Effect of CNTF on Retinal Ganglion Cell Survival in Experimental Glaucoma

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PURPOSE. To assess the neuroprotective effect of virally mediated overexpression of ciliary-derived neurotrophic factor (CNTF) and brain-derived neurotrophic factor (BDNF) in experimental rat glaucoma.

METHODS. Laser-induced glaucoma was produced in one eye of 224 Wistar rats after injection of adenoassociated viral vectors (type 2) containing either CNTF, BDNF, or both, with saline-injected eyes and noninjected glaucomatous eyes serving as the control. IOP was measured with a hand-held tonometer, and semiautomated optic nerve axon counts were performed by masked observers. IOP exposure over time was adjusted in multivariate regression analysis to calculate the effect of CNTF and BDNF.

RESULTS. By multivariate regression, CNTF had a significant protective effect, with 15% less RGC axon death (P < 0.01). Both combined CNTF-BDNF and BDNF overexpression alone had no statistically significant improvement in RGC axon survival. By Western blot, there was a quantitative increase in CNTF and BDNF expression in retinas exposed to single viral vectors carrying each gene, but no increase with sequential injection of both vectors.

CONCLUSIONS. These data confirm that CNTF can exert a protective effect in experimental glaucoma. The reason for the lack of observed effect in the BDNF overexpression groups is unclear, but may be a function of the level of neurotrophin expression achieved.

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BDNF alone or injections combined with additional measures prevent some experimental glaucoma injury.41,42 Yet, neuronal exposure to exogenous BDNF has been reported to downregulate its trkB receptor43 and to be toxic as well as beneficial.44,45 Although optic nerve transection downregulates the trkB receptor, overexpression of the trkB receptor delays RGC death after optic nerve transection.46

Ciliary-derived neurotrophic factor (CNTF), first identified by Adler et al.,57 has neuroprotective properties in various experimental injuries to retinal and central neurons.48–54 Including by intravitreal injection in experimental glaucoma.55 It is a secreted protein affecting neurons through a heterotrimeric membrane receptor.56 CNTF is expressed in cells of all retinal layers and in retinal pigment epithelium,57 as well as in the optic nerve head.58 Endogenous retinal expression of CNTF increases with retinal and optic nerve injury.59,60 but may decrease with experimental IOP elevation.61 Virally mediated overexpression of CNTF is protective of RGCs after optic nerve injury62,63 and of photoreceptors in retinal degeneration models,64–66 but depresses electoretinographic potentials at some dose levels. Human clinical trials are under way with intravitreal capsules containing immortalized pigment epithelial cells expressing CNTF.67

Some reports with BDNF found more RGC preservation when it was combined with other interventions.68 BDNF supplemented by a free radical scavenger was protective in rat glaucoma, but neither agent alone was effective.41 After optic nerve transection, regeneration into a nerve graft was greater with CNTF treatment alone than with BDNF alone.48 Combined treatment with CNTF and BDNF was superior to either alone in the rescue of rd photoreceptors49 and after laser retinal treatment.69 Both inhibition of free radicals and inhibition of nitric oxide synthase are known to potentiate the beneficial effect of BDNF.70 We studied the potentially neuroprotective effect of BDNF and CNTF further, in a rat glaucoma model, by overexpressing CNTF or BDNF alone or by overexpressing both in combination.

Methods

Comparison of BDNF, CNTF, BDNF+CNTF, or Saline

A total of 224 adult male Wistar rats (400–425 g) began the study, of which 38 died before the group was killed for the following reasons: Eighteen had anesthesia-related death, 11 were euthanatized for cataract after virus injection, 7 were euthanatized for other ocular complications, and 2 were euthanatized for overall poor health. After the remaining rats were killed, an additional 10 could not be used because of technical problems, leaving 176 for final analysis. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved and monitored by the Johns Hopkins University School of Medicine Animal Care and Use Committee.

The experimental groups for this protocol received one of the following four treatments: (1) overexpression of BDNF; (2) overexpression of CNTF; (3) overexpression of both BDNF and CNTF; or (4) saline injection. To produce these groups, we performed intravitreal injections of (1) adenovirus containing the BDNF gene and the woodchuck hepatitis enhancer element (AAV/BDNF/WPRE; n = 52 eyes), (2) AAV carrying the CNTF gene (AAV/CNTF; n = 45), (3) normal saline (n = 47), or (4) AAV/BDNF/WPRE followed by AAV/CNTF 2 weeks later (or vice versa; n = 47). In each group, experimental glaucoma was induced 2 weeks after the last viral vector injection. In addition, a laser-only group of 33 rats was included that did not receive any injection, leading to an overall total of 224 rats.

Animals were killed 4 weeks after induction of experimental glaucoma. Of the AAV/BDNF/WPRE group, 31 were used to estimate the number of surviving RGC axons and 5 were prepared for Western blot analysis of BDNF expression. In the AAV/CNTF group, 29 were used for RGC axon count and 6 for Western blot; in the normal saline group, 29 were used for RGC axon count and 6 for Western blot; and in the combination therapy group, 34 were used for RGC axon count and 6 for Western blot. In the laser-only group, 30 were used to estimate the number of surviving retinal ganglion cells, for a total of 176 animals in the final analysis. The laser treatments, IOP measurements, and optic nerve counts were performed in a masked fashion.

Plasmid Preparation

The CNTF plasmid68 expresses a secretory form of human CNTF which has a high affinity for the CNTF receptor α, with a hybrid chicken β-actin (CBA) promoter and cytomegalovirus (CMV) enhancer element that supports expression in photoreceptors, retinal pigment epithelium, and retinal ganglion cells.71

The pGFP plasmid72 was used to create the AAV/BDNF/WPRE. Flanked by AAV terminal repeats, the expression cassette of pGFP included a 1.7-kb sequence containing the hybrid CMV immediate early enhancer/chicken β-actin (CBA) promoter. The pGFP construct had GFP cDNA downstream of the CBA promoter and incorporated the woodchuck hepatitis posttranscriptional regulatory element (WPRE)73 that supports expression in photoreceptors, retinal pigment epithelium, and retinal ganglion cells.71

Vector Packaging and Titering

Packaging of the CNTF plasmid into rAAV2 was performed by the Retina Gene Therapy Group, University of Florida,76 and the BDNF and GFP plasmids were packaged in rAAV2 by the Johns Hopkins University Cystic Fibrosis Vector Core.77 Briefly, low-passage human 293 cells were cotransfected with the given plasmid (CNTF or BDNF), and the helper plasmid pDG that contains both the AAV genes (rep and cap) to produce biologically active rat BDNF.74,75

Functional Assay of AAV/BDNF/WPRE and AAV/CNTF

To test the bioactivity of AAV/BDNF/WPRE and AAV/CNTF, COS-7 cells were grown to 30% confluence in a 24-well culture plate in 1 mL of DMEM/F12 media per well containing 10% FBS and 1 U/mL penicillin/streptomycin. One microliter of AAV/CNTF or AAV/BDNF/WPRE (physical particle titers 4.79 × 1013 and 1.2 × 1013, respectively) or one microliter of a control AAV/GFP virus (1 × 1013)77 used as a virus control, was added per well in triplicate. At 72 hours, the media were exchanged. Twenty-four hours later, the media from the triplicate samples were harvested and pooled. Media from COS-7 cells that were not exposed to virus, but had the same media-exchange treatment, were used as the conditioned media control (CM).

RGCs from postnatal day (P)4 rats were enriched by immunoselection78 and plated at a density of 5000 cells/well in 96-well culture dishes in growth medium (Neurobasal medium [Invitrogen-Gibco, Grand Island, NY], B-27 supplement, glutamine 2 mM, and penicillin/streptomycin 1 U/mL), containing forskolin at a final concentration of 2.5 μM. Quadruplicate wells were supplemented with either 20 or 40 μL of the various conditioned media, after it had been centrifuged at 2000 g for 5 min.
14,000 rpm for 10 minutes. Two additional controls were: (1) RGC cultures grown in unsupplemented growth medium and (2) RGC grown in growth medium supplemented with 50 ng/mL recombinant BDNF (PeproTech, Rocky Hill, NJ). After 3 days in culture, the ganglion cells were stained with calcein AM, Hoescht, and ethidium homodimer. Twenty fields per well were imaged using an array scan (Cellomics VTI; Thermo Fisher Scientific, Pittsburgh, PA) and the images were analyzed and quantified with commercial software (Neuronal Profiling software application; Thermo Fisher Scientific).

**INTRAVITREAL INJECTIONS.**

Animals were anesthetized with a cocktail of intraperitoneal ketamine (75 mg/kg) and xylazine (5 mg/kg) and topical 1% proparacaine eye drops. Using an operating microscope, a peritomy was made superotemporally, and a partial thickness pilot hole was made in the sclera with a 30-gauge needle. A glass needle with a tip diameter between 30 to 50 μm was connected by polyethylene tubing to a 5-mL syringe (Hamilton Company, Reno, NV) to inject virus or saline. The assembly was prefilled with light mineral oil (Sigma-Aldrich, St. Louis, MO) before drawing up 3.5 μL of either normal saline or virus. The needle was left in place for 2 minutes to allow for dispersal of the material.

In animals receiving BDNF alone or CNTF alone, injections were given 2 weeks before the first laser treatment for IOP elevation. In animals that received injections of both vectors (BDNF and CNTF), the first injection was 4 weeks before laser treatment, and the second was 2 weeks before, with the order of the two vectors randomly chosen. After the injections, the retina was examined using an indirect ophthalmoscope and 90-D lens (Volk Optical, Mentor OH) to assure the lack of retinal detachment or injury.

**EXPERIMENTAL IOP ELEVATION.**

Two weeks after the intravitreal injection of virus or saline (4 weeks after the first vector injection in the CNTF/BDNF combined group), animals were anesthetized with intraperitoneal ketamine (75 mg/kg) and xylazine (5 mg/kg) and topical 1% proparacaine eye drops. A 532-nm diode laser was used to treat the trabecular meshwork with 40 to 50 spots at 50 μm at a power of 0.6 W and duration of 0.6 second. IOP was measured in each eye under anesthesia with a hand-held tonometer, using the machine-generated average (TonoLab; iCare Finland, Expoo, Finland). Readings were taken 1 day after laser and again immediately before retreatment. A laser second treatment was given 1 week later if the IOP difference between the treated and control eyes was less than 8 mm Hg. Additional weekly IOP measurements were made for the first month after the induction of experimental glaucoma. We calculated the mean IOP, peak IOP, peak IOP difference, and positive IOP integral, representing the area under the curve of IOP exposure in the glaucomatous eye relative to the fellow control eye over time. Expressed in units of mm Hg-days, the positive integral captures the cumulative exposure of IOP compared with the control.

**RGC AXON COUNTING.**

Animals used for nerve counts had tissues fixed by vascular perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer followed by 5% glutaraldehyde in 0.1 M phosphate buffer. Nerves were embedded in glutaraldehyde in 0.1M phosphate buffer. Nerves were embedded in resin, sectioned at 1 μm, and photographed with a 100× oil lens for image analysis (Cool Snap Camera and Metamorph Image Analysis software; Molecular Devices, Downingtown, PA). Ten random 50 × 50 μm fields, equivalent to a 10% sampling of the total nerve area, were counted to determine the average fiber density/mm². This value was multiplied by the total nerve area to estimate the total fiber number, which was then compared to a pooled control to generate the percentage of fiber loss per animal. Mean RGC axon counts from each of the treatment groups were compared to each of the control groups by univariate regression controlling for positive IOP integral.

**BIOSTATISTICAL ANALYSIS.**

Although animals are randomly assigned to treatment groups and given uniform laser exposure, the resultant mean IOP levels over time can differ between treatment groups. This fluctuation would bias the interpretation of data, potentially masking a treatment effect, or producing one that did not exist. Therefore, we calculated for every animal the mean, peak, and positive integral IOP (cumulative exposure above the fellow eye over time), as described earlier. In regression models in which axon loss was the dependent variable, the independent variables were treatment group and IOP exposure (mean, peak, or positive integral). The model with positive integral most accurately captures the potential damaging effect of IOP on RGCs, and this was the model used to determine whether the treatment groups differed significantly.

**WESTERN BLOT ANALYSIS.**

Retinas were flash frozen on dry ice and stored at −80°C until homogenization in 300 μL of 20 mM Tris buffer with 10% sucrose and protease inhibitor (Roche Diagnostics Corp., Indianapolis, IN) by sonication for 4 seconds at 4°C. The protein concentration was determined with a commercial assay (Bio-Rad, Hercules, CA). For a given treatment group’s Western blot analysis, the treated and contralateral control eyes of each animal were loaded in serial order and processed in a single gel. This method allowed for standardization of the processing steps within each treatment library and allowed a direct comparison between the experimental and fellow control eye. In a prior study by Martin et al., we compared noninjected control eyes to AAV-GFP control virus-treated eyes and found no increase in BDNF expression; therefore, we did not repeat those comparisons in this study. Proteins were separated on a 4% to 12% Bis-Tris gel using sodium dodecyl sulfate—polyacrylamide gel, transferred to a membrane with a 0.45-μm pore size and blocked for 1 hour at room temperature in 5% nonfat dry milk/PBS-T. To identify CNTF, membranes were probed overnight at 4°C with goat anti-human CNTF antibody (R&D Systems, Minneapolis, MN) at 1:250 dilution followed by peroxidase-conjugated donkey anti-goat secondary (R&D Systems) at 1:5000 dilution for 1 hour at room temperature. For BDNF, we probed at room temperature for 1 hour with rabbit polyclonal anti-BDNF N-20 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:350 dilution, followed by peroxidase-conjugated donkey anti-rabbit secondary (GE Healthcare, Bucks, UK) at 1:20,000 dilution.

Immunoblots were then detected (SuperSignal West Femto Maximum Sensitivity Substrate; Thermo Scientific, Waltham, MA, and BioMax autoradiograph film; Eastman Kodak, Rochester, NY). To pre-


TABLE 1. IOP Exposure in Glaucous and Control Eyes

<table>
<thead>
<tr>
<th>Treatment Group (n)</th>
<th>Mean IOP (mm Hg)</th>
<th>Peak IOP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glaucoma</td>
<td>Control</td>
</tr>
<tr>
<td>AAV/BDNF (n = 31)</td>
<td>17.7 (3.2)</td>
<td>10.1 (1.9)</td>
</tr>
<tr>
<td>AAV/CNTF (n = 29)</td>
<td>18.2 (4.7)</td>
<td>9.5 (1.4)</td>
</tr>
<tr>
<td>AAV/CNTF + AAV/BDNF (n = 34)</td>
<td>16.6 (3.5)</td>
<td>10.0 (1.7)</td>
</tr>
<tr>
<td>Saline (n = 29)</td>
<td>16.3 (3.2)</td>
<td>9.8 (2.0)</td>
</tr>
<tr>
<td>Laser only (n = 30)</td>
<td>17.3 (3.6)</td>
<td>10.7 (2.7)</td>
</tr>
</tbody>
</table>

Data are the mean (SD) for each treatment group. Mean IOP is the average IOP after induction of experimental glaucoma. Peak IOP is the IOP of the experimental and control eyes on the day that the IOP was most elevated after laser treatment.

IOP Exposure

Analysis of the laser-treated rats revealed that the IOP exposure among the experimental groups was not significantly different, in terms of mean IOP, peak IOP, and positive IOP integral difference of the experimental and control eyes (P > 0.05, ANOVA; Tables 1, 2).

Estimation of Surviving Retinal Ganglion Cell Axons

The group with overexpression of CNTF alone had 15% less fiber loss (greater survival) than the combined control groups (saline injection with glaucoma and glaucoma only), using regression analysis in which IOP exposure was entered as an independent variable (P = 0.018; Table 2). The group with combined overexpression of both CNTF and BDNF had 7% increased survival compared with the control, but the difference was not statistically significant (P = 0.19). BDNF overexpression alone did not change RGC axon survival (P = 0.44). We also calculated the ratio of percentage RGC loss as judged from axon counts to IOP exposure in mm Hg-days. This relationship provides a method of comparing the rate of axon loss as a function of IOP exposure between the various treatment groups. Note that the proportionate loss by this measure was most mitigated by CNTF treatment and was comparable in this group with the value obtained in a prior study of BDNF overexpression.

RESULTS

Bioactivity of AAV/BDNF/WPRE and AAV/CNTF

To ensure that the lots of AAV/BDNF/WPRE and AAV/CNTF that were being used for the in vivo experiments were capable of infecting cells and producing bioactive neurotrophins, we infected COS-7 cells with the same preparations of virus used in the animal experiments and tested the conditioned medium from the infected cells, and control conditioned medium that had not been exposed to virus, in an RGC-based assay. The assay involves culture of purified P4 rat RGCs for 3 days, with or without various test media, followed by automated image analysis to quantify neurite outgrowth. As can be seen in Figure 1, the level of total neurite length observed with the AAV/GFP virus–conditioned medium was similar to that seen with the uninfected conditioned medium (CM), whereas both the AAV/BDNF/WPRE and AAV/CNTF CM demonstrated a dose-dependent stimulation of total neurite length, thus demonstrating that the viruses could indeed produce bioactive neurotrophins.

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Westen Blot Analysis of CNTF and BDNF Expression

Western blot analysis for whole retina CNTF and BDNF showed significant increases for both molecules in the appropriate single vector overexpression groups, with nearly threefold increase in CNTF in experimental compared with control fellow eyes and almost a doubling of BDNF levels (Table 3). However, neither neurotrophin was significantly increased in the double-injection group (CNTF/BDNF). Furthermore, the injection of saline led to a mild, significant increase in CNTF levels in experimental eyes, though the specific overexpression with the CNTF vector caused nearly twice the increase in CNTF as did the saline injection (P = 0.006). Values that were normalized against the amount of actin present in each specimen were not different in any substantive way from those shown without normalization.

DISCUSSION

These experiments support past reports cited above that found CNTF to increase RGC survival after injury and represents the first report to our knowledge that overexpression of CNTF using viral vectors could have therapeutic potential to reduce...
TABLE 3. Western Blot Assay for Retinal CNTF and BDNF Expression

<table>
<thead>
<tr>
<th>Neurotrophin</th>
<th>Treatment Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAV/CNTF</td>
</tr>
<tr>
<td>CNTF (25 kDa)</td>
<td>2.82 (0.73)*</td>
</tr>
<tr>
<td>BDNF (14 kDa)</td>
<td>1.21 (0.35)</td>
</tr>
</tbody>
</table>

Values are mean ratio (SD) of experimental to control eye density data from blots for 6 animals in the CNTF, CNTF/BDNF, and saline groups and five animals in the BDNF group.

* Significant: CNTF in CNTF group (P = 0.0017); CNTF in saline group (P = 0.01); BDNF in BDNF group (P = 0.0022).

RGC loss in an animal model of glaucoma. Other investigators have given CNTF by injection into the vitreous or have over-expressed the gene in other optic nerve injury models with significant neuroprotective effects. Of note, we found that CNTF levels in eyes that had saline injection were significantly higher than in fellow control eyes, but this increase, which was less than that seen with viral-mediated overexpression, did not measurably increase RGC survival. It has been reported that lens injury leads to an increase in retinal CNTF and extends RGC life after mechanical optic nerve injury.81 We excluded from analysis any rats with visible lens injury, but undetected injury or other effects of the injection procedure could have had a similar effect.82

We did not see increased RGC axon survival when CNTF and BDNF vectors were injected together, but the Western blot data suggest that there was lower CNTF expression in this combined group than in the CNTF alone group. We speculate that the level of CNTF expression may have been diminished by dual injections, either by detrimental effects of the second injection or by competition or an interaction between the viral vectors. Because we found no evidence of increased RGC axon survival in the two groups with lesser CNTF increase (CNTF- BDNF combined and saline control) and a significant effect in the CNTF alone group, our data may indicate the threshold for a short-term beneficial effect on experimental glaucoma, as they represent a dose–response comparison. The issue of the appropriate dose of CNTF for RGC neuroprotection is particularly important in light of the several reports that administration of CNTF to the retina can result in decreased retinal function as measured by both acuity (optokinetic response) and electrophysiological parameters.83

Mc Gill et al.85 have recently shown that lower doses of CNTF have protective effects in models of outer retinal disease without toxicity. Clearly, the possible future use of CNTF as a human therapeutic agent will require monitoring for such functional deficits and determining the dose that best optimizes efficacy and minimizes unwanted side effects.

The mechanisms by which CNTF acts to decrease RGC death may be similar to the effects reported in other models. These studies have found that it activates receptors on Müller cells, astrocytes,86,87 and RGCs,88 leading to upregulation of Stat 3,89 which is known to be activated in experimental glaucoma injury.86 Additional research recently found that CNTF acts to increase RGC survival both in vitro and in vivo through mechanisms involving both Stat 3 and the mitogen-activated kinase pathway.87 It is increasingly clear that glaucoma and other RGC injuries simultaneously upregulate both survival and death mechanisms.88

In these experiments, we did not detect a beneficial effect of BDNF overexpression, but the level of retinal BDNF was increased only 44% compared with that in untreated eyes. By contrast, in our previous study in which we observed a significant RGC neuroprotective effect with AAV/BDNF/WPRE, retinal BDNF levels were increased approximately fivefold.21 We had attempted to produce the same quality vector in the present work as in that prior study, but it was evident in pilot tests of the newer vector preparations that the efficiency of the AAV/GFP was less than we had observed previously. In addition, perhaps explaining the difference in neuroprotective activity and relative expression levels between the AAV/BDNF/WPRE and AAV/CNTF vectors, the BDNF vector had a lower physical particle titer than the CNTF vector and at the 20-μL dose it also had less neurite promoting activity. This finding again emphasizes the importance of dose and expression effects in conducting and interpreting gene therapy studies.

Acknowledgments

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