Intravitreally Transplanted Dental Pulp Stem Cells Promote Neuroprotection and Axon Regeneration of Retinal Ganglion Cells After Optic Nerve Injury

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Purpose. To investigate the potential therapeutic benefit of intravitreally implanted dental pulp stem cells (DPSCs) on axotomized adult rat retinal ganglion cells (RGCs) using in vitro and in vivo neural injury models.

Methods. Conditioned media collected from cultured rat DPSCs and bone marrow–derived mesenchymal stem cells (BMSCs) were assayed for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) secretion using ELISA. DPSCs or BMSCs were cocultured with retinal cells, with or without Fc-TrK inhibitors, in a Transwell system, and the number of surviving βIII-tubulin$^+$ retinal cells and length/number of βIII-tubulin$^+$ neurites were quantified. For the in vivo study, DPSCs or BMSCs were transplanted into the vitreous body of the eye after a surgically induced optic nerve crush injury. At 7, 14, and 21 days postlesion (dpl), optical coherence tomography (OCT) was used to measure the retinal nerve fiber layer thickness as a measure of axonal atrophy. At 21 dpl, numbers of Brn-3a$^+$ RGCs in parasagittal retinal sections and growth-associated protein-43$^+$ axons in longitudinal optic nerve sections were quantified as measures of RGC survival and axon regeneration, respectively.

Results. Both DPSCs and BMSCs secreted NGF, BDNF, and NT-3, with DPSCs secreting significantly higher titers of NGF and BDNF than BMSCs. DPSCs, and to a lesser extent BMSCs, promoted statistically significant survival and neuritogenesis/axogenesis of βIII-tubulin$^+$ retinal cells in vitro and in vivo where the effects were abolished after TrK receptor blockade.

Conclusions. Intravitreal transplants of DPSCs promoted significant neurotrophin-mediated RGC survival and axon regeneration after optic nerve injury.

Keywords: dental pulp stem cells, mesenchymal stem cells, axon regeneration, neuroprotection, cell transplantation

Trauma is the most common cause of central nervous system (CNS) injury with, in America alone, 11,000 people a year suffering a spinal cord injury (SCI),[1] 80,000 a year suffering severe traumatic brain injury,[2] and between 0.5% and 5.0% of head injuries resulting in traumatic optic neuropathy.[3] Chronic degenerative diseases are another leading cause of CNS damage, including glaucoma, a condition that affects retinal ganglion cells (RGCs) and is the second leading cause of blindness worldwide.[4] Lost neurons are not replaced, and severed axons do not regenerate after CNS injury, and, thus, recovery of lost sensory and motor function is severely limited.

The failure of CNS axons to regenerate after injury is partly attributed to a nonpermissive trophic environment composed of both a paucity of neurotrophic growth factors and an abundance of axon growth inhibitory molecules.[5] Neurotrophins, a class of neurotrophic factors (NTF), include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3). They promote regeneration of injured axons and the survival of axotomized neurons after binding to the tropomyosin receptor kinase-A, -B, and -C (TrK) receptors, respectively.[6] Inhibitory ligands, which derive from degenerate myelin[7,8] and scar tissue[6,9] in CNS lesion sites, induce receptor-mediated growth cone collapse of regenerating injured axons.

Thus, inducing changes to the microenvironment of injured neurons/axons to promote neuronal survival and disinhibited axon regeneration represents a potential treatment approach. The delivery of NTF to neuron somata rather than to the lesion site has proved a successful therapeutic strategy.[6] For example, several studies have successfully promoted RGC survival after intravitreal delivery of exogenous NTF to the vitreous after optic nerve injury.[10,11] To promote a significant effect, however, repeated injections of NTF combinations are necessary, which are highly invasive for the patient, indicating that a continuous delivery mechanism is preferred.[12,13] Moreover, bolus administration of neurotrophins act to downregulate the TrK receptors,[14,15] an effect that may be avoided by opting for a lower but continuous delivery regimen. Cellular therapy is regarded as a promising means of altering the trophic environment of damaged CNS neurons, such as RGCs. This strategy has met with some success; for example, using intravitreally administered fibroblasts genetically altered to

release NTF combinations[16] after optic nerve crush (ONC). ONC acts as an effective model of CNS injury in general and retinal neuron disease in particular.[6]

As an alternative to engineered cells, naturally occurring stem cells have been used to promote CNS repair, providing a source of either replacement neurons[17,18] or NTF combinations that promote endogenous neuron survival and axon regeneration by altering the local trophic microenvironment.[19] Stem cell–based CNS studies have increasingly used NTF-secreting bone marrow–derived mesenchymal stem cells (BMSCs) as a cellular therapy.[20,21] Moreover, BMSC-conditioned medium is neuroprotective in culture,[22] and intravitreal BMSC transplantation is neuroprotective for RGCs after optic nerve injury[23] and glaucoma.[24]

However, an emerging alternative stem cell source is the dental pulp, which contains self-renewing and pluripotent stem cells.[25] Dental pulp stem cells (DPSCs) are isolated from the dental pulp of both infant and adult mammalian teeth with relative ease of access and few ethical hurdles. Thus, DPSCs represent a potential autologous and allogeneic cellular therapy for CNS injury, particularly because recent evidence suggests that they are more potent than BMSCs at promoting functional recovery after spinal cord injury.[21] Although largely uncharacterized, a few studies have explored their potential to play a direct role in neuronal replacement owing to their neural crest origin.[26] DPSCs differentiate into neurons under defined in vitro conditions[27,28] and their integration into the CNS after transplantation has been described.[29]

Less focus has been given to exploiting DPSCs as an indirect NTF therapy (i.e., using DPSC-derived NTF to promote endogenous CNS neuron survival and axon regeneration). DPSCs express mRNA for NGF, glial cell line–derived neurotrophic factor (GDNF), and BDNF.[30–32] When transplanted into the hippocampus, DPSCs secrete ciliary neurotrophic factor (CNTF), VEGF, NGF, and FGF-2,[33] which could explain the findings of Sakai et al,[21] who demonstrated some functional recovery after complete transection of the spinal cord by transplanting DPSCs into the lesion site. The authors witnessed both an improvement in locomotory BBB[34] (Basso, Beattie, Bresnahan) scores and axon growth into the cell implant and across the lesion site at greater levels than after BMSC transplant. This observation, along with the greater expression of neurotrophic factor mRNA by DPSCs compared with BMSCs[21] indicates that DPSCs produce higher titers of neurotrophic factors compared with BMSCs. DPSCs transplanted into a cerebral infarct site after middle cerebral artery occlusion also promoted significant recovery in forelimb sensorimotor function. The transplanted DPSCs differentiated into astrocyte-like cells, suggesting that DPSCs contributed to neural regeneration as supportive cells through NTF secretion.[35]

In the present study, we investigated the neuroprotective and axogenic properties of primary adult rat DPSCs for axotomized RGCs. We carried out in vitro coculture studies of DPSCs with primary adult rat retinal cultures and compared βIII-tubulin$^+$ retinal cell survival and neurite outgrowth in these cultures with that in BMSC/retinal cell cocultures. Using specific Fc-TrK fusion protein blockers of the neurotrophin receptors, we determined a βIII-tubulin$^+$ retinal cell neuroprotective and axogenic role for DPSC-derived neurotrophins. In addition, we used an in vivo model of ONC injury to determine the effects of intravitreal stem cell transplantation on Brn-3a$^+$ RGC survival and axon regeneration. Our findings demonstrate that DPSCs promote RGC survival and axon regeneration through the secretion of neurotrophins to a greater extent than do BMSCs, and, hence, we propose that DPSCs have potential as a cellular therapy to treat RGC injury and degenerative disease.

Methods

All reagents were purchased from Sigma (Poole, UK) unless otherwise specified.

DPSC Isolation and Culture

Three adult male Sprague-Dawley rats weighing 170 to 200 g (Charles River, Kent, UK) were housed under Home Office guidelines and killed by "Schedule 1 Methods" before extraction of both upper and lower incisors. The dental pulp was removed under sterile conditions in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies, Gibco, Paisley, UK) supplemented with 1% penicillin/streptomycin (P/S), sliced into 1-mm^3 fragments and incubated in 4 mL 0.25% trypsin-EDTA for 30 minutes at 37°C. Trypsin was inactivated by adding an equal volume of DMEM containing 1% P/S and 10% fetal bovine serum (FBS). A single cell population was obtained by passing the cell suspension through a 70-μm cell strainer (BD Biosciences, Oxford, UK), which was centrifuged at 150*g* for 5 minutes. Cell pellets were resuspended in DMEM containing 1% P/S and 10% FBS and seeded into T25 flasks (Corning, Amsterdam, The Netherlands) in a total volume of 5 mL. Cultures were maintained at 37°C in 5% CO_2 and medium was changed 24 hours after seeding, and every 3 days thereafter, with cells passaged when 80% confluent using 0.05% trypsin. Each animal provided stem cells for separate cultures to supply conditioned medium for the ELISA before cells from three cultures were pooled for the in vitro coculture/in vivo transplantation experiments.

BMSC Isolation and Culture

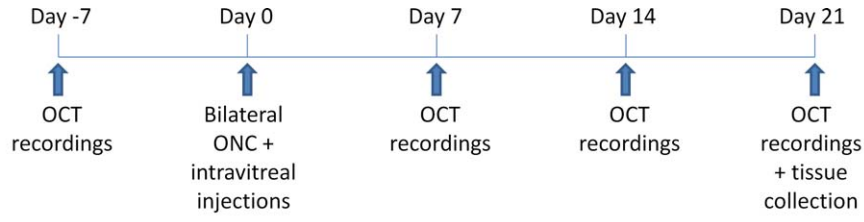
BMSCs were isolated from femurs removed from the same animals described above. In sterile conditions, the ends of the femurs were detached, and the bone marrow flushed with 10 mL DMEM. Cell aspirates were centrifuged at 150*g* for 5 minutes before cells were resuspended in DMEM containing 1% P/S and 10% FBS. Cell suspensions were seeded into T25 flasks in a total volume of 5 mL. Cultures were maintained at 37°C in 5% CO_2 and medium was changed 24 hours after seeding and every 3 days thereafter, with cells passaged when 80% confluent. Each animal provided stem cells for separate cultures to supply conditioned medium for the ELISA before cells from three cultures were pooled for the in vitro coculture/in vivo transplantation experiments.

NGF/BDNF/NT-3 ELISA

To quantify the neurotrophins produced by BMSCs and DPSCs, conditioned medium was taken from cells at passages two to four, cultured for 48 hours, and assayed using E_{MAX} Immunoassay kits (Promega, Southampton, UK) for rat NGF, BDNF, and NT-3, as well as CNTF (R&D Systems, Abingdon, UK), according to the manufacturers' instructions. Briefly, a standard curve was constructed using the provided neurotrophin standards, and test samples of conditioned medium at varying dilutions were run in duplicate after acid treatment, with neurotrophin concentrations extrapolated from the standard curve.

Retinal Cell Coculture

Cell culture 24-well plates (BD Biosciences) were coated for 60 minutes with 100 μg/mL poly-D-lysine and then for 30 minutes with 20 μg/mL laminin. After terminal anesthesia, eyes were removed from three male Sprague-Dawley rats weighing 170 to 200 g (Charles River) and the retinae minced in 1.25 mL papain



Number of animals (eyes)	Treatment Left eye	Right eye	Measured endpoint
6 (12)	Dead DPSC + ONC	DPSC + ONC	RGC counts, axon counts and RNFL thickness
6 (12)	Dead BMSC + ONC	BMSC + ONC	RGC counts, axon counts and RNFL thickness
6 (12)	PBS + ONC	PBS + intact	RGC counts, axon counts and RNFL thickness

FIGURE 1. Experimental design used for in vivo experiment. Time line of the in vivo experiment detailing the times when the OCT recordings and tissue collections were undertaken, in relation to the day of the ONC and DPSC/BMSC transplantation.

(Worthington Biochem, Lakewood, NJ) containing 62.5 μL DNase I (Worthington Biochem) and incubated for 90 minutes at 37°C. The retinal cell suspension was centrifuged at 300*g* for 5 minutes and the pellet resuspended in a solution containing 1.35 mL EBSS (Earle's balanced salt solution; Worthington Biochem), 150 μL reconstituted albumin ovomucoid inhibitor (Worthington Biochem), and 75 μL DNase I. The retinal cell suspension was layered onto 2.5 mL of albumin ovomucoid inhibitor to form a discontinuous density and centrifuged at 70*g* for 6 minutes. The resulting retinal cell pellet was resuspended in 1 mL supplemented Neurobasal-A (24.2 mL Neurobasal-A [Gibco] supplemented with 500 μL B27 supplement [Life Technologies, Invitrogen, Paisley, UK], 62.5 μL L-glutamine [200 mM; Invitrogen], and 125 μL gentamycin [Invitrogen]) and seeded at a density of 125,000 cells/800 μL in each well of the 24-well plate.

DPSCs and BMSCs were used at passages two to four and plated at a density of 50,000 cells/200 μL into a 0.4-μm porous cell culture insert (Millicell; Millipore, Watford, UK) that was inserted into each of the 24 wells containing retinal cells, to give a total volume of 1 mL of medium per well. Particular wells containing retinal cell cultures were also treated with 5 μg/mL Fc-TrKA, Fc-TrKB, and/or Fc-TrKC (single or combinatorial treatments; R&D Systems) fusion TrK-specific protein inhibitors,[36] as well as the general kinase inhibitor k252a (50 nM). A combination of recombinant human NGF, BDNF, and NT-3 was also added to selected retinal cell cultures (all at 60 ng/mL) to act as a positive control.

Cocultures were incubated for 4 days at 37°C before immunocytochemical staining of retinal cells for βIII-tubulin. All experiments were repeated on three separate occasions. Each of the treatment groups in each of the three experimental runs comprised three replicate wells containing retinal cells harvested from one animal. The DPSCs/BMSCs tested in each of the three experimental runs represented pooled cells from three animals.

In Vivo Experimental Design

The experimental design for the in vivo experiment is detailed in Figure 1. Briefly, 18 animals (36 eyes) were divided into six groups of six eyes. The first six animals (12 eyes) received a bilateral ONC and DPSC transplanted intravitreally, living cells in the right eye and dead cells in the left. The next six animals (12 eyes) received the same allocation, but BMSCs were transplanted instead of DPSCs. The final six rats (12 eyes) received a unilateral ONC to the left eye, whereas the right eye served as an intact control. Both eyes in each animal of this group received an intravitreal control injection of PBS instead of cell suspension to control for the transplantation procedure. Optical coherence tomography (OCT) was used to measure retinal nerve fiber layer thickness (RNFL) of animals every 7 days, including 7 days before the surgery and excluding the day of the surgery. Animals were killed at 21 days after ONC/cell transplantation.

Animals

All animal procedures were performed in strict accordance to the UK Home Office Animals Scientific Procedures Act, 1986, ARVO statement for the use of animals in ophthalmic and vision research, and approved by the University of Birmingham Ethical Review Sub-Committee. Eighteen adult female Sprague-Dawley rats weighing 150 to 200 g (Charles River) were housed in conditions of 21°C and 55% humidity under a 12-hour light and dark cycle, given food/water ad libitum, and were under constant supervision from trained staff. Anesthesia was induced with 5% isoflurane/1.5 L per minute O_2 (National Veterinary Supplies, Stoke, UK) and was maintained at 3.5% during surgery.

Surgical Procedures

Following anesthetic induction as described above, a subcutaneous injection of buprenorphine (0.1 mL/100 g; National Veterinary Supplies) was given and the animal secured in a head-holding frame. Intraorbital ONC was performed as described previously.[37] Briefly, the optic nerve was surgically exposed and crushed using forceps 1 mm posterior to the lamina cribrosa with no damage to retinal blood vessels. Immediately after ONC, a glass micropipette, produced in-house from a glass capillary rod (Harvard Apparatus, Edenbridge, Kent, UK) using a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) preloaded with 150,000 cells

TABLE. Antibodies Used in Immunohistochemistry and Immunocytochemistry

Antigen	Dilution	Supplier	Catalog No.
BDNF	1:200	Promega	G1641
NT-3	1:200	Millipore	AB1780SP
GFAP	1:200	Sigma	G9269
RBMY	1:100	Santa Cruz, Santa Cruz, CA	SC-14572
βIII-tubulin	1:500	Sigma	T8660
Brn3a	1:200	Santa Cruz	SC-31984
GAP-43	1:400	Zymed Laboratories, San Francisco, CA	33-5000
Laminin	1:200	Sigma	L9393
Mouse IgG,. Fluor 488	1:400	Molecular Probes, Paisley, UK	A-21202
Rabbit IgG, Fluor 488	1:400	Molecular Probes	A-21206
Rabbit IgG, Fluor 594	1:400	Molecular Probes	A-21207
Goat IgG, Fluor 594	1:400	Molecular Probes	A-11058

suspended in 5 μL of PBS, was used to inject living or dead cells (killed by heating for 30 minutes at 80°C; or PBS alone in controls) into the vitreous of the eye. After surgery, animals were placed in heated recovery cages and monitored for recovery of normal behavior, after which they were returned to home cages.

OCT of RNFL

Every 7 days, including 7 days before the surgery but excluding the week of the surgery (Fig. 1), OCT was performed on rats (anesthetized as detailed above) using a Spectralis HRA3 confocal scanning laser ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany). OCT images were taken of the retina around the optic nerve head and the in-built software was used to segment the gathered images and quantify the RNFL thickness.

Tissue Preparation

At 21 dpl, animals were given an intraperitoneal injection of 1 mL sodium pentobarbital (National Veterinary Supplies) and perfused intracardially with 4% paraformaldehyde (PFA; TAAB, Reading, UK) in PBS while under terminal anesthesia. Eyes and optic nerves were removed and immersion fixed in 4% PFA in PBS for 2 hours at 4°C before cryoprotection in 10%, 20%, and 30% sucrose solution in PBS for 24 hours with storage at 4°C. Eyes and optic nerves were then embedded using optimal cutting temperature embedding medium (Thermo Shandon, Runcorn, UK) in peel-away mold containers (Agar Scientific, Essex, UK) by rapid freezing under crushed dry ice and were stored at −80°C. After embedding, eyes and optic nerves were sectioned on a cryostat microtome (Bright, Huntingdon, UK) at −22°C at a thickness of 20 μm and 15 μm, respectively, and mounted on positively charged glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA). Longitudinal optic nerve and parasagittal eye sections were left to dry on slides overnight at 37°C before storage at −30°C. Optic nerve sections were chosen at random for analysis, whereas eye sections were chosen with the optic nerve head visible.

Immunohistochemistry

Mounted tissue sections were equilibrated to room temperature, hydrated in PBS for 2 × 5 minutes, permeabilized in 0.1% Triton X-100 in PBS for 20 minutes at room temperature, and washed for 2 × 5 minutes in PBS before isolation with a hydrophobic PAP pen (Immedge pen; Vector Laboratories, Peterborough, UK). Nonspecific protein-binding sites in sections were blocked by incubation in blocking buffer (75 μL; 0.5% bovine serum albumin [g/mL], 0.3% Tween-20, 15% normal goat/donkey serum [Vector Laboratories] in PBS) in a humidified chamber for 30 minutes at room temperature and then sections were drained and incubated with primary antibody diluted in antibody-diluting buffer (ADB; 0.5% bovine serum albumin, 0.3% Tween-20 in PBS) overnight at 4°C. The following day, slides were washed for 3 × 5 minutes in PBS. Tissue sections were then incubated with secondary antibody diluted in ADB for 1 hour in a hydrated incubation chamber at room temperature. After 1 hour, slides were washed for 3 × 5 minutes in PBS, mounted in Vectorshield mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and stored at 4°C before microscopic analysis. Antibodies used in this staining are detailed in the Table.

Immunocytochemistry

Cells in 24-well plates were fixed in 4% PFA for 10 minutes, washed for 3 × 10 minutes of PBS, blocked in blocking solution as described above for 20 minutes, and incubated with primary antibody diluted in ADB for 1 hour at room temperature. After 1 hour, cells were washed for 3 × 10 minutes in PBS, incubated with the secondary antibody diluted in ADB for 1 hour at room temperature, washed for 3 × 10 minutes in PBS, mounted in Vectorshield mounting medium containing DAPI, and stored at 4°C. Antibodies used in this staining are detailed in the Table.

Microscopy and Analysis

Fluorescently stained sections were analyzed by an operator blinded to treatment groups, using a Zeiss Axioplan-2 fluorescent microscope (Carl Zeiss, Ltd., Hertfordshire, UK). For immunocytochemistry, all retinal cells that were positive for the neuronal marker βIII-tubulin,[38] with or without neurites, were counted over each entire well of the 24-well plate, with the number of βIII-tubulin$^+$ retinal cells with neurites and the total number of βIII-tubulin$^+$ retinal cells being recorded. Neurite outgrowth was measured in images taken at ×20 magnification using an Axiocam HRc camera (Carl Zeiss, Ltd.). Each well was divided into nine equal sectors and the length of the longest neurite per βIII-tubulin$^+$ retinal cell in each sector was measured using Axiovision software (Carl Zeiss, Ltd.).

For immunohistochemistry, Brn3a$^+$ RGCs[39] were counted in 20-μm-thick sections of the retina, along a 250-μm linear region of the ganglion cell layer, stretching out horizontally on either side of the optic nerve. Four sections per retinae and six retinae from six different animals per treatment group were quantified.

For in vivo quantification of axon regeneration, ×20 magnification images were taken of growth-associated protein-43 (GAP-43)–stained longitudinal sections of the optic nerves and composite images were constructed in Photoshop CS3 (Adobe Systems, Inc., San Jose, CA). Photoshop CS3 was used to contrast-enhance selected images to improve the visibility of GAP-43$^+$ axons, with all manipulations kept identical across the treatment groups. RGC axon regeneration in vivo was quantified in the composite images by counting the number of GAP-43$^+$ axons extending across a line set at 90° across the optic nerve at 100, 200, 400, 800, and 1200 μm distal (toward the chiasm) to the center of the crush site (identified by laminin$^+$ staining) of six optic nerves from six different animals per treatment group and three sections per optic nerve. By measuring the diameter of the nerve at each measurement point, the number of axons/mm width was

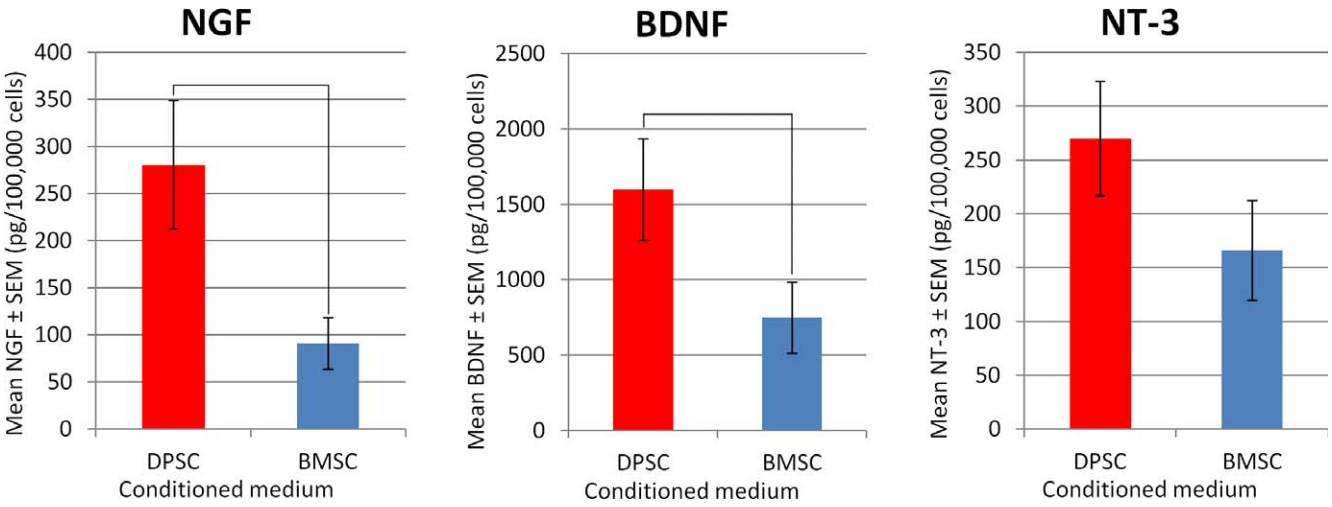


Figure 2. NGF, BDNF, and NT-3 secretion from DPSCs and BMSCs. DPSC- and BMSC-conditioned medium, collected after 48 hours of cell culture, was assayed using specific ELISAs for rat NGF, BDNF, and NT-3 ($n = 3$; *black lines* indicate significant difference at $P < 0.05$).

calculated. This value was then used to derive $\sum ad$, the total number of axons extending distance d in an optic nerve with radius r using the formula described by others[40]:

$$\sum ad = \pi r^2 \times \frac{\text{average number of axons/mm width}}{\text{section thickness (0.015 mm)}}.$$

Statistics

All statistical tests were performed using SPSS 17.0 (IBM SPSS, Inc., Chicago, IL) and data were presented as mean ± SEM. The Kolmogorov-Smirnov test was used to ensure all data were normally distributed before parametric testing using a one-way ANOVA with a Tukey post hoc test. Statistical difference was considered significant at P values less than 0.05.

Results

DPSCs Secreted NGF, BDNF, and NT-3

DPSCs secreted NGF (281 ± 68 pg/24 h/10^5 cells), BDNF (1600 ± 338 pg/24 h/10^5 cells), and NT-3 (270 ± 53 pg/24 h/10^5 cells) in culture, as analyzed by ELISA (Fig. 2). These neurotrophic titers were 2- to 3-fold higher than those detected in conditioned medium from BMSC cultures (91.3 ± 24.2, 749 ± 237, 166 ± 46 pg/24 h/10^5 cells, respectively) with the differences for NGF and BDNF being statistically significant ($P < 0.05$). CNTF was undetectable in all samples tested (data not shown).

DPSCs Promoted βIII-Tubulin$^+$ Retinal Cell Survival and Neuritogenesis in a Coculture Assay

DPSCs promoted a significant ($P < 0.05$) increase in the survival of cocultured βIII-tubulin$^+$ retinal cells (340.3 ± 10.4 cells/well) compared with retinal cells cultured alone (92.7 ± 20.8 cells/well), cocultured with BMSCs (227 ± 27.6 cells/well) or treated with recombinant human NGF, BDNF, and NT-3 (278.7 ± 8.0 cells/well; Fig. 3).

DPSCs also promoted a significant ($P < 0.05$) increase in the number of βIII-tubulin$^+$ retinal cells with neuritis, as well as the neurite length (161.6 ± 5.8 μm, 172.7 ± 9.5 μm, respectively; Fig. 3), compared with either retinal cells cultured alone (36.0 ± 5.2 μm, 22.7 ± 5.2 μm) or cocultured with BMSCs (137.8 ± 2.3 μm, 91.0 ± 12.6 μm; Fig. 3). The combination of recombinant human NGF, BDNF, and NT-3 significantly ($P < 0.05$) increased the number of βIII-tubulin$^+$ retinal cells with neurites (142.3 ± 10.1 cells/well), as well as the neurite length (155.4 ± 27.4 μm) compared with retinal cells cultured alone, or when cocultured with BMSCs ($P > 0.05$).

Fc-TrK Receptor Blockers Attenuated the Survival and Neuritogenic Effects of DPSCs

The number of βIII-tubulin$^+$ retinal cells surviving in DPSC cocultures (340.3 ± 10.4 cells/well) was significantly ($P < 0.05$) decreased after treatment with Fc-TrKA (182.7 ± 16.4 cells/well), Fc-TrKB (165.3 ± 3.0 cells/well), and Fc-TrKC (193 ± 17.1 cells/well) used alone or in combination (99.3 ± 9.0 cells/well; Fig. 3). In BMSC cocultures, βIII-tubulin$^+$ retinal cell survival (227.0 ± 27.6 cells/well) was significantly ($P < 0.05$) reduced with Fc-TrKA (145.3 ± 5.4 cells/well), Fc-TrKB (138.0 ± 5.5 cells/well), or Fc-TrKA, -B, and -C together (85.7 ± 17.1 cells/well), but not after adding Fc-TrKC (158.3 ± 10.3 cells/well; $P > 0.05$).

Fc-TrKA, -B, and -C used individually, significantly ($P < 0.05$) decreased both the number of neurite-bearing cells (84.0 ± 9.5, 64.0 ± 5.3, 74.7 ± 12.9 cells/well, respectively) as well as the length (112.4 ± 9.1 μm, 86.7 ± 9.0 μm, 103.7 ± 1.1 μm, respectively) of neurites in DPSC/retinal cell cocultures compared with DPSCs/retinal cells cocultured without inhibitors (Fig. 3). Combining the Fc-TrK inhibitors further attenuated the number of βIII-tubulin$^+$ retinal cells with neurites (38.0 ± 4.9 cells/well) as well as neurite length (53.9 ± 7.9 μm) seen in the DPSC/retinal cell coculture. Similar effects, although less exaggerated, were seen in the BMSC/retinal cell cocultures. Accordingly, a statistically significant ($P < 0.05$) reduced neurite length from 137.8 ± 2.3 μm to 65.4 ± 2 μm was seen only when the three neurotrophin inhibitors were combined in the BMSC/retinal cell coculture, but not when each inhibitor was used in isolation.

DPSC Transplants Preserved RNFL Thickness for Up to 14 Days After ONC Injury

All transplanted animals and eyes survived the experiment with no observable adverse effects.

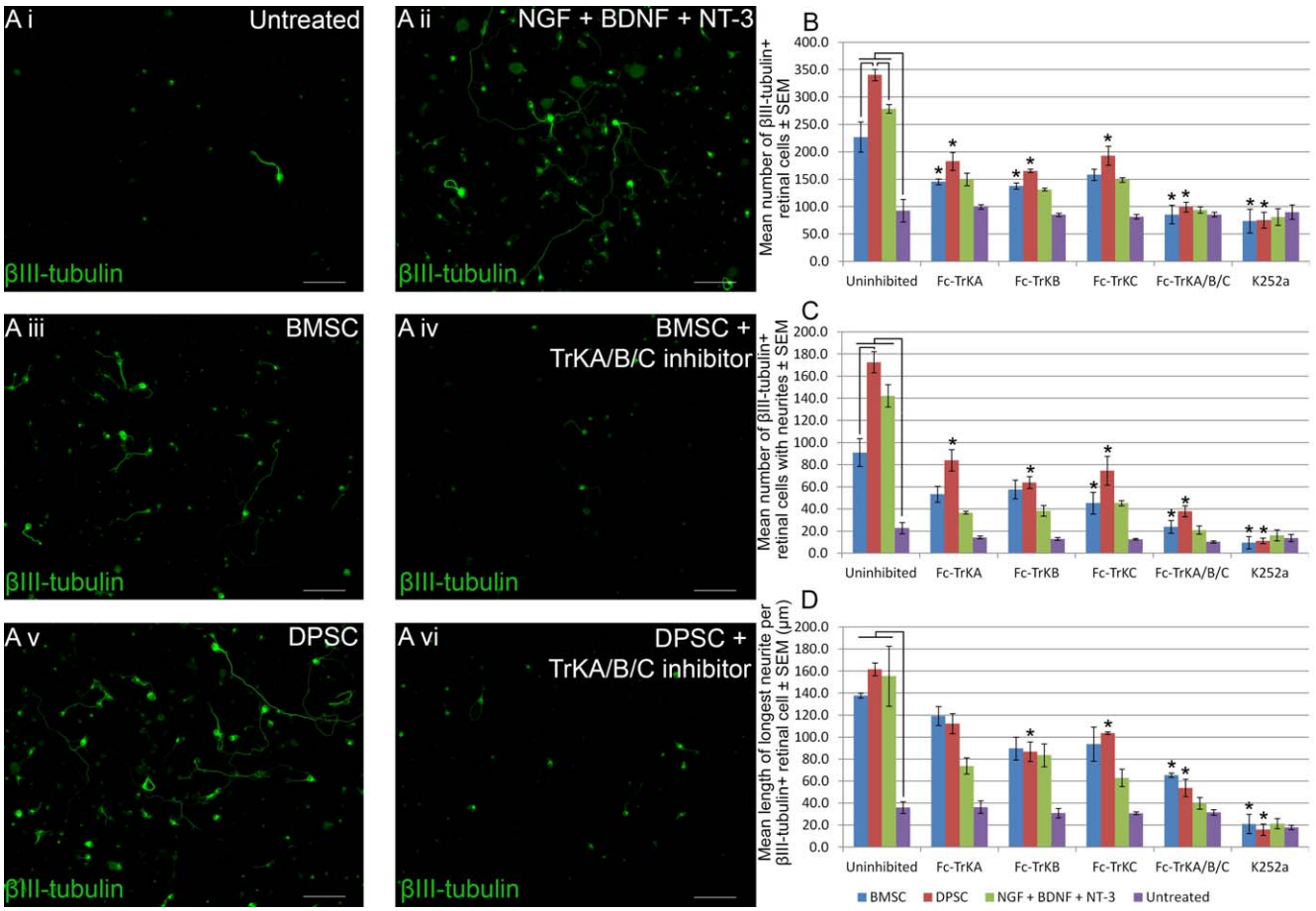


Figure 3. Effects of DPSCs and BMSCs on βIII-tubulin$^+$ retinal cells. In vitro βIII-tubulin$^+$ retinal cells, cultured either alone (**Ai**), with exogenous neurotrophins (**Aii**), with BMSCs (with or without TrK inhibitors [**Aiii**, **Aiv**, respectively]) or with DPSCs (with or without TrK inhibitors [**Av**, **Avi**, respectively]). All images are representative of the entire culture, nine separate culture wells per treatment with every three wells using a different animal (*scale bars*: 100 μm). The number of surviving βIII-tubulin$^+$ retinal cells (**B**), number of βIII-tubulin$^+$ retinal cells with neurites (**C**), and the length of the longest βIII-tubulin$^+$ retinal cell neurite (**D**) when retinal cells were cocultured with BMSCs (*blue bars*), DPSCs (*red bars*), exogenous neurotrophins (*green bars*), or alone (*purple bars*). *Black lines* indicate significant difference at $P < 0.05$. The effects of TrKA, -B, and -C Fc-inhibitors, as well as K252a on βIII-tubulin$^+$ retinal cell survival and neuritogenesis in DPSC and BMSC cocultures are shown (points marked with an *asterisk* indicate significant difference from uninhibited cultures at $P < 0.05$).

Because the RNFL comprises RGC axons that pass over the surface of the retina toward the optic disk, RNFL thickness was used to measure postaxotomy RGC axonal atrophy and did not significantly ($P < 0.05$) change in uninjured animals over time. In ONC animals, RNFL thickness was reduced significantly ($P < 0.05$) from 49.3 ± 2.1 μm to 30.2 ± 1.5 μm at 7 dpl, 21.4 ± 1.6 μm at 14 dpl, and 17.0 ± 1.2 μm at 21 dpl (Fig. 4). Animals receiving dead DPSC/BMSC transplantations showed a similar thinning in RNFL thickness with no significant ($P < 0.05$) difference from ONC alone. However, there was no significant ($P < 0.05$) RNFL thinning at 7 dpl in animals that were injected with living DPSCs/BMSCs (46.2 ± 1.4 μm, 46.0 ± 2.1 μm, respectively) compared with intact animals at 7 dpl (45.7 ± 1.2 μm), indicating a neuroprotective effect of the DPSCs. At 14 dpl, RNFL thickness of the DPSC-transplanted animal had decreased to 32.8 ± 0.7 μm, which was significantly ($P < 0.05$) lower than that in intact animals (45.4 ± 0.2 μm), but still significantly ($P < 0.05$) higher than in untreated animals (21.4 ± 1.6 μm). This is in contrast to animals that received BMSCs in which RNFL thickness decreased to 28.5 ± 1.6 μm by 14 dpl, which was not significantly ($P > 0.05$) different from untreated animals. By 21 dpl, the RNFL in animals receiving either DPSCs or BMSCs (24.0 ± 1.3 μm, 22.0 ± 1.8 μm, respectively) had reduced to a thickness not significantly ($P > 0.05$) different from that seen in untreated animals at 21 dpl (17.0 ± 1.2 μm).

Transplanted Intravitreal DPSCs Survived In Vivo for 21 Days

Viable DPSCs were detected in the vitreous at 21 dpl associated with elevated levels of BDNF and NT-3 in the retina at 21 dpl compared with eyes transplanted with dead DPSCs (Fig. 5). Activated glial fibrillary acidic protein$^+$ (GFAP) glia were also observed in eyes transplanted with DPSCs but not with dead DPSCs. Similar findings were observed with BMSCs (data not shown).

Intravitreal DPSC Transplants Protected RGCs From Death After ONC

Intravitreal DPSC transplantation after ONC significantly increased ($P < 0.05$) RGC survival at 21 dpl (27.9 ± 2.0 RGCs/mm of retina) compared with animals receiving BMSC

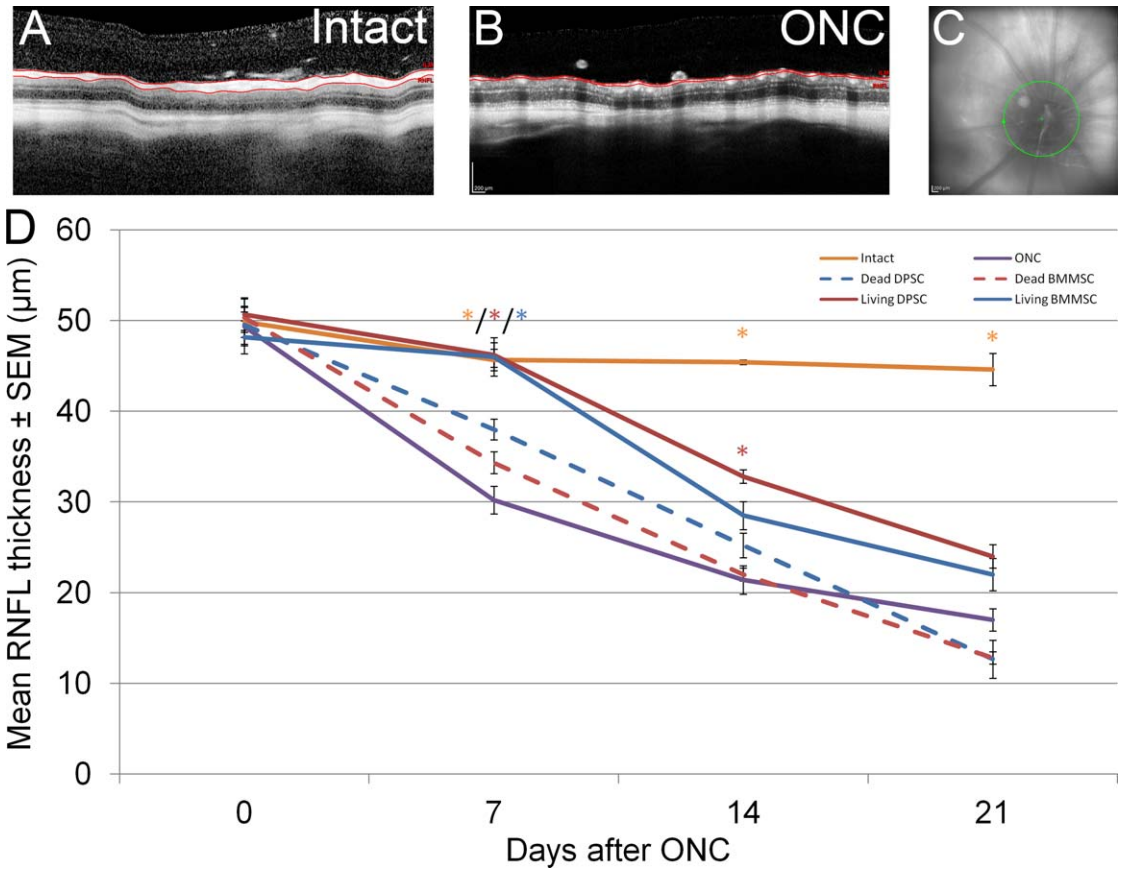


FIGURE 4. RNFL thickness after ONC. OCT images of retina from an uninjured rat (**A**) and a rat 21 days after ONC (**B**) are shown with *red lines* outlining the RNFL. OCT images were taken of the retinal section surrounding the optic nerve head, indicated by the *green line* (**C**). Images are representative of the six animals used in each treatment group (*scale bar*: 200 μm). The graph (**D**) depicts changes in RNFL thickness over time for uninjured optic nerves (*orange line*), DPSC transplanted eyes (*red line*), BMSC transplanted eyes (*blue line*), dead DPSC transplanted eyes (*dashed red line*), and dead BMSC transplanted eyes (*dashed blue line*). Points marked with an *asterisk* indicate significant difference from untreated/dead cell transplanted animals at $P < 0.05$.

transplants (16.2 ± 1.3 RGCs/mm of retina), dead DPSC transplants (5.7 ± 0.6 RGCs/mm of retina), or ONC alone (6.9 ± 1.1 RGCs/mm of retina; Fig. 6), as determined by Brn3a$^+$ staining. Nonetheless, RGC survival after BMSC transplantation was also significantly ($P < 0.05$) greater than in animals receiving dead BMSC transplants (8.4 ± 1.1 RGCs/mm of retina) or in untreated animals, demonstrating that BMSCs exerted some neuroprotective effect for RGCs, although at a lower level than did DPSCs.

Intravitreal DPSC Transplants After ONC-Promoted RGC Axon Regeneration

At distances of 100, 200, 400, 800, and 1200 μm distal to the crush site, the number of regenerating GAP-43$^+$ RGC axons was significantly ($P < 0.05$) increased (284.7 ± 33.0, 221.0 ± 23.3, 214.5 ± 26.0, 181.9 ± 42.0, 115.9 ± 25.6 axons/nerve, respectively) after intravitreal transplantation of DPSCs compared with BMSCs (133.7 ± 21.1, 115.9 ± 25.0, 85.4 ± 19.8, 77.2 ± 10.4, 50.4 ± 10.3 axons/nerve, respectively), dead DPSCs (68.7 ± 19.6, 54.4 ± 11.0, 42.7 ± 8.6, 31.7 ± 15.3, 9.5 ± 4.9 axons/nerve, respectively), or untreated (78.1 ± 16.9, 48.6 ± 7.2, 34.9 ± 6.0, 11.7 ± 3.7, 2.5 ± 1.5 axons/nerve, respectively; Fig. 7) at 21 dpl. BMSC-transplanted animals had significantly ($P < 0.05$) greater numbers of regenerating RGC axons in the distal optic nerve compared with untreated animals at all distances and significantly ($P < 0.05$) greater numbers of regenerating axons compared with animals receiving dead BMSCs (59.7 ± 6.5, 45.5 ± 8.6, 46.7 ± 9.2, 40.4 ± 9.9, 18.2 ± 5.3 axons/nerve, respectively) at distances of 100 and 200 μm distal to the crush site.

DISCUSSION

This study provides evidence that DPSCs, through secretion of neurotrophins, significantly increase both survival and neuritogenesis of primary adult rat βIII-tubulin$^+$ retinal cells in an in vitro coculture assay. Furthermore, when transplanted into the vitreous body of adult rats after ONC, DPSCs significantly promote Brn-3a$^+$ RGC survival and axon regeneration. Noteworthy, the neuroprotective and proregenerative effects of DPSCs seen in these in vitro and in vivo models was greater than that observed with BMSCs, which can be related to their enhanced neurotrophic profile as determined by ELISA and suggests that DPSCs have a greater potential to repair CNS/retinal injury.

Our findings are consistent with a recent study that demonstrated greater positive effects of locally transplanted DPSCs on locomotory recovery from SCI than did BMSC transplants.[21] Moreover, the improvement in locomotory function after cell transplantation into an SCI site occurred in the absence of local neuronal differentiation, suggesting that the transplanted cells acted indirectly, creating a more

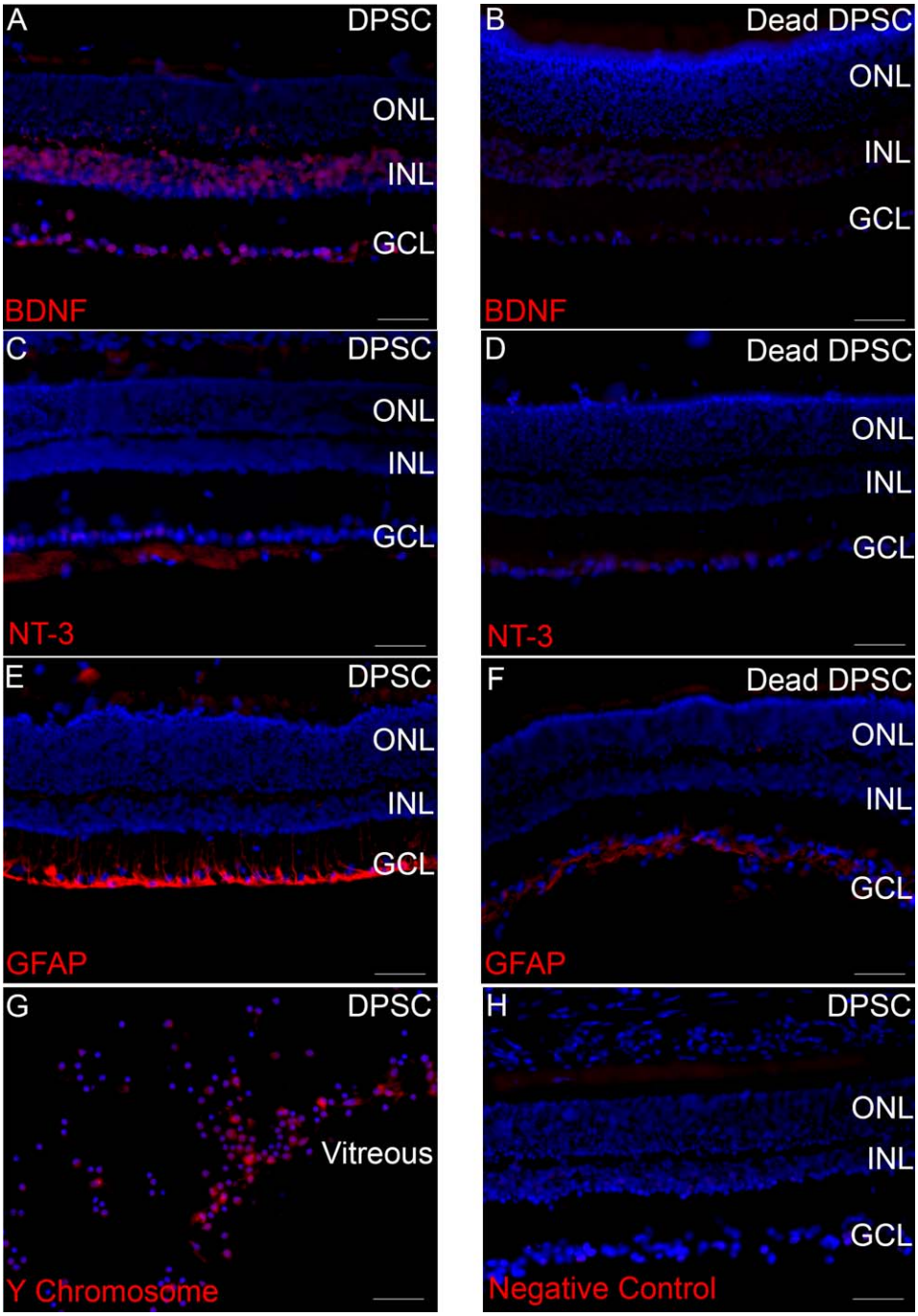


Figure 5. DPSC survival and trophic effects 21 days after ONC/cell transplantation. Immunohistochemically stained 20-μm-thick parasagittal sections of retina and vitreous, stained for BDNF (**A**, **B**), NT-3 (**C**, **D**), GFAP (**E**, **F**), and Y chromosome (**G**) 21 days after ONC and intravitreal transplantation of DPSCs (**A**, **C**, **E**, **G**) or dead DPSCs (**B**, **D**, **F**) with outer nuclear layer (ONL), inner nuclear layer (INL), and ganglion cell layer (GCL) labeled. A negative control with the primary antibodies omitted is included (**F**). All images are representative of the two images per section, four sections per retina, six retinae from six different animals per treatment group. DAPI was used as a nuclear counterstain (*scale bars*: 100 μm).

supportive trophic environment for endogenous axonal sprouting/growth.

Our finding that DPSCs enhanced βIII-tubulin$^+$ retinal cell survival and neurite outgrowth in a coculture model can be attributed to the release of soluble factors, as the two populations of cells were separated by a porous membrane. Moreover, the use of specific Fc-TrK inhibitors enabled us to identify DPSC-derived NGF, BDNF, and NT-3 as important NTFs responsible for this neuroprotective and neuritogenic effect. Use of individual Fc-TrK inhibitors as opposed to combined, demonstrated that NGF, BDNF, and NT-3 each had equally important neuroprotective and neuritogenic effects. The ELISA measurements confirmed the secretion of these factors by the DPSCs, corroborating previous work showing that DPSCs

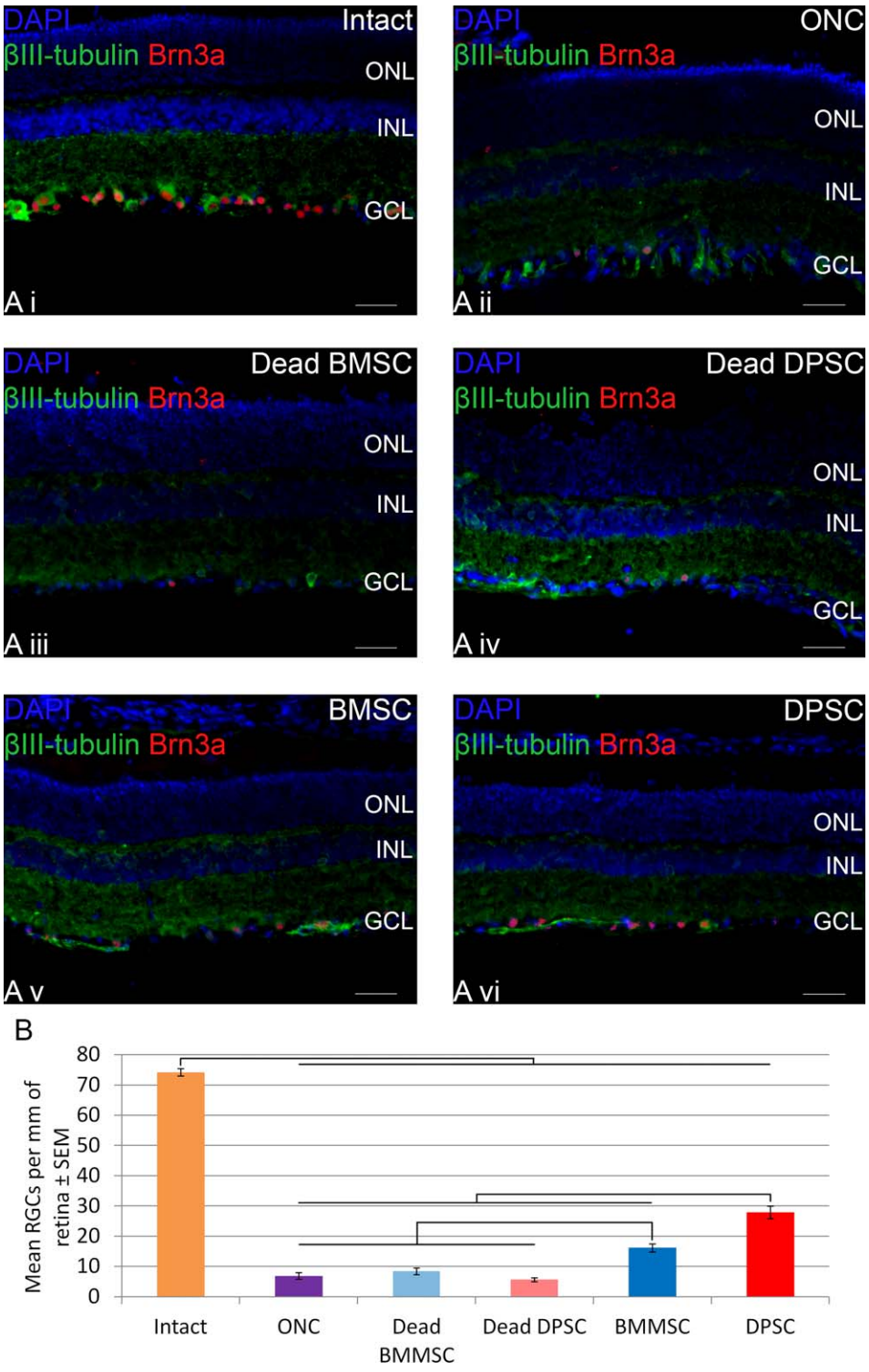


Figure 6. RGC survival 21 days after ONC/cell transplantation. Immunohistochemically stained 20-μm-thick parasagittal sections of retina, stained for βIII-tubulin (*green*) and Brn3a (*red*) in intact animals (**Ai**) and 21 days after ONC (**Aii**) and intravitreal transplantation of dead BMSCs (**Aiii**), dead DPSCs (**Aiv**), living BMSCs (**Av**), and living DPSCs (**Avi**) with ONL, INL, and GCL labeled. All images are representative of the two images per section, four sections per retina, six retinae from six different animals per treatment group. DAPI was used as a nuclear counterstain (*scale bars*: 100 μm). In (**B**), the number of Brn3a$^+$ RGCs, counted in a 1-mm region of the GCL 21 dpl is shown. *Black lines* indicate significant difference at $P < 0.05$.

express multiple NTF mRNA, including neurotrophins.[21,31–33] Interestingly, BMSCs exhibited a less potent neurotrophic effect on cultured βIII-tubulin$^+$ retinal cells than DPSCs; and this novel observation can be related to their reduced neurotrophin profile. Of note, K252a, a nonspecific blocker of TrK receptors, as well as other protein kinases, further reduced the neuritogenic effect of DPSCs/BMSCs compared with Fc-Trk blockade. These findings suggest that other TrK-

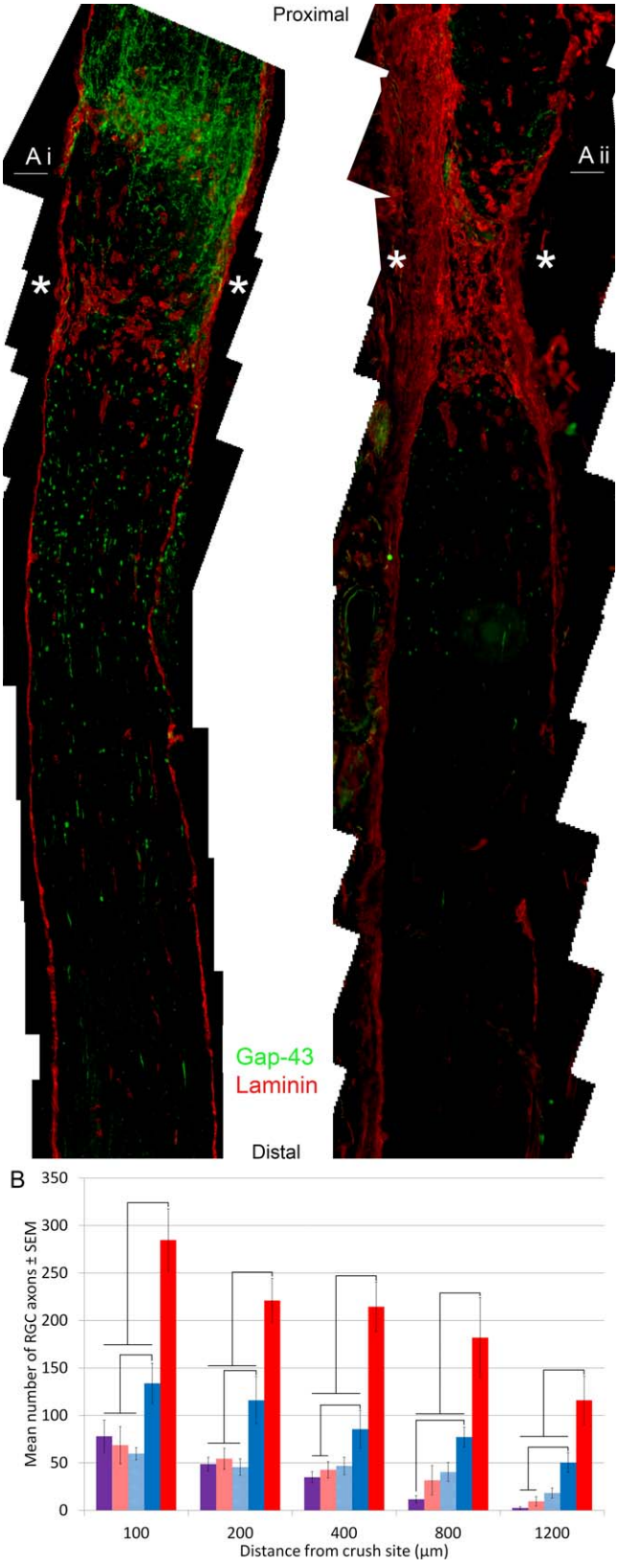


FIGURE 7. Regeneration of RGC axons in the optic nerve, 21 days after ONC/cell transplantation. Immunohistochemically stained 15-μm-thick longitudinal sections of optic nerves, stained for GAP-43 (*green*) and laminin (*red*) 21 days after ONC and DPSC (**Ai**) or dead DPSC (**Aii**) transplantation with the crush site marked by an *asterisk*. All images are representative of three sections per nerve, six nerves from six different animals per treatment group (*scale bars*: 100 μm). The number of regenerating axons was measured at 100, 200, 400, 800, and 1200 μm from the ONC site at 21 dpl in untreated animals (*purple bars*), animals receiving intravitreal dead DPSC transplants (*red dashed bars*), dead BMSCs (*blue dashed bars*), living BMSCs (*blue bars*), and living DPSCs (*red bars*); *black lines* indicate significant difference at $P < 0.05$. Note GAP-43$^+$ axons outside basal lamina of optic nerve = peripheral innervation of the tissue.

independent growth factors may also mediate the neurotrophic effects of DPSCs/BMSCs. Indeed, DPSCs express other trophic factors, such as GDNF.[30] By contrast, neuroprotection was similarly reduced after both K252a and TrK blockade, suggesting that the stem cell–derived neurotrophins NGF, BDNF, and NT-3 were the primary RGC neuroprotective agents.

Axotomy interrupts the supply of retrogradely transported neuroprotective NTF and, in many cases, the neuron subsequently dies, with RGCs being exquisitely sensitive to such adversities.[10,41] Neurotrophins also play an important role in growth cone formation/elongation and are relatively abundant in the peripheral nervous system compared with the CNS, possibly explaining the disparity between the axon regenerative response of the two sites. DPSCs/BMSCs provide an alternative source of NTF for axotomized RGCs, protecting them from death and promoting RGC axogenesis.

After ONC, RGCs begin dying from 7 dpl[42] with 80% to 90% dead by 2 to 3 weeks,[6,10,41] thus making this a suitable in vivo model to assess DPSC-mediated effects on RGC survival. We used two methods of assessing RGC number in our in vivo model. First, OCT was used to measure the thickness of the RNFL, which is composed of the axons of the RGCs. These are lost concomitantly with RGC death and thus provide a means of monitoring axonal atrophy in real time. Second, Brn3a$^+$ RGCs in the ganglion cell layer of retinal sections were counted at 21 dpl, a method that excludes amacrine cells and astrocytes from the counts.[39]

OCT recordings showed that in intact animals, RNFL thickness remained constant over time, whereas after ONC, RNFL thickness was progressively and significantly reduced. DPSC or BMSC transplantation resulted in 100% RGC neuroprotection for up to 7 dpl, but by 14 dpl, significant neuroprotection was seen only in animals treated with DPSCs. By 21 dpl, RNFL thickness was decreased in all ONC groups, suggesting that cell-mediated neuroprotection was failing. Thus, the OCT data suggest that RGC death was significantly delayed but not entirely averted. Reasons for the transient neuroprotective effect of the transplanted cells may be ligand-mediated downregulation of the TrK receptors[14,15] and/or gradual loss of the grafted cells with concomitant loss of neurotrophin-mediated protection of RGCs. However, Y chromosome$^+$ immunohistochemical staining indicated that DPSCs persisted in the vitreous of rats 21 days after transplantation. Further studies are required to analyze in detail the survival and fate of the transplanted stem cells in the vitreous of the eye.

Corroborating the OCT results, significantly more Brn3a$^+$ RGCs were present in the retinae of animals that received intravitreal transplants of either BMSCs or DPSCs compared with controls (i.e., untreated animals or those receiving dead BMSCs/DPSCs). This corroborates the RNFL thickness data, suggesting that OCT is a valid method for monitoring RGC survival, although immunocytochemical analysis proves a more direct as well as a more sensitive approach. RGC survival was more pronounced in animals receiving DPSCs compared with those receiving BMSC transplants, correlating with our in vitro coculture results as well as ELISA data, highlighting higher titers of neurotrophins produced by the DPSCs. These findings

are also consistent with well-documented data demonstrating therapeutic short-term effects of injected recombinant neurotrophins.[10,11]

This study provides new evidence that DPSCs are neuroprotective for RGCs and is supported by the reports of reduced numbers of apoptotic neurons seen after SCI when DPSCs are transplanted into the lesion site.[21] Three other studies have shown significant RGC survival after intravitreal cell transplantation. The first two used BMSCs in an animal model of glaucoma[24] and optic nerve transection,[23] and the other study used intravitreally transplanted fibroblasts genetically modified to express NTF in the same ONC rat model used in this study.[16] All these studies showed significant, although short-term, RGC survival, and attribute this effect to the release of NTF by the transplanted cells. In particular, it was reported that BMSC transplantation resulted in RGC survival of 66% compared with 46% in untreated animals at 8 dpl.[23] This protection appears substantially less than that achieved in the current study (complete protection after 7 days), as assessed by OCT, but can be explained by the fact that the authors[23] transplanted BMSCs 3 days before the ONC, meaning that the RGC counts were done 11 days after BMSC transplantation. It is likely that the efficacy of the transplanted cells diminished significantly by 11 days and that the neuroprotective effect was equally diminished. This also concurs with our findings that the neuroprotective effects of the transplanted cells became less pronounced over time.

The promising neurite outgrowth stimulated by the DPSCs seen in the in vitro coculture experiments were supported by the GAP-43$^+$ RGC axon regenerative response seen in the in vivo ONC experiment. Accordingly, intravitreal transplantation of DPSCs increased the number of GAP-43$^+$ axons in the proximal stump, with many crossing the lesion site and regenerating into the distal optic nerve. As well as more pronounced axon regeneration through the lesion site, the distal nerve stump contained significantly more GAP-43$^+$ axons that persisted for long distances through the putative axon growth inhibitory environment of the distal optic nerve. Finally, less laminin$^+$ scar tissue was seen at crush sites traversed by regenerating axons, which is a well-documented correlation.[6,43] Indeed, in all the DPSC/BMSC-transplanted animals with regenerating RGC axons, no scar tissue was present at the lesion site. This phenomenon has been attributed to secretion of metalloproteinases and plasminogen by the regenerating axons that block meningeal fibroblast migration into the wound and degrade scar tissue.[6,44] Thus, the lack of scar tissue is an additional indication of DPSC-induced RGC axon regeneration.

This study demonstrates the potential therapeutic benefit of DPSCs to stimulate the growth of axons along the long nonpermissive distances required to restore neural function. Our finding also suggests that the regenerating axons were disinhibited by the DPSC-derived neurotrophins, presumably through regulated intramembrane proteolysis of inhibitory receptors and dissolution of chondroitin sulfate proteoglycans,[45] and corroborates a previous ONC study in which a significant number of RGC axons regenerated into the distal optic nerve after intravitreal transplantation of fibroblasts genetically modified to express FGF-2, BDNF, and NT-3.[16] Our results also support the recent work that concluded that the transplantation of DPSCs promoted axonal regeneration across an SCI lesion site.[21]

It cannot be ruled out, and is not mutually exclusive in the aforementioned explanation, that the neuroprotective and neuritogenic/axogenic effects seen in this study are attributable to an indirect interaction between the stem cell–derived neurotrophins and the βIII-tubulin$^+$ retinal cells mediated by GFAP$^+$ retinal glia, which also secrete NTF. In addition, inflammation triggers the release of CNTF from GFAP$^+$ retinal glia, resulting in RGC neuroprotection and axogenesis.[46,47] In this study, we show glial cell activation 21 days after stem cell transplantation, which suggests that glia have a role in the induction of stem cell–directed neuroprotection/axogenesis, although increased neurotrophin titers in eyes at 21 dpl may be stem cell–derived, glial-derived, or a combination of both. Thus, it is possible that upregulation of glial NTF production contributed to the neuroprotective and axogenic effects seen after stem cell transplantation.

We report here for the first time that intravitreal BMSCs promoted a small but significant regeneration of RGC axons, even at 1200 μm distal to the crush site. Nonetheless, DPSCs promoted significantly greater regeneration of RGC axons than did BMSCs, reflecting their elevated neurotrophin secretion profile and underlining the potential benefit of DPSCs above other mesenchymal cell sources.

An important future consideration would be to develop a safe and more sustained delivery mechanism for the cells. In the present study, cells were injected as a suspension, which carries with it certain risks, such as migration of the cells into endogenous tissue and their uncontrolled proliferation. Encapsulation of cells in biologically compatible materials for transplantation into the vitreous has already been shown with a retinal cell line that had been genetically modified to release CNTF in both animal models[48] and patients.[49] Not only did the encapsulated cells survive for 6 months,[49] but they were also retrievable. Further studies are ongoing in our laboratory to develop a similar delivery mechanism for adult human DPSCs.

Conclusions

We demonstrate here for the first time that DPSCs secrete multiple neurotrophins that were at least in part responsible for promoting axotomized RGC neuroprotection and neuritogenesis/axogenesis, both in vitro and in vivo. DPSCs were more effective than BMSCs, which is likely due to the higher titers of neurotrophin secretion by the DPSCs. DPSCs may be a promising alternative for a CNS regenerative cell therapy.

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