to their correct target zone in the tectum. However, BDNF was found to have additional effects that adversely affected the quality of regeneration. It promoted premature branching at ectopic locations, diminished the growth rate of axons through the tectum, and resulted in the formation of ectopic collaterals. Thus, although BDNF has robust effects on axonal behavior, it is, nevertheless, compatible with axonal regeneration, axon navigation and the formation of terminal arbors.

Keywords

In Vivo Imaging; Trophic Factor; Visual System; Injury; Axon

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Conflict of Interest

The authors have no conflict of interest in this study.

Role of Authors

Amy Dawson performed the in vivo imaging. Jill Miotke performed the anatomical studies. All authors participated in the design, analysis and writing.

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1. Introduction

Many injuries and diseases of the CNS such as spinal cord injury (SCI), closed head trauma, and glaucoma involve axonal injury. Promoting survival of the axotomized neurons and stimulating axons to regenerate in the adult human could potentially cure these conditions. This has been a major, though elusive, goal in basic and applied neuroscience for many decades. Although regenerative failure is complex, one frequently cited reason for it is a lack of sufficient trophic factor support in the adult CNS (Alto et al., 2009; Benowitz and Popovich, 2011; Blesch and Tuszynski, 2009; Harvey et al., 2009; Lu and Tuszynski, 2008). In response, a large number of experimental studies have attempted to stimulate regeneration by delivering exogenous trophic factors, either alone or in combination with other treatments. Significant beneficial effects have been observed in a variety of models including SCI (Blesch and Tuszynski, 2009; Lu and Tuszynski, 2008) and optic nerve injury (Benowitz and Yin, 2007; Benowitz and Popovich, 2011; Berry et al., 2008; Harvey et al., 2009; Zhang et al., 2005).

BDNF is a member of the neurotrophin family originally identified for its role in cell survival during development (Binder and Scharfman, 2004). It has been widely used in regeneration studies, most notably in the visual system, for its neuroprotective effects. When the optic nerve is crushed in rodents, many retinal ganglion cells are rapidly lost by apoptosis. By 2 weeks, about 90% of ganglion cells have died (Benowitz and Popovich, 2011; Berry et al., 2008; Cohen et al., 1994; Di Polo et al., 1998; Harvey et al., 2009). BDNF has been found to be a potent protector against ganglion cell death. If BDNF is delivered intraocularly by injection or viral vector delivery, as many as 50% survive at 2 weeks (Benowitz and Popovich, 2011; Berry et al., 2008; Cohen et al., 1994; Di Polo et al., 1998; Harvey et al., 2009; Mansour-Robaey et al., 1994; Sawai et al., 1996; Zhang et al., 2005; Zhi et al., 2005) and this is further enhanced with trk B gene transfer (Cheng et al., 2002).

For regeneration and injury, research on BDNF and other trophic factors has primarily focused on outcome with the goal of achieving the strongest effect such as the greatest cell survival or most regeneration (Benowitz and Popovich, 2011; Blesch and Tuszynski, 2009; Harvey et al., 2009; Reeves and Keirstead, 2012). Comparatively little attention has been paid to possible side effects that might interfere with recovery. For BDNF, there is strong evidence for such side effects. Although regeneration is normally not observed in the optic nerve, severed ganglion cell axons do show an abortive regenerative response and their distal ends remain near the injury site. When BDNF is given intraocularly, these axons retract into the eye and sprout extensively within the retina (Mansour-Robaey et al., 1994; Sawai et al., 1996). With adjunctive therapy significant regeneration occurs within the optic nerve. Lens injury, zymosan injection, or delivery of putative factors thought to be produced during inflammation, stimulate substantial regeneration beyond the crush site (Benowitz and Popovich, 2011; Leon et al., 2000). This regeneration is completely prevented when BDNF is delivered intraocularly, even when a minimally damaging “microcrush” is used. Instead, axons form hypertrophic swellings proximal to the crush site and fail to regenerate beyond the injury site (Pernet and Di Polo, 2006).

The reason for this BDNF-mediated inhibition of regeneration is not clear. To further explore this issue, we asked if BDNF might similarly inhibit CNS regeneration in a system that normally regenerates spontaneously (Lee-Liu et al., 2013; Matsukawa et al., 2004). To do this, we used the retinotectal system of the adult goldfish because its optic axons can regenerate after axotomy and can reform accurate and functional connections with the optic tectum, their major projection site. Also, developmental studies have shown that BDNF and its receptor *trkB* are present in the visual system of lower vertebrates (Cohen-Cory and Fraser, 1994; Marshak et al., 2007) like they are in mammals (Cabelli et al., 1995; Isenmann et al., 1999; Marler et al., 2014) and play similar roles in regulating growth and branching, making this a valid model to investigate the effects of BDNF.

In this study, the optic nerve of the goldfish was crushed and BDNF was chronically administered to the retinal ganglion cells during optic nerve regeneration. At 5 weeks, when regenerating axons have normally grown into tectum and have arrived at their normal target area in tectum, the structure and projection pattern of optic axons were examined anatomically. For this, a small number of retinal ganglion cells from one spot in retina were fluorescently labeled with DiI and the axons were visualized in tectal whole mounts. Axons within retina were also examined anatomically in retinal flat mounts. To also investigate effects of BDNF on the dynamic behavior of regenerating axons, axons were similarly labeled with DiI at 3 weeks and the axons were imaged in the living fish over several hours. The rate of growth, retraction and branch formation of axons regenerating within tectum were documented in sequential images obtained in each fish.

2. Results

2.1 Anatomical Whole Mount Study: Retina

In adult rats, it has been reported that intraocular BDNF and NT4/5 stimulated optic axons to branch within the retina following optic nerve section (Cui et al., 2003; Sawai et al., 1996). Since growth and branching within retina might adversely affect the capacity of axons to regenerate to targets, we looked for this in retina. For this, we crushed the optic nerve and at 5 weeks prepared retinal whole mounts to examine axonal structure in retina. Optic axons were labeled with a spot injection of DiI into retina (Danks et al., 1994; Dawson and Meyer, 2001; Wang and Meyer, 2000). In these whole mounts, the site of DiI labeling was readily visible as a bright haze of fluorescence about 200 μ m in diameter in peripheral retina. In the vehicle control retinas, strongly labeled axons emanated from this zone and traversed a direct path to the optic disc without branching (Fig. 1A). In the BDNF-treated eye, axons were similarly brightly labeled and similarly followed a direct path to the optic nerve head. No branching or any other anatomical effect was observed (Fig. 1D). In short, in goldfish, BDNF had no detectable effect within retina.

2.2 Anatomical Whole Mount Study: Tectum

To analyze the pathway and structure of regenerating optic fibers and their capacity to grow to their target zone in the tectum, ventronasal fibers labeled with a spot injection of DiI were visualized at 5 weeks post-crush (Fig 1, 2). Five weeks was chosen since this is the time that regenerating axons arrive at their posterior target zone (Meyer and Kageyama, 1999). To do

this, tectal whole mounts were prepared from the same fish used for the retinal studies above. These were examined under high power microscopy (Fig 1B, C, D, F) as from reconstructions of individual axons from photomontages of the tectal whole mounts (Fig 2). In the vehicle control injected fish, DiI-labeled optic axons were readily visible in flat mounts of the tectum as they coursed across the tectum just below the surface (Fig. 1B, 1C, 2A). Most entered the medial brachia but then dispersed to follow independent trajectories across much of anterior tectum as shown in the drawing of the reconstructed axons (Fig. 2A). In addition, a significant minority entered the tectum through the lateral branch of the optic pathway and traversed unusual lateral to dorsal paths to posterior medial tectum (not shown). In anterior dorsal tectum (Fig 1B, 2A), very few branches were seen, and no branches at all were seen in fibers within lateral tectum either in the anterior or posterior half. In contrast, within posterior medial tectum, axons followed much more circuitous paths and branched extensively (Fig. 1C, 2A). Due to the large number of axonal processes in this zone, it was not possible to reconstruct individual axons.

In the BDNF-treated fish, the overall distribution and number of DiI-labeled regenerating fibers was comparable to that of the vehicle-injected controls (Fig. 1E, 1F, 2B). Similar numbers entered the medial and lateral branches of the optic pathway and followed similar routes to terminate in posterior medial tectum as seen in the tracings of the reconstructed axons (Fig 2B). Within posterior medial tectum, these axons followed tightly twisted paths and branched extensively in a manner similar to the controls (Fig. 1F, 2B). As in the controls, it was not possible to reconstruct individual axons because of the large number of processes. In contrast to controls, in anterior dorsal tectum, most axons branched (Fig 1E, 2B).

Branching was quantified in anterior medial tectum in axons that were most brightly labeled. In control fish, 49 axons were examined and only 10 branches found. In the BDNF-treated fish, 79 axons were examined, and 68 branches were found. The relative number of branches expressed as branches per fiber was 0.86 in the BDNF-treated versus 0.2 in the control. Thus, there was 4.3 times more branching with BDNF treatment. This difference was highly significant (binomial test, $p = 6.17 \times 10^{-7}$). Most of the branches that were seen in BDNF-treated fish (71%) were long processes that extended into posterior tectum without further branching, essentially bifurcations. The remaining branches were short extensions of < 50 μm . Although few branches were seen in the controls, most of these (80%) were also long bifurcations that extended into posterior tectum, and the remaining branches were also < 50 μm . The relative proportion of short branches versus long bifurcations was not significantly different between BDNF-treated and controls (two proportion z-test, $p = 0.81$).

In BDNF-treated fish, axons also coursed through the lateral branch of the optic pathway, entered the incorrect lateral half of tectum, then grew posteriorly and turned medially to terminate in the correct medial posterior half of the tectum (not shown). The number of these errant fibers and their pathways were indistinguishable from the vehicle controls. Interestingly, no ectopic branching was observed in any of these fibers within the lateral half of tectum.

2.3 In vivo Imaging: Regenerating optic fibers, control injections

In order to try to understand what kind of axonal behaviors might have led to the above described structural abnormalities, we turned to in vivo imaging of Dil-labeled optic axons. We imaged at 3 weeks after optic nerve crush since this is when these ventral nasal axons have entered the anterior dorsal tectum where the abnormal structures were observed in the above described tectal whole mount study (Fig 3). A total of 11 Dil-labeled optic fiber endings were observed in the middle to posterior third of dorsal tectum. None of the eleven optic fibers were branched and no new branches were formed during the imaging period.

The endings were imaged once every hour and their positions were compared to that seen in the initial time point to determine if they grew, retracted or were stable. These hourly changes were pooled and analyzed to see if the fibers used one behavior more than another. Fibers displayed a significantly larger number of growth epochs (44%) compared to stable epochs (23%; two-proportion z-test, $p = 0.05$). The frequency of growth epochs compared to epochs of retraction (33%) was not significantly different (two proportion z-test, $p = 0.37$) and the number of retraction and stable epochs also did not differ significantly (two-proportion z-test, $p = 0.39$). All 11 endings showed retraction, growth and stability during the imaging period.

The net translocation was then analyzed. For this, the overall distance each individual fiber grew or each fiber retracted was measured. Seven of the eleven fibers had a positive net translocation (growth), averaging $+43 \mu\text{m}$. Four fibers had negative net translocation (retraction) averaging $-22 \mu\text{m}$. There was no significant difference between these two groups (Student's t-test, $p = 0.52$). The overall (average) net translocation of all the fibers was $+19 \mu\text{m}$. A measurement of motility ("gross movement") was calculated by summing the absolute value of the distances each fiber traveled at every 60-minute interval regardless of whether it was growth or retraction. The average gross movement of all axons was $101 \mu\text{m}$ (Table 1).

2.4 In Vivo Imaging: Regenerating optic fibers: BDNF injections

Exactly the same injection protocol was used to administer BDNF as was used for the vehicle control. A total of 58 fiber endings were observed for 5 – 7 hours (Fig 4, 5). The most distinguishing feature of these fibers was branching. Branches were seen in every animal except one. Of the 58 fiber endings, 47 of these (81%) were branches that originated from a parent fiber in the field of view. In addition, 15 branches actually formed during the imaging period, either as a bifurcation of an ending or as a branch that initiated from an axonal shaft (Figs. 4, 5).

The epochs of growth, retraction and stability were measured for these fibers as in section 2.3 above. BDNF-treated fibers displayed epochs of growth (24%) in equal frequency as epochs of retraction (24%; two-proportion z-test, $p = 0.91$). However, these fibers displayed significantly more episodes of stability (52%) than either growth or retraction (24% in both cases; two-proportion z-test, $p = <0.001$).

The net translocation movements of the BDNF-treated optic fibers were also analyzed as described previously. Twenty-four of the 58 endings (41%) showed positive net

translocation (growth) that averaged +25 μm , while 18 endings (31%) showed negative net translocation (retraction), which averaged -14 μm . Sixteen of the 58 optic fibers (28%) displayed net stability, which included fibers that showed no movement during the entire imaging period (11 endings) and those that grew and retracted by equal distances so as to be within 5 μm of their original position at the end of the imaging period. BDNF-treated optic fibers did not significantly differ in their positive and negative net growth averages (Mann Whitney rank sum test, $p = 0.25$). Overall net movement for all fibers averaged +6 μm . Gross movement (absolute values of growth plus retraction) averaged 26 μm for regenerating optic fibers treated with BDNF (Table 1).

2.5 In Vivo Imaging: Comparison of BDNF to vehicle-treated fibers

The data is summarized in Table 1. The BDNF group differed qualitatively in branching from the vehicle control group. In the BDNF-treated fish, 32 branches were seen at the beginning of the imaging period versus 0 in the vehicle control fish (two-proportion z-test, $p = <0.001$). In addition, during the imaging period, 15 branches formed in the BDNF-treated group compared to none in the vehicle control group (two-proportion z-test, $p = <0.001$). Altogether, 81% of the monitored fiber endings in the BDNF-treated fish were determined to have branches compared to 0% in the controls (Table 1).

In the frequency of different episodic behaviors, BDNF-treated fibers had significantly more stable epochs than vehicle control fibers (two-proportion z-test, $p = <0.001$) and significantly fewer growth episodes than vehicle control fibers (two-proportion z-test, $p = 0.01$). The frequency of retraction episodes did not differ between the two groups (two-proportion z-test, $p = 0.26$). Also, the number of fibers that showed no net movement during the entire imaging period was much greater in the BDNF group.

The magnitude (distance) of movement also differed. The primary motility measure, the average gross movement was 4 times less in the BDNF-treated fibers than the vehicle controls and this was a highly significant difference (Mann Whitney rank sum test, $p = <0.001$). The overall average net translocation was numerically less in the BDNF group, but this was not significantly different (Mann Whitney rank sum test, $p = 0.57$).

Analysis of individual fibers grouped according to those showing net growth and those showing net retraction displayed a trend consistent with less movement in BDNF-treated fibers. Neither of these was statistically significant, however, possibly due to there being fewer fibers in the vehicle control group. Since the vehicle-treated fibers were indistinguishable in morphology and translocation behavior (Student's t-test, $p = 0.44$) from regenerating fibers observed in a previous experiment (Dawson and Meyer, 2001), the data were pooled with the present controls for statistical testing. This yielded a net positive translocation of +56 μm for the control growth group and this was significantly greater than the +25 μm of the BDNF group (Mann Whitney rank sum test, $p = 0.05$). The net negative translocation measure was not significantly different.

2.6 In Vivo Imaging: Ectopic fibers

In the preceding experiments, retinal ganglion cells in ventral retina were labeled and visualized in dorsal tectum. Thus the effects of BDNF were shown only for fibers in the correct mediolateral half of tectum. Since regenerating axons also invade the incorrect mediolateral half of tectum (Becker and Cook, 1987; Cook, 1983; Olson and Meyer, 1991), it is possible to ask if BDNF has effects on these highly ectopic axons. Since the lateral half of tectum is inaccessible for in vivo imaging, this was done by labeling dorsal nasal retina and imaging these fibers in dorsal tectum.

In the vehicle treated fish, 8 fiber endings were visualized in the dorsal tectum to examine their behavior and morphology. These formed simple unbranched endings. No branches were seen (Fig. 6). In the BDNF treated fish, fourteen ectopic fiber endings were visualized at 3 weeks. Of these, 11 (79%) constituted branches. In addition, branch formation was observed during the imaging period (Fig. 7). Thus BDNF had comparable effects on ectopic fibers.

3. Discussion

3.1 BDNF in early regeneration: branching and decreased motility

In early regeneration at 3 weeks after optic nerve crush, we previously found that fibers are simple processes with few observable branches (Dawson and Meyer, 1996). Likewise, in the present study, fibers from the vehicle-injected eye were also simple processes displaying little if any branching. In the previous in vivo imaging study of 84 fibers at 3 weeks, only 9 fibers had detectable branches, and these were simple short branches averaging 56 μm in length. No branches formed during the imaging period. With the data from this previous study, this indicates that only 9% of normal regenerating fibers had branches at this time. In contrast, 81% of BDNF-treated fibers had branches at 3 weeks after nerve crush (present study), which represents a 9 fold increase in branched fibers caused by exogenous BDNF. In addition, 25% of the BDNF-treated fibers formed branches during the imaging period, compared to 0% in the vehicle controls. Also, 0% was seen in a previous study with no retina injection.

Many, if not all, of these BDNF-induced branches were ectopic, since at 3 weeks fibers from nasal ventral retina have not yet reached their normal termination site in posterior dorsal tectum. This was further confirmed by imaging axons from dorsal nasal retina in medial tectum. These fibers would be ectopic along both mediolateral and anterioposterior axes. In this case, 79% of observed BDNF-treated axons exhibited branches compared to 0% of the vehicle controls. Thus, BDNF strongly promoted premature branching at ectopic positions within the tectum.

BDNF also significantly decreased the motility of regenerating fibers both in distance moved and frequency of movement. BDNF decreased motility ("gross movement") by 75% and doubled the number of stable epochs. For fibers that showed overall growth during the imaging period, BDNF-treated fibers grew half as much as the controls. Thus, BDNF changed the behavior from one in which growth was the most frequent behavior to one of stability. BDNF-treated fibers grew less often and for shorter distances.

3.3 Comparison to BDNF effects during development

The effect of BDNF during development has been extensively studied, most notably in the retinotectal projection of *Xenopus* (Cohen-Cory and Fraser, 1994; Cohen-Cory and Fraser, 1995; Hu et al., 2005; Marshak et al., 2007). BDNF has been found to increase the number and length of branches of optic axons within tectum; conversely, blocking BDNF or trk signaling decreased branching (Cohen-Cory and Fraser, 1994; Cohen-Cory and Fraser, 1995; Marshak et al., 2007). Although this would seem to indicate that the effects of BDNF in development and regeneration are equivalent, there are some salient differences that suggest otherwise. In development, effects of BDNF were observed when it was applied to the tectum but no effect was found when it was delivered to the retina. In regeneration, we saw robust effects with intraocular BDNF administration. During development, BDNF increased the episodes of growth (Hu et al., 2005; Lom et al., 2002) whereas we found a decrease in growth episodes during regeneration. These divergent findings suggest that BDNF may have different effects in the adult than in development. This would not be surprising given the numerous differences in both intracellular and cell surface proteins in developing versus regenerating axons (Brecknell and Fawcett, 1996; Rossi et al., 2007), showing that regenerating axons do not simply revert to an embryonic phenotype. These differences point to the limitations of extrapolating the findings from development to regeneration, and to the importance of performing studies on regenerating axons.

3.4 BDNF in late regeneration

During development, optic axons sequentially grow into the tectum over an extended period following stereotypic spatial cues. As a result of this spatiotemporal order, axons exhibit considerable order in their paths that is maintained the adult fish. Thus, axons labeled with Dil from one small region of the retina in a normal fish can be seen to course together and to follow a fairly direct path to their target zone in the tectum. For example, ventronasal axons all enter the medial branch of the optic tract, traverse across anterior tectum as a loosely associated bundle running near the midline, and form terminal arbors within a small region of tectum a few hundred micrometers wide in the middle of posterior tectum near the medial edge (Danks et al., 1994; Johnson et al., 1999; Wang and Meyer, 2000). In contrast, regenerating axons grow in nearly simultaneously and have highly disorganized trajectories. In early regeneration, axons from one retinal locus are widely distributed across much of anterior tectum, including the wrong mediolateral half (Becker and Cook, 1987; Cook, 1983; Meyer et al., 1985). Axons subsequently traverse very diverse routes to navigate to their correct topographic positions where they form convergent arbors in late regeneration. As expected, disordered axonal pathway behavior was clearly evident in the tectal whole mounts at 5 weeks regeneration. In particular, in the vehicle control animals, regenerating axons from ventronasal retina traversed much of the anterior dorsal tectum to terminate in posterior dorsal tectum.

Since exogenous BDNF induced extensive ectopic branching and decreased growth rate in early regeneration (present study), one might expect that it could interfere with the ability of optic axons to grow to their correct target region. There is evidence that it does so during development. In mammals, optic fibers normally undergo a developmental rearrangement of their projections to superior colliculus and dorsal lateral geniculate (dLGN) to form the

mature projection pattern. Delivery of exogenous BDNF prevents these rearrangements, leaving ectopic connections (Isenmann et al., 1999). Similarly, delivery of BDNF has been shown to interfere with the formation of ocular dominance columns in visual cortex (Cabelli et al., 1995).

Somewhat surprisingly, we found that although BDNF-treated optic fibers had highly disordered pathways like the controls, they were able to regenerate to their correct topographic position at 5 weeks regeneration. Axons from nasal ventral retina formed overlapping arbors in posterior dorsal tectum that were indistinguishable from vehicle controls. This included axons that had invaded the wrong lateral half of tectum, and thus had to not only grow through anterior tectum but out of lateral tectum as well in order to reach their correct position. Thus BDNF did not inhibit axonal navigation.

There were, however, some residual abnormalities. With BDNF treatment most axons had branches in anterior dorsal tectum at 5 weeks regeneration compared to few in the controls, which resulted in a 4 fold increase in the number of branches with BDNF. The large majority of these branches were bifurcations that produced long processes extending into their correct position in posterior tectum. Apparently, these axons resolved most of the BDNF-induced ectopic branching that we observed at 3 weeks regeneration by extending parallel processes into posterior tectum. However, not all ectopic branching was resolved by 5 weeks. About 30% of the branches were relatively short with endings that were observed in anterior tectum, suggesting that these were retaining ectopic synapses that transiently form during regeneration (Meyer and Kageyama, 1999). These results are summarized in the diagram shown in Figure 7. Interestingly, for fibers that entered the lateral half of tectum instead of dorsal half, no branches were seen in lateral half of tectum in either BDNF-treated or vehicle controls, even though BDNF stimulated ectopic branching in these fibers at 3 weeks. Evidently, BDNF-treated fibers in the lateral tectum resolved these ectopic branches by branch retraction.

3.5 Implications for Regeneration

The most important result is that excellent regeneration can be obtained with chronic BDNF treatment. BDNF is not inherently incompatible with optic nerve regrowth, axonal navigation, and axonal restructuring which needs to take place for effective regeneration. There were, however, some “side effects”, that is, additional effects that adversely affected the quality of regeneration. BDNF strongly stimulated early ectopic branching and slowed axonal growth. Goldfish optic axons were able to largely overcome these initial difficulties, presumably due to the robust nature of regeneration in the lower vertebrates. For mammalian regeneration, particularly in humans where regeneration is much less robust and where the distances that axons need to grow are far greater, these side effects could be much more problematic. Assuming that a way to use BDNF to promote cell survival could be found so that it was compatible with regeneration, one solution to the side-effect problem might be to stop BDNF treatment once axons have reached their target and begin making synaptic connections. At this point, axons would be able to derive BDNF from their targets and might not need exogenous BDNF to promote cell survival.

Why BDNF inhibited *in situ* regeneration in rodents (Pernet and Di Polo, 2006) but not in the goldfish is not known. It seems unlikely that exogenous BDNF is shutting down the growth response of the axotomized ganglion cells, since it was associated with extensive axonal sprouting within the retina in rodents (Mansour-Robaey et al., 1994; Sawai et al., 1996) and with upregulation of GAP43 and α -tubulin (Klocker et al., 2001; Kwon et al., 2007). Also BDNF does not prevent the regeneration of ganglion cell axons into sciatic nerve implants, although it appears to reduce growth distance (Cui et al., 2003; Mansour-Robaey et al., 1994). BDNF increases nitric oxide in the rodent retina (Zhang et al., 2005) which could conceivably interfere with regeneration. An interesting possibility is that BDNF enhances signaling from inhibitory molecules found in the optic nerve of mammals. This might explain why BDNF not only prevented regeneration but also stimulated the retraction of axons from the myelinated optic nerve and back to the retina, which contains no myelin. This would also explain why BDNF-treated axons could regenerate into sciatic nerve implants, which are a permissive environment. In lower vertebrates, growth inhibitors are either not effective or are eliminated after nerve injury (Becker et al., 2000). Thus, in goldfish, BDNF might not be able to enhance the inhibitory signals that stop growth.

4. Conclusions

Unlike mammals where BDNF completely inhibited optic nerve regeneration (Pernet and Di Polo, 2006) and instead induced axonal retraction and sprouting within the retina (Mansour-Robaey et al., 1994; Sawai et al., 1996), in goldfish, no sprouting of optic axons was seen in the retina. Instead, optic axons regenerated into the tectum and navigated to their appropriate tectal region, where they formed terminal branches. However, significant side effects of exogenous BDNF that adversely affected the quality of regeneration were also observed. In early regeneration when axons were growing toward their target, *in vivo* imaging revealed BDNF-induced premature axonal branching and decreased axonal motility; and, in later regeneration, ectopic branches were retained.

5. Experimental Procedure

5.1 Animals

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

5.2 Optic nerve crush

Nerve crush was used to sever optic axons. This method reliably axotomizes these axons while constraining the axons within the optic sheath, thereby significantly facilitating regeneration within the optic pathway. Fish were anesthetized by immersion in 0.05% tricaine methanesulfonate (Sigma Chemical Co., St. Louis, MO). Under a stereomicroscope, the left optic nerve was crushed with fine forceps until a visibly clear zone was produced in the nerve, while leaving the optic sheath intact. Care was taken to avoid damaging the vasculature supplying the retina.

5.3 Intraocular BDNF injections

Intravitreal injections were used to deliver BDNF or vehicle control solution to the retina. Fish have a large, easily accessible vitreal space which makes it possible to deliver substances over a prolonged period with repeated injections without damaging the retina. The procedure used here is that same as previously used to deliver TTX for as long as 4 months without detectable retinal damage (Meyer, 1982; Olson and Meyer, 1991). Fish were lightly anesthetized in 0.05% tricaine methanesulfonate. Under a stereomicroscope, a hole was made in the dorsal limbus of the left eye with a sterile syringe needle. The tip of a 10 μ l syringe was inserted through the hole into the vitreous behind the lens. Then 1 μ l of BDNF solution (200 ng recombinant human BDNF/ml of teleost Ringer's, 0.05% DMSO and 1 mg/ml BSA) or vehicle control solution (teleost Ringer's with 0.05% DMSO and 1 mg/ml BSA) was injected into the vitreous. (rhBDNF: carrier protein-free, PeproTech, Inc., Rocky Hill, NJ; DMSO: Hybri-Max Cell Culture grade, Sigma Chemical Co., St. Louis, MO; BSA: cell-culture tested (A4161), Sigma Chemical Co.). Using the same entry hole, injections were repeated approximately every 48 hours starting at 7 days post optic nerve crush and continued until the fish was imaged or perfused. The schedule of these injections was rigidly maintained and was the same for both the BDNF and vehicle control solutions. Typically, the BDNF and control animals were run together as a cohort, where the animals were randomly assigned to their treatment group at the time of the first intraocular injection.

5.4 Dil injections

"Spot Injections" of Dil into retina were used to label optic axons in vivo. This technique labels a few optic axons that originate from a small region of retina. Axons are labeled along their entire length, thereby allowing one to visualize both pathway order and topography of the termination zone of these axons as well as their structure. Both normal and regenerating axons have been shown to be strongly labeled (Danks et al., 1994; Johnson et al., 1999; Wang and Meyer, 2000). For this, fish were anesthetized as for optic nerve crush and a 0.25% solution of Dil (Molecular Probes, Inc., Eugene, OR) in absolute EtOH was injected into a small spot in the left retina. After removing a small piece of sclera with a fine tungsten hook, a Dil-filled glass needle (15–20 μ m in diameter) mounted on a microliter syringe was inserted through the underlying pigmented epithelium. The needle was advanced into the retina by 100 μ m intervals and 1.6nl of Dil was injected at each step to a depth of 500–600 μ m for a total of 5–6 injections. Injections were typically made in nasal ventral left retina so as to label axons that normally terminate in dorsal posterior right tectum. Ventral retina was selected because only dorsal tectum was accessible for in vivo imaging. Nasal retina was selected because these fibers course across anterior tectum to terminate in posterior tectum and thus provide a good assay for axonal navigation. For one of the in vivo imaging studies, dorsal nasal retina was injected in order to visualize axons that were in the wrong dorsal ventral half of tectum, that is, were in dorsal tectum (ventral tectum is inaccessible). Fish were left to recover 4–6 days to allow for dye transport. (BDNF and vehicle control injections continued throughout the period of dye transport as described above.)

5.5 In Vivo Imaging

For this study, the optic nerve was crushed and beginning at 7 days the eye was injected with vehicle or BDNF every 48 hours until in vivo imaging. Imaging was performed at around 3 weeks since this is the period in which regenerating optic axons are in the process of growing across the anterior half of tectum toward their target zone in posterior tectum (Olson and Meyer, 1991). For fish with Dil-labeled ventral nasal optic fibers, the actual range for the BDNF-treated fish (n=11) was 17–28 days with a median of 24 days. Fish (n=11) with control injections were identically imaged at 17–28 days. In preparation for imaging, fish were anesthetized with tricaine methanesulfonate and d-Tubocurarine (2 µg/g fish, Sigma Chemical Co., St. Louis, MO) was injected into the epaxial (dorsal) musculature to insure immobilization during the imaging period. The tecta were exposed by excising the cranium covering the midbrain region and aspirating any overlying fat deposits. Fish were then immobilized in a holder similar to those used in electrophysiological studies (Meyer, 1977). Fish were respired by a tube placed in the mouth through which aerated water was pumped over the gills. Fish were sometimes anesthetized throughout the imaging period by inclusion of 0.01% tricaine methanesulfonate in the respiration water. For the actual imaging, 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 and the images were stored 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 plane of focus to ensure all pieces of the fibers were visualized. The depth of each fiber was estimated relative to the tectal surface by imaging at 4 µm intervals until the focal plane with the ending of the fiber in focus was reached. The tecta were illuminated only while collecting the Z-series and kept moist throughout the entire experiment with teleost Ringer's. Blood flow in the tecta was checked before and after each Z-series. The experiment was terminated if blood flow had slowed or stopped. Fish were sacrificed at the end of experiments by pithing through the open cranium.

5.6 Data analysis of in vivo imaging

The 12-bit image files (4094 gray scale values) from each Z-series time point were scaled to 8 bits (255 gray scale values) based on histogram analysis of intensity levels and converted to TIFF files with image processing software (ImagePro Plus, Media Cybernetics, Silver Spring, MD). The usable gray scale of the 12-bit image was always less than 8 bits in depth so it was possible to map the 12-bit image into 8-bit space without the loss of information. The image file that contained most of the fibers in focus was used as a template to create a composite in which all parts of the fibers were in focus. This was accomplished by selecting pieces from each 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 ("cut-and-paste" method). Z-series montages were done for each time point and the position of the end of each fiber was measured to determine changes of fiber length and direction of growth by comparing each time point with 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. Changes in fiber trajectories were scored qualitatively. Statistical analyses were performed using SigmaStat (SPSS Corporation, Chicago, IL) and the results were rounded to the nearest hundredths. Statistical significance was set at the $p < 0.05$ level.

5.7 Anatomical analysis of tectal and retinal whole mounts

Whole mounts of retina and tectum were used to analyze the pathway, structure and termination zone of optic axons. In the retina, optic axons occupy a narrow layer very near the inner surface immediately above the ganglion cell layer and in the tectum axons are found in two superficial layers, the SO (optic fiber layer) and SFGS (optic innervations layer), which begin at about 25 μm (SO) down to about 150 μm (lower SFGS). This superficial location makes it possible to visualize Dil-labeled axons in their entirety in flat mounts of retina and tectum without the necessity of reconstructing serial sections (Wang and Meyer, 2000).

At about 5 weeks (32–33 days) after left optic nerve crush, fish that had received repeated intraocular BDNF ($n=2$) or control vehicle injections ($n=2$) into the vitreous of the left eye that began 7 days after optic nerve axotomy (see section 5.3 above) were perfused with fixative, and tectal and retinal whole mounts were prepared by a modification of the method of Wang and Meyer (Wang and Meyer, 2000). In brief, at 28 days after optic nerve crush, fish received an injection of Dil into a single site at the periphery of the ventral nasal quadrant of the left retina (section 5.4 above) and were allowed to recover for 4–5 days to allow for dye transport. Preliminary studies of regenerates that had not received repeated intraocular injections found this method anterogradely labeled a small number of optic axons that terminated in the posterior medial quadrant of the right tectum at 5 weeks post-crush. After the dye transport period, fish were deeply anesthetized in tricaine methanesulfonate, and then perfused initially with 0.05% tricaine methanesulfonate in teleost Ringer's, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. The tectal lobes were dissected from the brain, partially slit anteriorly from the posterior pole to enable them to lie flat, sandwiched between two small square pieces of Telfa surgical dressing (Kendal Co., Boston, MA) and immersed in the same fixative for 15 minutes. A small square glass weight was placed on top of the Telfa pad sandwich to ensure it remained flat and submerged during postfixation. The retinas were removed from the left eyes and several radial cuts were made at their margins before flat-mounting onto a microscope slide. Tectal and retinal whole mounts were coverslipped with glycerol.

Immediately after preparation of the whole mounts of retina and tectum, digital images were acquired using a cooled CCD camera (Spot RT KE Monochrome, Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on an Olympus BX51 epifluorescence microscope equipped with rhodamine optics. For tectum, labeled fibers that were able to be clearly resolved using 10X/0.3 N.A. and 20X/0.5 N.A. dry objectives (Olympus) were systematically traced. For this, a series of images in the Z-axis was acquired that contained all focal planes in which the fibers were in focus. Each Z-series was stored as a set of individual 12-bit TIFF files. After high power images of all labeled fibers had been

acquired, low power images of the entire tectal whole mount were obtained using an Olympus 4X/0.1 N.A. objective so as to determine the position of these fibers within tectum. Photographs of retina were also obtained to confirm localized labeling.

The 12-bit TIFF files were imported into ImagePro Plus for image processing and analysis, which was performed blind with respect to treatment condition. In order to reconstruct the axonal projections over large areas of tectum or retina, it was necessary to create a montage by tiling together two-dimensional projections of 10X Z-series. For each individual Z-series, contrast and brightness were adjusted for optimal visualization, and the same conversion was used for each image within the same Z-series. A two-dimensional projection of in-focus fibers was reconstructed from each Z-series by the “cut and paste” method used for data analysis of fibers visualized by in vivo imaging (section 5.6 above). Final assembly of the projections into a montage was aided by landmarks visible in the low-power images of the whole mounts. For quantification of branching behavior, axons and branches were identified and manually marked on the montage based on careful examination of unprocessed 20X Z-series. The length of short axonal branches was measured by tracing them on the 10X projections following calibration of the images with a stage micrometer. The binomial test (Microsoft Excel 2007) was used to determine the statistical significance of the difference in the number of axon branches in BDNF-treated fish as compared to vehicle-treated fish. All other statistical analyses were performed using SigmaStat, as above. Statistical significance was set at the $p < 0.05$ level.

Tracings were also made of labeled axons at low power in order to create a two-dimensional reconstruction of their distribution in the medial half of the tectum. For this, a “cut and paste” montage of the entire medial hemi-tectum was assembled. In Image Pro Plus, the above described reconstructions of each 10X Z-series were appropriately scaled, tiled, and pasted onto the 4X images of the tectal whole mount. This low power digital montage was then imported into Adobe Illustrator CS6, and labeled fibers were manually traced using a digital tablet and pen (Wacom). Only well-labeled fibers were selected for tracing. In the posterior half of the tectum, axons branched extensively and formed early stage terminal arbors within their normal termination zone. These branches were so numerous and overlapped so extensively that many of these small branches could not be reliably traced. These are not represented in the drawings.

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References

- Alto LT, Havton LA, Conner JM, Hollis li ER, Blesch A, Tuszynski MH. Chemotropic guidance facilitates axonal regeneration and synapse formation after spinal cord injury. *Nat Neurosci.* 2009; 12:1106–13. [PubMed: 19648914]
- Becker DL, Cook JE. Initial disorder and secondary refinement of regenerating axons in the optic tract of the goldfish: Signs of a new role for axon collateral loss. *Development.* 1987; 101:323–337.
- Becker T, Anliker B, Becker C, Taylor J, Schachner M, Meyer R, Bartsch U. Tenascin-R inhibits regrowth of optic fibers in vitro and persists in the optic nerve of mice after injury. *Glia.* 2000; 29:330–346. [PubMed: 10652443]

- Benowitz LI, Yin Y. Combinatorial treatments for promoting axon regeneration in the CNS: Strategies for overcoming inhibitory signals and activating neurons' intrinsic growth state. *Dev Neurobiol.* 2007; 67:1148–65. [PubMed: 17514713]
- Benowitz LI, Popovich PG. Inflammation and axon regeneration. *Curr Opin Neurol.* 2011; 24:577–83. [PubMed: 21968547]
- Berry M, Ahmed Z, Lorber B, Douglas M, Logan A. Regeneration of axons in the visual system. *Restor Neurol Neurosci.* 2008; 26:147–74. [PubMed: 18820408]
- Binder DK, Scharfman HE. Brain-derived neurotrophic factor. *Growth Factors.* 2004; 22:123–31. [PubMed: 15518235]
- Blesch A, Tuszynski MH. Spinal cord injury: plasticity, regeneration and the challenge of translational drug development. *Trends Neurosci.* 2009; 32:41–7. [PubMed: 18977039]
- Brecknell J, Fawcett J. Axonal regeneration. *Biol Rev Camb Philos Soc.* 1996; 71:227–255. [PubMed: 8616210]
- Cabelli RJ, Hohn A, Shatz CJ. Inhibition of ocular dominance column formation by infusion of NT-4/5 or BDNF. *Science.* 1995; 267:1662–1666. [PubMed: 7886458]
- Cheng L, Sapieha P, Kittlerova P, Hauswirth WW, Di Polo A. TrkB gene transfer protects retinal ganglion cells from axotomy-induced death in vivo. *J Neurosci.* 2002; 22:3977–86. [PubMed: 12019317]
- Cohen-Cory S, Fraser SE. BDNF in the development of the visual system of *Xenopus*. *Neuron.* 1994; 12:747–761. [PubMed: 8068082]
- Cohen-Cory S, Fraser SE. Effects of brain-derived neurotrophic factor on optic axon branching and remodelling in vivo. *Nature.* 1995; 378:192–6. [PubMed: 7477323]
- Cohen A, Bray GM, Aguayo AJ. NT-4/5 increases adult rat retinal ganglion cell survival and neurite outgrowth *in vitro*. *Journal of Neurobiology.* 1994; 25:953–959. [PubMed: 7964706]
- 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.
- Cui Q, Yip HK, Zhao RC, So KF, Harvey AR. Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. *Mol Cell Neurosci.* 2003; 22:49–61. [PubMed: 12595238]
- 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; 345:253–66. [PubMed: 7929900]
- Dawson A, Meyer R. Characterization of the dynamics of retinal fibers during early regeneration in adult goldfish. *Soc Neurosci.* 1996; 22:1726.
- 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]
- Di Polo A, Aigner LJ, Dunn RJ, Bray GM, Aguayo AJ. Prolonged delivery of brain-derived neurotrophic factor by adenovirus-infected Muller cells temporarily rescues injured retinal ganglion cells. *Proc Natl Acad Sci U S A.* 1998; 95:3978–83. [PubMed: 9520478]
- Harvey AR, Hellstrom M, Rodger J. Gene therapy and transplantation in the retinofugal pathway. *Prog Brain Res.* 2009; 175:151–61. [PubMed: 19660654]
- Hu B, Nikolakopoulou AM, Cohen-Cory S. BDNF stabilizes synapses and maintains the structural complexity of optic axons in vivo. *Development.* 2005; 132:4285–98. [PubMed: 16141221]
- Isenmann S, Cellerino A, Gravel C, Bahr M. Excess target-derived brain-derived neurotrophic factor preserves the transient uncrossed retinal projection to the superior colliculus. *Mol Cell Neurosci.* 1999; 14:52–65. [PubMed: 10433817]
- 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]
- Klocker N, Jung M, Stuermer CA, Bahr M. BDNF increases the number of axotomized rat retinal ganglion cells expressing GAP-43, L1, and TAG-1 mRNA--a supportive role for nitric oxide? *Neurobiol Dis.* 2001; 8:103–13. [PubMed: 11162244]
- Kwon BK, Liu J, Lam C, Plunet W, Oschipok LW, Hauswirth W, Di Polo A, Blesch A, Tetzlaff W. Brain-derived neurotrophic factor gene transfer with adeno-associated viral and lentiviral vectors

prevents rubrospinal neuronal atrophy and stimulates regeneration-associated gene expression after acute cervical spinal cord injury. *Spine (Phila Pa 1976)*. 2007; 32:1164–73. [PubMed: 17495772]

- Lee-Liu D, Edwards-Faret G, Tapia VS, Larrain J. Spinal cord regeneration: Lessons for mammals from non-mammalian vertebrates. *Genesis*. 2013; 51:529–544. [PubMed: 23760835]
- Leon S, Yin Y, Nguyen J, Irwin N, Benowitz L. Lens injury stimulates axon regeneration in the mature rat optic nerve. *J Neurosci*. 2000; 20:4615–4626. [PubMed: 10844031]
- Lorn B, Cogen J, Sanchez AL, Vu T, Cohen-Cory S. Local and target-derived brain-derived neurotrophic factor exert opposing effects on the dendritic arborization of retinal ganglion cells in vivo. *J Neurosci*. 2002; 22:7639–49. [PubMed: 12196587]
- Lu P, Tuszynski MH. Growth factors and combinatorial therapies for CNS regeneration. *Exp Neurol*. 2008; 209:313–20. [PubMed: 17927983]
- Mansour-Robaey S, Clarke DB, Wang YC, Bray GM, Aguayo AJ. Effects of ocular injury and administration of brain-derived neurotrophic factor on survival and regrowth of axotomized retinal ganglion cells. *Proc Natl Acad Sci U S A*. 1994; 91:1632–6. [PubMed: 8127857]
- Marler KJ, Suetterlin P, Dopplapudi A, Rubikaite A, Adnan J, Maiorano NA, Lowe AS, Thompson ID, Pathania M, Bordey A, Fulga T, Van Vactor DL, Hindges R, Drescher U. BDNF promotes axon branching of retinal ganglion cells via miRNA-132 and p250GAP. *J Neurosci*. 2014; 34:969–79. [PubMed: 24431455]
- Marshak S, Nikolakopoulou AM, Dirks R, Martens GJ, Cohen-Cory S. Cell-autonomous TrkB signaling in presynaptic retinal ganglion cells mediates axon arbor growth and synapse maturation during the establishment of retinotectal synaptic connectivity. *J Neurosci*. 2007; 27:2444–56. [PubMed: 17344382]
- Matsukawa T, Arai K, Koriyama Y, Liu ZW, Kato S. Axonal regeneration of fish optic nerve after injury. *Biological & Pharmaceutical Bulletin*. 2004; 27:445–451. [PubMed: 15056844]
- Meyer RL. Eye-in-water electrophysiological mapping of goldfish with and without tectal lesions. *Exp Neurol*. 1977; 56:23–41. [PubMed: 558902]
- Meyer RL. Tetrodotoxin blocks the formation of ocular dominance columns in goldfish. *Science*. 1982; 218:589–91. [PubMed: 7123262]
- 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]
- 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]
- 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; 303:412–23. [PubMed: 2007657]
- Pernet V, Di Polo A. Synergistic action of brain-derived neurotrophic factor and lens injury promotes retinal ganglion cell survival, but leads to optic nerve dystrophy in vivo. *Brain*. 2006; 129:1014–26. [PubMed: 16418178]
- Reeves A, Keirstead HS. Stem cell based strategies for spinal cord injury repair. *Adv Exp Med Biol*. 2012; 760:16–24. [PubMed: 23281511]
- Rossi F, Gianola S, Corvetti L. Regulation of intrinsic neuronal properties for axon growth and regeneration. *Prog Neurobiol*. 2007; 81:1–28. [PubMed: 17234322]
- Sawai H, Clarke DB, Kittlerova P, Bray GM, Aguayo AJ. Brain-derived neurotrophic factor and neurotrophin-4/5 stimulate growth of axonal branches from regenerating retinal ganglion cells. *J Neurosci*. 1996; 16:3887–94. [PubMed: 8656282]
- Wang Z, Meyer R. Fine retinotopic organization of optic terminal arbors in the tectum of normal goldfish. *Vis Neurosci*. 2000; 17:723–735. [PubMed: 11153652]
- Zhang CW, Lu Q, You SW, Zhi Y, Yip HK, Wu W, So KF, Cui Q. CNTF and BDNF have similar effects on retinal ganglion cell survival but differential effects on nitric oxide synthase expression soon after optic nerve injury. *Invest Ophthalmol Vis Sci*. 2005; 46:1497–503. [PubMed: 15790921]

Zhi Y, Lu Q, Zhang CW, Yip HK, So KF, Cui Q. Different optic nerve injury sites result in different responses of retinal ganglion cells to brain-derived neurotrophic factor but not neurotrophin-4/5. *Brain Res.* 2005; 1047:224–32. [PubMed: 15904902]

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Highlights

BDNF is permissive for optic nerve regeneration in situ

BDNF is permissive for axonal navigation and pathfinding

BDNF alters dynamic growth behavior of regenerating axons

BDNF stimulates premature ectopic branching

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Figure 1.

Whole mount analysis of Dil- labeled optic fibers in the retina (A, D) and the contralateral medial (dorsal) tectum (B, C, E, F) in fish treated with vehicle (A–C) or BDNF (D–F) at 5 weeks regeneration. A, D: Spot injection of Dil in a single site in peripheral ventronasal retina anterogradely labels a small number of optic fibers that project to the optic disc without branching in both vehicle-treated (A) and BDNF-treated (D) fish. Peripheral is to the left in each panel. B, E: Optic fibers traversing the anterior half of medial tectum are unbranched in vehicle-treated fish (B). In contrast, BDNF-treated optic fibers (E) exhibit ectopic branches. The arrow marks a short branch (~30 μ m) emanating from an axonal shaft. The arrowheads indicate axons that have bifurcated into two long branches. Anterior is at the top and medial to the left of each panel. C, F: At 5 weeks regeneration, optic fibers begin to form terminal arbors in the posterior half of medial tectum in both vehicle-treated (C) and BDNF-treated (D) fish. Anterior is at the top and medial to the left of each panel. Scale bar represents 50 μ m in A and D; 100 μ m in all other panels.

Figure 2.

Reconstruction of Dil-labeled optic axons in medial (dorsal) tectum in vehicle control injected (A) and BDNF injected (B) fish. Tracings were made of selected well labeled axons from montages of photomicrographs of the medial half of tectum (lateral half not shown). In posterior medial tectum, axons branched extensively forming many small overlapping processes. Some of these could not be reliably drawn, and thus are not represented. Scale bar represents 500 μm . A = anterior; P = posterior; M = medial; L = lateral.

Figure 3.

In vivo imaging of retinotopic optic fibers from ventronasal retina at 20 days regeneration. These fibers were treated with vehicle. The arrow marks one of the fibers followed during the imaging period. It grew for a total of 27 μm . Anterior is to the top and medial to the left of each panel. Scale bar represents 20 μm .

Figure 4.

In vivo imaging of retinotopic optic fibers at 17 days regeneration. These fibers were treated with BDNF. The arrowhead indicates one of the many endings followed during the experiment, which was a branch. It bifurcated by the 11:45 a.m. time point. Notice the other branches within the field. The arrow labels a fiber that also bifurcated by the 10:40 a.m. time point. Anterior is to the top and medial to the left of each panel. Scale bar represents 20 μ m.

Figure 5.

In vivo imaging of retinotopic optic fibers at 18 days regeneration. These are BDNF-treated fibers. The arrowhead indicates an ending that bifurcated by the 11:00 a.m. time point, but had lost the bifurcation by the 1:00 p.m. time point. The arrow labels another branch within the field. Anterior is to the top and medial to the left of each panel. Scale bar represents 20 μm .

Figure 6.

In vivo imaging of ectopic optic fibers at 21 days regeneration. The fibers were treated with vehicle. The arrow indicates an ending that was followed for the entire imaging period. Note the simple structure. Anterior is at the top and medial to the left of each panel. Scale bar represents 20 μ m.

Figure 7.

In vivo imaging of ectopic optic fibers at 24 days regeneration. The fibers were treated with BDNF. The thin arrow indicates an area along an axonal shaft that had branches for the entire imaging period. The thick arrow indicates another area of branches within the field. Anterior is to the top and medial to the left of each panel. Scale bar represents 20 μ m.

2 WEEKS**5 WEEKS** **CONTROL** **BDNF****Figure 8.**

Diagram of main results comparing axonal morphology between control (upper panel) and BDNF-treated (lower panel) at 3 weeks and 5 weeks regeneration. Dorsal tectum is shown in outline. Lateral half of tectum is omitted for simplicity. Regenerating optic fibers invade at the anterior end of tectum at the left and eventually terminate in near the periphery of posterior dorsal tectum at lower right.

Table 1**TRANSLOCATION MOVEMENTS AND BRANCH FORMATION**

“Branches” are the number of branches observed extending from all axons in the imaging field at the beginning of imaging or formed during the imaging period and total of both of these. “Epochs” compare each observed fiber between 2 sequential images, that is, images taken 1 hour apart. (E.g., a fiber that always grew would have 100% growth epochs. One that grew half of the time would have 50% etc. A stable epoch is no observed change of position.) “Net Translocation” is the change in the position of the ending of an axon observed between the first image and the last image of the entire imaging session (5–7 hours). “Gross Movement (Motility)” measures the change in the position of the ending of each axon that occurred between each image (1h intervals) given as an absolute distance (same number regardless of growth or retraction, e.g., 10 μm growth = 10 μm retraction). These distance changes are then summed for each observed axon and averaged for all axons to give “Gross Movement”.

	Vehicle-Treated Fibers	BDNF-Treated Fibers
Branches At Beginning Imaging	0	32 [*]
Branches Formed During Imaging	0	15 [*]
Total Number of Branches	0	47 [*]
Growth Epochs	44%	24% ⁺
Retraction Epochs	33%	24%
Stable Epochs	23%	52% [*]
Positive Net Translocation	+43 μm (n = 7) (+56 μm) ^I	+25 μm (n = 24) [°]
Negative Net Translocation	−22 μm (n = 4)	−14 μm (n = 18)
Zero Net Translocation	N = 0	N = 16 [*]
Overall Net Translocation	+19 μm	+6 μm
Gross Movement (Motility)	101 μm	26 μm [*]

^{*} p = <0.001, two proportion z-test;

⁺ p = <0.01, two proportion z-test;

[°] p = <0.05 Mann Whitney rank sum test.

^I Data pooled as per text.