

# Transplantation of BDNF-Secreting Mesenchymal Stem Cells Provides Neuroprotection in Chronically Hypertensive Rat Eyes

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**PURPOSE.** To evaluate the ability of mesenchymal stem cells (MSCs) engineered to produce and secrete brain-derived neurotrophic factor (BDNF) to protect retinal function and structure after intravitreal transplantation in a rat model of chronic ocular hypertension (COH).

**METHODS.** COH was induced by laser cauterization of trabecular meshwork and episcleral veins in rat eyes. COH eyes received an intravitreal transplant of MSCs engineered to express BDNF and green fluorescent protein (BDNF-MSCs) or just GFP (GFP-MSCs). Computerized pupillometry and electroretinography (ERG) were performed to assess optic nerve and retinal function. Quantification of optic nerve damage was performed by counting retinal ganglion cells (RGCs) and evaluating optic nerve cross-sections.

**RESULTS.** After transplantation into COH eyes, BDNF-MSCs preserved significantly more retina and optic nerve function than GFP-MSC-treated eyes when pupil light reflex (PLR) and ERG function were evaluated. PLR analysis showed significantly better function ( $P = 0.03$ ) in BDNF-MSC-treated eyes (operated/control ratio = 63.00%  $\pm$  11.39%) than GFP-MSC-treated eyes (operated/control ratio = 31.81%  $\pm$  9.63%) at 42 days after surgery. The BDNF-MSC-transplanted eyes also displayed a greater level of RGC preservation than eyes that received the GFP-MSCs only (RGC cell counts: BDNF-MSC-treated COH eyes, 112.2  $\pm$  19.39 cells/section; GFP-MSC-treated COH eyes, 52.21  $\pm$  11.54 cells/section;  $P = 0.01$ ).

**CONCLUSIONS.** The authors have demonstrated that lentiviral-transduced BDNF-producing MSCs can survive in eyes with chronic hypertension and can provide retina and optic nerve functional and structural protection. Transplantation of BDNF-producing stem cells may be a viable treatment strategy for glaucoma. (*Invest Ophthalmol Vis Sci.* 2011;52:4506–4515) DOI:10.1167/iovs.11-7346

Glaucoma is an optic neuropathy resulting in progressive retinal ganglion cell (RGC) death and loss of visual function. Although the underlying causes of glaucoma have not been clearly elucidated, many factors that may contribute to the neurodegeneration of RGCs have been identified, including reactive changes in optic nerve head glial cells,<sup>1</sup> a decrease in retrograde transport of vital trophic factors,<sup>2</sup> oxidative stress mediated by the generation of reactive oxygen species (ROS),<sup>3,4</sup> and excessive activation of different immune system components.<sup>5–7</sup> Considering that glaucoma is one of the most frequent causes of blindness worldwide, there is an enormous need to develop therapeutic strategies that may protect optic nerve function and structure in this patient population.

Cell transplantation has been proposed as an experimental strategy to treat the diseased and injured central nervous system (CNS), including the retina. Multipotent bone marrow-derived mesenchymal stem cells (MSCs) hold great potential for the delivery of therapeutic proteins to treat the damaged or diseased CNS. Transplantation of MSCs has attracted considerable attention in efforts to develop cell-based therapies because they are readily obtained from the patient. Promising results have been reported with the use of MSCs in animal models for a number of different diseases, including spinal cord injury,<sup>8,9</sup> stroke,<sup>10</sup> and myelin deficiency.<sup>11</sup> In addition, MSCs have the ability to survive and migrate when transplanted to CNS tissues,<sup>12–15</sup> to differentiate into neural-like cells in vitro,<sup>13,15–18</sup> and to display electrophysiological properties consistent with mature neurons.<sup>19,20</sup> Naive MSCs have also shown the potential to be neuroprotective when used as a therapeutic modality in animal models of retinal degeneration<sup>21–26</sup> and glaucoma.<sup>27</sup> Engineering of stem cells to produce neurotrophic growth factors has been explored as an attractive mode of long-term delivery of neuroprotective substances to the injured CNS in different animal models.<sup>28–30</sup>

Brain-derived neurotrophic factor (BDNF) is a 14-kDa neuroprotective protein<sup>31</sup> that preferentially binds to the high-affinity TrkB<sup>32</sup> receptor. Target-derived BDNF from the thalamus is essential for correct RGC development<sup>33,34</sup> and ex vivo maintenance of RGCs.<sup>35,36</sup> It has been shown that retrograde transport of target-derived BDNF to the retina is decreased in an animal model of acute elevation of intraocular pressure (IOP).<sup>2</sup> Supplemental delivery of BDNF in different animal models has been shown to have beneficial effects on the preservation of the retina and optic nerve structure,<sup>37–41</sup> pro-

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viding hope that the therapeutic use of BDNF may become a viable option for long-term treatment of glaucoma.

The principal purpose of this study was to evaluate whether transplanted MSCs can survive in eyes with chronic hypertension and provide protection for the retina and for optic nerve function and structure. Additionally, we wanted to determine whether MSCs engineered to produce and secrete BDNF would provide better functional and structural outcomes in eyes with chronic hypertension than in eyes that received only control MSCs.

## MATERIALS AND METHODS

### Animals

All animal studies were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and procedures were approved by the Iowa State University Committee on Animal Care. Adult Brown Norway rats (10 months of age, retired breeders;  $n = 28$ ) and E17 rat pups ( $n = 36$ ) were used for experiments. Rats were kept under a 12-hour light/12-hour dark regimen.

### Rat Mesenchymal Stem Cells

Rat MSCs isolated from adult rats (obtained from Tulane Center for Gene Therapy, New Orleans, LA) were maintained as an adherent cell line in  $\alpha$ -modified Eagle's medium ( $\alpha$ -MEM; 12561-049; Invitrogen, Carlsbad, CA) containing 20% hybridoma-qualified FBS (S11595; Atlanta Biologicals, Norcross, GA), 2 mM L-glutamine, and antibiotic-antimycotic solution (1%, 15240-096; Invitrogen; 10,000 U/mL penicillin, 10,000  $\mu$ g/mL streptomycin, 25 ng/mL amphotericin B). Cells were maintained as low-density cultures plated at 75–150 cells/cm<sup>2</sup>. When cultures reached 70%–80% confluence, MSCs were gently lifted from the dish using 0.25% trypsin and 0.1% EDTA solution (Invitrogen) and were pelleted at 800g. MSCs were subsequently plated into 150-mm culture dishes at 75–150 cells/cm<sup>2</sup>. Cultures were maintained by supplementing the dish with fresh medium every other day.

### Engineering Stem Cells Ex Vivo with Lentiviral Vectors

MSCs were engineered to produce and secrete brain-derived neurotrophic factor (BDNF; rat cDNA) using lentiviral vectors, as previously reported.<sup>42</sup> Briefly, MSCs were plated in six-well plates at a density of 1000–1200 cells per well and were allowed to adhere for 12 hours. After adhering to the plate, the growth medium was substituted in each well with  $\alpha$ -MEM containing 2% FBS and 12  $\mu$ g/mL gelatinous solid (Sequa-brene, 152667; Sigma-Aldrich, St. Louis, MO). Two separate lentiviral constructs encoding BDNF (LV-BDNF; CMV-BDNF-IRES-GFP) and green fluorescence protein (GFP, LV-GFP; CMV-GFP) were added simultaneously to MSCs at a multiplicity of infection (MOI) of 15 for each vector. The LV-GFP vector was used as an additional reporter because of inefficient IRES-GFP expression. A population of control MSCs was engineered with only the LV-GFP vector at an MOI of 30 to match the viral titer of the BDNF/GFP-engineered MSCs. Viral particles were removed after 8 hours of exposure, and medium was changed to fresh growth medium. Engineered MSCs were subsequently maintained as previously described.<sup>42</sup>

### ELISA of BDNF Production

Enzyme-linked immunosorbent assay (ELISA) was used to quantify the amount of BDNF released from BDNF-MSCs and GFP-MSCs, respectively. A BDNF assay (E<sub>max</sub> Immunoassay System, G7610; Promega Corporation, Madison, WI) was used to detect BDNF in media conditioned for 24 hours by BDNF-MSCs and GFP-MSCs, as reported previously.<sup>42</sup> Initially, 350,000 cells were plated onto 150-mm<sup>2</sup> plates and allowed to adhere for 24 hours. Media were replaced with fresh growth media, and cells were grown for an additional 24 hours.

Conditioned media (CM) were collected and frozen at  $-20^{\circ}\text{C}$  for subsequent analysis. Anti-BDNF monoclonal antibody in carbonate buffer was used to coat the wells of a 96-well plate overnight at  $4^{\circ}\text{C}$ . Wells were incubated with sample buffer and blocker for 1 hour at room temperature. Samples from CM and a purified BDNF standard curve (0–500 pg/well) were prepared and incubated in wells on a shaker for 2 hours. Wells were subsequently washed, an anti-BDNF polyclonal antibody was added to the wells, and plates were incubated again on a shaker for 2 hours. An anti-IgY horseradish peroxidase-conjugated antibody was incubated in the wells on a shaker for 1 hour at room temperature and subsequently washed. Chromagen substrate 100  $\mu$ L (TMB-One solution; Promega) was added to each well and reacted for 10 minutes at room temperature. Reactions were stopped by the addition of 100  $\mu$ L of 1 N HCl per well. Plates were read using a microplate reader (EL800 Universal Microplate Reader; BioTek Instruments, Winooski, VT) at 450 nm, and data were analyzed (KC Junior Software; BioTek Instruments).

### Neurotrophin Bioactivity Assay

Primary cultures of rat embryonic day (E) 17 dorsal root ganglia (DRG) were used to assess the bioactivity of BDNF released from MSCs because DRGs have been shown to elongate neurites in response to neurotrophin exposure. Briefly, E17 rat pups were decapitated, rinsed in ice-cold 70% ethanol, and subsequently placed in ice-cold Dulbecco's modified Eagle's medium (DMEM). Spinal columns were dissected through a ventral approach in ice-cold L-15 media (21683-027; Invitrogen), and lumbar DRGs were dissected and subsequently pooled. After dissection, DRGs were rinsed with ice-cold L15 and transferred to warm DRG growth media containing DMEM, L-glutamine, penicillin-streptomycin, and 10% FBS. Three DRG explants were transferred in approximately 75  $\mu$ L media onto a poly-L-ornithine-coated coverglass placed in 24-well culture plates. DRGs were allowed to adhere for 8 hours, and wells were flooded with experimental media. The experimental media in which DRGs were cultured included MSC growth media, MSC media conditioned by BDNF-MSCs or GFP-MSCs, respectively, or MSC media containing 50 ng recombinant human BDNF (rhBDNF; 450-02; PeproTech, Rocky Hill, NJ). Explants were allowed to grow for an additional 48 hours and subsequently were fixed with 4% paraformaldehyde in 0.1 M PO<sub>4</sub> buffer (pH 7.4). Neurite outgrowth was visualized in cultures by staining with an anti-neurofilament antibody (RMO.308, 1:50; Virginia Lee, University of Pennsylvania). Images were taken and semiquantitatively scored by seven naive observers to judge the extent and density of neurite outgrowth in each condition. Each DRG was scored on a scale from 1 to 5, with 1 representing modest neurite growth in terms of length and density and 5 representing long, dense neurite arborization from the DRGs. Data from this analysis were pooled, and statistical analysis was performed.

### Laser-Induced Chronic Ocular Hypertension

Chronic ocular hypertension (COH) was induced in the left eye of all animals, as previously described, while the opposite (right) eyes were left untouched (no laser surgery or cell transplant was performed) to serve as a control for all experiments.<sup>43</sup> Briefly, rats were anesthetized with 2.5% isoflurane + 100% oxygen; body temperature was maintained with a heating pad. Indocyanine green (10  $\mu$ L, 10 mg/mL; Sigma) was injected into the anterior chamber of the eye at a very slow rate to avoid abrupt elevation of the IOP from the procedure. Animals were pretreated with 4% pilocarpine hydrochloride eye drops to increase outflow of the dye into the trabecular meshwork and episcleral veins. The pilocarpine treatment also caused miosis, which protected the posterior pigmented structures of the eye from the diode laser energy (the pigmented iris served as a barrier for any potential stray energy). Twenty minutes after injection, a diode laser (DioVet; Iridex Corporation, Mountain View, CA) was used to externally deliver 810-nm energy pulses through a 50- $\mu$ m fiberoptic probe to the region of the trabecular meshwork and episcleral veins in close proximity to the limbal region. Careful positioning of the fiberoptic probe ensured

that the orientation of the laser was away from the pigmented structures of the retina. Between 50 and 60 laser spots through a 300° range of the limbal radius (350-mW energy, 1500-ms pulse time) were delivered. Rats were given acetaminophen (100 mg/kg) and codeine (75 mg/kg) in the drinking water for 7 days after induction to control pain. Topical antibiotic ointment was applied to the cornea to prevent excessive drying and corneal ulcers.

### Cell Transplantation

Two days after the induction of COH, a small puncture was made in a dorsolateral location in the eye with a 30-gauge needle. A beveled glass microelectrode attached to a Hamilton syringe was inserted into this opening, and approximately 200,000 cells were delivered intravitreally in 2  $\mu$ L of Earle's balanced salt solution. A small amount of antibiotic ointment was applied to the cornea after the transplantation procedure. Cells were only transplanted into eyes that received COH induction.

### Electroretinography

Rats were dark adapted for at least 12 hours before recording. Animals were initially anesthetized with 3.5% halothane, 30% N<sub>2</sub>O, and 70% O<sub>2</sub> until unresponsive to the righting reflex and were subsequently maintained using 1.25% halothane, 30% N<sub>2</sub>O, and 70% O<sub>2</sub> for the duration of the recording. Pupils were dilated using 2.5% phenylephrine, after which 1% tropicamide and topical anesthetic (1% proparacaine) was applied to the cornea. Animals were positioned on a heated sliding stage to maintain body temperature during anesthesia and recording sessions. Ground and reference electrodes were placed subcutaneously in the tail and forehead of the animal, respectively. A solid custom-made gold ring recording electrode (Roland Consult, Brandenburg, Germany) was placed on each eye in contact with the cornea, and a thin layer of methylcellulose was used to maintain contact between the cornea and the electrode and to decrease the recording noise. ERG recordings were performed (Reti-Port system; Roland Consult). Scotopic rod responses (illumination 0.22 cd/m<sup>2</sup>, cutoff frequency 1–300 Hz;  $n = 8$  responses were averaged) were collected first, followed by scotopic maximum combined response (78 cd/m<sup>2</sup>, 1–300 Hz;  $n = 8$  responses were averaged), oscillatory potentials (78 cd/m<sup>2</sup>, 200–500 Hz;  $n = 8$  responses were averaged), photopic cone response (21 cd/m<sup>2</sup> rod bleaching background light in combination with 78 cd/m<sup>2</sup> stimulation, 1–300 Hz;  $n = 8$  responses were averaged), and flicker ERG (78 cd/m<sup>2</sup>, 1–300 Hz;  $n = 50$  responses were averaged). ERG amplitudes were subsequently analyzed (RETIport32 software; Roland Consult).

### Computerized Pupillometry

Computerized pupillometry was used to assess pupil light reflex (PLR), as reported previously.<sup>43,44</sup> Briefly, animals were anesthetized initially with 4% halothane, 30% N<sub>2</sub>O, and 70% O<sub>2</sub>. A light plane of anesthesia was maintained with 1% halothane, 30% N<sub>2</sub>O, and 70% O<sub>2</sub> to avoid suppression of the PLR response caused by the use of higher doses of anesthetic. A two-channel computerized pupillometer (Neurooptics, San Clemente, CA) was used to record the movement of the pupil from the control (nonoperated) eye and the operated eye while a computer-controlled stimulus light (white light-emitting diodes) was alternately turned on in front of the control or operated eye (on time, 0.2 seconds; off time, 5 seconds). The stimulus light intensity used for pupil recordings was 78 cd · m<sup>2</sup>. The change in the pupil area of the control (nonoperated) eye was evaluated after alternating light stimulus between the operated and the control eye, whereas pupil responses were recorded only from the control eye. Pupil responses from the nonoperated control eye were expressed as a ratio between PLR response after illuminating the operated eye and PLR response after illuminating the control eye (PLR ratio % = operated/control), as previously reported.<sup>43,45</sup>

### Tonometry

Goldmann applanation tonometry with a modified prism for rat recordings was used to monitor the IOP in experimental and control eyes, as previously described.<sup>46,47</sup> Animals were anesthetized with a very light plane of anesthesia (1.0% halothane, 30% N<sub>2</sub>O, and 70% O<sub>2</sub>) to decrease possible anesthetic effects on the IOP.<sup>48</sup> Animals that did not develop an increase in IOP or that developed ulcerated corneas were excluded from the study.

### Eye Tissue Preparation

Rats were deeply anesthetized with halothane and quickly decapitated 42 days after the induction of COH. Eyes were dissected and fixed with 4% paraformaldehyde (pH 7.4). Optic nerves were dissected from the eye before fixation and immersed in 2% paraformaldehyde/2% glutaraldehyde solution to provide adequate fixation for cross-sectional optic nerve analysis. Eye globes were sectioned for histology and immunohistochemistry analysis.

### Optic Nerve Quantification

Optic nerves were collected in 3-mm-thick segments obtained 1 mm posterior to the globe. Optic nerves were rinsed in cacodylate buffer, postfixed in 2% osmium tetroxide in cacodylate buffer, dehydrated in alcohol, and embedded in epoxy resin. Cross-sections (2  $\mu$ m) were cut with an ultramicrotome, mounted on glass slides, and stained with 1% paraphenylenediamine. The degree of optic nerve damage was evaluated using a grading scheme similar to that used by other investigators.<sup>6,7,49</sup> Optic nerves were assigned to 1 of 5 grades of damage, with 1 representing healthy optic nerves and 5 representing severely damaged optic nerves. Analysis was performed in a masked manner.

### Retinal Ganglion Cell Quantification

Immunohistochemical procedures were used to quantify the number of RGCs in the retina. Briefly, cryosections were rehydrated in potassium phosphate-buffered solution (KPBS) and incubated in blocking solution containing 0.1% bovine serum albumin (A9647; Sigma), 0.04% Triton X-100 (Fisher), and 5% normal donkey serum (017-000-121; Jackson ImmunoResearch, West Grove, PA) in KPBS. Anti-Brn-3a antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in blocker and incubated overnight at room temperature. Tissue was rinsed with KPBS containing Triton X-100, and incubated with Cy3-conjugated secondary antibodies for 2 hours. After rinses, 4', 6'-diamidino-2-phenylindole, dilactate (DAPI, 1  $\mu$ g/mL, D3571; Sigma) was applied for 30 minutes at room temperature to visualize nuclei. Slides were mounted with anti-fade mounting media (Gelmount; Sigma) and sealed. Negative controls were processed in parallel by omission of the primary or secondary antibody. Tissue was imaged using a microscope (Microphot; Nikon, Melville, NY). Micrographs were prepared using commercial software (Photoshop, version 9.0 [Adobe, San Jose, CA] and Macromedia Freehand, version 10.0 [Macromedia, San Francisco, CA]). Quantification of RGCs was performed by counting Brn-3a-immunolabeled cells in serial sections (five per eye) of control and hypertensive rat eyes.

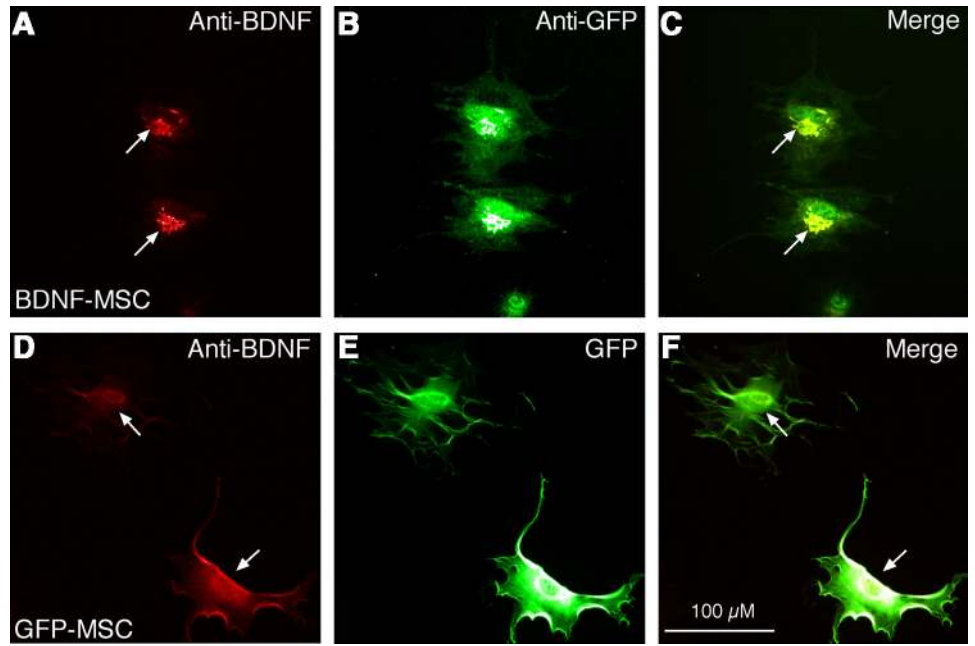
### Statistical Analysis

Statistical analysis was performed by using Student's *t*-test, paired *t*-test, and one-way ANOVA with Bonferroni posttest (as indicated in the text) graphing and statistics software (GraphPad, San Diego, CA).  $P < 0.05$  was considered significant.

## RESULTS

### Evaluation of BDNF Production from MSCs

Lentiviral vectors were used to engineer MSCs to produce the neurotrophic factors BDNF and GFP or GFP alone. The infection rate of the lentiviral vectors was evaluated by counting

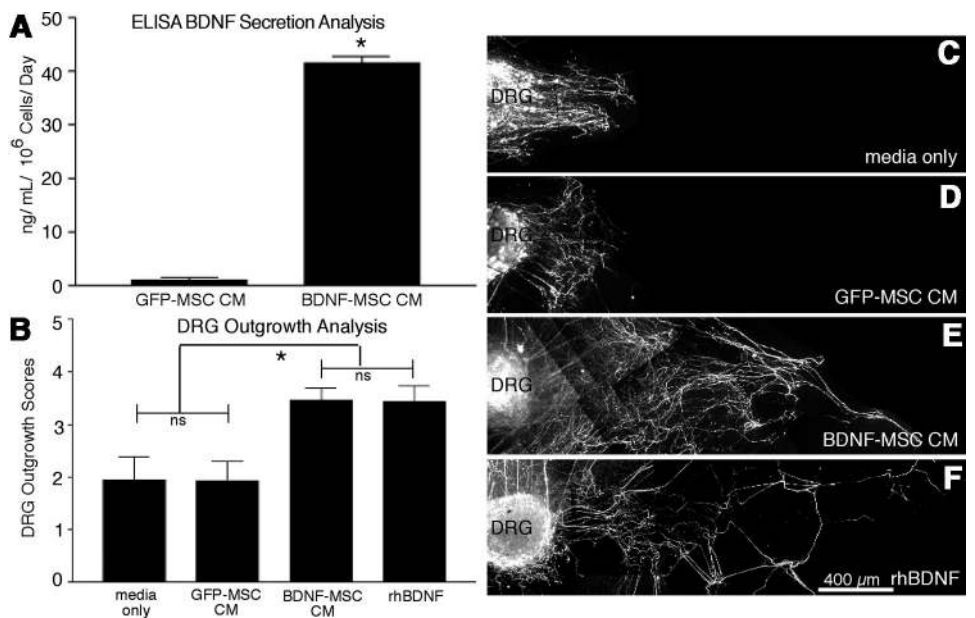


**FIGURE 1.** MSCs engineered with lentiviral vectors express GFP (green) and BDNF (red). MSCs transduced with lentiviral BDNF vectors (A–C) showed increased expression of BDNF (A, arrows). Merged image (C) demonstrates perinuclear localization of BDNF immunoreactivity in BDNF-MSCs (arrows). Control MSCs transduced with lentiviral GFP vectors (D–F) show diffuse BDNF immunoreactivity throughout the cell (D, arrows). Robust GFP expression is apparent using conventional fluorescence microscopy (E). Merged image (F) demonstrates the BDNF localization in GFP-MSCs.

DAPI-stained cell nuclei and GFP-positive cells in eight fields. The observed infection rate in our experiments was  $99.05\% \pm 0.65\%$ . We have shown that MSCs engineered to express the BDNF construct displayed intense anti-BDNF immunoreactivity (IR) in the perinuclear area within the cell body (Fig. 1A). To ensure that the intense IR was due to transduction by a viral construct, anti-GFP antibodies were used to identify GFP produced by the IRES-GFP site and to examine the cellular localization of BDNF immunoreactivity (Figs. 1B, 1C). In contrast, MSCs transduced with LV-GFP vectors displayed less intense BDNF immunoreactivity than BDNF-MSCs but expressed high levels of GFP (Figs. 1D–F), demonstrating BDNF upregulation in MSCs is due to BDNF gene delivery and not an artifact of general lentiviral transduction.

To determine whether transduced MSCs were capable of secreting BDNF, ELISA analysis of media conditioned by engineered MSCs was performed. Secretion of BDNF from control GFP-MSCs was  $0.97 \pm 0.52$  ng BDNF/mL per  $10^6$  cells/day, which was significantly less than  $41.40 \pm 1.32$  ng/mL per  $10^6$  cells/day secreted by lentiviral transduced BDNF-MSCs (Fig. 2A;  $P < 0.0001$ , Student's *t*-test). Rat embryonic DRG cultures were used to investigate the bioactivity of the secreted BDNF, as we have previously described.<sup>42</sup> The length and density of neurites extending from DRGs cultured in control media, or media conditioned by engineered MSCs, was assessed and quantified in the masked manner previously described.<sup>42</sup> On treatment with media only, DRGs elaborated relatively short neurites (Figs. 2B, 2C). A similar level of modest outgrowth was

**FIGURE 2.** MSCs secrete bioactive BDNF. ELISA analysis revealed BDNF-MSCs secrete  $41.40 \pm 1.32$  ng BDNF/ $10^6$  cells/day, which was significantly higher than control GFP-MSCs secreting  $0.97 \pm 0.52$  ng BDNF/ $10^6$  cells/day (A). Media conditioned by BDNF-MSCs or GFP-MSCs were collected and applied to E17 rat dorsal root ganglia explant cultures to assay and quantitate BDNF bioactivity (B). DRGs were treated with MSC growth media (C), media conditioned by GFP-MSCs (D) or BDNF-MSCs (E), or MSC growth media supplemented with 50 ng rhBDNF (F). Montage images of DRGs were collected and subsequently scored by naive observers (B). No significant difference was observed in the modest outgrowth of neurites induced by MSC media only (C) or GFP-MSC-conditioned media (B, D;  $P > 0.05$ ). BDNF-MSC conditioned media induced robust outgrowth of neurites from DRGs (E) and did not differ significantly from DRG outgrowth induced by rhBDNF-supplemented media (B, F;  $P > 0.05$ ). Each group of DRGs exposed to BDNF, however, had significantly more neurite outgrowth than each of the control groups ( $P < 0.05$ ). Scale bar, 400  $\mu$ m (C–F). GFP-MSC CM, GFP-MSC conditioned media; BDNF-MSC CM, BDNF-MSC conditioned media; rhBDNF, recombinant human brain-derived neurotrophic factor. \* $P < 0.05$ .



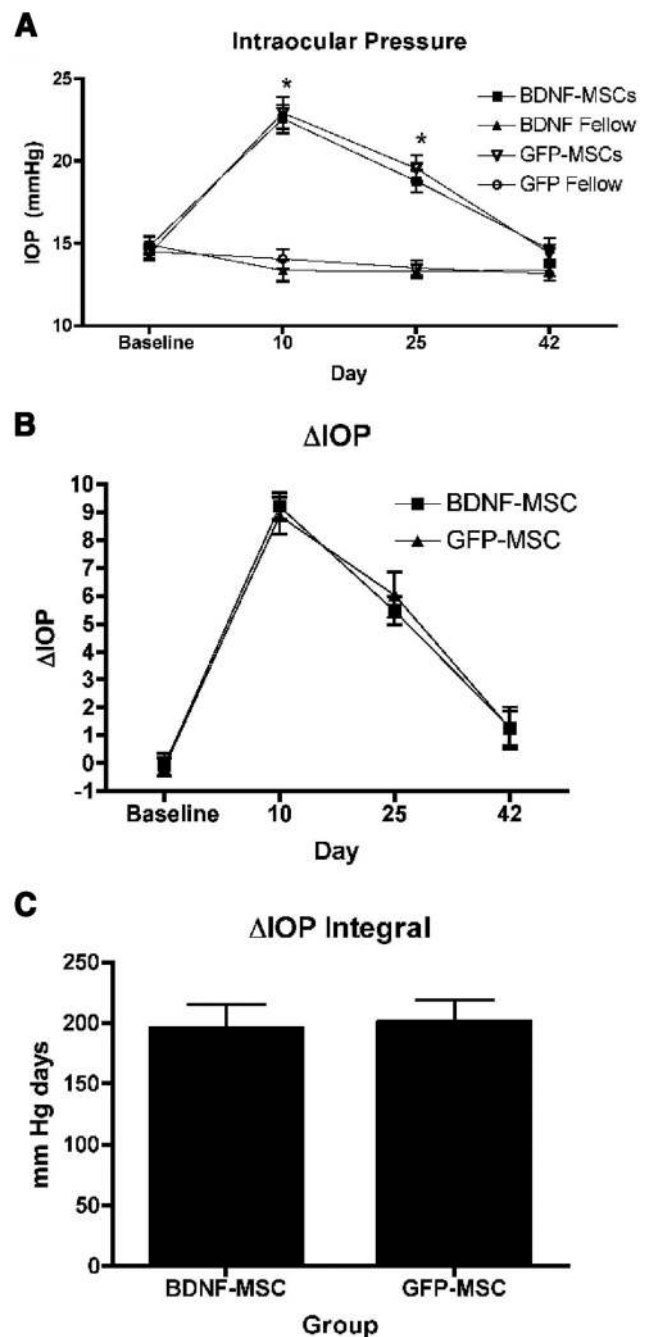
observed in DRGs treated with GFP-MSC-conditioned media (CM) (Fig. 2D), and statistical analysis revealed no significant difference between these two groups (Fig. 2B;  $P > 0.05$ , one-way ANOVA with Bonferroni posttest). In contrast, DRGs that were treated with BDNF-MSC CM (Fig. 2E) and DRGs treated with 50 ng rhBDNF (Fig. 2F) displayed extensive neurite outgrowth and often elaborated processes in excess of 1.6 mm from the explant. Neurite outgrowth in DRGs treated with BDNF-MSC CM is similar in those treated with rhBDNF ( $P > 0.05$ , one-way ANOVA with Bonferroni posttest). Both these groups displayed significantly greater neurite outgrowth scores than DRGs treated with media or GFP-MSC CM (Fig. 2B,  $P < 0.05$ , one-way ANOVA with Bonferroni posttest). Together these results demonstrated that the MSCs transduced with the lentiviral BDNF vectors were capable of producing and secreting BDNF with potent neurite outgrowth-promoting bioactivity.

### Evaluation of the IOP in a Rat Model of COH

The rat model of COH was used to evaluate the possible beneficial effects of MSC transplantation on retinal and optic nerve function and structure. The IOP for eyes of BDNF-MSC- and GFP-MSC-treated animals before COH induction were not significantly different:  $14.0 \pm 0.5$  and  $15.0 \pm 0.5$  mm Hg, respectively (Fig. 3A;  $P = 0.4523$ , Student's *t*-test). A significant increase in IOP was observed after the induction of COH (Fig. 3A). The IOP for BDNF-MSC- and GFP-MSC- transplanted eyes 10 days after induction was  $22.6 \pm 0.9$  and  $23 \pm 1$  mm Hg, respectively, and was not significantly different between groups (Fig. 3A;  $P = 0.8167$ , Student's *t*-test). The IOP was significantly higher in laser-treated eyes than in fellow control eyes, which were  $13.4 \pm 0.6$  for BDNF-MSC fellow eyes (Fig. 3A;  $P < 0.0001$ , paired *t*-test) and  $14 \pm 0.6$  mm Hg for GFP-MSC fellow eyes (Fig. 3A;  $P < 0.0001$ , paired *t*-test). The increased IOP in laser-treated eyes was also apparent 25 days after induction: BDNF-MSC,  $18.8 \pm 0.6$  mm Hg; GFP-MSC,  $19.6 \pm 0.8$  mm Hg; fellow control eyes,  $13.3 \pm 0.4$  ( $P < 0.0001$ , paired *t*-test) and  $13.5 \pm 0.4$  mm Hg ( $P < 0.0001$ , paired *t*-test), respectively (Fig. 3A). The increased IOP returned to near baseline levels by 42 days after induction, with IOPs of  $14.7 \pm 0.7$  and  $14.4 \pm 0.5$  mm Hg for BDNF-MSC and GFP-MSC transplant recipient eyes, respectively. Additionally, these values were not significantly different from those of fellow eyes for BDNF-MSC-treated ( $13.4 \pm 0.4$  mm Hg;  $P = 0.1579$ ) and GFP-MSC-treated ( $13.2 \pm 0.4$  mm Hg;  $P = 0.0749$ ) animals. To ensure there was no significant difference in the relative change in IOP between BDNF-MSC- and GFP-MSC-treated eyes, the difference was calculated between the fellow IOP and the experimental eye IOP ( $\Delta$ IOP). This analysis revealed no significant difference in the baseline  $\Delta$ IOP ( $P = 0.5541$ ) or at 10 ( $P = 0.5350$ ), 25 ( $P = 0.7570$ ), or 42 ( $P = 0.4782$ ) days after the elevation of IOP (Fig. 3B). The calculated  $\Delta$ IOP integral for COH eyes treated with BDNF-MSCs ( $195.5 \pm 19.59$  mm Hg days) and GFP-MSCs ( $200.6 \pm 18.50$  mm Hg days) was not significantly different ( $P = 0.8512$ , Student's *t*-test).

### Analysis of Retinal and Optic Nerve Function: ERG and PLR Analysis

Electroretinography analysis revealed significant preservation of retinal electrical activity in COH eyes that received BDNF-MSC transplants compared with those experimental eyes that received control GFP-MSCs. At 20 days after transplantation, BDNF-MSC-recipient animals had significantly greater ERG amplitudes (amplitudes were expressed as a ratio between operated eye and fellow control eye ERG amplitudes) compared with GFP-MSC-recipient animals for the rod b-wave (BDNF,  $41.21\% \pm 9.90\%$ ; GFP,  $18.37\% \pm 4.70\%$ ;  $P = 0.0403$ , Student's



**FIGURE 3.** Laser cauterization of trabecular meshwork and episcleral veins resulted in the development of COH. (A) Laser cauterization resulted in the significant IOP increase in operated eyes at 10 and 25 days after surgery ( $*P < 0.05$ ). BDNF-MSCs, hypertensive eyes that received transplants of BDNF-producing MSCs; BDNF-Fellow, opposite (control eye) that did not receive laser surgery or an MSC transplant; GFP-MSCs, hypertensive eyes that received transplants of GFP-expressing MSCs not modified to produce BDNF; GFP-Fellow, opposite (control eye) that did not undergo laser surgery or MSC transplantation. (B) Statistical analysis revealed no difference in the  $\Delta$ IOP (IOP operated eye – IOP control eye) between BDNF-MSC and GFP-MSC groups at different time points during the experiment (mean  $\pm$  SEM). (C) Analysis of the IOP integral revealed no significant difference in the  $\Delta$ IOP integral between BDNF-MSC- and GFP-MSC-treated groups.

*t*-test), cone b-wave (BDNF,  $58.49\% \pm 9.60\%$ ; GFP,  $35.26\% \pm 4.22\%$ ;  $P = 0.0315$ ), maximum combined b-wave (BDNF,  $53.68\% \pm 10.39\%$ ; GFP,  $25.09\% \pm 5.76\%$ ;  $P = 0.0064$ ), and

flicker amplitude (BDNF, 51.44% ± 9.81%; GFP, 31.17% ± 4.08%; *P* = 0.0447, Fig. 4A). There was not a significant difference in the ERG amplitude ratios for the cone a-wave (BDNF, 72.10% ± 15.5%; GFP, 85.46% ± 17.20%; *P* = 0.8205) or the maximum combined a-wave (BDNF, 49.35% ± 13.58%; GFP, 26.94% ± 5.34%; *P* = 0.0900) amplitudes. Analysis of oscillatory potentials did not reveal a statistically significant difference between BDNF-MSC and GFP-MSC-treated animals despite a trend toward higher amplitudes in the BDNF-treated group (BDNF, 56.65% ± 12.20%; GFP, 24.28% ± 4.62%; *P* = 0.146; Fig. 4A).

Sustained preservation of ERG responses was also observed at 40 days after transplantation for the rod b-wave (BDNF, 43.23% ± 7.88%; GFP, 20.88% ± 5.41%; *P* = 0.0245), maximum-combined b-wave (BDNF, 61.16% ± 8.45%; GFP, 34.71% ± 8.66%; *P* = 0.0349), cone b-wave (BDNF, 59.30% ± 7.99%; GFP, 29.33% ± 5.05%; *P* = 0.0033), and flicker responses (BDNF, 68.58% ± 7.54%; GFP, 40.57% ± 5.58%; *P* = 0.0054; Fig. 4B). There was not a significant difference in the ERG amplitude ratios for any measured a-wave parameters, cone (BDNF, 74.02% ± 14.09%; GFP, 78.51% ± 11.31%; *P* = 0.8035)

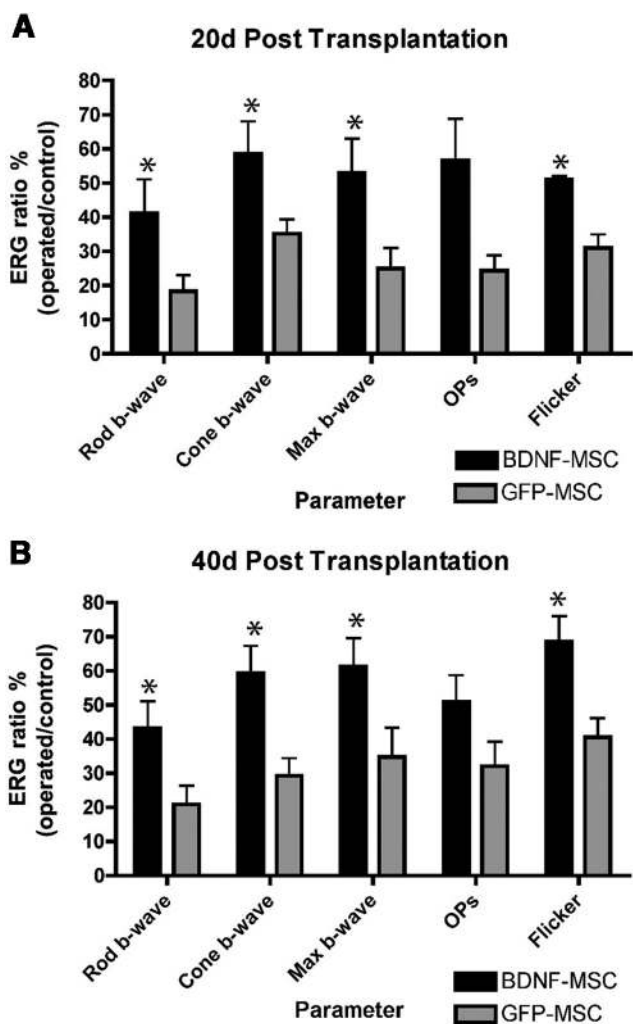


FIGURE 4. Transplantation of BDNF-MSCs preserved retinal function in COH eyes. Retinal electrical activity was significantly higher in BDNF-MSC-treated eyes than in GFP-MSC-treated eyes at 20 (A) and 40 (B) days after transplantation for the rod b-wave, cone b-wave, maximum combined b-wave (Max b-wave), and flicker responses. Oscillatory potentials (OPs) were not significantly different between BDNF-MSC- and GFP-MSC-treated animals. \**P* < 0.05.

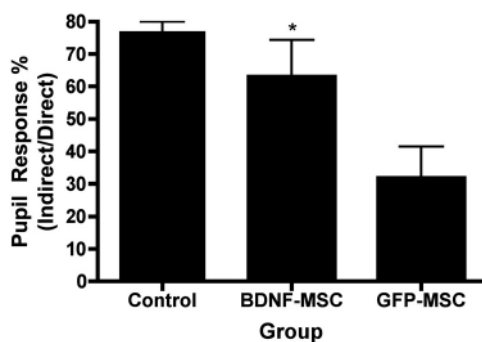


FIGURE 5. Computerized pupillometry analysis of the pupil light response at 42 days after transplantation showed significantly better pupil constriction in BDNF-MSC-treated eyes than in GFP-MSC-treated eyes (\**P* < 0.05). The pupil light reflex is presented as a ratio of constriction amplitudes measured from the control (nonoperated) eye after illumination of the operated and control eyes.

or the maximum combined a-wave (BDNF, 61.16% ± 14.36%; GFP, 34.75% ± 6.48%; *P* = 0.8035) amplitudes. The oscillatory potentials in BDNF-MSC- and GFP-MSC-treated animals were not significantly different (BDNF, 51.01% ± 7.74%; GFP, 32.11% ± 7.21%; *P* = 0.0869). Computerized pupillometry was used to assess the combined function of the different retinal layers and the optic nerve. There was a significantly better afferent pupil response in BDNF-MSC-treated eyes (63.00% ± 11.39%) than in GFP-MSC-treated eyes (31.81% ± 9.63%; *P* = 0.0343) at 42 days after transplantation (Fig. 5).

### MSCs Survive after Transplantation into Chronically Hypertensive Eyes

Examination of the ocular sections demonstrated that MSCs were capable of surviving in hypertensive eyes with 17.62% ± 2.52% (BDNF-MSC) or 15.89% ± 3.73% (GFP-MSC) survival rates at the termination of the experiment (BDNF-MSC, 35,233 ± 5041 cells; GFP-MSC, 31,780 ± 7469 cells). MSCs were counted per tissue section at the termination of the experiment. Transplanted MSCs were capable of surviving in COH eyes and were predominantly found adjacent to the ganglion cell layer (GCL) (Figs. 6A-C), integrated into inner retinal layers (Figs. 6D-F), or in the vitreous of transplanted

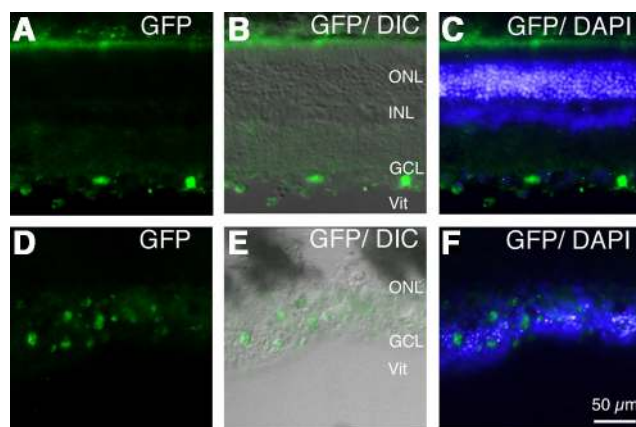


FIGURE 6. MSCs survive after transplantation into hypertensive eyes. Lentiviral-transduced MSCs were detected in the retina using an anti-GFP antibody at the end of the experiment (A, D). MSCs (BDNF-MSCs, A-C; GFP-MSCs, D-F) were predominantly detected in the retinal ganglion cell layer (B, C) and the inner retinal layers at (E, F). Autofluorescence was detected in the outer retina associated with photoreceptor outer segments (A-C). DIC, differential interference contrast.

eyes 42 days after transplantation (Supplementary Fig. S1, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7346/-/DCSupplemental>). Immunohistochemical analysis of retinal tissue sections collected from eyes with chronic hypertension that underwent BDNF-GFP-MSC transplantation at the termination of the experiment showed strong BDNF and TrkB expression within the retina (Supplementary Figs. S2A, S2B, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.11-7346/-/DCSupplemental>).

### Retinal Ganglion Cell and Optic Nerve Quantification

Retinal ganglion cells (RGCs) were identified based on immunoreactivity for the RGC-specific transcription factor Brn3a<sup>50</sup> and were counted to determine the level of neuroprotection provided to RGCs from BDNF-MSCs. Analysis of baseline measurements of the numbers of Brn3a-IR RGCs in the GCL in fellow (nonoperated) eyes for BDNF-MSC-treated ( $200.8 \pm 40.1$ /section) and GFP-MSC-treated ( $176.6 \pm 40$ /section) rats revealed no significant difference between groups (Figs. 7A, 7B, 7G;  $P = 0.6743$ ; Student's *t*-test). Quantification of Brn3a-IR RGCs in BDNF-MSC-treated eyes revealed a significantly higher number of RGCs ( $112.2 \pm 19.3$ /section) than in GFP-MSCs ( $52.2 \pm 11.5$ /section; Figs. 7C–G;  $P = 0.0106$ , Student's *t*-test). Statistical analysis revealed that both groups had significantly fewer RGCs than fellow control eyes (Fig. 7G; BDNF,  $P = 0.0142$ ; GFP,  $P = 0.0056$ ; both paired *t*-tests).

Optic nerve integrity was examined to compare the optic nerves of the untreated control eye with those of the BDNF-MSC- and GFP-MSC-transplanted eyes. Masked analysis revealed no significant difference in the appearance (grade) of optic nerves from COH eyes that received the BDNF-MSCs ( $2.5 \pm 0.34$ ; Fig. 8A) and their fellow control eyes ( $1.73 \pm 0.38$ ; Figs. 8B, 8E;  $P = 0.0948$ , paired *t*-test). In contrast, there was a significant difference in optic nerve integrity when comparing optic nerves of COH eyes that received the GFP-MSCs ( $3.25 \pm 0.37$ , Fig. 8C) with those of their fellow control eyes ( $1.08 \pm 0.07$ ; Figs. 8D, 8E;  $P < 0.001$ ). However, analysis of optic nerve scores between BDNF-MSC-treated ( $2.5 \pm 0.34$ ) and GFP-MSC-treated ( $3.25 \pm 0.37$ ) eyes did not reach a statistically significant difference, despite a trend toward higher values in the BDNF-MSC group ( $P = 0.1493$ ).

### DISCUSSION

Current medical and surgical interventions for glaucoma are focused on IOP reduction and the modification of factors, such as blood pressure, to improve optic nerve blood flow and to prevent the progression of vision loss. However, visual field deficits continue to develop in many patients despite adequate IOP control, which dictates a need for the development of novel therapeutic approaches that may provide direct protection of optic nerve function and structure. Different neurotrophic growth factors have been tested in animal models of glaucoma and optic nerve damage as possible therapeutic agents for preserving the function and structure of damaged tissues, with a particular focus on the use of BDNF.<sup>37,40,44,51–55</sup> RGCs<sup>56</sup> and astrocytes<sup>57</sup> have an intrinsic capacity to produce BDNF, and the retina can also receive BDNF from the thalamus by way of retrograde transport. BDNF binds predominantly to TrkB receptors, which activate prosurvival signaling cascades by dimerization or internalization<sup>58</sup> of the receptor. It has been shown that TrkB expression is decreased by negative feedback loops<sup>59,60</sup> after bolus injections of BDNF, which could attenuate any potential benefit derived from delivering large amounts of supplemental BDNF to the retina.

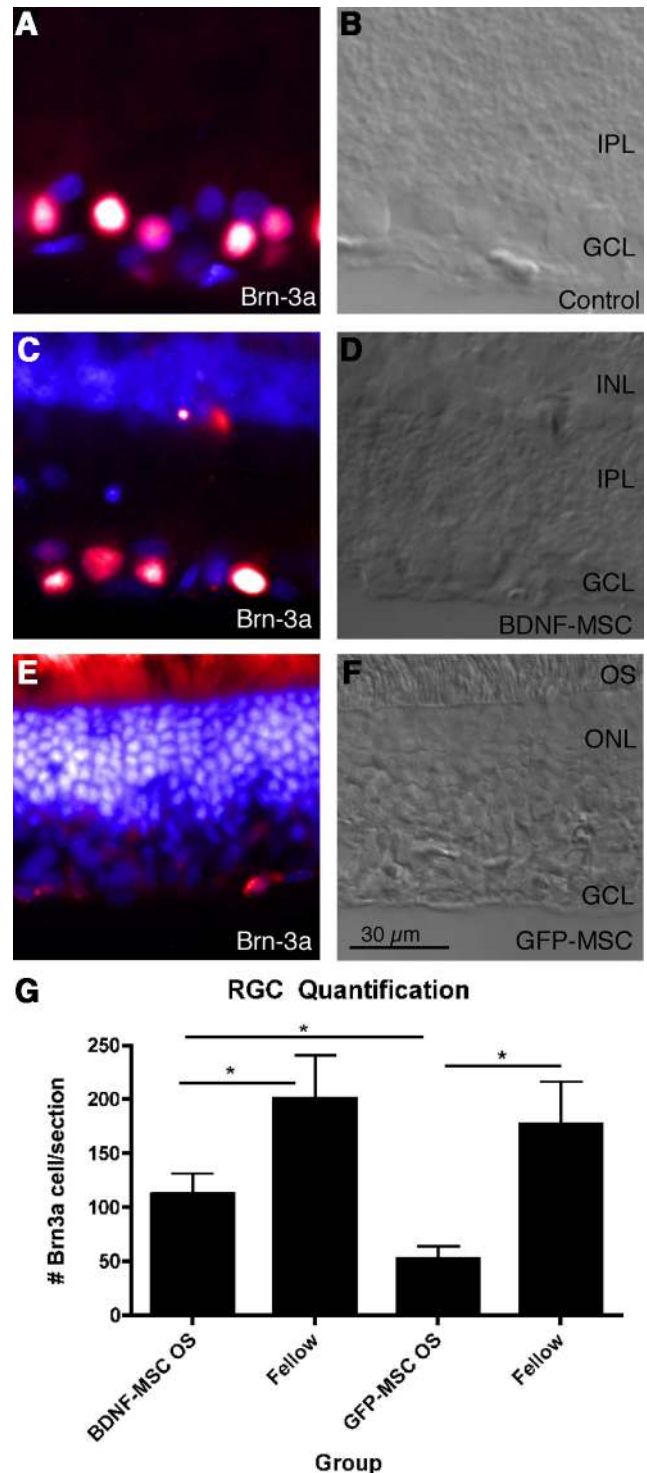
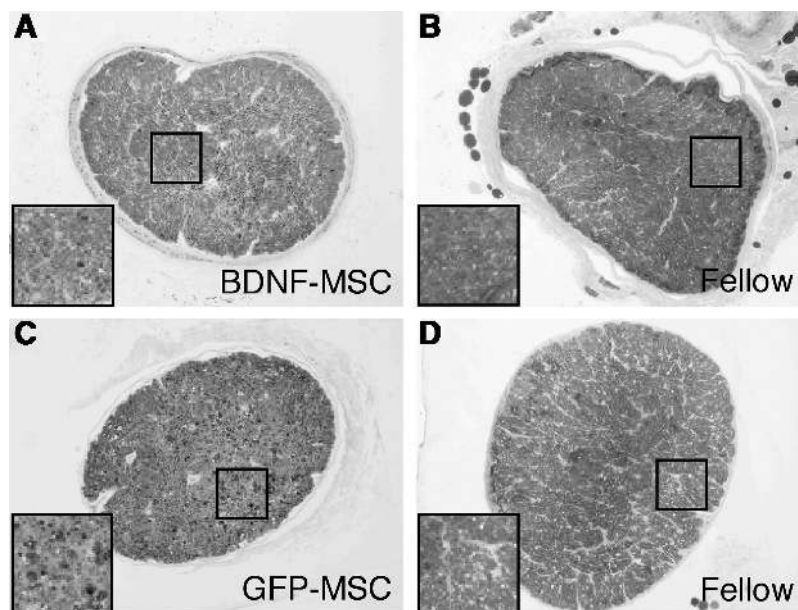
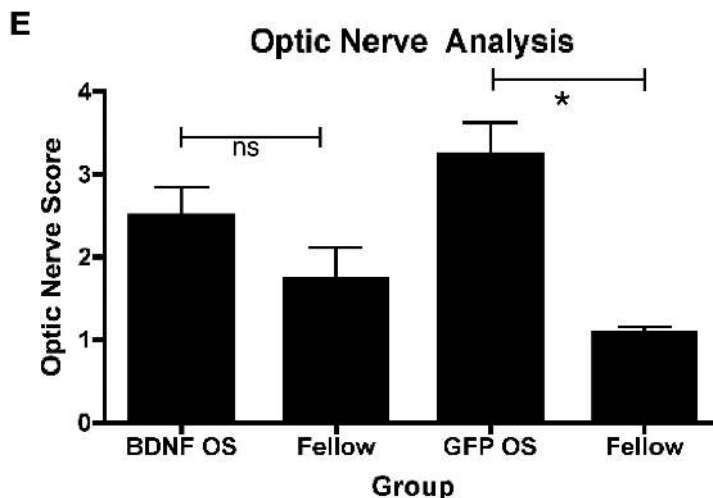


FIGURE 7. BDNF-MSC-transplanted cells served to protect Brn3a-IR RGCs in COH eyes. BDNF-MSC-treated COH eyes had significantly fewer Brn3a-IR cells per section than untreated fellow eyes (A–D;  $P < 0.05$ ). GFP-MSC-treated COH eyes also had significantly fewer Brn3a-IR cells per section than untreated fellow eyes (E, F;  $P < 0.01$ ). However, quantitative analysis of BDNF-MSC- and GFP-MSC-treated eyes revealed significantly more Brn3a-IR cells per section in BDNF-MSC-treated eyes than in GFP-MSC-treated COH eyes (G;  $*P < 0.05$ ).



**FIGURE 8.** Effect of BDNF-MSC transplants on optic nerve integrity in COH eyes. Optic nerves were assigned to 1 of 5 grades of damage, with 1 representing healthy optic nerves and 5 representing severely damaged optic nerves. No significant difference in optic nerve grades was observed between the optic nerves of COH eyes treated with BDNF-MSCs (A) and the fellow optic nerves (B). A significant difference was observed between optic nerves of COH eyes treated with GFP-MSCs (C) and control optic nerves of fellow, untreated eyes (D,  $P < 0.001$ ). However, analysis of optic nerve scores between BDNF-MSC- and GFP-MSC-treated eyes did not reveal a statistically significant difference (E).



In our study, we used MSCs to deliver a constant, low level of BDNF and demonstrated that this approach has potential for functional and structural retinal protection in eyes with chronic hypertension.

We have previously demonstrated that chronic intravitreal release of low BDNF doses from polymer microspheres does provide effective functional protection in rat retinas exposed to the aggressive acute elevation of IOP.<sup>44</sup> Other groups have demonstrated similar positive effects in terms of decreased optic nerve damage in a rat model of COH<sup>54</sup> and the DBA-2J mouse model of glaucoma<sup>55</sup> using long-term delivery of relatively low doses of neurotrophic growth factors. Approaches that use viral-mediated gene therapy have also been shown to be successful in preventing RGC degeneration in rodent models of optic nerve transection<sup>38</sup> and ocular hypertension,<sup>37</sup> whereas the transplantation of naive MSCs into rats with COH also showed positive effects.<sup>27</sup> Although results from these studies showed anatomic preservation of RGCs and optic nerve structure, the effects on visual system functional status have not been addressed.

In this study, we have used a similar approach with the particular goal of evaluating the functional properties of chronic hypertensive rat eyes after BDNF-MSC transplantation.

We have shown that MSCs can be used as cellular vehicles for delivering neuroprotective proteins to the retina and are readily transformed using lentiviral vectors to secrete bioactive BDNF. Importantly, transplanted MSCs are capable of surviving in eyes with COH and do not appear to cause any undesirable side effects. Aside from the ability to survive for prolonged periods of time in hypertensive eyes, MSCs can be used in the form of autologous transplants, which would dramatically reduce risks associated with the rejection of transplanted cells and the potential transmission of infectious agents between donors and recipients.

One important finding of this study is that BDNF-MSC transplants confer robust protection of retinal and optic nerve function and, to some extent, optic nerve structure. Although structural preservation may provide potential evidence of the successful therapeutic approach, ultimately the functional status of damaged tissue dictates potential for the preservation or recovery of visual function. This study clearly demonstrated that chronic low-dose delivery of BDNF results in significant preservation of retinal and optic nerve functional parameters observed by ERG and PLR analysis, despite relatively small effects on optic nerve structural protection (observed by grading of optic nerve cross-sections), which might have been



limited by the semiquantitative nature of the grading scale method.

In conclusion, the use of these easily obtained, adult-derived stem cells as vectors to deliver neuroprotective proteins may become an attractive therapeutic approach for the treatment of chronic neurodegenerative retinal and optic nerve diseases in the future.

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