Adult Hamsters
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Regeneration of Axotomized Retinal Ganglion Cells in Adult Hamsters

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xonal injuries to the mammalian central nervous system (CNS) often result in the loss of axons and progressive degeneration of neurons. Injured CNS neurons have a very limited ability to regenerate their axons in developing animals but are generally unable to do so in adults. The death of the neurons and the failure of axonal regeneration after axotomy have been attributed to an interruption of the supply of the appropriate trophic factors (TFs) derived from the cells in their central targets and on their pathways; the activation of an intrinsic suicide program leading to apoptosis; and an unfavorable environment surrounding the neurons and the axons, presumably the presence of inhibitory factors. Recent evidence also suggests that the lack of intrinsic growth potential in the CNS neurons also plays an important role.

Because the somata and the myelinated axons of retinal ganglion cells (RGCs) can be easily accessed and separately manipulated, neuronal viability and axonal regeneration in mammalian visual systems have been intensively studied for many years. In rats, for example, most RGCs are lost within 2 weeks of proximal transection of the optic nerve (ON). The surviving RGCs fail to reestablish functional connection in their former targets, and only abortive axonal sprouting has been observed at the transected end of the ON. The site of the ON lesion appears to have different effects on the extent of the axonal regeneration and the loss of RGCs. Generally, the severity of the axonal degeneration and the loss of RGCs decrease with increasing distance of the lesion from the eye. However, the capability of RGC axonal regeneration also is decreased as the distance increases.

Recently, data from our laboratory have shown that intravitreal implantation of a short segment of peripheral nerve (PN) could effectively increase axonal regeneration of the distally or proximally axotomized RGCs in young adult hamsters, suggesting that some of the TFs released from the PN graft are capable of promoting axonal regeneration. However, the factors involved in this regeneration-promoting effect have not been identified. Many studies of the biological functions of TFs in the visual system have been directed at neuronal viability, with little information available on axonal regeneration. For example, brain-derived neurotrophic factor (BDNF), neurotrophin-4/5 (NT-4/5), and basic fibroblast growth factor (bFGF) were all shown to promote the survival of developing RGCs, adult RGCs, or both; but their effects on axonal regeneration were limited—they only enhanced neurite outgrowth in vitro and axonal branching and sprouting in vivo but no long-distance axonal regeneration. Studies on nerve growth factor (NGF), neurotrophin-3 (NT-3), and ciliary neurotrophic factor (CNTF) revealed no significant effects on RGC neurite outgrowth and inconclusive results on the viability of RGCs, although they may exert different effects on axonal regeneration in other systems. The present study was undertaken to clarify the ability of different TFs to promote axonal regeneration of RGCs into a sciatic nerve (SN)
A total of 118 young adult hamsters (Mesocricetus auratus, 6 to 8 weeks old) were used in this study. The experimental procedures have been described elsewhere. This investigation adhered to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All operations were carried out under Sagatal (sodium pentobarbitone; 60 mg/kg body wt) anesthesia.

**Distal Axotomy Model.** To cut the ON intracranially, a small hole (ca. 2 × 2 mm) was drilled in the left frontal bone just rostral to the bregma. Neural tissues below the craniotomy were removed by aspiration until the left ON was exposed; care was taken to avoid damaging the blood vessels around the ON during this procedure. A pair of small iridectomy scissors was used to completely transect the ON (approximately 7 mm from the optic disc. Fig. 1A). After the ON transection, a 1.5-cm-long segment of autologous SN was dissected from the left leg. The proximal tip of the SN segment was desheathed and dismantled. With the aid of a glass micropipette, this part of the SN segment was guided into the brain cavity and apposed thoroughly onto the proximal stump of the severed ON (Fig. 1A). The connection of the transplanted SN graft with the transected ON was maintained by packing the cavity with pieces of GelFoam (UpJohn). The remaining portion of the SN (distal part) was placed along the wall of the cavity and on top of the skull. The end of the SN graft was tied with a 5-0 suture and secured to the connective tissue on the skull. The interface of the ON and the SN graft was checked in previous experiments by examining sections of the decalcified head with the brain in situ, and direct contact of the majority, if not the whole, of the ON stump with the SN graft was consistently achieved.

**Proximal Axotomy Model.** To cut the ON intraorbitally, the dural sheath of the ON was opened with a 27-gauge ½ needle and a pair of fine forceps. Complete transection of the ON was made inside the dura at a site approximately 0.5 mm from the optic disc. A 1.5-cm-long segment of autologous SN graft was sutured with 10/0 suture to the proximal stump of the ON, and the distal part of the SN graft was placed under the scalp (Fig. 1B).

**Experimental Groups**

**Distal Axotomy Model.** All animals received an intracranial transection of the ON and a transplantation of a 1.5-cm segment of autologous SN but were otherwise divided into nine groups for regeneration studies in the distal ON axotomy model (Table 1). Group 1 received no further treatment and group 2 received intravitreal injections of phosphate-buffered saline (PBS); these two groups served as controls. In group 3 animals, a 2-mm segment of autologous peroneal branch of SN was removed from the hind limb and implanted into the vitreous of the left eye (Fig. 1A). Groups 4 through 9 received intravitreal injections of various TFs (CNTF, NGF, BDNF, NT-3, and NT-4/5, Regeneron; bFGF, Chemicon). The eye injections were made through a glass micropipette in the most peripheral part of the lower temporal retina. The volume of each injection was 2 µl. In all cases, the first eye injection was made right before the transection of the ON. Four additional injections of the same molecule in each animal were performed at the same site at a time interval of every 5 days. The dosages of each injection of the TFs for CNTF, BDNF, NT-3, NT-4/5, NGF, and bFGF were 1, 5, 5, 3, 3, and 1 µg, respectively. These dosages had been shown to be effective in previous studies. Because we initially found that CNTF promoted axonal regeneration of distally axotomized RGCs, different dosages of CNTF (0.5 µg and 2 µg) were used to test its efficacy to promote axonal regeneration. We also examined whether CNTF had effects on RGC survival in this distal axotomy model by comparing animals injected with either CNTF (1 µg × 5) or its carrier (distilled water).

**Proximal Axotomy Model.** To further confirm the enhancement effect of CNTF on axonal regeneration, experiments were also carried out in animals with proximal axotomy of the ON. All animals received an intraorbital transection of the ON, and a 1.5-cm-long autologous SN graft was sutured to the transected ON. The animals were divided into four groups. Group 1 received intraocular injections of CNTF carrier (distilled water) and served as the control. Groups 2 through 4 received intraocular injections of different dosages of CNTF (0.5 µg, 1 µg, and 2 µg). Four intraocular injections of the same molecules were made at a time interval of every 5 days starting from the day the lesion appeared.
Visualization of Regenerating RGCs

All animals were allowed to survive for 4 weeks in the studies using the distal axotomy model and 3 weeks in the studies using the proximal axotomy model. Regenerating or surviving RGCs were labeled by applying Fluoro-Gold (FG) 3 days before they were euthanatized. In the regeneration studies, FG was applied in gelfoam (6%, distal axotomy model, Fig. 1A) or injected using a micropipette (2% in 1 μl, proximal axotomy model, Fig. 1B) into the distal end of the SN graft to label the RGCs that had regenerated their axons to the site of dye application. In the survival studies, a piece of gelfoam soaked with 6% FG was applied to the newly transected site right behind the optic disc to label the surviving RGCs. All animals were killed with an overdose of Sagatal 3 days after the dye application, and the left retinas were dissected in 4% paraformaldehyde and postfixed in the same fixative for 1 hour before they were flat-mounted with 30% glycerin and examined under a fluorescence microscope.

Statistical Analysis

For statistical analysis of the results in the regeneration studies, data were analyzed using one-way ANOVA followed by a two-tailed Dunnnett test. Dunnnett’s test was used to compare the mean values of experimental groups against the controls. The Bonferroni test was also used to compare treated animals at different CNTF concentrations (0.5 μg, 1 μg, and 2 μg) for axonal regeneration, whereas a two-tailed Student’s t-test was used to compare the CNTF group with the vehicle group for survival effects.

RESULTS

The eyes with multiple intravitreal injections showed no obvious infections. Animals were discarded when the surgery caused severe bleeding in the brain. Histologic examination of the intravitreal PN grafts at 1 month postimplantation revealed the presence of viable cellular components, including macrophages laden with myelin debris.°

Effects of SN Grafts and of Intraocular Injections in Distal Axotomy Model

As expected in the distal ON axotomy model, the extent of axonal regeneration in animals receiving graft only or graft plus intraocular PBS injection was limited. A representative fluorescence photomicrograph of FG-labeled RGCs in a “graft only” retinal wholemount is shown in Figure 2A; only one labeled RGC was observed in this field. On the average, the number of RGCs whose axons had reached the site of dye application 1 month after the operations was 4.67 per retina (n = 9) in “graft only” animals, and 4.83 per retina (n = 6) in “graft plus PBS injection” animals. The difference between the two groups was not significant (P > 0.05, Fig. 3), suggesting that the eye injection method used in this study did not by itself influence the outcome of the results.

Table 1. Experimental Conditions and Results

<table>
<thead>
<tr>
<th>Graft</th>
<th>Transsection Site of ON</th>
<th>No. of Animals</th>
<th>Dosage of TFs</th>
<th>No. of Regenerating RGCs ± SEM</th>
</tr>
</thead>
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<tr>
<td>PBS</td>
<td>Distal</td>
<td>9</td>
<td>2 μl × 5</td>
<td>4.67 ± 1.44</td>
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<tr>
<td>PN in eye</td>
<td>Distal</td>
<td>6</td>
<td>2 mm</td>
<td>4.83 ± 1.83</td>
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<td>CNTF</td>
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<td>71.29 ± 17.91</td>
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<tr>
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<td>5</td>
<td>1 μg × 5</td>
<td>56 ± 15.6</td>
</tr>
<tr>
<td>CNTF</td>
<td>Distal</td>
<td>9</td>
<td>2 μg × 5</td>
<td>119.89 ± 27.72</td>
</tr>
<tr>
<td>BDNF</td>
<td>Distal</td>
<td>7</td>
<td>5 μg × 5</td>
<td>112.6 ± 40.41</td>
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<td>NT-3</td>
<td>Distal</td>
<td>12</td>
<td>5 μg × 5</td>
<td>15.25 ± 10.43</td>
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<td>NT-4/5</td>
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<td></td>
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<td>5</td>
<td>2 μl × 4</td>
<td>24.14 ± 12.32</td>
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<td>bFGF</td>
<td>Distal</td>
<td>7</td>
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<td>Distilled water</td>
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<td>3 μg × 5</td>
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<td>1 μg × 5</td>
<td>4812 ± 366</td>
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<tr>
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<td>4</td>
<td>0.5 μg × 4</td>
<td>15.059 ± 2608</td>
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<tr>
<td>CNTF</td>
<td>Proximal</td>
<td>5</td>
<td>1 μg × 4</td>
<td>13.186 ± 585</td>
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<tr>
<td>CNTF</td>
<td>Proximal</td>
<td>7</td>
<td>2 μg × 4</td>
<td>19.155 ± 1504</td>
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</table>

* Values are means ± SEM.

Figure 2. Fluorescence photomicrographs of FG-labeled RGCs with their dendrites in retinal wholemounts of “graft only” (A) and “graft plus CNTF treatment” (B) animals in distal ON axotomy model and in “graft only” (C) and “graft plus CNTF treatment” (D) animals in proximal ON axotomy model. Scale bar, 50 μm.
Effect of Intravitreal PN Implants in Distal Axotomy Model

In animals treated with intravitreal implantation of a 2-mm PN segment, the number of regenerating RGCs into the SN graft was substantially increased, because the average number of FG-labeled RGCs observed 1 month after the implantation was 71.28 per retina \((n = 14)\), an increase of 15-fold. This figure was significantly higher than either the “graft only” group or the “graft plus PBS injection” group \(P < 0.01\), Fig. 3.

Effect of Intravitreal Injections of CNTF in Distal Axotomy Model

The highest degree of RGC axonal regeneration was observed in animals receiving intravitreal CNTF injections. After five injections of 1 \(\mu g\) CNTF, the average number of FG-labeled RGCs was 119.89 per retina \((n = 9)\), a 25-fold increase, which was significantly different from the “graft only” \(P < 0.01\), Fig. 3) and “graft plus PN implant” (Bonferroni test, \(P < 0.05\)) animals. As shown in Figure 2B, the number of FG-labeled RGCs in CNTF-treated animals was higher than in “graft only” animals. The effects of CNTF on axonal regeneration appeared to be dose related. The mean number of regenerating RGCs in animals receiving 0.5 \(\mu g\) CNTF injections was 66.42 per retina \((n = 5\), Table 1\), which was not significantly different from the “graft only” control group \((P > 0.05\), Fig. 4\). The optimal effect of CNTF was achieved at the 1 \(\mu g\) dose \((mean = 119.89)\), and a higher dose \((2 \mu g)\) of CNTF did not further increase the average number of regenerating RGCs \((mean = 112.6, n = 7\), Table 1\), despite the fact that the highest number of regenerating axons reached 346 in one of the animals from the latter group. Both mean values were significantly higher than the “graft only” control group \((P < 0.01\), Fig. 4\). Because relatively high SDs occurred in these three CNTF-treated groups, no significant difference was detected among them (Bonferroni test, \(P > 0.05\), Fig. 4).

Effect of Intravitreal Injections of CNTF on RGC Survival in Distal Axotomy Model

The average number of surviving RGCs in the distal axotomy model was 6634 per retina \((n = 6)\) in distilled water-treated animals, whereas the number surviving in animals treated with five injections of 1 \(\mu g\) CNTF was 5557 per retina \((n = 7)\). These two values were not significantly different from each other (Student’s \(t\)-test, \(P > 0.05\), Fig. 5).

Effects of Intravitreal Injections of Neurotrophins and bFGF in Distal Axotomy Model

BDNF, NT-3, and NGF treatments failed to promote axonal regeneration of distally axotomized RGCs into the SN graft; their respective values for FG-labeled RGCs were 15.25 \((n = 12)\), 0.8 \((n = 5)\), and 5.29 \((n = 7)\) per retina, which were not significantly different from values in “graft only” or “graft plus PBS injection” groups \((P > 0.05\), Fig. 3\). The mean number of FG-labeled RGCs in NT-4/5-treated animals was slightly increased, to 24.14 \((n = 7)\) per retina, but this increase was also not significantly different from the controls \((P > 0.05\), Fig. 3\). bFGF promoted survival of RGCs after injury \(^{14}\) but also failed to enhance axonal regeneration in this study \(mean\) number of labeled RGCs = 1.75, \(n = 4\); \(P > 0.05\), Fig. 3\).

Effect of Intravitreal Injections of CNTF on RGC Survival in Distal Axotomy Model

The average number of surviving RGCs in the distal axotomy model was 6634 per retina \((n = 6)\) in distilled water-treated animals, whereas the number surviving in animals treated with five injections of 1 \(\mu g\) CNTF was 5557 per retina \((n = 7)\). These two values were not significantly different from each other (Student’s \(t\)-test, \(P > 0.05\), Fig. 5).
Figure 6. The average numbers and SEMs of FG-labeled RGCs that had regenerated axons to the site of dye application in animals treated with different dosages of CNTF after proximal ON axotomy. The comparisons were made against the distilled water-treated control (Dunnett’s test, *P < 0.01). Significant difference among treatment groups was detected between 1 µg and 2 µg treatment groups (Bonferroni test, *P < 0.05).

Effect of Intraocular Injections of CNTF in Proximal Axotomy Model

Similar to previous results, the proximally axotomized RGCs showed a higher capability to regenerate axons into a SN graft. The average number of FG-labeled RGCs in control animals treated with distilled water was 4812 per retina (n = 5) 3 weeks after the ON lesion; in contrast, the average numbers of FG-labeled RGCs in animals receiving injections of 0.5 µg or 1 µg CNTF were 15,059 (n = 4) and 13,186 (n = 5) per retina, respectively. Both values were significantly higher than that of the control (P < 0.01, Fig. 6). The effect of CNTF on axonal regeneration in the proximal ON axotomy model also appeared to be dose related, because the highest concentration of CNTF (2 µg) used in this study yielded the highest degree of axonal regeneration; the average number of regenerating RGCs in these animals was 19,155 per retina (n = 7), which was significantly higher than that of the control (P < 0.01, Fig. 6). Among these three CNTF-treated groups, a significant difference was detected only between 1 µg and 2 µg treatment groups (Bonferroni test, *P < 0.05). Note that a high SD occurred in the 0.5-µg treatment group. Representative fluorescence photomicrographs of FG-labeled RGCs in retinal whole-mounts treated with distilled water and CNTF (1 µg × 4) are shown in Figures 2C and 2D, respectively.

Discussion

Effects of Intravitreal Applications of CNTF and PN Segment on Axonal Regeneration

In this study, we used both distal and proximal ON axotomy models to study the capability of axotomized RGCs to regenerate. Among the TFs tested in this study, CNTF is the only effective factor in promoting axonal regeneration of axotomized RGCs in young adult hamsters. The magnitude of the regeneration enhancement by CNTF treatment was achieved by a 25-fold increase in the distal ON axotomy paradigm and by a 4-fold increase in the proximal ON axotomy paradigm. Thus, the results demonstrate that injured RGCs are able to regenerate in response to intravitreal administration of appropriate TFs, and CNTF is at least one of the TFs that can promote axonal regeneration. Because ON contains a large amount of myelin and oligodendrocytes (rich sources of inhibitory molecules), the distally axotomized RGCs are generally less capable of regenerating axons than proximally axotomized RGCs. As a result, the enhancement effect of an appropriate TF on axonal regeneration, as indicated by magnitude in this study, could be observed more easily in the distal than in the proximal model (a 25-fold versus a 4-fold increase). The proximally axotomized RGCs appeared to be more sensitive to CNTF than the distally axotomized RGCs for regeneration—CNTF treatments at 0.5 µg did not significantly enhance axonal regeneration, and 2 µg CNTF treatments did not further increase axonal regeneration from that of 1 µg CNTF treatments in the proximal axotomy model. However, in the proximal axotomy model, CNTF treatments started to promote axonal regeneration at the dose of 0.5 µg, with the highest degree of axonal regeneration achieved at the 2-µg dose (Table 1).

It had been reported that electrophysiological responses to light by 500 regenerating RGCs were detected in the PN grafts in rats. In a similar experiment in hamsters, the electrophysiological responses to light by a small number of the regenerating RGCs were also recorded in the superior colliculus. Accordingly, the regenerating axons in this study may have the potential to form functional synapses and to restore some of the lost vision.

Intravitreal implantation of a PN segment has also significantly enhanced axonal regeneration (a 15-fold increase) of distally axotomized RGCs in this study. This observation confirmed a similar finding in previous studies. Because CNTF is a major TF secreted from PN, the positive effect of intravitreal implants of a PN segment on axonal regeneration may be due to the action of CNTF. However, it is also possible that other factors or a combination of factors from the PN implant are also involved in the promotion of axonal regeneration of the axotomized RGCs.

The level of CNTF expression was previously shown to change substantially after neuronal trauma in both the central and peripheral nervous systems. In the retina, the changes in CNTF expression were an early marker of neuronal injury; the increase or decrease in the level of CNTF expression was correlated with the degeneration of photoreceptor cells after injury or the enhancement of photoreceptor cell survival by intravitreal administration of BDNF. Recently, both CNTF-Rα and its mRNA have been shown to express in high levels in the developing retinas, indicating a significant role during the development of the visual system. This evidence suggests that CNTF may play an important role in the visual system, particularly in the response to injury.

Effects of NGF, BDNF, NT-4/5, NT-3, and bFGF on Axonal Regeneration

Although RGCs have been shown to express trkA, the preferred receptors for NGF, BDNF, NT-4/5, and NT-3, respectively, and although these TFs have been shown to promote the survival of RGCs in vitro and in vivo, they did not increase axonal regeneration of the distally axotomized RGCs in the present study. Similarly, bFGF has also been shown to increase the survival of RGCs, and both bFGF and FGF receptor-1 in the retina have been found to...
be upregulated after injury, but the intravitreal applications of bFGF did not enhance axonal regeneration of distally axotomized RGCs in the present study.

**Possible Mechanisms Underlying the Failure of RGC Axonal Regeneration**

It has been shown that many of the RGC axons that had retracted into the retina do not extend across or into the ON head but rather were diverted at its edges. Because the ON head does not contain oligodendrocytes, which is a rich source of axonal regeneration inhibitors, the failure of the RGC axons to grow back to the ON suggested that intraretinal molecules unrelated to oligodendrocytes and myelin might impair their growth. This may be the case in the failure of the majority of the RGCs to regenerate in our proximal axotomy model. The candidates for these possible inhibitory molecules are chondroitin sulfate proteoglycan, tenascin, and collagen. The possibility of these inhibitory factors playing a part in the failure of the RGC axonal regeneration in our distal axotomy model cannot be ruled out. However, an inhibitory role may also be played by the inhibitors derived from oligodendrocytes in the distal axotomy model, because there was still approximately a 7-mm-long segment of ON attached to the eye, and this segment of the ON contained a large number of oligodendrocytes. Although the mechanism of CNTF on promoting axonal regeneration of axotomized RGCs remains to be elucidated, it is possible that CNTF maintains more axons in the ON stump after axotomy, enhances intrinsic regenerative potential of injured RGCs, increases survival of a regeneration-sensitive subpopulation of the RGCs, changes the behavior of growth cones and ability to help the regenerating axons to negotiate through the inhibitory environment, and increases the level of CNTF receptor expression in the retina, on the glial cells along the ON, and on the growth cones.

The observations that CNTF, BDNF, NT-4/5, and bFGF had differential effects on RGC survival and axonal regeneration in the present study and in other studies suggest that distinct mechanisms are responsible for these two aspects of neural repair. This hypothesis is supported by in vitro observations that neuronal viability and neurite outgrowth were mediated by different domains of preferred receptors and involved the activation of distinct cellular pathways.

Our results provide further evidence to support the neurotrophic factor concept, which includes the local action of neurotrophic proteins, because intravitreal injections of CNTF in the study promoted axonal regeneration of axotomized RGCs. The mechanisms behind the enhanced regeneration of the RGCs by CNTF certainly deserve further investigation, and one of the important issues is to clarify the changes in the level of CNTF receptor in the retinas after injury and during regeneration. Because studies have shown that cytokines and neurotrophins can interact synergistically, we have begun to test the effects of combined application of CNTF and neurotrophins on axonal regeneration. The outcome of these investigations may bring important implications of using TFs as therapeutic agents in the clinical treatment of neuronal degenerative diseases and neurotrauma.

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**References**


