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Intravitreal injections of GDNF-loaded biodegradable microspheres are neuroprotective in a rat model of glaucoma

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Purpose: To evaluate the efficacy of intravitreal injection of GDNF-loaded biodegradable microspheres in promoting the survival of retinal ganglion cells (RGCs) and their axons in a rat model of chronically elevated intraocular pressure (IOP).

Methods: Chronic elevation of IOP was induced in Brown Norway rats through injection of hypertonic saline (1.9 M) into the episcleral veins. After injection, IOP was measured twice a week in rats using topical anesthesia. Poly DL-lactide-co-glycolide (PLGA) microspheres were fabricated using a modified version of the spontaneous emulsification technique. Two and ten percent of volume solutions of microspheres loaded with glial cell line-derived neurotrophic factor (GDNF) were injected into the vitreous cavity of rats with elevated IOP, with injections of blank microspheres and PBS serving as controls. Histological analysis was used to quantify surviving RGCs and axons and provide comparison among different groups. In addition, the thickness of the retinal inner plexiform layer (IPL) and the level of glial fibrillary acidic protein (GFAP) expression within the retina and optic nerve were quantitatively compared.

Results: IOP was significantly increased in eyes with episcleral vein injection over untreated eyes ($p < 0.001$) but did not show a significant difference among groups that received intravitreal injections of GDNF microspheres, blank microspheres, or PBS ($p = 0.1852$). The duration of IOP elevation in this experiment was eight weeks. Expression of GDNF and its receptors localizes to the adult rat RGCs. Ten percent of the GDNF microsphere treatment significantly increased RGC survival and axon survival ($p < 0.001$), reduced the loss of retinal IPL thickness ($p < 0.001$), and decreased glial cell activation in the retina and optic nerve ($p < 0.001$) compared with blank microspheres and PBS. In addition, GDNF microsphere treatment moderately reduced cupping of the optic nerve head.

Conclusions: Delivery of GDNF via biodegradable microspheres significantly increased the survival of RGCs and their axons, preserved IPL thickness, and decreased retina and optic nerve glial cell activation in an experimental glaucoma model. This study suggests that GDNF delivered by PLGA microspheres may be useful as a neuroprotective tool in the treatment of glaucomatous optic neuropathy.

Glaucoma is the second leading cause of blindness worldwide and the prevalence of this disease is expected to grow as the population ages [1,2]. In this disease, progressive death of the retinal ganglion cells (RGCs) leads to optic nerve degeneration and vision loss. Therefore, a major therapeutic aim is to facilitate the survival of RGCs [3]. Currently, glaucoma treatment relies on pharmacological or surgical reduction of intraocular pressure (IOP). However, IOP can be difficult to control and many patients suffer progressive visual field loss despite what appears to be adequate control of IOP. For these reasons, it is critical to develop treatment that actively prevents the death of RGCs, which are at risk in this condition [4-11].

One approach has been delivery of neurotrophic factors to the inner retina. Neurotrophic factors have the ability to promote the survival of neurons and to influence the growth of neurons. Furthermore, retrograde axonal transport of neurotrophic factors synthesized in target structures has been spe-

cifically associated with RGC survival. For instance, blocked transport of brain-derived neurotrophic factor (BDNF) has been observed in glaucoma models and intravitreal injection of BDNF has been known to increase RGC survival. Therefore, neurotrophic factor deprivation has been proposed as one mechanism leading to RGC death in glaucoma [12-17]. In addition, the growing recognition that glaucoma is a form of optic neuropathy suggests that neuroprotection, therapy directed at preventing neuronal loss, may represent an efficacious adjunctive therapy in this setting [5].

Glial cell line-derived neurotrophic factor (GDNF) is a 20 kDa glycosylated homodimer belonging to the TGF- β superfamily that was first recognized for its ability to increase the survival of dopaminergic neurons in animal models of Parkinson disease [18]. Recent work has established that GDNF signals directly through the cell surface receptor, GFR- α , and indirectly through the transmembrane Ret receptor, tyrosine kinase [19]. Both receptors have been identified on embryonic chick RGCs as well as on amacrine and horizontal cells [20]. Exogenous GDNF also increased RGC survival in axotomized rats and in mice following liquid injection and adenoviral transmission [21-24]. Studies in our laboratory showed that intravitreal microsphere-delivered GDNF signifi-

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cantly increased long-term RGC survival in the DBA/2J mouse glaucoma model [25].

Effective neuroprotection in glaucoma likely requires the consistent availability of the active agent, such as GDNF, for prolonged periods of time. Biodegradable microspheres are especially attractive as drug delivery vehicles for several reasons. First, they are relatively inert in the vitreous cavity, inciting only a minimal host immune response. Furthermore, they can be formulated in ways so as to alter the duration and magnitude of drug release. In addition, they can be reproduced with a high consistency and at a low cost [25-27]. In this study, we induced chronic elevation of IOP in rats through episcleral vein injection of hypertonic saline [28,29] and used this model to investigate the neuroprotective activity of Poly DL-lactide-co-glycolide (PLGA) biodegradable microsphere-delivered GDNF on the survival of RGCs and their axons.

METHODS

Animals: All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all experimental protocols were approved by the Animal Care and Use Committee of the Schepens Eye Research Institute. Adult male Brown Norway rats (350-450 g, Charles River Laboratories, Wilmington, MA) were housed with a 12 h light/dark cycle and with water and food provided *ad libitum*. All rats were acclimatized to the environment and to IOP measurements for at least two weeks before experiments.

Intraocular pressure elevation: Unilateral elevation of IOP was produced in adult male Brown Norway rats as previously described by Morrison [28,29]. In brief, anesthesia was induced by intraperitoneal injection consisting of ketamine (75 mg/kg), xylazine (10 mg/kg), and acepromazine (2 mg/kg; all from Phoenix Pharmaceutical Inc., St. Joseph, MO). Hypertonic saline (1.9 M) was injected into the episcleral vein of the left eye through a glass micro-needle (generated in a pipette puller) while the right eye served as a normal control. A syringe pump (Harvard apparatus, Holliston, MA) was used to standardize the injection pressure, volume, and duration. Two weeks later, the injection procedure was repeated on a second episcleral vein on the opposite side of the same eye in all treated animals.

Intraocular pressure measurement: IOP was measured using a TonoPen XL tonometer (Medtronic Ophthalmics, Jacksonville, FL). Baseline IOP was obtained before the first hypertonic saline injection and twice per week thereafter. All IOP measurements were performed in conscious rats using only topical corneal anesthesia (0.5% Proparacaine Hydrochloride, Akorn Inc. Buffalo Grove, IL). Fifteen consecutive readings were taken, and the average served as the measured IOP. In addition, all IOP measurements were performed around 2:00 PM to minimize fluctuations due to diurnal variability [30,31].

Fabrication of microspheres: Microspheres containing GDNF were fabricated using a modification of a spontaneous emulsion technique described previously [32]. To summarize,

200 mg of 50:50 PLGA (DURECT Corp., Birmingham, AL) was dissolved in 5 ml of a solution containing trifluoroethanol and dichloromethane at a volume ratio of 4:1. Additionally, 8 mg magnesium hydroxide was added to minimize protein aggregation during encapsulation. As obtained from the manufacturer, 10 µg GDNF (R&D Systems, Minneapolis, MN) was reconstituted in 300 µl of a solution containing 7 mg bovine serum albumin (BSA) and 100 mg docusate sodium (Sigma-Aldrich, St. Louis, MO) dissolved in 3 ml PBS. The two solutions were vortexed briefly, forming a fine emulsion that was poured into 200 ml of gently stirring 1% (w/v) polyvinyl alcohol (PVA, 88% hydrolyzed). After stirring for three hours to allow microspheres to harden, they were collected via centrifugation and washed three times to remove residual PVA. The microspheres were then rapidly frozen in liquid nitrogen, lyophilized for 72 h, and stored in a desiccator at -20 °C. Blank microspheres were made in the same way except that additional BSA was added in lieu of GDNF. Resulting GDNF and blank microspheres exhibited average diameters of approximately 8 µm as determined by a particle size.

Intravitreal injection: One week after the first hypertonic saline injection, eyes were randomly chosen to receive intravitreal injections of either blank microspheres, microspheres loaded with GDNF, or PBS alone. Intravitreal injection was performed under general anesthesia using an ophthalmic operating microscope (Möller-Wedel GmbH, Wedel, Germany) and beveled glass micro-needles with an outer diameter of approximately 100 µm. Two and ten percent (w/v) suspensions of microspheres were prepared in PBS and briefly vortexed immediately before each injection to ensure a uniform dispersion of microspheres in the injected fluid. A 30-gauge hypodermic needle was used to perforate the sclera 1.5 mm behind the limbus. Five microliters of test sample was then injected by way of this passage into the vitreous using a 50 µl Hamilton Syringe (Hamilton Co, Reno, NV). Care was taken not to damage the lens. Following intraocular injections, the needle was held in place for one min and withdrawn slowly. In addition, paracentesis was simultaneously performed to relieve pressure and thereby prevent reflux. Animals with retinal bleeding or lens injury following the injection procedure were excluded from the study.

Microspheres labeled with the fluorescent marker rhodamine were injected into the vitreous to verify the injection technique. Fundus images were examined *in vivo* using epifluorescence microscopy.

Tissue preparation and histopathology: Rats were killed by CO₂ inhalation eight weeks after the second hypertonic saline injection. Eucleated eyes were immediately fixed in 4% paraformaldehyde (PFA). Optic nerve specimens were taken 1 mm posterior to the globe and placed into a fixative consisting of 2.5% glutaraldehyde and 2% formaldehyde in PBS.

The eyes, together with an additional subset of optic nerve (ON) segments were fixed in 4% PFA overnight, cryoprotected in serial sucrose solution, frozen in optimal cutting temperature compound (Tissue-Tek, Miles Diagnostic Division,

Elkhart, IN), sectioned in their entirety at 10 μm , mounted on Superfrost Plus slides (VWR Scientific, West Chester, PA), and stored at -80°C for further study.

ON segments were washed in 0.1 M cacodylate buffer and post-fixed in 2% aqueous osmium oxide. The segments were then dehydrated in graded alcohols and embedded in epon. One micrometer section was cut and stained with 1% toluidine blue in 1% borate buffer. Optic axons were counted in nine fields per ON cross section viewed at 1000X magnification. Axon survival percentage was calculated based on the number of ON axons in elevated IOP eyes divided by the number of axons in untreated contralateral eyes.

For immunohistochemical analysis, rabbit anti-GDNF antibody (1:100), goat anti-GFR α -1 antibody (1:50), goat anti-GFR α -2 antibody (1:50), goat anti-Ret antibody (1:50), goat anti-CD45 (1:100; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-Neuronal Nuclei (1:100, Chemicon, Temecula, CA), mouse anti-Neurofilament 200KD (1:100, Chemicon, Temecula, CA), and rabbit anti-GFAP (1:200, Invitrogen, Carlsbad, CA) were used on retina and ON frozen sections. FITC-conjugated AffiniPure Goat anti-Mouse IgG (1:200), FITC-conjugated AffiniPure Goat anti-Rabbit IgG (1:200), FITC-conjugated AffiniPure Mouse anti-Goat IgG (1:200), FITC-conjugated AffiniPure Goat anti-Mouse IgG (1:200), Cy3-conjugated AffiniPure Donkey anti-Goat IgG (1:800), Cy3-conjugated AffiniPure Goat anti-Mouse IgG (1:800), and Cy3-conjugated AffiniPure Goat anti-Rabbit IgG (1:800; All from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as secondary antibodies. Negative controls for immunostaining consisted of substituting normal serum in place of the primary antibodies.

Anti-NeuN positive cells in the RGC layer were counted from the superior aspect of an average of five vertical sections through the optic disc for each eye. A grid was placed

over the retinal section and the mean number of NeuN+ cells in the equivalent area of RGC layer from each eye was calculated.

The fluorescence intensity of glial fibrillary acidic protein (GFAP) expression in the retina and optic nerve cross section was measured by ImageJ.

Statistical Analysis: The data were expressed as means \pm SD. Data between groups was compared using Student's t-test. The significance of RGC and axon rescue was assessed by one-way ANOVA. Statistical significance was declared at $p<0.05$.

RESULTS

Hypertonic saline injection elevates intraocular pressure: Normal control eyes in Brown Norway rats had an average IOP of 21.2 ± 3.6 mmHg. The saline injection method resulted in significant IOP elevation at all time points ($p<0.001$) with pressure levels consistently approaching double that of control eyes by the third week (Figure 1). Overall IOP exposure (in mmHg-days, defined as the integral of the IOP difference between the experimental and control eyes during the follow-up) was not significantly different between treatment groups which are those that received intravitreal injection of GDNF (Figure 1B,C), blank microspheres (Figure 1D), or PBS (Figure 1E) on the first week ($p=0.1852$). The IOP elevation seen in this study was sustained up until the end of the experiment at the 10 week time point, eight weeks beyond the second hypertonic saline injection.

Injection technique delivers microspheres to the vitreal cavity: The technique used for intravitreal injection of microspheres was verified by in vivo fundus imaging. Injected microspheres were visualized in the vitreous cavity, posterior to the lens, immediately following injection. Diffusion of microspheres throughout the vitreous was evident from using

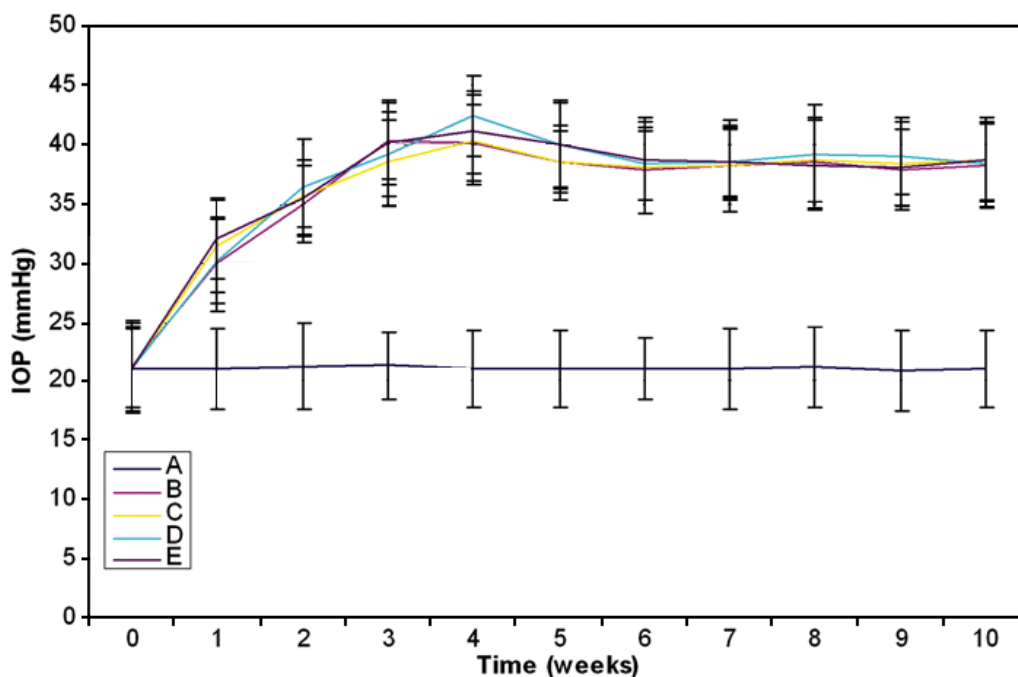


Figure 1. Time course of intraocular pressure in Brown Norway rats. IOP significantly increased in eyes with episcleral vein injection of hypertonic saline on weeks 0 and 2 (B, C, D, E) compared with eyes without injection (A; $p<0.001$). Overall IOP exposure was not statistically, significantly different among groups that received intravitreal injection on week 1 of GDNF microspheres (B, C), blank microspheres (D), or PBS alone (E; $p=0.1852$). Data are expressed as the mean \pm SD (n=9).

rhodamine-labeling four days and 10 days after injection. These microspheres could still be observed 30 days later after injection (data not shown).

GDNF, GFR α -1, GFR α -2, and Ret are expressed in the rat retina: Immunoreactivity for GDNF was mainly detected in the ganglion cell layer, inner nuclear layer, and inner segment of photoreceptors (Figure 2A,D,G). Immunoreactivities for GFR α -1 and Ret were mainly localized to the ganglion cell layer (Figure 2B,H) while immunoreactivities for GFR α -2 mainly localized to the ganglion cell layer and inner nuclear layer (Figure 2E).

GDNF microspheres reduce retinal damage due to chronic intraocular pressure elevation: Chronic IOP elevation resulted in substantial cupping of the optic nerve head (ONH; Figure 3D,E) compared with normal ONH architecture (Figure 3A). Ten percent of GDNF microspheres (Figure 3B) reduced ONH excavation and 2% GDNF microspheres (Figure 3C) moderately reduced the ONH excavation compared with either blank microspheres (Figure 3D) or PBS alone (Figure 3E).

Anti-neurofilament 200 labeling was used to evaluate nerve fiber layer (NFL) thickness in the vicinity of the optic disc. Compared to normal rat eyes (Figure 3F), eyes with chronic IOP elevation and treated with blank microspheres (Figure 3I) or PBS (Figure 3J) showed substantially diminished NFL thickness. Ten percent of GDNF microspheres (Figure 3G) decreased the degree of NFL loss whereas 2% GDNF microspheres (Figure 3H) did not preserve the NFL to the same extent.

Chronic IOP elevation also resulted in substantially diminished retinal thickness (Figure 3L,M,N,O) compared with normal retinal architecture (Figure 3K). The average thickness of the retinal inner plexiform layer (IPL) in normal animals was $108.4 \pm 4.9 \mu\text{m}$, and chronic IOP elevation resulted in a significant loss of IPL thickness (Figure 3P; $p < 0.001$). GDNF microspheres were associated with significant preservation of the IPL with a thickness of $78.5 \pm 3.8 \mu\text{m}$ seen following 10% GDNF microspheres. In comparison, 2% GDNF microspheres resulted in an IPL of $57.9 \pm 3.3 \mu\text{m}$ thickness,

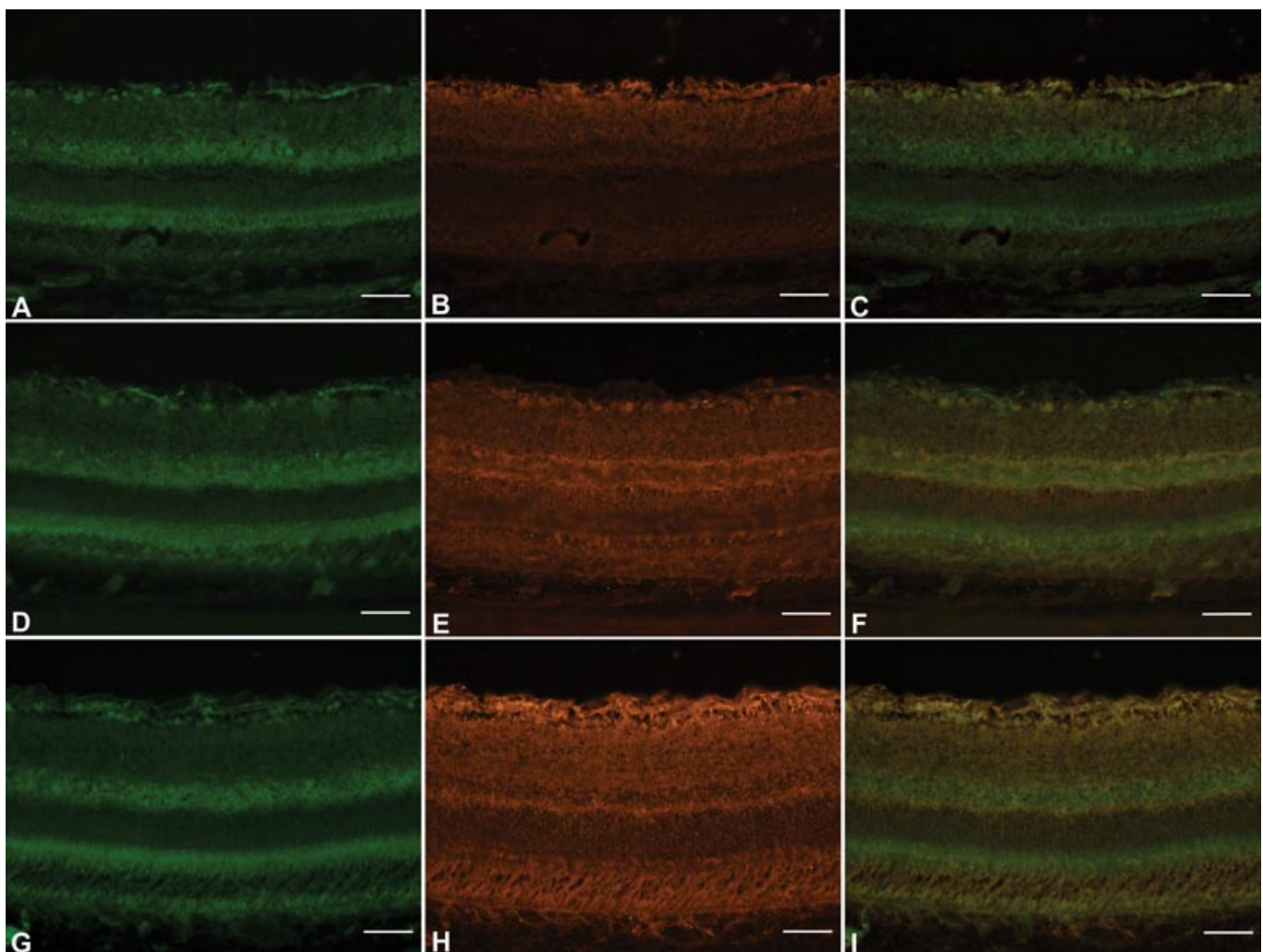


Figure 2. Expression of GDNF and its receptors. Immunoreactivities of GDNF are shown on A, D, and G, respectively. (B), (E), and (H) illustrate the immunoreactivities of GFR α -1, GFR α -2, and Ret, respectively. Merged photos of GDNF and GFR α -1 (C), GDNF and GFR α -2 (F), and GDNF and Ret (I) are also shown. Scale bars represent 100 μm .

which was statistically greater than the $52.7 \pm 2.7 \mu\text{m}$ seen with PBS treatment alone ($p < 0.01$) but not significantly greater than blank microspheres ($p > 0.05$).

GDNF microspheres increase retinal ganglion cell survival: The effects of chronic IOP elevation and GDNF

microspheres on RGC survival are shown in Figure 4A-E and Figure 4P. Eight weeks of IOP elevation resulted in substantial loss of RGCs as labeled with the anti-neuronal nuclear antibody NeuN (Figure 4B-E) when compared to eyes without IOP elevation (Figure 4A).

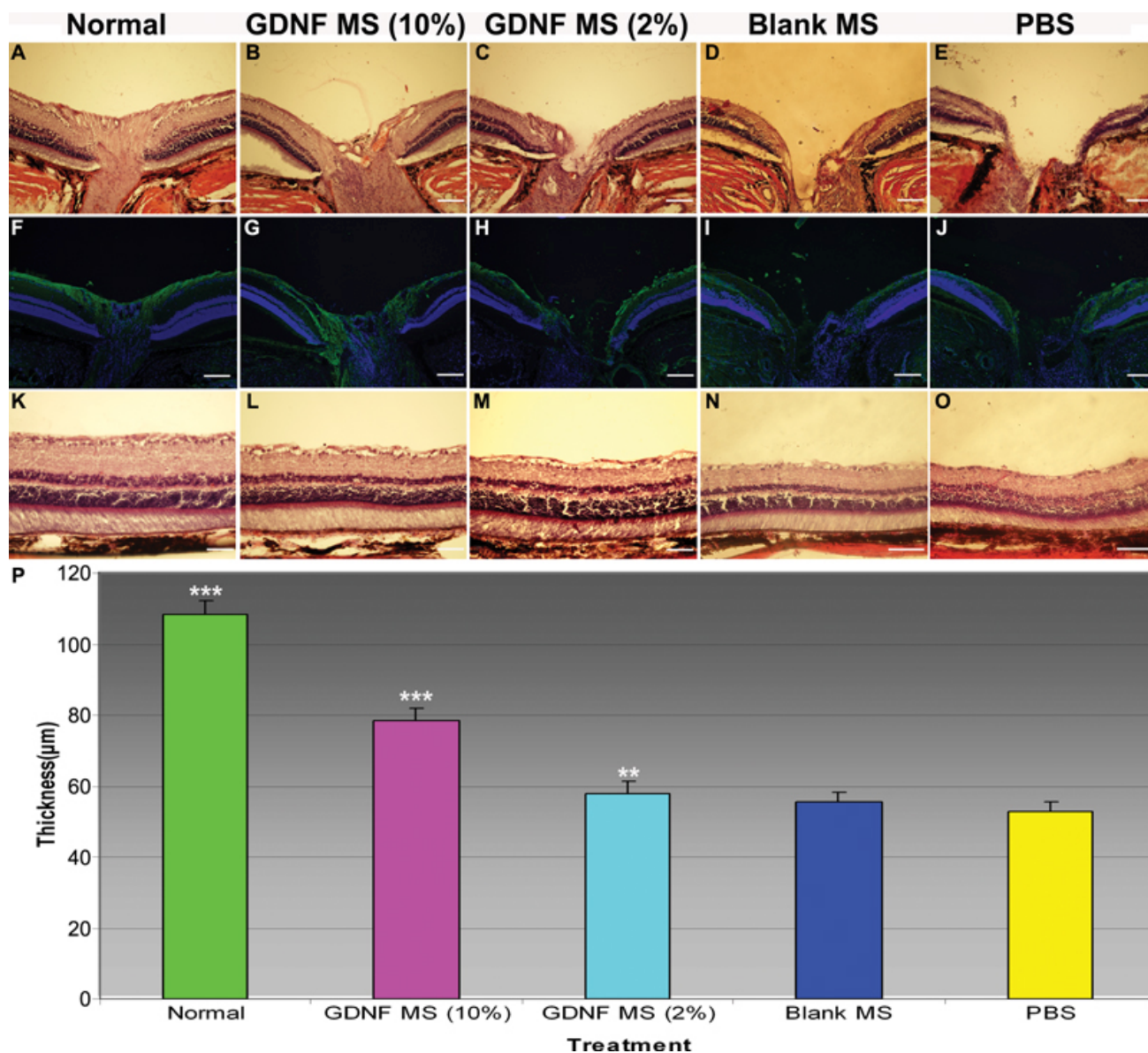


Figure 3. GDNF microspheres reduce retinal damage due to chronic intraocular elevation. **A-E** Chronic IOP elevation resulted in the cupping of the ONH (upper and middle row, scale bar: $200 \mu\text{m}$): **A** shows the normal ONH architecture. Ten percent of GDNF microsphere (MS) treatment (**B**) reduced the ONH excavation, and 2% GDNF MS treatment (**C**) moderately reduced the ONH excavation compared with blank MS treatment (**D**) and PBS (**E**). **F-J** (Neurofilament 200 kDa, middle row): **F** also illustrates the normal ONH architecture. Ten percent of GDNF MS treatment (**G**) decreased the loss of NFL, and 2% GDNF MS treatment (**H**) moderately decreased the loss of NFL compared with blank MS treatment (**I**) and PBS treatment (**J**). **K-O** Effects of GDNF MS treatment on preservation of the thickness of IPL after chronic IOP elevation (lower row, scale bar: $100 \mu\text{m}$): **K** shows the normal retinal architecture. Ten percent of GDNF MS treatment (**L**) resulted in the preservation of the thickness of IPL, and 2% GDNF MS treatment (**M**) moderately resulted in a protection of IPL compared with blank MS (**N**) and PBS treatment (**O**). **(P)** shows the quantitative analysis of GDNF MS treatment on the thickness of IPL. Chronic IOP elevation resulted in a significant loss of the thickness of IPL compared with that of the normal retina ($p < 0.001$). Ten percent of GDNF MS treatment resulted in a significant reservation of the thickness of IPL compared with 2% GDNF MS, blank MS, and PBS treatment ($p < 0.001$). Two percent of GDNF MS treatment resulted in significant reservation of the thickness of IPL compared with PBS treatment ($p < 0.01$). There were no significant differences between groups treated with 2% GDNF MS versus blank MS nor between groups treated with blank MS versus PBS ($p > 0.05$). Three asterisks indicate $p < 0.001$ and a double asterisk denotes $p < 0.01$. In the figure, MS represents microsphere.

The effects of GDNF microspheres on RGC survival were quantified by counting anti-NeuN positive cells in the ganglion cell layer. In normal eyes without IOP elevation, the average number of anti-NeuN positive cells in the GCL was $64.2 \pm 3.6/\text{mm}$. Eight weeks of IOP elevation resulted in a significant loss of the RGCs: $23.1 \pm 2.1/\text{mm}$ with blank

microsphere treatment and $22.2 \pm 2.4/\text{mm}$ with PBS treatment. GDNF microsphere treatment (started one week after the first hypertonic saline injection) resulted in dose-dependent preservation of RGCs: $41.1 \pm 2.2/\text{mm}$ with 10% GDNF microspheres (25 ng/eye) compared with $25.9 \pm 2.6/\text{mm}$ with 2% GDNF microspheres (5 ng/eye).

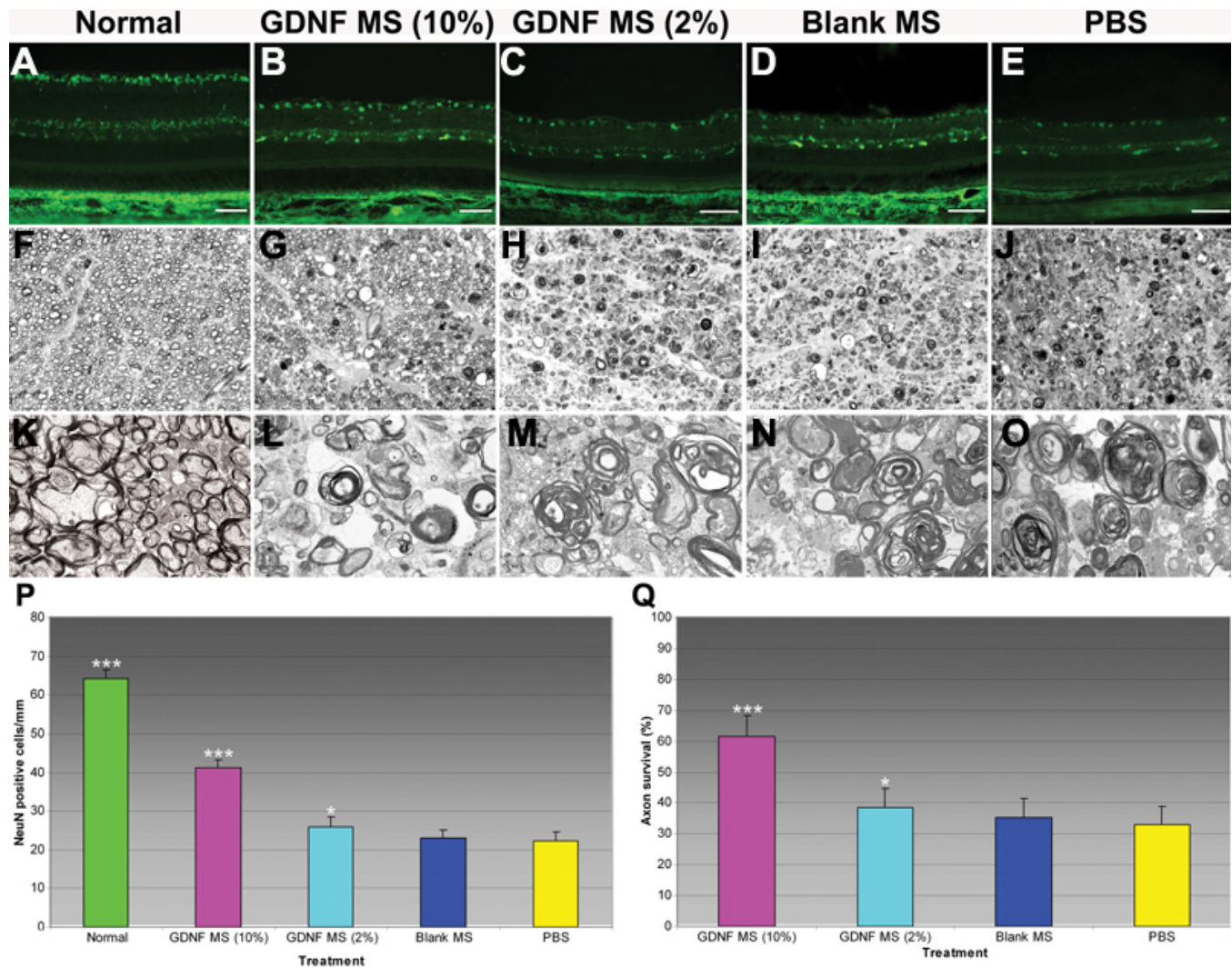


Figure 4. GDNF microspheres increased retinal ganglion cells and their axons survival. **A-E** Effects of GDNF microsphere (MS) treatment on the survival of RGCs (anti-NeuN positive cells) due to chronic IOP elevation (scale bars represent 100 μm): **A** shows a normal retina without IOP elevation. Ten percent of GDNF MS treatment (**B**) resulted in the preservation of RGCs, and 2% GDNF MS treatment (**C**) resulted in moderate preservation of RGCs compared with blank MS (**D**) and PBS treatment (**E**). **F-O** Effects of GDNF MS treatment on axon survival due to chronic IOP elevation: **F** illustrates normal ON axons without IOP elevation. Ten percent of GDNF MS treatment (**G**) resulted in a preservation of axons, and 2% GDNF MS treatment (**H**) resulted in a moderate preservation of axons compared with blank MS (**I**) and PBS treatment (**J**). Degenerating axons occupied nearly the entire mass of the ON of rats treated with blank MS (**I**) and PBS (**J**). **K-O** are corresponding representative EM photos. **F-J**: magnification 1000X; **K-O**: magnification 7100X. **P** shows the quantitative analysis of GDNF MS treatment on the survival of RGCs. Chronic IOP elevation resulted in a significant loss of the RGCs ($p < 0.001$). Ten percent of GDNF MS treatment significantly increased the RGC survival compared with 2% GDNF MS treatment, blank MS treatment, and PBS treatment ($p < 0.001$). Two percent of GDNF MS treatment resulted in significant reseration of RGCs compared with PBS treatment ($p < 0.05$). There were no significant differences between groups treated with 2% GDNF MS versus blank MS and between groups treated with blank MS versus PBS ($p > 0.05$). **Q** illustrates the quantitative analysis of GDNF MS treatment on the ON axon survival. The survival percentage was 61.58% with 10% GDNF MS treatment compared with 38.56% with 2% GDNF MS treatment, 35.25% with blank MS, and 33.12% with PBS treatment ($p < 0.001$). Two percent of GDNF MS treatment increased the survival percentage compared with PBS treatment ($p < 0.05$). There were no significant differences between groups treated with 2% GDNF MS versus blank MS and between groups treated with blank MS versus PBS ($p > 0.05$). Three asterisks indicate $p < 0.001$ and one asterisk denotes $p < 0.05$. In the figure, MS represents microsphere.

Chronic IOP elevation resulted in significant loss of RGCs compared to uninjected control eyes without IOP elevation ($p < 0.001$). Ten percent of GDNF microspheres significantly increased RGC survival compared with either 2% GDNF microspheres, blank microspheres, or PBS treatment ($p < 0.001$). Two percent of GDNF microspheres resulted in significant preservation of RGCs compared with PBS treatment ($p < 0.05$). There were no significant differences between groups treated with 2% GDNF microspheres versus blank microspheres or with blank microspheres versus PBS ($p > 0.05$).

GDNF microspheres increase optic nerve axon survival: The effects of chronic IOP elevation and GDNF microspheres

on optic axon survival are shown in Figure 4F-O and Figure 4Q. Figure 4F shows a semi-thin cross section of a normal ON with axons stained using 1% toluidine blue. After eight weeks of IOP elevation, there was evidence of substantial axon degeneration (Figure 4G-J) compared with untreated normotensive controls (Figure 4F). Axonal degeneration can be identified by the appearance of swollen axons that lack apparent axoplasm as well as axons that appear dark due to the collapse of the myelin sheath. Degenerating axonal profiles such as these occupied nearly the entire mass of the ON in eyes treated with blank microspheres or PBS alone (Figure 4I,J).

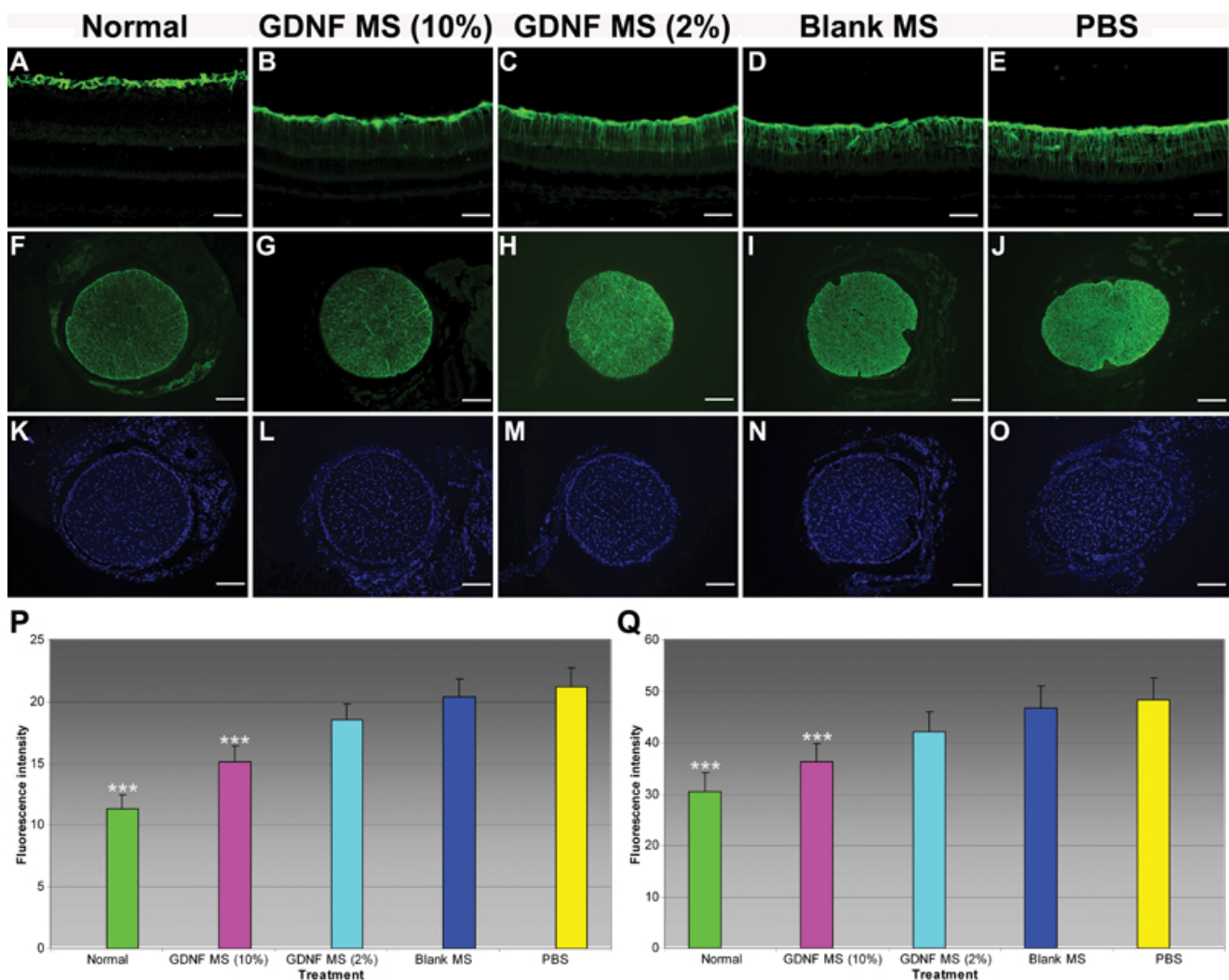


Figure 5. GDNF microspheres decreased GFAP expression of retina and optic nerve. GFAP expression was mainly localized to the inner limiting membrane in normal retina (A). Chronic IOP elevation resulted in increased GFAP expression (B-E). Scale bars represent 100 μ m. Chronic IOP elevation increased the GFAP expression in an ON cross section (G-J) compared with that of normal ON without IOP elevation (F). Ten percent of GDNF microsphere (MS) treatment (G) decreased the GFAP expression, and 2% GDNF MS treatment (H) moderately decreased the GFAP expression compared with blank MS (I) and PBS treatment (J). K-O illustrate the corresponding optic nerve section stained with DAPI. Scale bars represent 200 μ m. Quantitative analysis of GDNF MS treatment on the GFAP expression of retina (P) and ON (Q). Chronic IOP elevation resulted in significantly increased GFAP expression in the retina ($p < 0.001$) and optic nerve ($p < 0.001$) compared with that of normal tension eyes. Ten percent of GDNF microspheres significantly decreased the IOP-induced GFAP overexpression in both the inner retina ($p < 0.001$) and ON ($p < 0.001$) while 2% GDNF microspheres were more difficult to distinguish from treatment with blank microspheres and PBS alone ($p > 0.05$). Three asterisks indicate $p < 0.001$. In the figure, MS represents microsphere.

The effects of GDNF microspheres on axon survival were quantified by counting intact axonal profiles (Figure 4Q). The percentage of axon survival was calculated from the number of axons in elevated IOP eyes compared to the number of axons in the contralateral eye without IOP elevation. The survival percentage was 61.58% with 10% GDNF microspheres compared to 38.56% with 2% GDNF microspheres, 35.25% with blank microspheres, and 33.12% with PBS alone ($p < 0.001$). Two percent of GDNF microspheres were associated with significantly increased axonal survival compared with PBS treatment ($p < 0.05$). However, there were no significant differences comparing 2% GDNF to blank microspheres or comparing blank microspheres to PBS alone ($p > 0.05$).

GDNF microspheres decrease GFAP expression of retina and optic nerve: GFAP expression was mainly localized to the inner limiting membrane in normal retina (Figure 5A). Chronic IOP elevation resulted in significantly increased GFAP expression in the retina (Figure 5B-E) and optic nerve (Figure 5G-J) compared with untreated control eyes (Figure 5A,F; $p < 0.001$). Ten percent of GDNF microspheres significantly decreased the IOP-induced GFAP overexpression in both the inner retina (Figure 5P; $p < 0.001$) and optic nerve (Figure 5Q; $p < 0.001$) while 2% GDNF microspheres were more difficult to distinguish from treatment with blank microspheres and PBS alone ($p > 0.05$).

DISCUSSION

This study demonstrates that intravitreal GDNF, delivered by way of biodegradable PLGA microspheres, results in significant sparing of retinal ganglion cells in a rat model of glaucoma. This phenomenon includes preservation of IPL thickness as well as the sparing of optic axons both within the nerve fiber layer of the retina and within the orbital portion of the optic nerve. This sparing effect is dependent upon the dose of GDNF microspheres used and is not replicated by blank microspheres or injections of PBS alone. The glial activation that accompanies IOP-associated injury in the inner retina and ON is also mitigated by treatment with GDNF-laden microspheres, as evidenced by dose-dependent suppression of GFAP expression in these regions, further underscoring the beneficial effects of the treatment strategy used here.

The inner retinal sparing seen in the present study can be attributed to the neuroprotective effects of GDNF together with the sustained drug delivery properties conferred by the incorporation of peptides in PLGA microspheres. While the mechanism underlying the effects of GDNF on RGCs has yet to be entirely delineated, it is known that both endogenous and exogenous neurotrophic factors have neuroprotective effects on cells of the rodent retina. With respect to retinal ganglion cells, there is evidence to suggest that retrograde transport of neurotrophins from retinorecipient regions may be essential for survival [12,13]. Furthermore, obstructed axonal transport of BDNF has been observed in glaucoma models, and intravitreal injection of BDNF increases RGC survival [14-17]. Adult rat RGCs are capable of taking up exogenous GDNF placed in the superior colliculus and of retrogradely transporting this molecule to their cell bodies in the inner retina [21].

Both the GDNF-binding GFR α surface receptor and transmembrane Ret receptor, tyrosine kinase, are expressed by RGCs as well as other retinal neurons [19,20]. GDNF and the GDNF receptor complex are present in the human optic nerve head [33]. Studies have shown that GDNF interacts with a GPI-linked cell surface receptor, GFR α . In turn, GFR α together with bound GDNF interacts with the tyrosine kinase receptor, Ret. The binding of GDNF to the cell surface receptor activates the Ret tyrosine kinase [34,35]. Available data suggest that GDNF must interact with GDNF α initially, after which it may interact with Ret directly. In the present study, we have provided evidence that the expression of GDNF and its receptors localizes to the RGCs of the adult rat.

Effective neuroprotection in glaucoma likely requires the consistent availability of the active agent for prolonged periods of time. Neurotrophic factors present in the vitreous humor are rapidly degraded by free extracellular proteases including any released as a consequence of RGC degeneration. Additionally, neurotrophic factors may be taken up and degraded in the retina by resident microglia. Repeated injections of unprotected neurotrophic factors over the life of the patient might not be sufficient to consistently confer a significant visual advantage and could be expected to result in an unacceptable rate of serious complications such as retinal detachment and endophthalmitis.

One strategy for improving the sustainability of peptide compounds *in vivo* is to protect them from endogenous proteases and endocytotic activity via incorporation into biodegradable polymers. Much progress has recently been made in the field of polymeric drug delivery with biodegradable microspheres emerging as one promising platform for use in chronic neurodegenerative diseases [36-38]. Previous analysis of the microspheres used in the current study indicated a cumulative GDNF release of 35.4 ng/mg over 71 days *in vitro* [25]. This delivery is not ideal, yet it represents a significant improvement over previous examples. In particular, the burst release of GDNF in the first two days was only 59% for these microspheres compared with the previous reported burst release of 98% over the same interval [27]. More importantly, this delivery profile provides sufficiently sustained delivery of GDNF to protect mammalian RGCs *in vivo* as demonstrated here and in previous studies [25].

GDNF selectively enhances the survival and development of dopaminergic neurons with an ED₅₀ of 40 pg/ml *in vitro* [18], however, it can be difficult to determine the optimal *in vivo* dosages for an experimental drug therapy such as the microsphere delivery system. Previous studies have demonstrated significant rescue of axotomized RGCs following treatment with 1 μ g of GDNF in adult rats [21,22]. However, phase I clinical trials have shown that high doses of neurotrophins can be associated with significant side effects, including chronic pain [39]. An advantage of a slow release system is the ability to deliver low levels of the drug that achieves therapeutic relevance via continuous presence in the target microenvironment. Furthermore, the considerable expense of neurotrophic factors means that the slow-release of small doses is more economically feasible. A previous study in our labora-

tory has demonstrated that 1 μ l of a 2% suspension of GDNF microspheres (total theoretical release of GDNF=0.707 ng) significantly increased long-term RGC survival in the DBA/2J mouse glaucoma model [25]. In the present study, using a rat glaucoma model, we have shown that the rescue effect of intravitreally administered biodegradable microsphere-loaded GDNF is dose-dependent. Further dosage analysis of microsphere-delivered neurotrophins in the vitreous would be a helpful step toward achieving maximum pharmacological effect.

Increased expression of GFAP by glial cells is a nonspecific marker of neuronal injury [40], and glial cell activation has been proposed as an important factor contributing to RGC death in glaucoma [41,42]. In the normal retina, GFAP expression is mainly localized to the vicinity of the inner limiting membrane and nerve fiber layer. As with a variety of insults, we found that chronic IOP elevation resulted in significantly increased GFAP expression in the rat retina and the retro-orbital ON, and the treatment with GDNF microspheres significantly diminished this response in a dose-dependent fashion. While the mechanism underlying this phenomenon remains to be revealed, it is reasonable to note the potential association between the decreased expression of GFAP seen following treatment with GDNF-containing microspheres and the preservation of RGCs and their axons.

In summary, this study shows that PLGA microsphere-delivered GDNF represents an important neuroprotective strategy in the experimental treatment of glaucomatous optic neuropathy. Further studies will be important to demonstrate the functional consequences of this treatment and to extend this work to large animal models.

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