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Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury

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Running title: Dental pulp stem cells for optic nerve injury.

Precis: The neural regenerative effects of intravitreally transplanted dental stem cells were analysed after optic nerve crush. The results demonstrated significant neuroprotective and axogenic effects by dental cells, which were significantly more pronounced than that elicited by bone marrow-derived MSC.

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Abbreviations: ADB, antibody diluting buffer; ANOVA, one-way analysis of variance; BDNF, brain-derived neurotrophic factor; BMSC, bone marrow-derived mesenchymal stem cells; BSA, bovine serum albumin; CNS, central nervous system; CNTF, ciliary neurotrophic factor; dpl, days post-lesion; DPSC, dental pulp stem cells; FBS, foetal bovine serum; FGF-2, fibroblast growth factor-2; GAP-43, growth-associated protein-43; GFAP, glial fibriliary acidic protein; GDNF, glial cell line-derived neurotrophic factor; NGF, nerve growth factor; NT-3, neurotrophin-3; NTF, neurotrophic factors; OCT, optical coherence tomography; ONC, optic nerve crush; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RGC, retinal ganglion cell; RNFL, retinal nerve fibre layer; SCI, spinal cord injury; standard error of the mean, SEM; TrK, tropomyosin receptor kinase.

Abstract

Purpose

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

Methods

Conditioned media collected from cultured rat DPSC and bone marrow-derived mesenchymal stem cells (BMSC) were assayed for nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) secretion using ELISA. DPSC or BMSC were co-cultured 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, DPSC or BMSC were transplanted into the vitreous body of the eye after a surgically-induced optic nerve crush injury. At 7, 14 and 21 days post-lesion (dpl), optical computerized tomography (OCT) was used to measure the retinal nerve fibre 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 DPSC and BMSC secreted NGF, BDNF and NT-3, with DPSC secreting significantly higher titres of NGF and BDNF than BMSC. DPSC, and to a lesser extent BMSC, 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.

Conclusion

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

Key words: Dental pulp stem cells; Mesenchymal stem cells; Axon regeneration; Neuroprotection; Cell transplantation.

Introduction

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 to 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 2nd 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 non-permissive trophic environment comprised 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 axotomised 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 down-

regulate the TrK receptors[14, 15], an effect that may be avoided by opting for a lower but continuous delivery regime. 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), which 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 (BMSC) 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 (DPSC) are isolated from the dental pulp of both infant and adult mammalian teeth with relative ease of access and few ethical hurdles. Thus, DPSC represent a potential autologous and allogeneic cellular therapy for CNS injury, particularly since recent evidence suggests that they are more potent than BMSC at promoting functional recovery after spinal cord injury[21]. Although largely uncharacterised, a few studies have explored their potential to play a direct role in neuronal replacement due to their neural crest origin[26]. DPSC 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 DPSC as an indirect NTF therapy, i.e. using DPSC-derived NTF to promote endogenous CNS neuron survival and axon regeneration. DPSC

express mRNA for NGF, glial cell line-derived neurotrophic factor (GDNF) and BDNF[30-32]. When transplanted into the hippocampus, DPSC secrete ciliary neurotrophic factor (CNTF), vascular endothelial growth factor, NGF and fibroblast growth factor-2 (FGF-2)[33], which could explain the findings of *Sakai et al, 2012* who demonstrated some functional recovery after complete transection of the spinal cord by transplanting DPSC into the lesion site[21]. The authors witnessed both an improvement in locomotory BBB[34] 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 DPSC compared to BMSC[21] indicates that DPSC produce higher titres of neurotrophic factors compared to BMSC. DPSC transplanted into a cerebral infarct site after middle cerebral artery occlusion also promoted significant recovery in forelimb sensorimotor function. The transplanted DPSC differentiated into astrocyte-like cells suggesting DPSC contributed to neural regeneration as a supportive cell through NTF secretion[35].

In the present study, we investigated the neuroprotective and axogenic properties of primary adult rat DPSC for axotomised RGCs. We carried out *in vitro* co-culture studies of DPSC 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 co-cultures. Using specific TrK-Fc 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 DPSC promote RGC survival and axon regeneration through the secretion of neurotrophins to a greater extent than do BMSC and hence we propose that DPSC have potential as a cellular therapy to treat RGC injury and degenerative disease.

Experimental Procedures

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

DPSC isolation and culture

Three adult male Sprague-Dawley rats weighing 170-200g (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 DMEM (Life Technologies, Gibco, UK) supplemented with 1% penicillin/streptomycin (P/S), sliced into 1mm^3 fragments and incubated in 4ml of 0.25% trypsin-EDTA for 30min at 37°C. Trypsin was inactivated by adding an equal volume of DMEM containing 1% P/S and 10% foetal bovine serum (FBS). A single cell population was obtained by passing the cell suspension through a 70µm cell strainer (BD Biosciences, Oxford, UK), which was centrifuged at 150xg for 5min. Cell pellets were resuspended in DMEM containing 1% P/S and 10% FBS and seeded into T25 flasks (Corning, Amsterdam, NL) in a total volume of 5ml. Cultures were maintained at 37°C in 5% CO_2 and medium was changed 24h after seeding, and every 3d 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 3 cultures were pooled for the *in vitro* co-culture/*in vivo* transplantation experiments.

BMSC isolation and culture

BMSC 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 10ml of DMEM. Cell aspirates were centrifuged at 150xg for 5min 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 5ml. Cultures were maintained at 37°C in 5% CO_2 and medium was changed 24h after seeding and every 3d 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 3 cultures were pooled for the *in vitro* co-culture/*in vivo* transplantation experiments.

NGF/BDNF/NT-3 ELISA

To quantify the neurotrophins produced by BMSC and DPSC, conditioned medium was taken from cells at passage 2-4, cultured for 48h and assayed using E_{MAX} Immunoassay kits (Promega, Southampton, UK) for rat NGF, BDNF and NT-3 as well as CNTF (R&D systems, UK) according to the manufacturer's 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 co-culture

Cell culture 24-well plates (BD Biosciences) were coated for 60min with 100µg/ml poly-D-lysine and then for 30min with 20µg/ml laminin. After terminal anaesthesia, eyes were removed from 3 male Sprague-Dawley rats weighing 170-200g (Charles River) and the retinae minced in 1.25ml of papain (Worthington Biochem, NJ, USA) containing 62.5µl of DNase I (Worthington Biochem) and incubated for 90min at 37°C. The retinal cell suspension was centrifuged at 300xg for 5min and the pellet resuspended in a solution containing 1.35ml of EBSS (Worthington Biochem), 150µl of reconstituted albumin ovomucoid inhibitor (Worthington Biochem) and 75µl of DNase I. After adding to the top of 2.5ml of albumin ovomucoid inhibitor to form a discontinuous density gradient, the retinal cell suspension was centrifuged at 70xg for 6min. The resulting retinal cell pellet was resuspended in 1ml of supplemented Neurobasal-A (24.2ml Neurobasal-A (Gibco) supplemented with 500µl of B27 supplement (Life Technologies, Invitrogen, UK), 62.5µl of L-glutamine (200mM; Invitrogen) and 125µl of gentamycin (Invitrogen)) and seeded at a density of 125,000 cells/800µl in each well of the 24 well plate.

DPSC and BMSC were used at passage 2-4 and plated at a density of 50,000 cells/200µl into a 0.4µm porous cell culture insert (Millicell, Millipore, UK) that was inserted into each of the 24 wells containing retinal cells to give a total volume of 1ml of medium per well. Particular wells containing retinal cell cultures were also treated with 5µg/ml TrKA-Fc, TrKB-Fc and/or TrKC-Fc (single or combinatorial treatments; R&D systems) fusion TrK-specific protein inhibitors[36] as well as the general kinase inhibitor k252a (50nM). A combination of recombinant human NGF, BDNF and NT-3 was also added to selected retinal cell cultures (all at 60ng/ml) to act as a positive control.

Co-cultures were incubated for 4d at 37°C before immunocytochemical staining of retinal cells for βIII-tubulin. All experiments were repeated on 3 separate occasions. Each of the treatment groups in each of the 3 experimental runs comprised 3 replicate wells containing retinal cells harvested from one animal. The DPSC/BMSC tested in each of the 3 experimental runs represented pooled cells from 3 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 6 groups of 6 eyes. The first 6 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 6 animals (12 eyes) received the same allocation but BMSC were transplanted instead of DPSC. The final 6 rats (12 eyes) received a unilateral ONC to the left eye, while the right eye served as an intact control. Both eyes in each animal of this group received an intravitreal control injection of phosphate-buffered saline (PBS) instead of cell suspension to control for the transplantation procedure. Optical coherence tomography (OCT) was used to measure retinal nerve fibre layer thickness (RNFL) of animals every 7d, including 7d 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 150-200g (Charles River) were housed in conditions of 21°C and 55% humidity under a 12h light and dark cycle, given food/water *ad libitum* and were under constant supervision from trained staff. Anaesthesia was induced with 5% Isoflurane/1.5L per minute O_2 (National Veterinary Supplies, Stoke, UK) and was maintained at 3.5% during surgery.

Surgical procedures

Following anaesthetic induction as described above, a subcutaneous injection of buprenorphine (0.1ml/100g; 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 1mm 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, California, USA) preloaded with 150,000 cells suspended in 5μl 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 behaviour, after which they were returned to home cages.

OCT of RNFL

Every 7d, including 7d before the surgery but excluding the week of the surgery (Figure 1), OCT was performed on rats (anaesthetised 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 1ml sodium pentobarbital (National Veterinary Supplies) and perfused intracardially with 4% paraformaldehyde (PFA; TAAB, Reading, UK) in PBS while under terminal anaesthesia. Eyes and optic nerves were removed and immersion fixed in 4% PFA in PBS for 2h at 4°C before cryoprotection in 10%, 20% and 30% sucrose solution in PBS for 24h 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 mould 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 20µm and 15µm, respectively, and mounted on positively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, USA). 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 X 5min, permeabilized in 0.1% triton x-100 in PBS for 20min at room temperature and washed for 2 X 5min in PBS before isolation with a hydrophobic PAP pen (Immedge pen; Vector Laboratories, Peterborough, UK). Non-specific protein binding sites in sections were blocked by incubation in blocking buffer (75µl; 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 30min 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 X 5min in PBS. Tissue sections were then incubated with secondary antibody diluted in ADB for 1h in a hydrated incubation chamber at room temperature. After 1h, slides were washed for 3 X 5min in PBS, mounted in Vectorshield mounting medium containing DAPI (Vector Laboratories) and stored at 4°C before microscopic analysis. Antibodies used in this staining are detailed in Table 1.

Immunocytochemistry

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

Microscopy and analysis

Fluorescently stained sections were analysed 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 20X magnification using an Axiocam HRc camera (Carl Zeiss Ltd). Each well was divided into 9 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 20μm thick sections of the retina, along a 250μm linear region of the ganglion cell layer, stretching out horizontally

either side of the optic nerve. Four sections per retinae and 6 retinae from 6 different animals per treatment group were quantified.

For *in vivo* quantification of axon regeneration, 20X 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, USA). 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 1200μm distal (towards the chiasm) to the centre of the crush site (identified by laminin$^+$ staining) of 6 optic nerves from 6 different animals per treatment group and 3 sections per optic nerve. By measuring the diameter of the nerve at each measurement point, the number of axons/mm width was 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]:

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

Statistics

All statistical tests were performed using SPSS 17.0 and data were presented as mean ± standard error of the mean (SEM). The Kolmogorov-Smirnov test was used to ensure all data were normally distributed before parametric testing using a one-way analysis of variance (ANOVA) with a Tukey *post-hoc* test. Statistical difference was considered significant at p values < 0.05.

Results

DPSC secreted NGF, BDNF and NT-3

DPSC secreted NGF (281 ± 68pg/24h/10^5 cells), BDNF (1600 ± 338pg/24h/10^5 cells) and NT-3 (270 ± 53pg/24h/10^5 cells) in culture, as analysed by ELISA (Figure 2). These neurotrophic titres were 2-3 fold higher than those detected in conditioned medium from BMSC cultures (91.3 ± 24.2, 749 ± 237, 166 ± 46pg/24h/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).

DPSC promoted βIII-tubulin$^+$ retinal cell survival and neuritogenesis in a co-culture assay

DPSC promoted a significant ($p<0.05$) increase in the survival of co-cultured βIII-tubulin$^+$ retinal cells (340.3 ± 10.4 cells/well) compared with retinal cells cultured alone (92.7 ± 20.8 cells/well), co-cultured with BMSC (227 ± 27.6 cells/well) or treated with recombinant human NGF, BDNF and NT-3 (278.7 ± 8 cells/well; Figure 3).

DPSC also promoted a significant ($p<0.05$) increase in the number of βIII-tubulin$^+$ retinal cells with neurites as well as the neurite length (161.6 ± 5.8 μm, 172.7 ± 9.5μm, respectively; Figure 3) compared with either retinal cells cultured alone (36 ± 5.2 μm, 22.7 ± 5.2μm) or co-cultured with BMSC (137.8 ± 2.3 μm, 91 ± 12.6μm; Figure 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 ± 27.4μm) compared with retinal cells cultured alone, or when co-cultured with BMSC ($p>0.05$).

Fc-TrK receptor blockers attenuated the survival and neuritogenic effects of DPSC

The number of βIII-tubulin^{+} retinal cells surviving in DPSC co-cultures (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 cells/well) and Fc-TrKC (193 ± 17.1 cells/well) used alone or in combination (99.3 ± 9 cells/well, Figure 3). In BMSC co-cultures, βIII-tubulin^{+} retinal cell survival (227 ± 27.6 cells/well) was significantly ($p<0.05$) reduced with Fc-TrKA (145.3 ± 5.4 cells/well), Fc-TrKB (138 ± 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 ± 9.5, 64 ± 5.3, 74.7 ± 12.9 cells/well, respectively) as well as the length (112.4 ± 9.1μm, 86.7 ± 9μm, 103.7 ± 1.1μm) of neurites in DPSC/retinal cell co-cultures compared with DPSC/retinal cells co-cultured without inhibitors (Figure 3). Combining the Fc-TrK inhibitors further attenuated the number of βIII-tubulin^{+} retinal cells with neurites (38 ± 4.9 cells/well) as well as neurite length (53.9 ± 7.9μm) seen in the DPSC/retinal cell co-culture. Similar effects, although less exaggerated, were seen in the BMSC/retinal cell co-cultures. Accordingly, a statistically significant ($p<0.05$) reduced neurite length from 137.8 ± 2.3μm to 65.4 ± 2μm was only seen when the three neurotrophin inhibitors were combined in the BMSC/retinal cell co-culture, but not when each inhibitor was used in isolation.

DPSC transplants preserved RNFL thickness for up to 14 days after optic nerve crush injury

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

Since the RNFL comprises RGC axons that pass over the surface of the retina towards the optic disk, RNFL thickness was used to measure post-axotomy 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 ± 2.1μm to 30.2 ± 1.5μm at 7 dpl,

21.4 ±1.6μm at 14 dpl and 17 ± 1.2μm at 21 dpl (Figure 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 DPSC/BMSC (46.2 ± 1.4μm, 46 ± 2.1μm, respectively) compared with intact animals at 7 dpl (45.7 ± 1.2μm) indicating a neuroprotective effect of the DPSC. At 14 dpl, RNFL thickness of the DPSC transplanted animal had decreased to 32.8 ± 0.7μm, which was significantly ($p<0.05$) lower than that in intact animals (45.4 ± 0.2μm) but still significantly ($p<0.05$) higher than in untreated animals (21.4 ± 1.6μm). This is in contrast to animals that received BMSC in which RNFL thickness decreased to 28.5 ± 1.6μm by 14dpl, which was not significantly ($p>0.05$) different from untreated animals. By 21 dpl, the RNFL in animals receiving either DPSC or BMSC (24 ± 1.3μm, 22 ± 1.8μm, respectively) had reduced to a thickness not significantly ($p>0.05$) different to that seen in untreated animals at 21 dpl (17 ± 1.2μm).

Transplanted intravitreal DPSC survived *in vivo* for 21 days

Viable DPSC were detected in the vitreous at 21dpl associated with elevated levels of BDNF and NT-3 in the retina at 21dpl compared to eyes transplanted with dead DPSC (Figure 5). Activated glial fibriliary acidic protein^{+} (GFAP) glia were also observed in eyes transplanted with DPSC but not with dead DPSC. Similar findings were observed with BMSC (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 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; Figure 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 BMSC exerted some neuroprotective effect for RGC, although at a lower level than did DPSC.

Intravitreal DPSC transplants after ONC promoted RGC axon regeneration

At distances of 100, 200, 400, 800 and 1200µm 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 DPSC compared with BMSC (133.7 ± 21.1, 115.9 ± 25.0, 85.4 ± 19.8, 77.2 ± 10.4, 50.4 ± 10.3 axons/nerve, respectively), dead DPSC (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; Figure 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 to animals receiving dead BMSC (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 200µm distal to the crush site.

Discussion

This study provides evidence that DPSC, through secretion of neurotrophins, significantly increase both survival and neuritogenesis of primary adult rat βIII-tubulin$^+$ retinal cells in an *in vitro* co-culture assay. Furthermore, when transplanted into the vitreous body of adult rats after ONC, DPSC significantly promote Brn-3a$^+$ RGC survival and axon regeneration. Noteworthy, the neuroprotective and pro-regenerative effects of DPSC seen in these *in vitro* and *in vivo* models was greater than that observed with BMSC, which can be related to their enhanced neurotrophic profile as determined by ELISA and suggests that DPSC 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 DPSC on locomotory recovery from SCI than did BMSC transplants[21]. Moreover, the improvement in locomotory function after cell transplantation into a SCI site occurred in the absence of local neuronal differentiation, suggesting that the transplanted cells acted indirectly, creating a more supportive trophic environment for endogenous axonal sprouting/growth.

Our finding that DPSC enhanced βIII-tubulin$^+$ retinal cell survival and neurite outgrowth in a co-culture model can be attributed to the release of soluble factors, since 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 NTF 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 DPSC, corroborating previous work showing that DPSC express multiple NTF mRNA, including neurotrophins[21, 31-33]. Interestingly, BMSC exhibited a less potent neurotrophic effect on cultured βIII-tubulin$^+$ retinal cells than DPSC; and this novel observation can be related to their reduced neurotrophin profile. Of note, K252a, a non-specific blocker of TrK receptors as well as other protein kinases, further reduced the neuritogenic effect of DPSC/BMSC compared to Fc-Trk blockade. These findings suggest that other TrK-independent growth factors may also mediate the neurotrophic effects of DPSC/BMSC. Indeed, DPSC 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. DPSC/BMSC provide an alternative source of NTF for axotomised RGCs, protecting them from death and promoting RGC axogenesis.

After ONC, RGCs begin dying from 7 dpl[42] with 80-90% dead by two to three weeks[6, 10, 41], thus making this a suitable *in vivo* model to assess DPSC-mediated effects on RGC survival. We utilised two methods of assessing RGC number in our *in vivo* model, firstly OCT was used to measure the thickness of the RNFL, which is comprised 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. Secondly, $Brn3a^+$ 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 overtime, 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 only seen in animals treated with DPSC. 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 down-regulation 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 DPSC persisted in the vitreous of rats 21 days after transplantation. Further studies are required to analyse 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 BMSC or DPSC compared with controls (i.e. untreated animals or those receiving dead BMSC/DPSC). 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 DPSC compared with those receiving BMSC transplants, correlating with our *in vitro* co-culture results as well as ELISA data, highlighting higher titres of neurotrophins produced by the DPSC. 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 DPSC are neuroprotective for RGCs and is supported by the reports of reduced numbers of apoptotic neurons seen after SCI when DPSC are transplanted into the lesion site[21]. Three other studies have shown significant RGC survival after intravitreal cell transplantation. The first two used BMSC 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, though 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 BMSC 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 DPSC seen in the *in vitro* co-culture experiments were supported by the GAP-43$^+$ RGC axon regenerative response seen in the *in vivo* ONC experiment. Accordingly, intravitreal transplantation of DPSC 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 DPSC to stimulate the growth of axons along the long non-permissive distances required to restore neural function. Our finding also suggest that the regenerating axons were disinhibited by the DPSC-derived neurotrophins, presumably through regulated intramembrane proteolysis of inhibitory receptors and dissolution of chondroitin sulphate 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 DPSC promoted axonal regeneration across a 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 titres in eyes at 21 dpl may be stem cell-derived, glial-derived or a combination of both. Thus, it is possible that up-regulation 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 BMSC promoted a small but significant regeneration of RGC axons, even at 1200µm distal to the crush site. Nonetheless, DPSC promoted significantly greater regeneration of RGC axons than did BMSC, reflecting their elevated neurotrophin secretion profile and underlining the potential benefit of DPSC 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 DPSC.

Conclusions

We demonstrate here for the first time that DPSC secrete multiple neurotrophins which were at least in part responsible for promoting axotomised RGC neuroprotection and neuritogenesis/axogenesis, both *in vitro* and *in vivo*. DPSC were more effective than BMSC,

which is likely due to the higher titres of neurotrophin secretion by the DPSC. DPSC may be a promising alternative for a CNS regenerative cell therapy.

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Legends

Figure 1: Experimental design used for *in vivo* experiment

Timeline 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.

Figure 2: NGF, BDNF and NT-3 secretion from DPSC and BMSC

DPSC and BMSC conditioned medium, collected after 48h of cell culture, was assayed using specific ELISAs for rat NGF, BDNF and NT-3 ($n = 3$; Black lines indicate significant difference at $p<0.05$).

Figure 3: Effects of DPSC and BMSC on βIII-tubulin$^+$ retinal cells *in vitro*

βIII-tubulin$^+$ retinal cells, cultured either alone (A i), with exogenous neurotrophins (A ii), with BMSC (with or without TrK inhibitors, A iii and A iv, respectively) or with DPSC (with or without TrK inhibitors, A v and A vi, respectively). All images are representative of the entire culture, 9 separate culture wells per treatment with every 3 wells using a different animal (scale bars = 100μm). 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 co-cultured with BMSC (blue bars), DPSC (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 co-cultures are shown (points marked with an * indicate significant difference from uninhibited cultures at $p<0.05$).

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 6 animals used in each treatment group (scale bar = 200μm). 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 * indicate significant difference from untreated/dead cell transplanted animals at $p<0.05$.

Figure 5: DPSC survival and trophic effects 21 days after ONC/cell transplantation

Immunohistochemically stained 20μm thick parasagittal sections of retina and vitreous, stained for BDNF (A and B), NT-3 (C and D), GFAP (E and F) and Y chromosome (G) 21 days after ONC and intravitreal transplantation of DPSC (A, C, E and G) or dead DPSC (B, D and F) with outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) labelled. A negative control with the primary antibodies omitted is included (F). All images are representative of the 2 images per section, 4 sections per retina, 6 retinae from 6 different animals per treatment group. DAPI was used as a nuclear counter stain (scale bars = 100μm).

Figure 6: RGC survival 21 days after ONC/cell transplantation

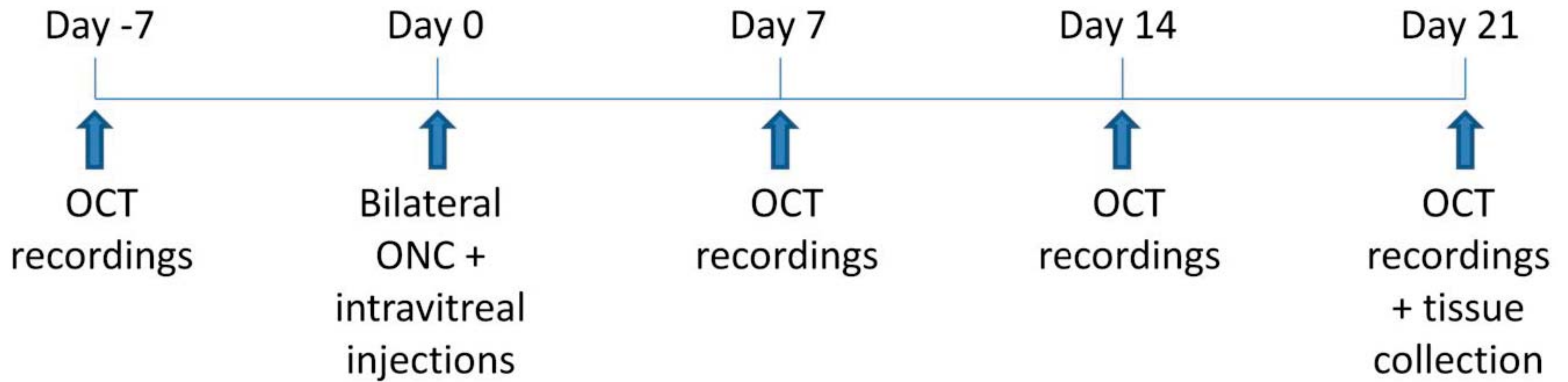
Immunohistochemically stained 20μm thick parasagittal sections of retina, stained for βIII-tubulin (green) and Brn3a (red) in intact animals (A i) and 21 days after ONC (A ii) and intravitreal transplantation of dead BMSC (A iii), dead DPSC (A iv), living BMSC (A v) and living DPSC (A vi) with outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) labelled. All images are representative of the 2 images per section, 4 sections per retina, 6 retinae from 6 different animals per treatment group. DAPI was used as a nuclear counter stain (scale bars = 100μm). In panel B, the number of $Brn3a^+$ RGCs, counted in a 1mm region of the GCL 21 dpl is shown. Black lines indicate significant difference at $p<0.05$.

Figure 7: Regeneration of RGC axons in the optic nerve, 21 days after ONC/cell transplantation

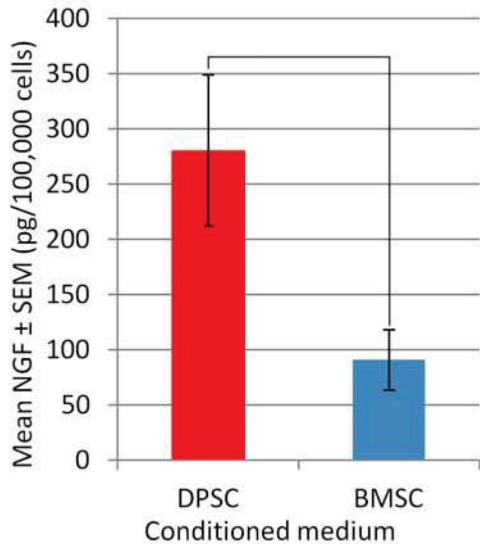
Immunohistochemically stained 15μm thick longitudinal sections of optic nerves, stained for GAP-43 (green) and laminin (red) 21 days after ONC and DPSC (A i) or dead DPSC (A ii) transplantation with the crush site marked by an *. All images are representative of 3 sections per nerve, 6 nerves from 6 different animals per treatment group (scale bars = 100μm). The number of regenerating axons was measured at 100, 200, 400, 800 and 1200μm from the ONC site at 21 dpl in untreated animals (purple bars), animals receiving intravitreal dead DPSC transplants (red dashed bars), dead BMSC (blue dashed bars), living BMSC (blue bars) and living DPSC (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.

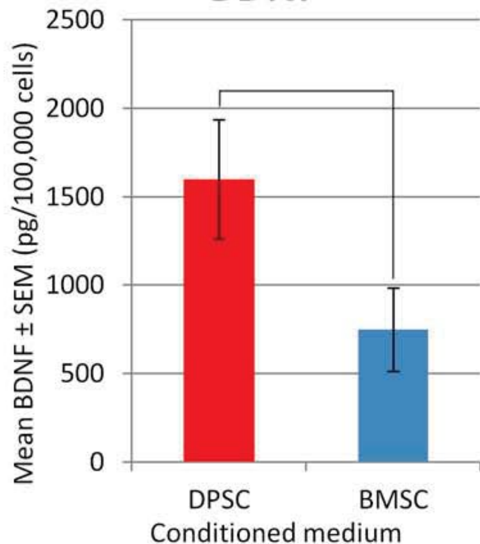
Antigen	Dilution	Supplier	Catalogue no.
BDNF	1:200	Promega	#G1641
NT-3	1:200	Millipore	#AB1780SP
GFAP	1:200	Sigma	#G9269
RBMY	1:100	Santa Cruz	#SC-14572
βIII-tubulin	1:500	Sigma	#T8660
Brn3a	1:200	Santa Cruz	#SC-31984
GAP-43	1:400	Zymed Laboratories	#33-5000
Laminin	1:200	Sigma	#L9393
Mouse IgG (Fluor 488)	1:400	Molecular probes	#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

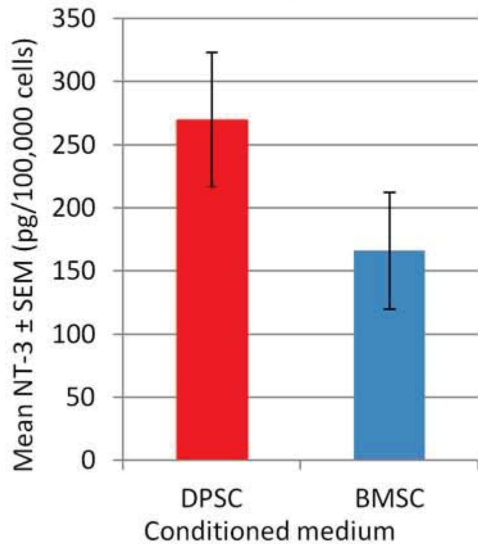
Table 1: Antibodies used in immunohistochemistry and immunocytochemistry – see text for definitions of abbreviations

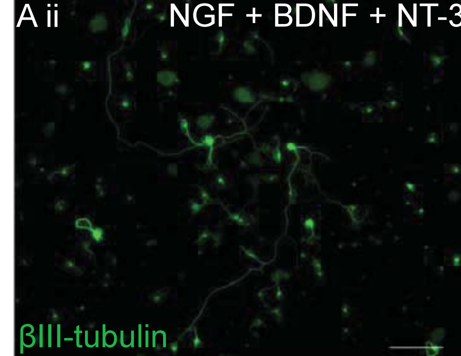
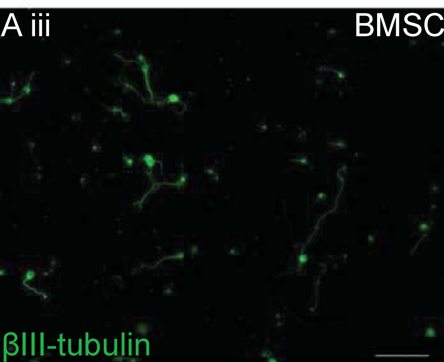
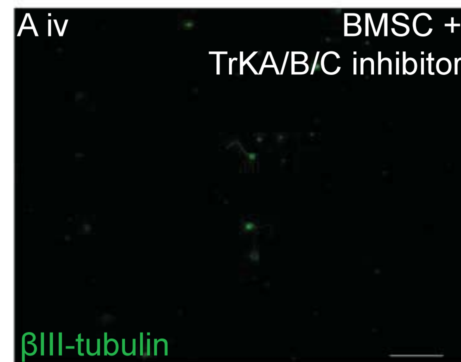
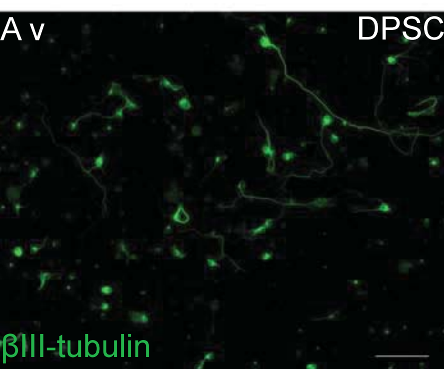
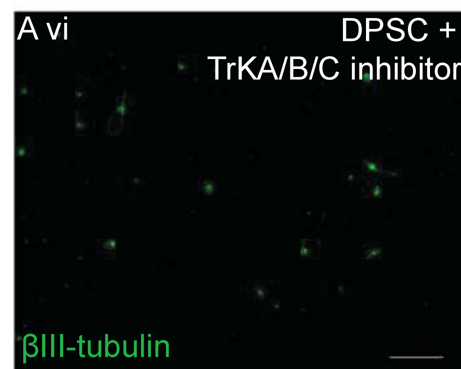
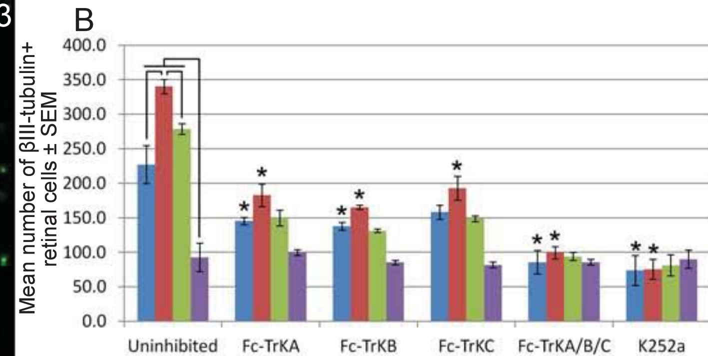
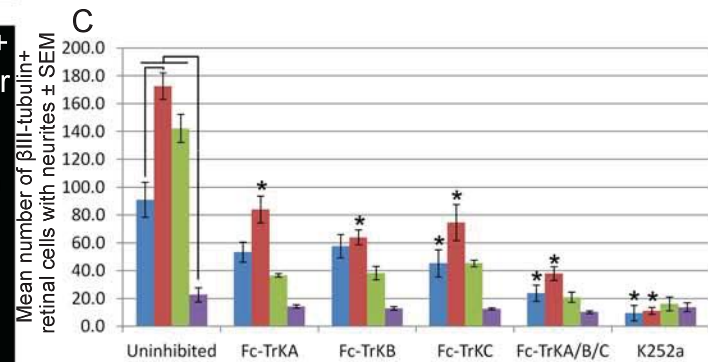
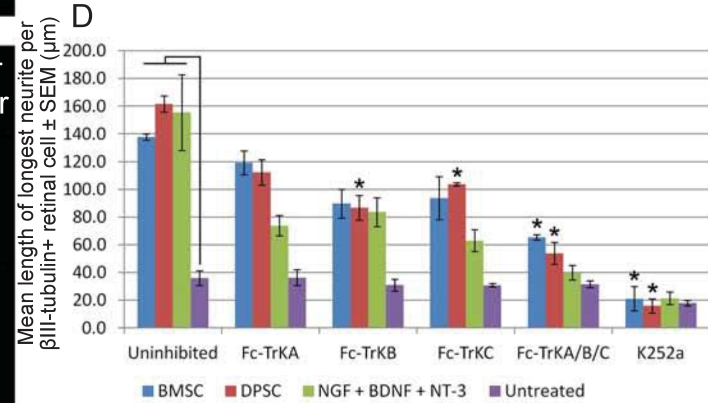


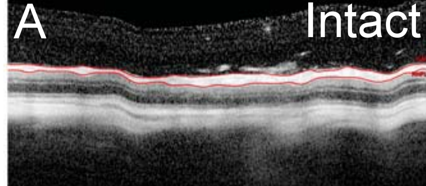
Number of animals (eyes)	Treatment		Measured endpoint
	Left eye	Right eye	
6 (12)	Dead DPSC + ONC	DPSC + ONC	RGC counts, axon counts and RNFL thickness
6 (12)	Dead BMSC + ONC	BMSC + ONC	RGC counts, axon counts and RNFL thickness
6 (12)	PBS + ONC	PBS + intact	RGC counts, axon counts and RNFL thickness

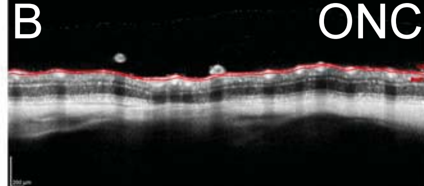

NGF
Mean NGF ± SEM (pg/100,000 cells)
400
350
300
250
200
150
100
50
0
DPSC
BMSC
Conditioned medium

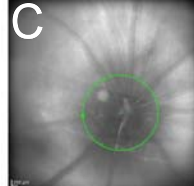

BDNF
Mean BDNF ± SEM (pg/100,000 cells)
2500
2000
1500
1000
500
0
DPSC
BMSC
Conditioned medium


NT-3
Mean NT-3 ± SEM (pg/100,000 cells)
350
300
250
200
150
100
50
0
DPSC
BMSC
Conditioned medium

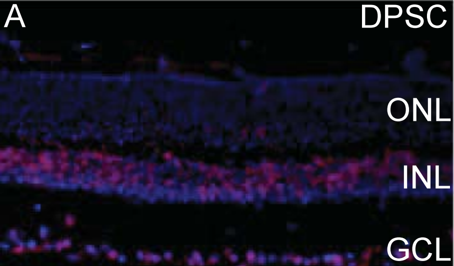
A i
Untreated
βIII-tubulin

A ii
NGF + BDNF + NT-3
βIII-tubulin

A iii
BMSC
βIII-tubulin

A iv
BMSC +
TrKA/B/C inhibitor
βIII-tubulin

A v
DPSC
βIII-tubulin

A vi
DPSC +
TrKA/B/C inhibitor
βIII-tubulin

B
Mean number of βIII-tubulin+ retinal cells ± SEM
400.0
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
Uninhibited
Fc-TrKA
Fc-TrKB
Fc-TrKC
Fc-TrKA/B/C
K252a

C
Mean number of βIII-tubulin+ retinal cells with neurites ± SEM
200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Uninhibited
Fc-TrKA
Fc-TrKB
Fc-TrKC
Fc-TrKA/B/C
K252a

D
Mean length of longest neurite per βIII-tubulin+ retinal cell ± SEM (μm)
200.0
180.0
160.0
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Uninhibited
Fc-TrKA
Fc-TrKB
Fc-TrKC
Fc-TrKA/B/C
K252a
BMSC
DPSC
NGF + BDNF + NT-3
Untreated

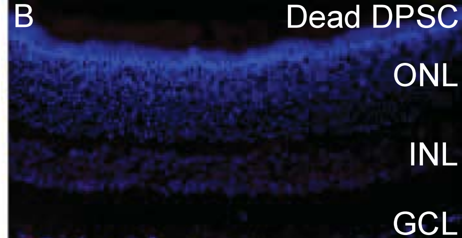

A
Intact

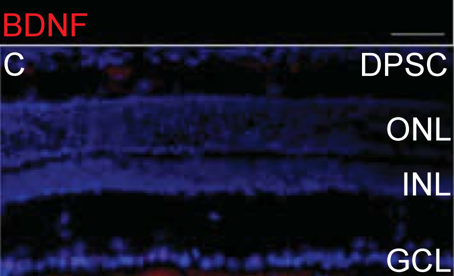

B
ONC

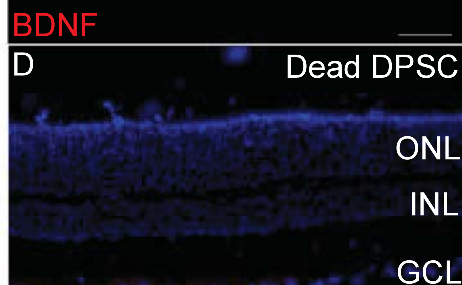

C

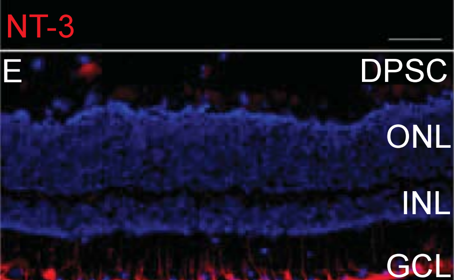
D
Mean RNFL thickness ± SEM (μm)
60
50
40
30
20
10
0
Intact
ONC
Dead DPSC
Dead BMMSC
Living DPSC
Living BMMSC
//*
*
*
*
0
7
14
21
Days after ONC

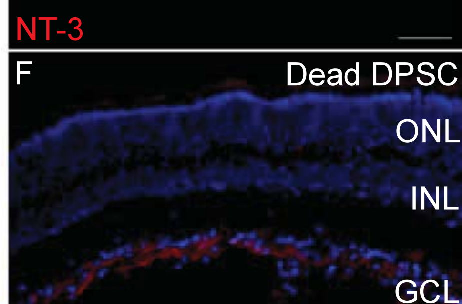

A
DPSC
ONL
INL
GCL
BDNF

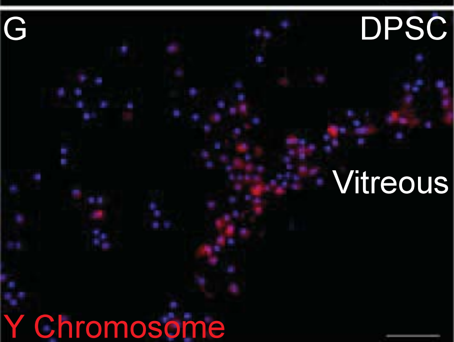

B
Dead DPSC
ONL
INL
GCL
BDNF

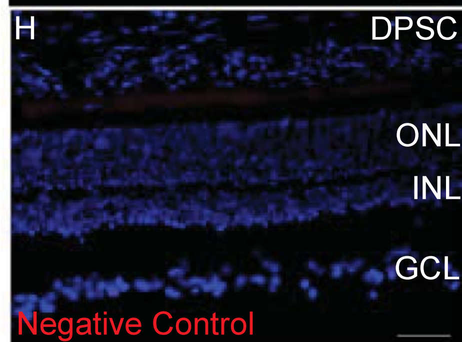

C
DPSC
ONL
INL
GCL
NT-3

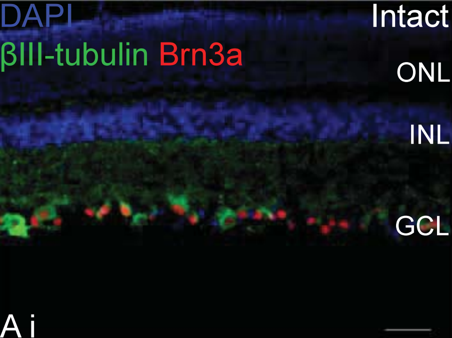

D
Dead DPSC
ONL
INL
GCL
NT-3

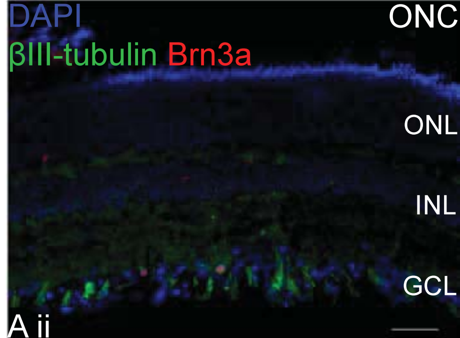

E
DPSC
ONL
INL
GCL
GFAP

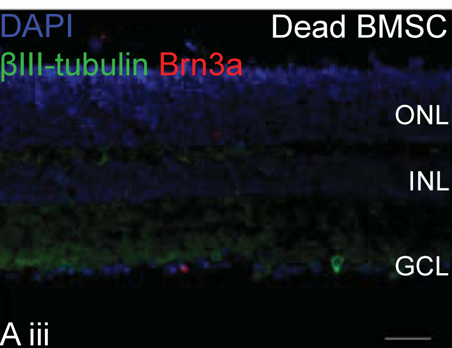

F
Dead DPSC
ONL
INL
GCL
GFAP

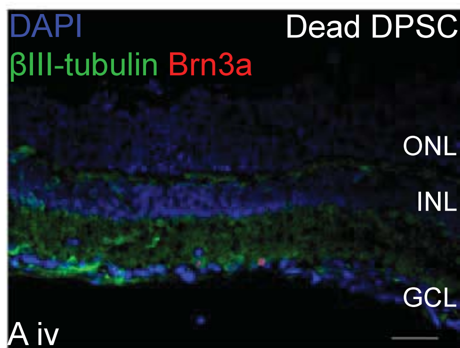

G
DPSC
Vitreous
Y Chromosome

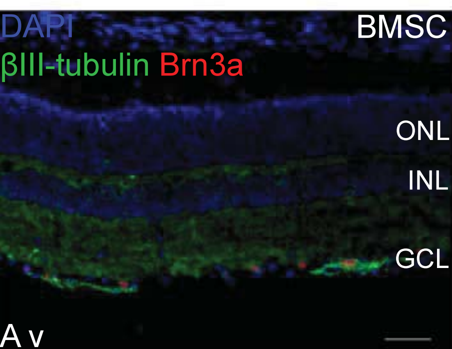

H
DPSC
ONL
INL
GCL
Negative Control

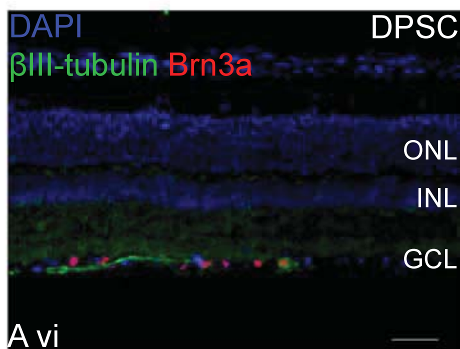

DAPI
βIII-tubulin Brn3a
Intact
ONL
INL
GCL
A i

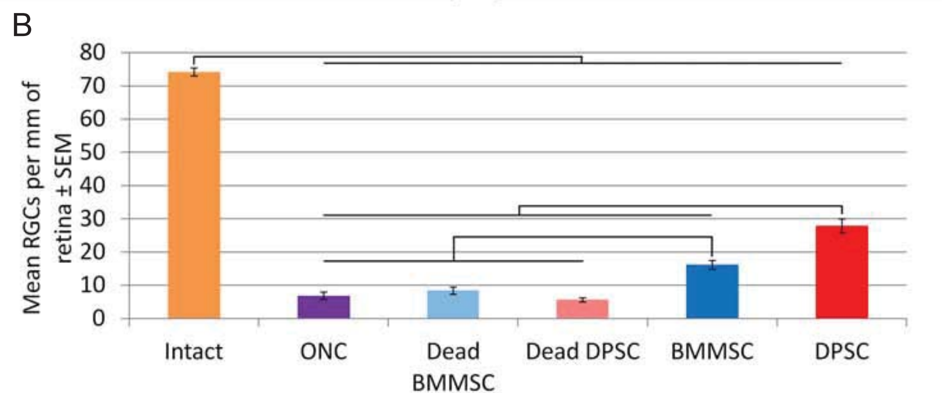

DAPI
βIII-tubulin Brn3a
ONC
ONL
INL
GCL
A ii


DAPI
βIII-tubulin Brn3a
Dead BMSC
ONL
INL
GCL
A iii


DAPI
βIII-tubulin Brn3a
Dead DPSC
ONL
INL
GCL
A iv


DAPI
βIII-tubulin Brn3a
BMSC
ONL
INL
GCL
A v


DAPI
βIII-tubulin Brn3a
DPSC
ONL
INL
GCL
A vi


B
Mean RGCs per mm of retina ± SEM
0
10
20
30
40
50
60
70
80
Intact
ONC
Dead BMMSC
Dead DPSC
BMMSC
DPSC

Proximal

A i

A ii

Gap-43
Laminin

Distal