MESENCHYMAL STEM CELL THERAPY FOR TRAUMATIC AND DEGENERATIVE EYE DISEASE

By

Ben Mead, BSc, MRes

Supervisors: Dr Ben Scheven & Dr Wendy Leadbeater

A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

ABSTRACT

Aims

Degenerative eye diseases such as glaucoma are a leading cause of irreversible blindness, and traumatic optic neuropathies affect up to 5% of all patients with traumatic head injuries. Treatments are limited making cell based neuroprotective/neuroregenerative therapies a huge research interest. The aim of this PhD research project was to investigate the application of dental pulp stem cells (DPSC) as a treatment for traumatic and degenerative eye diseases. The accuracy and reliability of counting retinal ganglion cells (RGC) in radial retinal section was also assessed.

Methods

Numbers of RGC in radial retinal sections were compared to numbers in retinal wholemounts. To determine the feasibility of DPSC as a treatment for RGC loss they were cultured together and survival and neuritogenesis were quantified. DPSC were also transplanted intravitreally into rat models of optic neuropathy (optic nerve crush) and glaucoma and surviving RGC and regenerated axons were quantified in radial retinal sections. Finally, to determine the neurotrophic secretory profiles of mesenchymal stem cells and if this has a role in the mechanism of the neuroprotective and axogenic properties of DPSC, ELISA, PCR and inhibitory assays were performed. Adipose and bone marrow-derived mesenchymal stem cells (ADSC; BMSC) were used as comparative cells to the DPSC.

Results

Quantifying RGC in radial retinal sections was as reliable and accurate as the current gold standard Thus, retinal wholemounts with Brn3a proved to be the most reliable marker for RGC. DPSC proved to be more efficacious that BMSC/ADSC in protecting RGC from optic nerve crush-/glaucoma-induced death, promoting significant regeneration of RGC axons in the former and preserving visual function (as measured by electroretinography) in the latter. The mechanism of action, as determined *in vitro*, appeared to be through the secretion of multiple neurotrophic factors (NTF) including nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3 and platelet-derived growth factor.

Conclusions

In conclusion, DPSC is a more potent cell therapy than BMSC/ADSC for the treatment of traumatic and degenerative disease, mostly acting through paracrine-mediated mechanisms involving the secretion of multiple NTF, and preserve visual function in glaucomatous rats thus making them a potentially useful treatment for preventing blindness in glaucoma patients.

ACKNOWLEDGEMENTS

There are a great many people I would like to thank for helping me get this far and this small acknowledgment doesn't do justice how thankful I am to everyone in supporting me up to this point. Firstly I would like to thank Prof Ann Logan, who has supported me on a huge number of projects starting from when I was still an undergraduate. Although you were not officially my supervisor, your advice, support and faith in me is greatly appreciated and working in your lab as made my PhD a pleasure. Next I would like to thank my supervisor Dr Ben Scheven, who supported me through these years, and provided the perfect balance of guidance and advice while also allowing me to make my own decisions and direct the research. You have been very flexible in all aspects of my PhD and I have nothing but good things to say about my experience with you as my supervisor. I'd also like to thank my other supervisor, Dr Wendy Leadbeater, who not only helped me with technical advice but also provided excellent support when I supervised undergraduate students. I have had an enjoyable experience teaching and supervising and those opportunities were thanks to you. I would also like to thank Prof Martin Berry, who never complained when I asked to read through my papers, despite some of them being in a not-so-great state and Dr Zubair Ahmed whose your technical help was greatly appreciated. There are many other people in the lab I would like to thank. Dr Lisa Hill, I will never forget one minute you being my supervisor, the next we are interviewing for the same PhD (thankfully they had a few places spare!). I am going to miss your invaluable aid, either when it is helping me in the animal house or supporting me when I have to make critical decisions that may or may not result in career suicide. Adam who enjoyed me supervising him so much he came back for more over the summer, I greatly appreciated your help with our paper and I hope you forgive me for all the cell counts I asked you to do. Jenna, Hannah, Keira, Felicity, Maryam, I highly doubt I will find a write up room as fun to work in as ours, you all helped in your own way and I will miss you all.

Finally I would like to thank my family, particularly my Mom, who has believed in me and gave me the best possible upbringing she could to get me to where I am today and my partner Helen, who has put up with the ups and downs that come with doing a PhD and stuck with me through it all.

PUBLICATIONS & PRESENTATIONS

Full research papers directly from this doctoral research

- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA (2013). Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. *Invest Ophthalmol Vis Sci* 2013;54:7544-7556 (Chaper 3)
- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA (2014). Dental pulp stem cells, a paracrine-mediated therapy for the retina. *Neural Regen Res* 2014;(6):577-578 (Appendix 1)
- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA (2014). Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells. 2014;Plos One 9: e109305. (Chapter 4)
- **Mead B**, Thompson A, Scheven BA, Logan A, Berry M, Leadbeater W (2014). Comparative evaluation of methods for estimating retinal ganglion cell number in retinal sections and whole mounts. 2014;Plos One 9(10): e110612. (Chapter 2)
- **Mead B** and Scheven BA (2014). Mesenchymal stem cell therapy for degenerative and traumatic eye disease. *Neural Regen Res*. 2015;(3):371-373 (Appendix 4)

Review papers directly from the doctoral research

- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA (2014). Stem cell treatment for CNS injury. Current Tissue Engineering. 2014;93-101 (9) (Appendix 2)
- **Mead B**, Berry M, Logan A, Scott RAH, Leadbeater W, Scheven BA. Stem cells for treatment of degenerative eye disease. Stem Cell Research. 2015;14:243-257 (Chapter 1; Appendix 3)

- In the above instances, Ben Mead conceived and conducted the experiments, analysed the data, wrote and submitted the manuscript as corresponding author.

Papers in preparation directly from the doctoral research

- **Mead B**, Hill LJ, Blanch RJ, Ward K, Logan A, Berry M, Leadbeater W, Scheven BA (2014). Stem cell-mediated neuroprotection and functional preseveration of retinal ganglion cells in a rodent model of glaucoma. STEM CELLS *Manuscript in writing*

Published peer-reviewed conference abstracts from this doctoral research

- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA. (2012) Dental pulp stem cells promote regeneration of retinal ganglion cells following injury. Research Showcase Event, University of Birmingham, UK, May 2012.
- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA. (2013) Dental pulp stem cells promote regeneration and survival of retinal ganglion cells after optic nerve crush. SfN, San Diego, November 2013.
- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA. (2013) Dental pulp stem cells promote regeneration and survival of retinal ganglion cells after optic nerve crush. BSODR, Bath, UK, September 2013.

Invited oral paper conference presentations from this doctoral research

- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA. (2013) The neuroprotective and proregenerative properties of dental pulp stem cells as a cellular therapy. Research Showcase Event, University of Birmingham, UK, June 2013 (Won best oral presentation award).
- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA. (2013) The neuroprotective and proregenerative effects of intravitreal of dental pulp stem cell/mesenchymal stem cell transplantation on retinal ganglion cells after optic nerve injury. University College London, UK, April 2013. (Won the Fairbairn award for the best oral presentation).
- **Mead B**, Logan A, Berry M, Leadbeater W, Scheven BA. (2014) Stem cell therapy for ocular disease. ARVO, Florida, May 2014
- **Mead B**, Hill LJ, Ward K, Blanch RJ, Logan A, Berry M, Leadbeater W, Scheven BA. (2015) Dental Pulp, Bone Marrow and Adipose-Derived Mesenchymal Stem Cell Therapies for Glaucoma. ARVO, Colorado, May 2015.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADSC	Adipose-derived stem cell
AMD	Age-related macular degeneration
BDNF	Brain-derived neurotrophic factor
BMSC	Bone marrow-derived mesenchymal stem cell
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DPSC	Dental pulp stem cell
ERG	Electroretinogram
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibriliary acidic protein
GSK-3β	Glycogen synthase kinase-3β
IGF	Insulin-like growth factor
IOP	Intraocular pressure
iPSC	Induced pluripotent stem cell
Ivit	Intravitreal
MSC	Mesenchymal stem cell
mTOR	Mammalian target of rapamycin
NGF	Nerve growth factor
NSC	Neural stem cell
NT-3	Neurotrophin-3
NTF	Neurotrophic factor
ONL	Outer nuclear layer
PDGF	Platelet-derived growth factor
RCS	Royal College of Surgeon
RGC	Retinal ganglion cell

RNFL	Retinal nerve fibre layer
RPE	Retinal pigment epithelium
SCI	Spinal cord injury
TBI	Traumatic brain injury
TIMP1	Tissue inhibitor of metalloproteinases-1
TON	Traumatic optic neuropathy
Trk	Tropomyosin related kinase
VEGF	Vascular endothelial growth factor

CHAPTER 1

GENERAL INTRODUCTION

1.1 The visual system

The visual system allows humans to perceive the world (visually) and is a vital aspect of independent functioning in the environment and in society. Loss of vision is a consequence of multiple traumatic and degenerative diseases and for many of these conditions, the resulting blindness is permanent. For example, 0.5-5% of traumatic head injuries result in optic neuropathies and blindness (Sarkies *et al*., 2004), and glaucoma is the leading cause of irreversible blindness, affecting 60 – 80 million worldwide (Quigley *et al*., 2006). No current treatments exist to alleviate the effects of optic neuropathy and treatment for glaucoma is symptomatic, restricted to the lowering of elevated intra-ocular pressure (IOP), even so 10% of treated patients still exhibit a permanent visual loss.

1.2 The retina and optic nerve

The retina is an outgrowth of the brain and is thus part of the central nervous system (CNS). Because of this, it is subject to the same limitations as other CNS tissue when damaged i.e. irreplaceable neurons and a lack of regenerative capacity. The retina (Figure 1) has three layers of neurons, an outer nuclear layer (ONL) composed of rod and cone photoreceptors, an inner nuclear layer composed of bi-polar cells/amacrine cells and a ganglion cell layer consisting of retinal ganglion cells (RGC). These layers make up the neural retina and sit upon the retinal pigment epithelium (RPE), adjacent to (but separated by Bruch's membrane) the choroid. The RPE is necessary for ensuring regular turnover and recycling of photoreceptor outer segments whereas the vascular choroid provides nourishment to the eye.

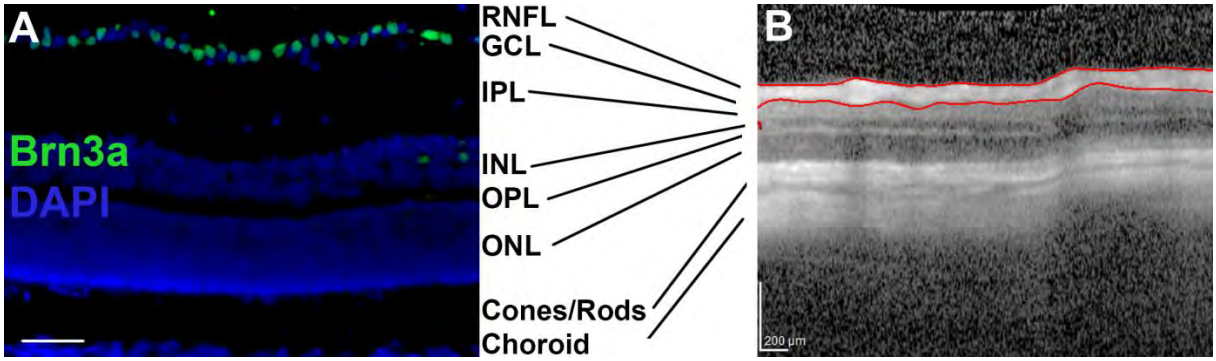


Figure 1: Immunohistochemical staining of the retina (A) and optical coherence tomography (OCT) imaging of the retina (B). Retina is stained for a marker of retinal ganglion cells (Brn3a; green) and a nuclear marker (DAPI; blue). Labelled are the retinal nerve fibre layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptor cones and rods, and the choroid. Retinal pigment epithelium and Bruch's membrane sit between the cones/rods and choroid (not shown). The differences in proportion are due to the significantly lower resolving power of OCT (B; scale bar: 200µm) in comparison to fluorescent microscopy (A; scale bar: 50µm).

Light, focussed on the retina by the lens, is detected by the cone and rod photoreceptors where it is converted into an electrochemical signal that travels through the bipolar cells to the RGC. Membrane depolarisations are propagated along the RGC axons in the retinal nerve fibre layer (RNFL) that exit the eye at the optic disc and course through the unmyelinated lamina cribrosa, then become myelinated by oligodendrocytes and make up the optic nerve. The axons of optic nerves of both eyes converge at the optic chiasm and decussate to project into contralateral optic tract before synapsing with relay neurons in the SC and LGN, from where signals are passed to the visual cortex, a feature necessary for stereoscopic vision (Berry *et al*., 2008).

1.3 Degeneration and the axon regenerative capacity of retinal neurons

The prevailing theory for the loss of RGC following traumatic or degenerative eye disease is the reduction in the vital supply of neurotrophic factors (NTF) which, under normal circumstances, are supplied from the innervated target and travel retrogradely along RGC axons to the RGC somata, acting as survival signals (Dawbarn and Allen, 2003). This is corroborated by a large body of literature on NTF-mediated neuroprotection of RGC (Barde, 1989, Sofroniew *et al.,* 2001, Jones *et al*., 2001, Morgan-Warren *et al*., 2013). During

development, neurons that fail to innervate their targets are starved of NTF survival signals and die by apoptosis (Butowt and von Bartheld, 2003). Similarly, many adult axotomised neurons atrophy and die after disconnection from target-derived NTF, but those with collaterals proximal to the transection site are protected by a supply of NTF derived from the targets of these collateral sites and also from local glia (Dougherty *et al*., 2000, Faulkner *et al*., 2004). Since axon collaterals are largely absent in the optic nerve, RGC are exquisitely sensitive to optic nerve damage, so that approximately 40% die within 7 days (Ahmed *et al*., 2011) and 90% are lost by 14-21 days (Mey and Thanos, 1993, Berkelaar *et al*., 1994). The loss of axotomised RGC detailed above is of relevance to diseases such as glaucoma and traumatic optic neuropathy (TON) where axonal damage is sustained (Figure 2).

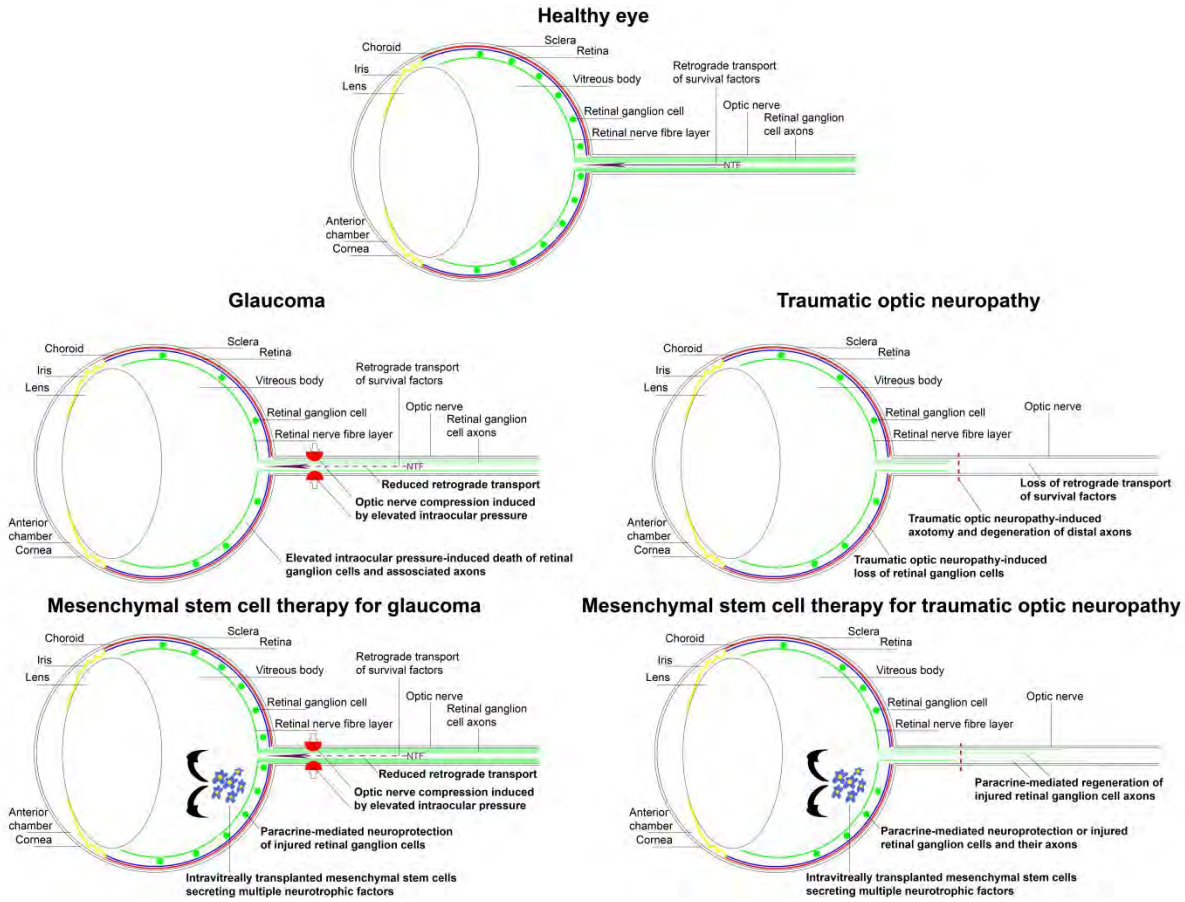


Figure 2: Schematic diagram of a parasagittal section of the eye and optic nerve demonstrating the neurodegenerative effects glaucoma and traumatic optic neuropathy and the potential for mesenchymal stem cells as a therapy (Adapted from Mead and Scheven. 2015).

The failure of adult CNS axotomised neurons to regenerate their axons is attributed to suppression of intrinsic axogenic machinery, the paucity of axogenic NTF (Berry *et al*., 2008) and the presence of myelin- and scar-derived axon growth inhibitory factors (Richardson *et al*., 1980, Sandvig *et al*., 2004) mediating growth cone collapse. This is in contrast to axons in the peripheral nervous system in which an abundance of Schwann cell-derived NTF contributes to the functional regeneration of their axons. The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) phosphorylate tyrosine residues (Dawbarn and Allen, 2003) after binding to the tropomyosin related kinase (TrK) receptor and promote RGC survival and axon growth (Berry *et al*., 2008) by activating intracellular signalling pathways (MAPK/PI3K/PKC; Figure 3), whilst the cytokine ciliary neurotrophic factor (CNTF) activates the JAK pathway after binding to the

heterotrimeric gp130 receptor complex. These and other NTF signal through phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) to activate the serine-threonine kinase mammalian target of rapamycin (mTOR), to promote axogenic protein synthesis and inhibit glycogen synthase kinase-3β (GSK-3β) which, amongst other roles, regulates growth cone dynamics (Morgan-Warren *et al*., 2013). Experimental activation of mTOR signalling in adult mice promotes RGC survival and axon regeneration after optic nerve transection (Park *et al*., 2008, Morgan-Warren *et al*., 2013). Promoting axon regeneration is relevant to scenarios in which either the optic nerve is injured or RGC precursors are transplanted into the ganglion cell layer (Hertz *et al*., 2014) which subsequently require long distance regeneration of their axons.

Along with loss of RGC, photoreceptor loss also occurs in many degenerative eye diseases such as age related macular degeneration (AMD) and retinitis pigmentosa. Photoreceptor outer segments are damaged by light and ultimately have a finite life, with 10% of the outer segments being recycled by RPE-mediated phagocytosis each day. RPE digestion of internalised phagosomes is not 100% efficient and toxic lysosomal proteins, such as lipofuscin, build up leading to RPE degeneration (Bharti *et al*., 2011). The thickening of the outer limiting membrane and successive reduction in the supply of diffusible factors to the RPE also contribute to the degeneration. The subsequent failure of the RPE to clear degenerate photoreceptor outer segments is the primary pathology in AMD and retinitis pigmentosa (Bharti *et al*., 2011).

1.3.1 Current methods for quantifying neuronal damage and repair in the retina

Animal models of traumatic and degenerative retinal eye disease are used to test potential neuroprotective and axogenic treatments. The optic nerve crush/transection (TON) and elevated IOP models of glaucoma, all experimentally induce chronic compression of the optic nerve. In TON, RGC axon regeneration can be measured in histological sections of the

optic nerve by staining for GAP-43. In contrast, the gold standard method of quantifying RGC loss and assessing the efficacy of neuroprotective treatments is to wholemount the retina and count the number of RGC in selected regions after staining the retina either with Brn3a or by back-labelling RGC with FluoroGold (Nadal-Nicolas *et al*., 2009). Unfortunately, the wholemounting technique is not always appropriate as it renders the tissue unusable for further analyses, meaning retinal sectioning is instead employed. The wide array of available models and analytical techniques now available has allowed researchers to test many different axogenic and neuroprotective treatments for ocular disease.

1.4 Cellular therapy for CNS/retinal injury

Treatments for long term neuroprotection and axon regeneration in damaged/diseased CNS tissue are limited and none are currently available in the clinic. Delivery of individual or combinations of NTF promotes incomplete and unsustained axon regeneration in the transected rat optic nerve (Logan *et al*., 2006) and spinal cord (Lu *et al*., 2004b). Accordingly, intravitreal (*ivit)* injection of recombinant BDNF and CNTF rescues axotomised RGC from death for up to 7 days (Mey and Thanos, 1993, Ahmed *et al*., 2011) but long term trophic support requires repeated low dose NTF injections (Ko *et al*., 2000, Ko *et al*., 2001) since transient high peak bolus delivery of NTF down-regulates TrK receptors (Sommerfeld *et al*., 2000, Chen and Weber, 2004). Injectable hydrogel formulations composed of collagen, alginate or chitosan are being developed (Pakulska *et al*., 2012) that continuously and slowly release low titres of NTF *in vivo* over several weeks. However, drug loading of implanted/injected hydrogels is limited and thus, for chronic neurodegenerative diseases like glaucoma, sustained NTF delivery requires repeated hydrogel implantation/injection, making FDA approval for clinical application a significant challenge. Alternative treatments, such as the transplantation of cells with extended longevity and engineered to continuously produce low levels of specific NTF combinations can remove the need for repeated injections and overcomes the problems of bolus NTF delivery regimes.

In contrast to this paracrine-mediated mechanism for neuronal rescue, cell therapy can be used to replace lost endogenous neurons and return, or prevent further loss of, function. For either of these two therapeutic approaches to achieve functional preservation, termed "indirect" and "direct" mechanisms, respectively, it has become apparent that stem cells are the most suitable candidates for this, as discussed below.

1.4.1 Direct treatment mechanism – stem cell differentiation for cell replacement

In AMD, replacement of RPE cells, either using the RPE cell line ARPE19 (Coffey *et al*., 2002) or surgical translocation of the macula to a healthy portion of RPE (da Cruz *et al*., 2007), preserves visual function, but a high rate of complications, such as retinal detachment and macular hole, means an alternative accessible source of exogenous RPE is preferred. Similarly, photoreceptor replacement by allogeneic subretinal transplantation of photoreceptor precursor cells isolated from retinae of *gfp*-expressing mice of various ages is effective (MacLaren *et al*., 2006). Experimentally transplanted photoreceptor precursors successfully survive, differentiate and integrate within the retina, improving retinal function and restoring vision, evident by the improvement in electroretinogram (ERG) performance and restoration of visual guided behaviour in animal models of photoreceptor loss (Pearson *et al*., 2012). However, the above approaches for photoreceptor/RPE cell replacement are not translatable as they utilize cell lines or primary cells from a deceased adult donor. Nevertheless, these proof-of-principle studies have led to research into the use of stem-derived retinal cells for the replacement of cells lost in degenerative conditions. However, the propensity to which stem cells differentiate differ greatly between the different stem cell types. Equally, some retinal neurons, such as photoreceptors, are more easily derived than others like RGC, and thus RGC replacement is still in its infancy.

1.4.2 Indirect treatment mechanism – paracrine-mediated neuroprotection and axon regeneration

Cells transfected with *ntf* genes or induced to secrete NTF have been grafted into the retina to treat retinal degeneration e.g. : (1), cells secreting BDNF, glial cell line-derived neurotrophic factor (GDNF) and NT-4 are RGC neuroprotective and improve visual function in cases of TON (Levkovitch-Verbin *et al*., 2010), sodium iodate-induced damage of the retina (Machalińska *et al*., 2013) and chronic ocular hypertension (Harper *et al*., 2011); (2), *ivit* transplantation of genetically engineered fibroblasts that over express fibroblast growth factor-2 (FGF-2), NT-3 and BDNF significantly increase RGC survival and axon regeneration after optic nerve crush (Logan *et al*., 2006); (3), cells engineered to secrete CNTF attenuate photoreceptor death in mouse models of retinitis pigmentosa (Jung *et al*., 2013); (4), neural progenitor cells transfected with crystallin-β-b2 promote both RGC and photoreceptor survival (Bohm *et al*., 2012); and (5), a glucagon-like peptide-1-secreting cell line promotes RGC survival after optic nerve crush (Zhang *et al*., 2011). Despite possible adverse effects, cell transplantation "mono-therapies" offer the potential advantages of continuous secretion of multiple NTF for the duration of the viability of the transplant, which bind to multiple receptors and thus signal survival and axon regeneration through multiple signalling pathways (Figure 3). Interestingly, many types of stem cells, in particular mesenchymal stem cells (MSC), innately secrete a wide array of NTF, making them a suitable paracrine-mediated cellular therapy (Figure 2).

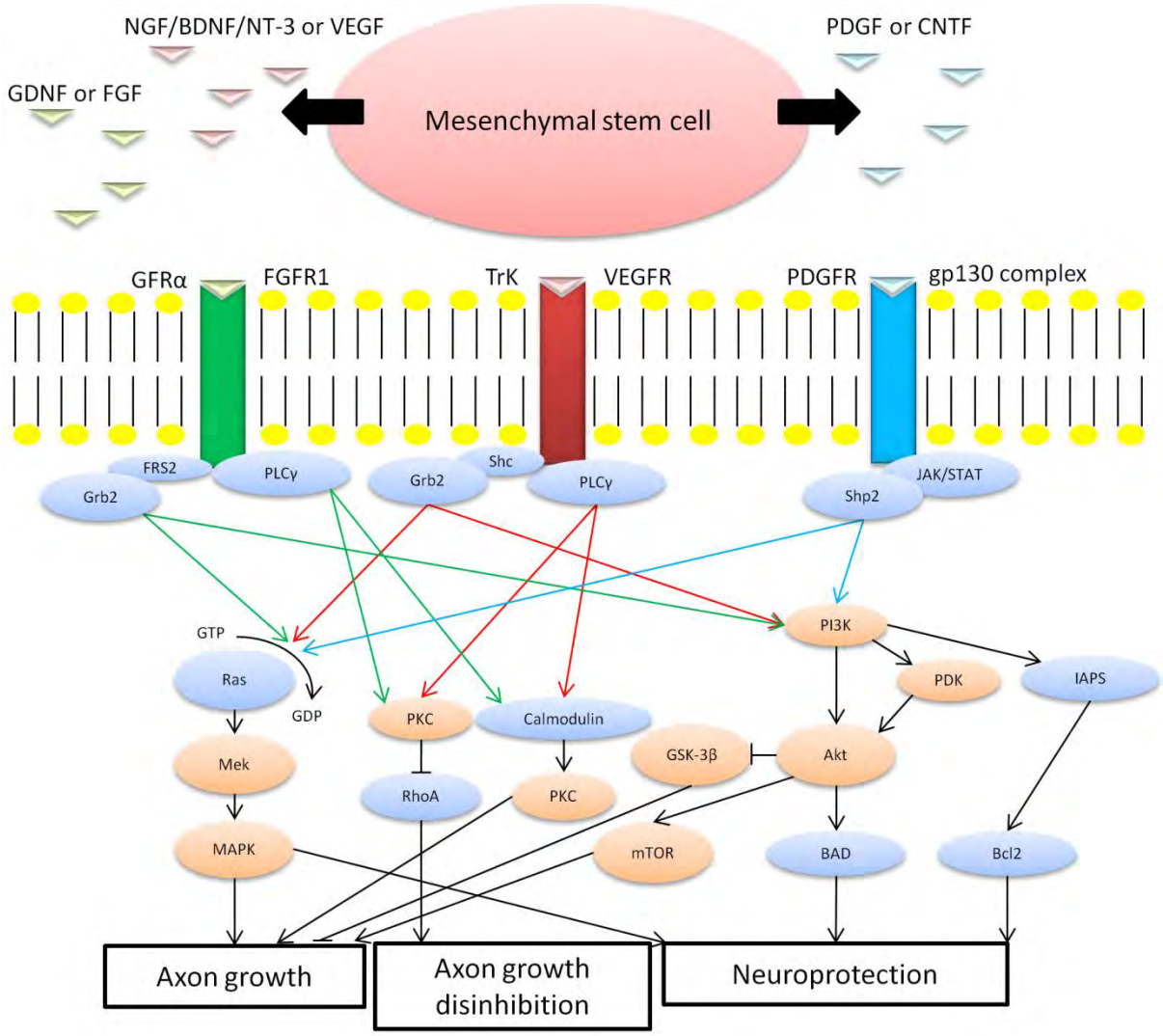


Figure 3: A schematic diagram showing the proposed mechanism by which MSC exert their neurotrophic effects on the injured CNS, including the retina, through secretion of NGF, BDNF, NT-3, CNTF GDNF, VEGF, FGF and PDGF which engage TrK A, B and C, CNTFα, GFRα, VEGFR, FGFR1 and PDGFR receptors, respectively, leading to the activation of intracellular pathways for axon growth, axon growth disinhibition and neuroprotection, accounting for the functional recovery seen in animals receiving MSC transplants after CNS/retinal injury. Some ligand-receptor interactions activate the same signalling pathways. (Abbreviations: BAD, bcl-2-assosciated death promoter; Bcl2, B-cell lymphoma 2; FGFR1, fibroblasts growth factor receptor 1; FRS2, fibroblast growth factor receptor substrate 2; GFRα, GDNF family receptor alpha; Grb2, growth factor receptor-bound protein 2; IAPS, inhibitor of apoptosis; MAPK, mitogen-activated protein kinase; Mek, mitogen-activated protein kinase kinase; PDK, phosphoinositide-dependant kinase; PKC, protein kinase C; PLCγ, phospholipase C-gamma). (Taken from Mead *et al*., 2015).

1.5 Characterization of different types of stem cells

Stem cells are defined as self-renewing cells with the capacity to differentiate into multiple cell types. Stem cells fall into the following categories: (1), totipotent stem cells can differentiate into both embryonic and extra-embryonic cells; whereas (2), pluripotent stem cells can only form cells from the three embryonic germinal layers, ectoderm, mesoderm and endoderm; (3), multipotent stem cells that are more restricted than pluripotent stem cells in their differentiation potential; finally (4), progenitors (which are not strictly stem cells) have already undergone commitment so that further differentiation is restricted to cells of a particular tissue, e.g. retinal progenitors can generate replacement cells in both the neural retina and RPE.

1.5.1 Embryonic stem cells/induced-pluripotent stem cells

Embryonic stem cells (ESC) are Tra-1-60^{+}/Tra-1-81^{+}/SSEA-3^{+}/SSEA-4^{+}/Oct4^{+}/Nanog^{+}/Sox2^{+} self-renewing pluripotent stem cells isolated from the inner cell mass of the blastocyst (Thomson *et al*., 1998) that can form cells from all three embryonic germinal layers, including mature neurons (Reubinoff *et al*., 2000). Induced pluripotent stem cells (iPSC) are ESC-like cells generated from somatic cell reprogramming by forcing the expression of the four transcription factors: Oct4, Sox2, Myc and Klf4 (Takahashi and Yamanaka, 2006). Although iPSC are characteristically similar to ESC, subtle differences in genetics/epigenetics have been observed (Doi *et al*., 2009, Kim *et al*., 2010).

1.5.2 Neural stem cells

Isolated from the CNS of adult and aborted foetus cadavers (Lu *et al*., 2012, McGill *et al*., 2012), neural stem cells (NSC) are self-renewing and multipotent with the potential to differentiate into neurons, astrocytes and oligodendrocytes, both in culture and *in vivo* (Clarke *et al*., 2000). NSC do not express unique markers and are identified by their

characteristics of neurosphere formation, differentiation potential and nestin expression (Bazan *et al*., 2004), a phenotypic marker for neuronal precursor cells (Lendahl *et al*., 1990).

1.5.3 Bone marrow-derived MSC

Originally described as fibroblast colony forming units (Friedenstein *et al*., 1966, Friedenstein *et al*., 1970), bone marrow-derived mesenchymal stem cells (BMSC; also referred to as bone marrow stromal cells) are $CD29^{+}/CD44^{+}/CD73^{+}/CD90^{+}/CD45^{-}$ (Minguell *et al*., 2001, Karaoz *et al*., 2011) self-renewing multipotent cells isolated from bone marrow aspirates. Although their precise biological function is uncertain, they can differentiate along chondrogenic, adipogenic and osteogenic lineages (Pittenger *et al*., 1999). Despite early reports of differentiation into non-mesodermal lineages such as neurons (Woodbury *et al*., 2000), later evidence disputes the neural differentiation capacity of MSC and conclusive evidence for the differentiation of BMSC into functional neurons is lacking (Lu *et al*., 2004a).

1.5.4 Adipose-derived MSC

Adipose-derived mesenchymal stem cell (ADSC) frequencies are higher in lipoaspirates from cosmetic liposuction surgery and abdominoplasty (Kern *et al*., 2006, Kalbermatten *et al*., 2011) than those of BMSC in bone marrow aspirates (Kingham *et al*., 2007) and thus are arguably the most readily available stem cells for use in regenerative medicine. Like BMSC, ADSC are multipotent $CD29^{+}/CD44^{+}/CD73^{+}/CD90^{+}/CD45^{-}$ (Kern *et al*., 2006, Zhou *et al*., 2013) with some evidence for the differentiation into neurons and glia (Kang *et al*., 2003), but evidence for these ADSC-derived neurons being functional is lacking.

1.5.5 Dental pulp stem cells

Neural crest-derived dental pulp stem cells (DPSC) (Chai *et al*., 2000) are $nestin^{+}/CD29^{+}/CD44^{+}/CD73^{+}/CD90^{+}/CD45^{-}$ (Kiraly *et al*., 2009, Kawashima, 2012, Sakai *et al*., 2012) cells with self-renewal and multipotent differentiation properties (Gronthos *et al*., 2002) and the potential to differentiate into neurons (Arthur *et al*., 2008, Kiraly *et al*., 2009).

They are readily isolated from the pulp of the 3rd/4th adult molars (Gronthos *et al*., 2000, Arthur *et al*., 2008) and can be expanded and stored for future use. Dental stem cells can also be isolated from the deciduous teeth of infants (Huang *et al*., 2009), which include exfoliated deciduous teeth (SHED) (Miura *et al*., 2003) and those from the apical papilla of immature teeth (SCAP) (Sonoyama *et al*., 2008). Cells from all origins have rapid proliferation rates and can differentiate along chondrogenic, adipogenic and osteogenic lineages (Sonoyama *et al*., 2008, Wang *et al*., 2012).

1.6 Fate of intraocular transplanted stem cells

The potential of stem cells to treat eye disease is dependent on their fate following *ivit* and sub-retinal transplantation and thus the incidence of immune rejection, differentiation into unpredicted phenotypes and unbridled migration within the neuropil of the retina, together with possible oncogenesis. Safeguards against these adverse outcomes include encapsulation of the stem implant (Zhang *et al*., 2011) and the employement of inducible suicide genes, such as viral-derived thymidine kinase, allowing selective destruction of the transplanted cells when organisms are treated with the toxic drug ganciclovir (Zhang *et al*., 2011). However, the potential risks of transplanting stem cells in the eye may have been exaggerated because intraocular cell movement is restrained and immune reactions muted.

1.6.1 Survival and migration of intraocular transplanted stem cells

In the eye, MSC survive for at least 3 - 5 weeks (Johnson *et al*., 2010, Levkovitch-Verbin *et al*., 2010, Haddad-Mashadrizeh *et al*., 2013), cluster in the vitreous body (Johnson *et al*., 2010, Haddad-Mashadrizeh *et al*., 2013) with a small number migrating into the retina with no observation of either tumorigenesis or uncontrolled proliferation (Johnson *et al*., 2010, Singh *et al*., 2012, Mendel *et al*., 2013, Mesentier-Louro *et al*., 2014, Tzameret *et al*., 2014, Tan *et al*., 2015). After subretinal transplantation, NSC remain immature for at least 7 months, barely proliferate and neither exhibit uncontrolled growth nor oncogenesis, but they do migrate from the injection site within the subretinal space (McGill *et al*., 2012, Lu *et al*.,

2013). By contrast, after *ivit* transplantation, NSC either attach to the retina and lens, where they remain (Jung *et al*., 2013), or integrate into the inner retinal layers (Grozdanic *et al*., 2006). ESC-derived RPE cells transplanted into the subretinal space of Royal College of Surgeon (RCS) rats (which spontaneously undergo RPE and subsequent photoreceptor degeneration) survive for over 200 days, preserve visual function with no evidence of either teratoma formation (Lu *et al*., 2009) nor proliferation (Vugler *et al*., 2008). Reactive retinal astrogliosis rather than penetration of the internal limiting membrane is proposed as a major limitation to retinal integration of ESC after *ivit* implantation (Banin *et al*., 2006); while, after subretinal grafting, cell migration is more extensive (Banin *et al*., 2006, Lamba *et al*., 2009) it is still hindered by the outer limiting membrane (West *et al*., 2008).

1.6.2 Immunological acceptance of intraocular transplanted stem cells

The vitreous cavity, like the anterior chamber of the eye, is an immunoprivileged environment (Jiang and Streilein, 1991) and thus amenable to cell transplantation. MSC fail to trigger an immune response when challenged with allogeneic lymphocytes and MSC-derived factors inhibit the proliferation of immunological cells (Kode *et al*., 2009, Singer and Caplan, 2011). These immunosuppressive/immunomodulatory actions of BMSC have led to Phase I (Le Blanc *et al*., 2004), Phase II (Le Blanc *et al*., 2008) and Phase III (Martin *et al*., 2010) clinical trials for the treatment of steroid refractory graft-*versus*-host disease. ADSC suppress the immune system with the same efficacy as BMSC *in vitro* (Puissant *et al*., 2005) and increase the survival rate of transplants in animal models of graft *versus* host disease (Yañez *et al*., 2006), whereas DPSC are as efficient as BMSC in the suppression of T cell proliferation *in vitro* (Pierdomenico *et al*., 2005). Thus, the failure of the host to launch immune reactions after *ivit*/subretinal implantation of MSC is probably explained by both the immune privileged status of these sites and the immunosuppressive properties of MSC. For example, immunosuppression is not required and adverse effects are not recorded after intravitreal transplantation of human BMSC (Johnson *et al*., 2010, Levkovitch-Verbin *et al*., 2010, Tzameret *et al*., 2014) and ADSC (Haddad-Mashadrizeh *et al*., 2013). Equally,

although not immunosuppressive, iPSC-derived from the somatic cells of the recipient would carry the same histocompatibility antigens as the host and do not require immunosuppression after transplantation. By contrast, ESC/NSC require immunosuppression when transplanted into the CNS since autologous transplantation is not possible (Cummings *et al*., 2005, Lu *et al*., 2012, Schwartz *et al*., 2012, Lu *et al*., 2013). For example, NSC transplantation into the subretinal space requires daily immunosuppressive treatment with cyclosporine A and dexamethasone (McGill *et al*., 2012). When transplanted into the vitreous without immunosuppression, NSC are detected in just 50% of transplanted eyes 32 days after grafting (Grozdanic *et al*., 2006), suggesting that the immunoprivileged environment of the vitreous does not sustain survival of NSC. ESC-derived RPE cells were one of the first ESC based therapies to be used in humans and early reports of subretinal transplantation as a treatment for AMD confirm their safety, although patients require immunosuppression throughout (Schwartz *et al*., 2012).

1.7 Potential of stem cells as a cellular therapy for traumatic and degenerative eye disease

A wealth of evidence exists on the use of stem cell for treating a wide array of traumatic and degenerative eye conditions which has led to a multitude of clinical trials.

1.7.1 Embryonic stem cells/induced-pluripotent stem cells

The greatest potential for cell replacement has been realised with ESC/iPSC, which are predifferentiated prior to transplantation into the eye, with the most success demonstrated in RPE/photoreceptor replacement for AMD.

ESC can be directed towards a retinal phenotype with developmental induction signals including bone morphogenetic protein (BMP) antagonists (Lamb *et al*., 1993), Wnt inhibition (Wilson and Houart, 2004) and insulin-like growth factor (IGF) treatment (Pera *et al*., 2001). Accordingly, 30% of ESC/iPSC differentiate into retinal progenitors (Ikeda *et al*., 2005),

increasing to 80% for both ESC (Lamba *et al*., 2006) and iPSC (Tucker *et al*., 2011) by incorporating BMP/Wnt inhibiton with IGF and FGF treatments. These ESC/iPSC-derived retinal progenitors successfully mature into photoreceptors as well as RPE cells and integrate into retinal explants after either co-culture with adult retina/retinal neurons (Osakada *et al*., 2008) or after the addition of a cocktail of small molecules (Osakada *et al*., 2009b). Comparisons of the gene expression profiles of ESC-derived retinal cells with primary developing foetal retinal cells using microarray analysis shows them to be highly conserved between the two cell sources throughout development (Lamba and Reh, 2011).

ESC-derived retinal progenitors, primed with FGF to form retinal neurons rather than RPE cells, successfully differentiate into photoreceptors (Hambright *et al*., 2012), integrate into the ONL (Lamba *et al*., 2009) and survive for over 3 months in the subretinal space of non-immunosuppressed mice with an intact blood-retinal barrier, with integration into the retina more significant when it is injured (Hambright *et al*., 2012). iPSC-derived photoreceptors transplanted into the subretinal space integrate into the ONL and increase retinal function as determined by ERG (Tucker *et al*., 2011). Ivit transplanted ESC topographically integrate into all the layers of the mouse retina, i.e. ESC-derived photoreceptors move to the ONL, and ESC-derived amacrine cells and RGC-like cells migrate to the inner nuclear layer/ganglion cell layer, respectively (Lamba *et al*., 2009, Reynolds and Lamba, 2013). However, integration is only possible up to 48 hours after birth, corroborating reports that in adult rats, *ivit* ESC-derived cells fail to integrate into the retina (Banin *et al*., 2006).

Both mouse (Eiraku *et al*., 2011) and human (Nakano *et al*., 2012) ESC can be induced to form a complete topographically organized retina, including the RPE. Developing photoreceptors, isolated from ESC-derived *ex vivo* retina, integrate after transplantation into mouse models of retinal degeneration (Gonzalez-Cordero *et al*., 2013). These findings have been replicated using iPSC showing the formation of a synaptically connected stratified retina (Phillips *et al*., 2012).

ESC/iPSC can be induced to differentiate into RPE cells using similar protocols to those described above, but with the omission/antagonism of FGF to bias the generation of RPE cells over neural retina (Meyer *et al*., 2009, Osakada *et al*., 2009a). These ESC/iPSC-derived RPE cells phagocytise photoreceptor outer segments (Carr *et al*., 2009a) and preserve retinal function in the RCS rats (Vugler *et al*., 2008, Carr *et al*., 2009b). A study comparing adult human ESC-derived RPE with foetal human RPE demonstrated a strong correlation in their gene expression profiles. However, iPSC-derived RPE have a distinct gene expression profile, indicating potential differences between ESC- and iPSC-derived retinal cells (Liao *et al*., 2010).

Subretinal transplantation of ESC/iPSC-derived RPE in cases of AMD require approximately 60,000 cells (Bharti *et al*., 2011) to restore RPE-mediated recycling of photoreceptor outer segments. In contrast to photoreceptor replacement, in this instance significant migration, integration and synaptogenesis is not required to achieve functional efficacy. The effectiveness of RPE replacement is already proven by the fact that current surgical intervention relies on the same principles i.e. translocating the macula to an adjacent, healthy portion of RPE (da Cruz *et al*., 2007). These attributes have led to the first clinical trial transplanting ESC-derived RPE cells in patients with AMD (Schwartz *et al*., 2012) (Figure 4).

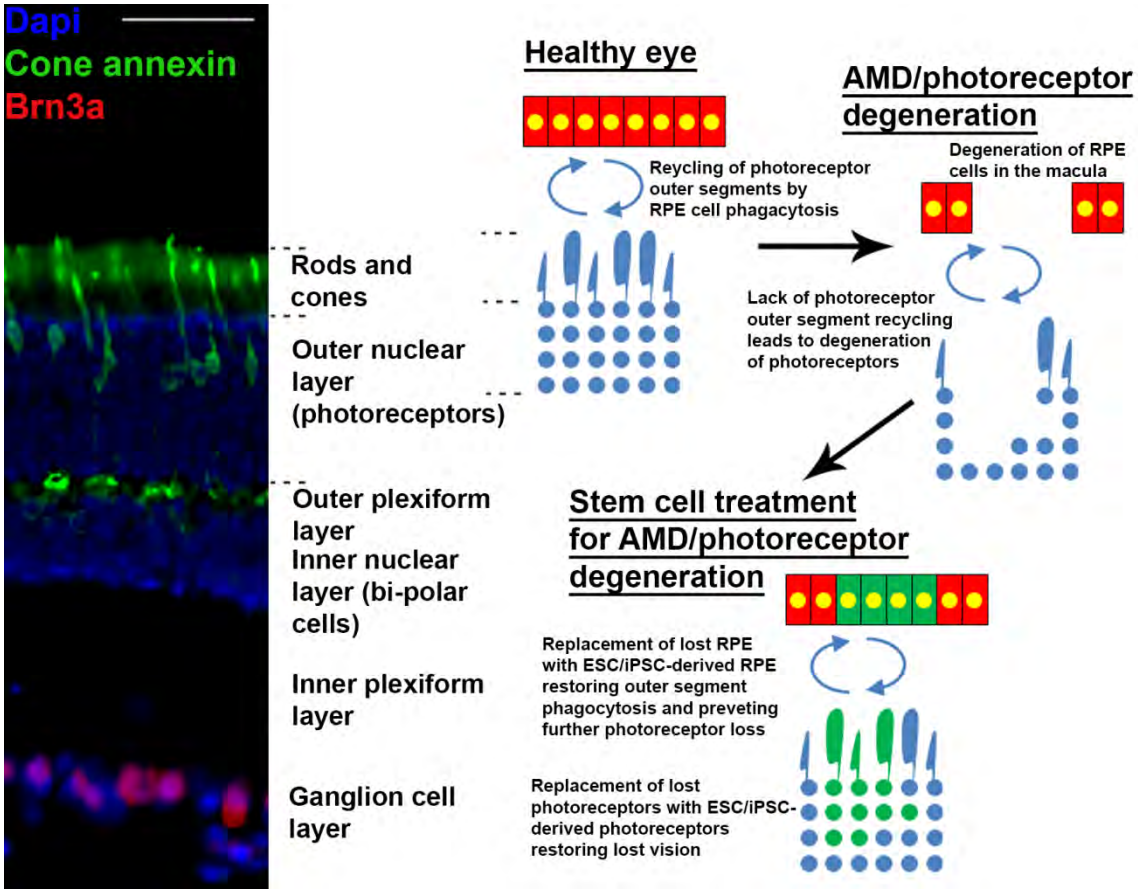


Figure 4: A diagram showing the proposed way in which ESC/iPSC-derived RPE and photoreceptors can be used to treat AMD/photoreceptor degeneration. The left panel shows a rat retina immunohistochemically stained for cone annexin (cone photoreceptor marker; green), Brn3a (RGC marker; red) and DAPI (nuclear marker; blue) with individual layers labelled (scale bar: 100µm). On the right, RPE is represented together with photoreceptor loss in AMD and the potential for cell replacement in preventing visual decline and restoring vision.

ESC/iPSC differentiate into RGC and, during the formation of ESC-/iPSC-derived retina *ex vivo*, RGC are the first cells to develop which mimics normal retinal development (Eiraku *et al*., 2011, Nakano *et al*., 2012, Phillips *et al*., 2012). The yield of RGC is enhanced by transfection of the stem cells with genes regulating RGC development, namely *math5* and *sox4* (Jiang *et al*., 2013). Similar to ESC/iPSC-derived photoreceptors integrating into the ONL, transplanted adult rat RGC integrate and survive in the ganglion cell layer (Hertz *et al*., 2014) but, unlike photoreceptors, the long distances over which RGC axons must regenerate to re-innervate central targets is unachievable (Sun *et al*., 2011).

Unlike MSC, the paracrine potential of ESC/iPSC for treating the injured retina/CNS is as yet unknown. Addition of TrK receptor blockers to ESC cultures perturbs their survival, indicating that neurotrophins are released and active in an autocrine fashion, but further analysis on the secretome is required (Pyle *et al*., 2006). *Ivit* transplantation of ESC-derived photoreceptors promotes the survival of nearby endogenous photoreceptors (Meyer *et al*., 2006). Similarly it is known that RPE cells secrete vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor, which may further explain how ESC-derived RPE protect photoreceptors from death (Strauss, 2005).

1.7.2 Neural stem cells

NSC transplantation is beneficial to recovery in a range of CNS injury models, including retinal degeneration (McGill *et al*., 2012), spinal cord injury (SCI) (Lu *et al*., 2003, Abematsu *et al*., 2010, Lu *et al*., 2012), stroke (Jeong *et al*., 2003) and traumatic brain injury (TBI) (Riess *et al*., 2002), although in many cases it is unclear if the improved functional recovery observed is attributable to replacement of lost cells and/or trophic support of surviving cells.

For example, when transplanted into injured CNS sites, such as those of SCI and TBI, NSC differentiate into neurons and glia (Riess *et al*., 2002, Jeong *et al*., 2003, Cummings *et al*., 2005, Martino and Pluchino, 2006, Abematsu *et al*., 2010, Lu *et al*., 2012), replacing lost cells and providing trophic support for damaged endogenous neurons (Lu *et al*., 2003). NSC differentiation is greatly enhanced by containment in a matrix loaded with multiple growth factors (Lu *et al*., 2012) and treatment with specific differentiation factors (Cao *et al*., 2001). In these instances, functional recovery is attributed to the generation of new NSC-derived neurons that directly integrate into the host neuronal circuitry (Martino and Pluchino, 2006, Abematsu *et al*., 2010) and not to the paracrine mediated axon regeneration and neuroprotection characteristic of MSC treatment.

Despite recent successes with NSC in other CNS injury models, few studies have shown the same effects in the eye. In rats, *ivit* transplantation of NSC after optic neuropathy induced by

elevated IOP, does not improve retinal function despite neuronal differentiation and integration into inner retinal layers (Grozdanic *et al*., 2006). A similar study using mice lacking RGC (apoptosis induced by removal of the superior colliculus) showed that NSC integrate into the retina but sparsely form βIII-tubulin^{+} mature neurons and do not form functional RGC (Mellough *et al*., 2004). Re-innervation of central targets by the axons of RGC replaced by stem cells is currently not possible. Indeed, more success has been seen in RCS rats in which the retinal degeneration is of the photoreceptors rather than the RGC (McGill *et al*., 2012). Subretinal transplantation of NSC protects photoreceptors from death in RCS rats (McGill *et al*., 2012) by phagocytosis of photoreceptor outer segments, a role usually restricted to RPE cells which, in RCS rats, are dysfunctional (Cuenca *et al*., 2013). Although a paracrine effect (i.e. secretion of NTF) has been suggested to mediate the effects of NSC in the retina, studies have only demonstrated this mechanism when NSC are genetically modified (e.g. to secrete CNTF (Jung *et al*., 2013)). The limited number of studies suggests that NSC are not currently viewed as useful for replacement of RGC. However, functional replacement of photoreceptors by NSC is more plausible because of their short synaptic distances. Despite this, integration of NSC into the ONL is not followed by differentiation into calbindin^{+}/rhodopsin^{+} mature photoreceptors (Nishida *et al*., 2000) and subretinal transplanted NSC protect, rather than replace photoreceptors (McGill *et al*., 2012, Jung *et al*., 2013).

Following transplantation into SCI lesion sites, NSC increase the expression of NGF, BDNF, NT-3 and GDNF within the lesion site (Gu *et al*., 2012, He *et al*., 2012) and promote axonal sprouting (Lu *et al*., 2003). However, the trophic support provided by undifferentiated NSC only minimally restores function when compared to strategies when they are induced to differentiate down a neuronal lineage before or after transplantation into SCI sites (Cao *et al*., 2001, Abematsu *et al*., 2010, Gu *et al*., 2012, He *et al*., 2012). In the eye, as stated above, *ivit* NSC transplanted into the vitreous fail to improve function in models of elevated IOP-induced RGC loss (Grozdanic *et al*., 2006) and axotomy (Flachsbarth *et al*., 2014), and

are neuroprotective only when transfected to secrete CNTF. However, in this study transplantation was made four weeks post-injury, so that it cannot be ruled out that NSC may be able to have a paracrine-mediated neuroprotective effect on RGC when transplanted at the time of RGC injury. Nonetheless, after subretinal transplantation of NSC in RCS rats, rather than being replaced, photoreceptors are protected against death by NSC-directed phagocytosis of photoreceptor outer segments (Cuenca *et al*., 2013) and induction of CNTF expression by Müller glia (Lu *et al*., 2013).

1.7.3 Bone marrow-derived MSC

Apparent *in vitro* neuronal differentiation and neuritogenesis of BMSC is probably artefactual, resulting from cell shrinkage and toxicity yielding cell morphologies characteristic of neurons (Lu *et al*., 2004a, Neuhuber *et al*., 2004). Undifferentiated BMSC co-express many functional ion channels (Li *et al*., 2006) as well as mature neuronal and glial markers, such as βIII-tubulin and glial fibrilliary acidic protein (GFAP), respectively (Karaoz *et al*., 2011, Tamaki *et al*., 2012) making successful phenotypic differentiation difficult to detect. The ability of BMSC to differentiate into neurons and replace those lost from injury is rarely reported *in vivo* (Vallières and Sawchenko, 2003). Their transplantation into the injury site after SCI promotes functional recovery without any evidence of neuronal replacement by BMSC differentiation (Kang *et al*., 2012).

In the eye, transplantation of BMSC into the vitreous after experimentally-induced glaucoma and optic nerve transection demonstrate no evidence of stem cell differentiation into mature retinal cells, despite some integration into the retina (Yu *et al*., 2006, Johnson *et al*., 2010, Levkovitch-Verbin *et al*., 2010, Mesentier-Louro *et al*., 2014). After transplantation into the subretinal space in RCS rats and mouse models of retinitis pigmentosa, some BMSC differentiate into cells with neuronal and glial characteristics, but not into mature photoreceptors or RPE cells (Zhang and Wang, 2010, Tzameret *et al*., 2014) and a

protective effect on endogenous photoreceptors and RPE cells is observed (Arnhold *et al*., 2007, Lu *et al*., 2010).

The neurotrophic secretome of BMSC, which includes NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF and platelet-derived growth factor (PDGF) is widely documented (Dormady *et al*., 2001, Chen *et al*., 2005, Wilkins *et al*., 2009, Ghorbanian *et al*., 2012, Sakai *et al*., 2012, Johnson *et al*., 2014) and places them as a candidate cellular therapy to combat ocular neurodegeneration. BMSC-mediated neuroprotection of RGC is reported to be mediated by PDGF (Johnson *et al*., 2014). The importance of BMSC-derived NTF for retinal neuron survival is confirmed by using TrK and PDGFR inhibitors which significantly diminish the RGC neuroprotection and/or neurite growth effects elicited by BMSC (Johnson *et al*., 2014). The vitreous does not permit the differentiation of BMSC into neurons (Hill *et al*., 2009). Nonetheless, *ivit* transplanted BMSC secrete diffusible factors directly protecting RGC from death in animal models of glaucoma (Yu *et al*., 2006, Johnson *et al*., 2010, Emre *et al*., 2015) and optic nerve transection (Levkovitch-Verbin *et al*., 2010, Mesentier-Louro *et al*., 2014, Tan *et al*., 2015), and these cells can also be indirectly effective by inducing Müller cell NTF production (Lee *et al*., 2012). Interestingly, BMSC also promote the regeneration of RGC axons after optic nerve crush (Mesentier-Louro *et al*., 2014, Tan *et al*., 2015), probably through the same NTF-mediated mechanisms (Berry *et al*., 2008). Subretinal and *ivit* BMSC transplantation in RCS rats and mouse models of retinitis pigmentosa significantly improves retinal function by preserving photoreceptor and RPE cell viability (Arnhold *et al*., 2007, Lu *et al*., 2010, Tzameret *et al*., 2014) and, although the underlying observations remain equivocal, a role for the NTF secretome in promoting cell survival is a likely explanation.

1.7.4 Adipose-derived MSC

There is conflicting evidence for the differentiation of ADSC into neurons *in vivo* and *in vitro* (Anghileri *et al*., 2008, Ye *et al*., 2010). BDNF/retinoic acid treatments induce the differentiation of ADSC into functional neurons, confirmed by patch clamp analysis and the

expression of phenotypic neuronal markers (Anghileri *et al*., 2008). The differentiation of ADSC into neuronal phenotypes, demonstrated in this and other studies (Ye *et al*., 2010) is only transient with de-differentiation occurring after withdrawal of the differentiation-inducing medium (Ye *et al*., 2010), explaining why ADSC-derived neurons are rarely seen *in vivo* after transplantation in animal models of stroke (Kang *et al*., 2003). Both studies (Kang *et al*., 2003, Ye *et al*., 2010) concluded that cerebrospinal fluid and the CNS neuropil do not sustain neuronal differentiation of ADSC. By contrast, ADSC pre-differentiated into $NG2^{+}/S100^{+}$ glia survive for up to 8 weeks after transplantation into rodent SCI sites (Arboleda *et al*., 2011) and thus, like other MSC, probably differentiate preferentially into glia *in vivo* (Cao *et al*., 2001, Cho *et al*., 2009, Leong *et al*., 2012).

ADSC survive for up to 90 days in the vitreous after transplantation, although their fate has not been studied (Haddad-Mashadrizeh *et al*., 2013). Interestingly, ADSC transplanted into the vitreous of mouse models of diabetic retinopathy preferentially differentiate into pericytes, associating with and conserving the retinal vasculature, suggesting a potentially unique role for ADSC in treating diabetic retinopathy (Mendel *et al*., 2013). The failure of ADSC to integrate into the retinal layers diminishes their potential for RGC and photoreceptor replacement (Mendel *et al*., 2013).

ADSC express NGF, BDNF, NT-3, GDNF, VEGF and PDGF (Kalbermatten *et al*., 2011, Zhou *et al*., 2013), with titres of BDNF and VEGF being significantly higher than those secreted by BMSC (Zhou *et al*., 2013). Despite this, ADSC are relatively untested in the eye but have efficacy as a paracrine-mediated therapy in other CNS injury animal models like SCI (Arboleda *et al*., 2011, Zhou *et al*., 2013) and stroke (Kang *et al*., 2003). A recent study demonstrated a neuroprotective effect of *ivit* transplanted ADSC in an experimental model of closed angle glaucoma with similar efficacy to that of BMSC (Emre *et al*., 2015). In co-culture, ADSC-derived NTF promote neuroprotection and neuritogenesis of injured RGC. In a mouse model of light induced photoreceptor damage, both *ivit* ADSC and ADSC-conditioned medium preserve ONL thickness and the amplitude of the a-wave of the ERG

(Sugitani *et al*., 2013, Tsuruma *et al*., 2014). Progranulin, tissue inhibitor of metalloproteinases-1 (TIMP1) and the secreted protein rich in cysteine (SPARC) are the active agents produced by ADSC *in vitro* and, after *ivit* transplantation, have similar effects to *ivit* ADSC/ADSC conditioned medium. Together, these data suggest that ADSC have therapeutic potential for neurodegenerative conditions through NTF production, with many of the active factors different from those produced by BMSC and DPSC.

1.7.5 Dental pulp stem cells

DPSC have been reported to differentiate into functionally active neurons *in vitro* (Arthur *et al*., 2008, Kiraly *et al*., 2009, Gervois *et al*., 2014). Following transplantation, DPSC integrate and survive in injured rat brain tissue for at least 4 weeks (Kiraly *et al*., 2011, Fang *et al*., 2013). Other studies demonstrate that, although DPSC-derived neurons express neuronal phenotypic markers, they neither generate action potentials nor form functional neuronal networks (Aanismaa *et al*., 2012). Like BMSC, they constitutively express mature neuronal and glial phenotypic markers even in an undifferentiated state and this may explain the contradictions in the literature, if these characteristics are taken as a read out of successful differentiation (Karaoz *et al*., 2011, Tamaki *et al*., 2012). *In* vivo, transplantation of DPSC into rat SCI lesion sites leads to functional recovery yet only glial, not neuronal differentiation is observed (Sakai *et al*., 2012), suggesting that differentiation of DPSC into neurons is possible *in vitro* but currently has not yet been realised *in vivo*.

Like other MSC, DPSC express (gene expression, as measured by PCR) multiple NTF including *ngf*, *bdnf*, *nt-3*, *gdnf*, *vegf* and *pdgf* (Nosrat *et al*., 1997, Nosrat *et al*., 2001, Gale *et al*., 2011, Sakai *et al*., 2012). Interestingly, DPSC express significantly greater amounts of *ngf*, *bdnf* and *nt-3* mRNA than BMSC (Sakai *et al*., 2012). DPSC-conditioned medium containing the above factors promotes neurite outgrowth of both cortical neurons (Sakai *et al*., 2012) and a neuroblastoma cell line (Ishizaka *et al*., 2013) with significantly greater efficacy than BMSC-conditioned medium. DPSC transplanted into mouse hippocampus

increase the basal expression levels of many NTF such as CNTF, VEGF, FGF-2 and NGF (Huang *et al*., 2008), although it is unknown if the transplanted DPSC directly expressed these NTF and/or indirectly promoted the expression of NTF by neighbouring cells in the surrounding neuropil.

DPSC transplantation into rat SCI lesion sites leads to greater functional improvement than after BMSC transplantation and, with a lack of observable neuronal differentiation, the data strongly suggests a paracrine-mediated mechanism (Sakai *et al*., 2012). Currently, no evidence exists for DPSC-mediated protection of retinal cells and/or regeneration of their axons. However, current evidence from other areas of the CNS strongly suggests that DPSC may be an applicable cellular therapy that warrants further research.

1.8 Aims and hypotheses

1.8.1 Aims

This project aimed to assess the efficacy of DPSC as a paracrine-mediated therapy for traumatic (TON) and degenerative (glaucoma) eye disease, in comparison to BMSC and ADSC, two more widely researched MSC. Firstly, the accuracy and reliability of counting RGC in radial sections of retina was assessed and compared to the current gold standard method of retinal wholemounts. This was necessary as retinal wholemounts are not ideal for assessing RGC numbers in stem cell transplanted animals as it renders the tissue unusable for further analyses, such as assessment of grafted stem cell survival and migration. The second aim was to compare the axogenic and neuroprotective properties of DPSC and BMSC after transplantation into the vitreous of an animal model of TON. The third aim was to determine the mechanism by which MSC including DPSC, BMSC and ADSC induced the survival of RGC and regeneration of their axons by co-culturing them with injured RGC with NTF receptor blockers. The final aim was to compare the potential benefits of DPSC, BMSC and ADSC as paracrine-mediated cellular therapies for glaucoma, assessing their ability not

only to protect RGC from death but also preserve the function of the retina and ultimately, vision.

1.8.2 Hypotheses

The first hypothesis (1) proposes that the technique used to quantify RGC numbers in radial sections of retina, is a reliable and accurate measure of RGC survival. The second hypothesis (2) proposes that, following transplantation into the vitreous of animals after an optic nerve crush, both BMSC and DPSC will promote survival of RGC and regeneration of their axons, with DPSC as the most efficacious. Further (3), the mechanism behind the neuroprotective and axogenic properties of DPSC/BMSC relates to a wide array MSC-derived NTF which activate their cognate receptors on RGC and promote survival and regeneration of their axons. Finally (4), it is hypothesised that, when transplanted into the vitreous of animals after induction of glaucoma, DPSC and, to a lesser extent BMSC and ADSC promote the protection of RGC and preserve retinal function.

1.9 Experimental approach

To test these hypotheses, (1) optic nerves were crushed and RGC death determined by quantification of $Brn3a^+$ RGC in radial retinal sections/wholemounts and retrogradle FluoroGold back-labelled RGC in wholemounts. Hypothesis 2 was tested by transplanting either DPSC or BMSC (dead or alive) into the vitreous of animals after optic nerve crush. Survival and regeneration were assessed using immunohistochemistry and OCT. Hypothesis 3 was tested by culturing the stem cells with primary injured RGC, separated from each other by a permeable membrane to allow only diffusible factors to be the means of interaction. Alongside this, highly specialised inhibitors of select NTF receptors were used to discover the relative importance of each secretable NTF. Hypothesis 4 was tested using a TGF-β mediated model of glaucoma in which DPSC, BMSC or ADSC were intravitreally transplanted. After 5 weeks, the retinae were analysed using ERG (function), OCT (morphology) and immunohistochemistry (RGC quantification).

References

Aanismaa R, Hautala J, Vuorinen A, Miettinen S, Narkilahti S (2012) Human dental pulp stem cells differentiate into neural precursors but not into mature functional neurons Stem Cell Discovery 2:85-91.

Abematsu M, Tsujimura K, Yamano M, Saito M, Kohno K, Kohyama J, Namihira M, Komiya S, Nakashima K (2010) Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. The Journal of Clinical Investigation 120:3255-3266.

Ahmed Z, Kalinski H, Berry M, Almasieh M, Ashush H, Slager N, Brafman A, Spivak I, Prasad N, Mett I, Shalom E, Alpert E, Di Polo A, Feinstein E, Logan A (2011) Ocular neuroprotection by siRNA targeting caspase-2. Cell Death & Disease 2 e173.

Anghileri E, Marconi S, Pignatelli A, Cifelli P, Galie M, Sbarbati A, Krampera M, Belluzzi O, Bonetti B (2008) Neuronal Differentiation Potential of Human Adipose-Derived Mesenchymal Stem Cells. Stem Cells and Development 17:909-916.

Arboleda D, Forostyak S, Jendelova P, Marekova D, Amemori T, Pivonkova H, Masinova K, Sykova E (2011) Transplantation of Predifferentiated Adipose-Derived Stromal Cells for the Treatment of Spinal Cord Injury. Cell Mol Neurobiol 31:1113-1122.

Arnhold S, Absenger Y, Klein H, Addicks K, Schraermeyer U (2007) Transplantation of bone marrow-derived mesenchymal stem cells rescue photoreceptor cells in the dystrophic retina of the rhodopsin knockout mouse. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie 245:414-422.

Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S (2008) Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 26:1787-1795.

Banin E, Obolensky A, Idelson M, Hemo I, Reinhardtz E, Pikarsky E, Ben-Hur T, Reubinoff B (2006) Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells. Stem Cells 24:246-257.

Barde Y-A (1989) Trophic factors and neuronal survival. Neuron 2:1525-1534.

Bazan E, Alonso FJM, Redondo C, Lopez-Toledano MA, Alfaro JM, Reimers D, Herranz AS, Paino CL, Serrano AB, Cobacho N, Caso E, Lobo MVT (2004) In vitro and in vivo characterization of neural stem cells. Histol Histopathol 19:1261-1275.

Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ (1994) Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. The Journal of Neuroscience : the official journal of the Society for Neuroscience 14:4368-4374.

Berry M, Ahmed Z, Lorber B, Douglas M, Logan A (2008) Regeneration of axons in the visual system. Restorative Neurology and Neuroscience 26:147-174.

Bharti K, Miller SS, Arnheiter H (2011) The new paradigm: retinal pigment epithelium cells generated from embryonic or induced pluripotent stem cells. Pigment Cell & Melanoma Research 24:21-34.

Bohm MRR, Pfrommer S, Chiwitt C, Bruckner M, Melkonyan H, Thanos S (2012) Crystallin-beta-b2-Overexpressing NPCs Support the Survival of Injured Retinal Ganglion Cells and Photoreceptors in Rats. Investigative Ophthalmology & Visual Science 53:8265-8279.

Butowt R, von Bartheld CS (2003) Connecting the dots: trafficking of neurotrophins, lectins and diverse pathogens by binding to the neurotrophin receptor p75(NTR). European Journal of Neuroscience 17:673-680.

Cao QL, Zhang YP, Howard RM, Walters WM, Tsoulfas P, Whittemore SR (2001) Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp Neurol 167:48-58.

Carr AJ, Vugler A, Lawrence J, Chen LL, Ahmado A, Chen FK, Semo M, Gias C, da Cruz L, Moore HD, Walsh J, Coffey PJ (2009a) Molecular characterization and functional analysis of

phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Molecular Vision 15:283-295.

Carr AJ, Vugler AA, Hikita ST, Lawrence JM, Gias C, Chen LL, Buchholz DE, Ahmado A, Semo M, Smart MJ, Hasan S, da Cruz L, Johnson LV, Clegg DO, Coffey PJ (2009b) Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. Plos One 4:e8152.

Chai Y, Jiang X, Ito Y, Bringas P, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM (2000) Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. Development 127:1671-1679.

Chen H, Weber AJ (2004) Brain-derived neurotrophic factor reduces TrkB protein and mRNA in the normal retina and following optic nerve crush in adult rats. Brain Res 1011:99-106.

Chen Q, Long Y, Yuan X, Zou L, Sun J, Chen S, Perez-Polo JR, Yang K (2005) Protective effects of bone marrow stromal cell transplantation in injured rodent brain: synthesis of neurotrophic factors. J Neurosci Res 80:611-619.

Cho S-R, Kim YR, Kang H-S, Yim SH, Park C-i, Min YH, Lee BH, Shin JC, Lim J-B (2009) Functional Recovery After the Transplantation of Neurally Differentiated Mesenchymal Stem Cells Derived From Bone Barrow in a Rat Model of Spinal Cord Injury. Cell transplantation 18:1359-1368.

Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlstrom H, Lendahl U, Frisen J (2000) Generalized potential of adult neural stem cells. Science 288:1660-1663.

Coffey PJ, Girman S, Wang SM, Hetherington L, Keegan DJ, Adamson P, Greenwood J, Lund RD (2002) Long-term preservation of cortically dependent visual function in RCS rats by transplantation. Nat Neurosci 5:53-56.

Cuenca N, Fernandez-Sanchez L, McGill TJ, Lu B, Wang SM, Lund R, Huhn S, Capela A (2013) Phagocytosis of Photoreceptor Outer Segments by Transplanted Human Neural Stem Cells as a Neuroprotective Mechanism in Retinal Degeneration. Investigative Ophthalmology & Visual Science 54:6745-6756.

Cummings BJ, Uchida N, Tamaki SJ, Salazar DL, Hooshmand M, Summers R, Gage FH, Anderson AJ (2005) Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc Natl Acad Sci U S A 102:14069-14074.

da Cruz L, Chen FK, Ahmado A, Greenwood J, Coffey P (2007) RPE transplantation and its role in retinal disease. Prog Retin Eye Res 26:598-635.

Dawbarn D, Allen SJ (2003) Neurotrophins and neurodegeneration. Neuropathology and applied neurobiology 29:211-230.

Doi A, Park IH, Wen B, Murakami P, Aryee MJ, Irizarry R, Herb B, Ladd-Acosta C, Rho J, Loewer S, Miller J, Schlaeger T, Daley GQ, Feinberg AP (2009) Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. Nat Genet 41:1350-1353.

Dormady SP, Bashayan O, Dougherty R, Zhang XM, Basch RS (2001) Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. Journal of hematotherapy & stem cell research 10:125-140.

Dougherty KD, Dreyfus CF, Black IB (2000) Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. Neurobiol Dis 7:574-585.

Eiraku M, Takata N, Ishibashi H, Kawada M, Sakakura E, Okuda S, Sekiguchi K, Adachi T, Sasai Y (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472:51-56.

Emre E, Yuksel N, Duruksu G, Pirhan D, Subasi C, Erman G, Karaoz E (2015) Neuroprotective effects of intravitreally transplanted adipose tissue and bone marrow-derived mesenchymal stem cells in an experimental ocular hypertension model. Cytotherapy.

Fang C-z, Yang Y-j, Wang Q-h, Yao Y, Zhang X-y, He X-h (2013) Intraventricular Injection of Human Dental Pulp Stem Cells Improves Hypoxic-Ischemic Brain Damage in Neonatal Rats. Plos One 8:e66748.

Faulkner JR, Herrmann JE, Woo MJ, Tansey KE, Doan NB, Sofroniew MV (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. The Journal of Neuroscience : the official journal of the Society for Neuroscience 24:2143-2155.

Flachsbarth K, Kruszewski K, Jung G, Jankwiak W, Riecken K, Wagenfeld L, Richard G, Fehse B, Bartsch U (2014). Neural stem cell-based intraocular administration of ciliary neurotrophic factor attentuates the loss of axotomized ganglion cells in adult mice. Invest Ophthalmol Vis Sci 2014;55:7029-7039

Friedenstein AJ, Piatetzky S, II, Petrakova KV (1966) Osteogenesis in transplants of bone marrow cells. Journal of embryology and experimental morphology 16:381-390.

Gale Z, Cooper PR, Scheven BAA (2011) Effects of Glial Cell Line-derived Neurotrophic Factor on Dental Pulp Cells. Journal of Dental Research 90:1240-1245.

Gervois P, Struys T, Hilkens P, Bronckaers A, Politis C, Brône B, Lambrichts I, and Martens W (2014) The human dental pulp as a source for stem cells with neurogenic differentiation potential. EACMFS Congress Proceedings 159-159

Ghorbanian MT, Haji Ghasem Kashani M, Lashkarblouki T, Hosseinpour L, Mirzaiyan L (2012) Characterization of in vitro cultured bone marrow and adipose tissue-derived mesenchymal stem cells and their ability to express neurotrophic factors. Cell Biol Int 36 (12):1239-1249.

Gonzalez-Cordero A, West EL, Pearson RA, Duran Y, Carvalho LS, Chu CJ, Naeem A, Blackford SJI, Georgiadis A, Lakowski J, Hubank M, Smith AJ, Bainbridge JWB, Sowden JC, Ali RR (2013) Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures integrate and mature within adult degenerate retina. Nat Biotech 31:741-747.

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S (2002) Stem cell properties of human dental pulp stem cells. J Dent Res 81:531-535.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America 97:13625-13630.

Grozdanic SD, Ast AM, Lazic T, Kwon YH, Kardon RH, Sonea IM, Sakaguchi DS (2006) Morphological integration and functional assessment of transplanted neural progenitor cells in healthy and acute ischemic rat eyes. Experimental Eye Research 82:597-607.

Gu YL, Yin LW, Zhang Z, Liu J, Liu SJ, Zhang LF, Wang TH (2012) Neurotrophin Expressions in Neural Stem Cells Grafted Acutely to Transected Spinal Cord of Adult Rats Linked to Functional Improvement. Cell Mol Neurobiol 32:1089-1097.

Haddad-Mashadrizeh A, Bahrami AR, Matin MM, Edalatmanesh MA, Zomorodipour A, Gardaneh M, Farshchian M, Momeni-Moghaddam M (2013) Human adipose-derived mesenchymal stem cells can survive and integrate into the adult rat eye following xenotransplantation. Xenotransplantation 20:165-176.

Hambright D, Park KY, Brooks M, McKay R, Swaroop A, Nasonkin IO (2012) Long-term survival and differentiation of retinal neurons derived from human embryonic stem cell lines in un-immunosuppressed mouse retina. Molecular Vision 18:920-936.

Harper MM, Grozdanic SD, Blits B, Kuehn MH, Zamzow D, Buss JE, Kardon RH, Sakaguchi DS (2011) Transplantation of BDNF-Secreting Mesenchymal Stem Cells Provides Neuroprotection in Chronically Hypertensive Rat Eyes. Investigative Ophthalmology & Visual Science 52:4506-4515.

He B-L, Ba Y-c, Wang X-y, Liu S-j, Liu G-d, Ou S, Gu Y-l, Pan X-h, Wang T-H (2012) BDNF expression with functional improvement in transected spinal cord treated with neural stem cells in adult rats. Neuropeptides 47(1):1-7.

Hertz J, Qu B, Hu Y, Patel RD, Valenzuela DA, Goldberg JL (2014) Survival and integration of developing and progenitor-derived retinal ganglion cells following transplantation. Cell Transplantation 23:855-872.

Hill AJ, Zwart I, Tam HH, Chan J, Navarrete C, Jen LS, Navarrete R (2009) Human umbilical cord blood-derived mesenchymal stem cells do not differentiate into neural cell types or integrate into the retina after intravitreal grafting in neonatal rats. Stem Cells Dev 18:399-409.

Huang AH-C, Snyder BR, Cheng P-H, Chan AWS (2008) Putative Dental Pulp-Derived Stem/Stromal Cells Promote Proliferation and Differentiation of Endogenous Neural Cells in the Hippocampus of Mice. STEM CELLS 26:2654-2663.

Huang GTJ, Gronthos S, Shi S (2009) Mesenchymal Stem Cells Derived from Dental Tissues vs. Those from Other Sources: Their Biology and Role in Regenerative Medicine. Journal of Dental Research 88:792-806.

Ikeda H, Osakada F, Watanabe K, Mizuseki K, Haraguchi T, Miyoshi H, Kamiya D, Honda Y, Sasai N, Yoshimura N, Takahashi M, Sasai Y (2005) Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc Natl Acad Sci U S A 102:11331-11336.

Ishizaka R, Hayashi Y, Iohara K, Sugiyama M, Murakami M, Yamamoto T, Fukuta O, Nakashima M (2013) Stimulation of angiogenesis, neurogenesis and regeneration by side population cells from dental pulp. Biomaterials 34:1888-1897.

Jeong SW, Chu K, Jung KH, Kim SU, Kim M, Roh JK (2003) Human neural stem cell transplantation promotes functional recovery in rats with experimental intracerebral hemorrhage. Stroke; a journal of cerebral circulation 34:2258-2263.

Jiang LQ, Streilein JW (1991) Immune privilege extended to allogeneic tumor cells in the vitreous cavity. Invest Ophthalmol Vis Sci 32:224-228.

Jiang Y, Ding Q, Xie X, Libby RT, Lefebvre V, Gan L (2013) Transcription factors SOX4 and SOX11 function redundantly to regulate the development of mouse retinal ganglion cells. The Journal of Biological Chemistry 288:18429-18438.

Johnson TV, Bull ND, Hunt DP, Marina N, Tomarev SI, Martin KR (2010) Neuroprotective Effects of Intravitreal Mesenchymal Stem Cell Transplantation in Experimental Glaucoma. Investigative Ophthalmology & Visual Science 51:2051-2059.

Johnson TV, Dekorver NW, Levasseur VA, Osborne A, Tassoni A, Lorber B, Heller JP, Villasmil R, Bull ND, Martin KR, Tomarev SI (2014) Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain 137:503-519.

Jones LL, Oudega M, Bunge MB, Tuszynski MH (2001) Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. The Journal of Physiology 533:83-89.

Jung GL, Sun J, Petrowitz B, Riecken K, Kruszewski K, Jankowiak W, Kunst F, Skevas C, Richard G, Fehse B, Bartsch U (2013) Genetically Modified Neural Stem Cells for a Local and Sustained Delivery of Neuroprotective Factors to the Dystrophic Mouse Retina. Stem Cells Translational Medicine 2:1001-1010.

Kalbermatten DF, Schaakxs D, Kingham PJ, Wiberg M (2011) Neurotrophic activity of human adipose stem cells isolated from deep and superficial layers of abdominal fat. Cell Tissue Res 344:251-260.

Kang KN, Kim da Y, Yoon SM, Lee JY, Lee BN, Kwon JS, Seo HW, Lee IW, Shin HC, Kim YM, Kim HS, Kim JH, Min BH, Lee HB, Kim MS (2012) Tissue engineered regeneration of completely transected spinal cord using human mesenchymal stem cells. Biomaterials 33:4828-4835.

Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, Jung JS (2003) Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Experimental Neurology 183:355-366.

Karaoz E, Demircan PC, Saglam O, Aksoy A, Kaymaz F, Duruksu G (2011) Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells. Histochemistry and Cell Biology 136:455-473.

Kawashima N (2012) Characterisation of dental pulp stem cells: A new horizon for tissue regeneration? Arch Oral Biol.

Kern S, Eichler H, Stoeve J, Kluter H, Bieback K (2006) Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells 24:1294-1301.

Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LI, Yabuuchi A, Takeuchi A, Cunniff KC, Hongguang H, McKinney-Freeman S, Naveiras O, Yoon TJ, Irizarry RA, Jung N, Seita J, Hanna J, Murakami P, Jaenisch R, Weissleder R, Orkin SH, Weissman IL, Feinberg AP, Daley GQ (2010) Epigenetic memory in induced pluripotent stem cells. Nature 467:285-290.

Kingham PJ, Kalbermatten DF, Mahay D, Armstrong SJ, Wiberg M, Terenghi G (2007) Adipose-derived stem cells differentiate into a Schwann cell phenotype and promote neurite outgrowth in vitro. Experimental Neurology 207:267-274.

Kiraly M, Kadar K, Horvathy DB, Nardai P, Racz GZ, Lacza Z, Varga G, Gerber G (2011) Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo. Neurochemistry International 59:371-381.

Kiraly M, Porcsalmy B, Pataki A, Kadar K, Jelitai M, Molnar B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G (2009) Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochemistry International 55:323-332.

Ko ML, Hu DN, Ritch R, Sharma SC (2000) The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. Invest Ophthalmol Vis Sci 41:2967-2971.

Ko ML, Hu DN, Ritch R, Sharma SC, Chen CF (2001) Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. Neuroscience Letters 305:139-142.

Kode JA, Mukherjee S, Joglekar MV, Hardikar AA (2009) Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. Cytotherapy 11:377-391.

Lamb TM, Knecht AK, Smith WC, Stachel SE, Economides AN, Stahl N, Yancopolous GD, Harland RM (1993) Neural induction by the secreted polypeptide noggin. Science 262:713-718.

Lamba DA, Gust J, Reh TA (2009) Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell stem cell 4:73-79.

Lamba DA, Karl MO, Ware CB, Reh TA (2006) Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc Natl Acad Sci U S A 103:12769-12774.

Lamba DA, Reh TA (2011) Microarray characterization of human embryonic stem cell--derived retinal cultures. Invest Ophthalmol Vis Sci 52:4897-4906.

Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, Lanino E, Sundberg B, Bernardo ME, Remberger M, Dini G, Egeler RM, Bacigalupo A, Fibbe W, Ringden O, M DCEGB (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371:1579-1586.

Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O (2004) Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 363:1439-1441.

Lee JY, Shin JM, Yeum CE, Chae GT, Chun MH, Oh SJ (2012) Intravitreal delivery of mesenchymal stem cells loaded onto hydrogel affects the regulatory expression of endogenous NGF and BDNF in ischemic rat retina. Tissue Eng Regen Med 9:249-258.

Leong WK, Henshall TL, Arthur A, Kremer KL, Lewis MD, Helps SC, Field J, Hamilton-Bruce MA, Warming S, Manavis J, Vink R, Gronthos S, Koblar SA (2012) Human Adult Dental Pulp Stem Cells Enhance Poststroke Functional Recovery Through Non-Neural Replacement Mechanisms. Stem Cells Translational Medicine 1:177-187.

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60:585-595.

Levkovitch-Verbin H, Sadan O, Vander S, Rosner M, Barhum Y, Melamed E, Offen D, Melamed S (2010) Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are

neuroprotective in rat eyes following optic nerve transection. Invest Ophthalmol Vis Sci 51:6394-6400.
Li GR, Deng XL, Sun H, Chung SS, Tse HF, Lau CP (2006) Ion channels in mesenchymal stem cells from rat bone marrow. Stem Cells 24:1519-1528.
Liao JL, Yu J, Huang K, Hu J, Diemer T, Ma Z, Dvash T, Yang XJ, Travis GH, Williams DS, Bok D, Fan G (2010) Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. Human Molecular Genetics 19:4229-4238.
Logan A, Ahmed Z, Baird A, Gonzalez AM, Berry M (2006) Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. Brain 129:490-502.
Lu B, Malcuit C, Wang S, Girman S, Francis P, Lemieux L, Lanza R, Lund R (2009) Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. Stem Cells 27:2126-2135.
Lu B, Morgans CW, Girman S, Luo J, Zhao J, Du H, Lim S, Ding S, Svendsen C, Zhang K, Wang S (2013) Neural Stem Cells Derived by Small Molecules Preserve Vision. Translational Vision Science & Technology 2:1.
Lu B, Wang S, Girman S, McGill T, Ragaglia V, Lund R (2010) Human adult bone marrow-derived somatic cells rescue vision in a rodent model of retinal degeneration. Exp Eye Res 91:449-455.
Lu P, Blesch A, Tuszynski MH (2004a) Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J Neurosci Res 77:174-191.
Lu P, Jones LL, Snyder EY, Tuszynski MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol 181:115-129.
Lu P, Wang Y, Graham L, McHale K, Gao M, Wu D, Brock J, Blesch A, Rosenzweig ES, Havton LA, Zheng B, Conner JM, Marsala M, Tuszynski MH (2012) Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell 150:1264-1273.
Lu P, Yang H, Jones LL, Filbin MT, Tuszynski MH (2004b) Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. The Journal of neuroscience : the official journal of the Society for Neuroscience 24:6402-6409.
Machalińska A, Kawa M, Pius-Sadowska E, Stępniewski J, Nowak W, Rogińska D, Kaczyńska K, Baumert B, Wiszniewska B, Józkowicz A, Dulak J, Machaliński B (2013) Long-Term Neuroprotective Effects of NT-4–Engineered Mesenchymal Stem Cells Injected Intravitreally in a Mouse Model of Acute Retinal Injury. Investigative Ophthalmology & Visual Science 54:8292-8305.
MacLaren RE, Pearson RA, MacNeil A, Douglas RH, Salt TE, Akimoto M, Swaroop A, Sowden JC, Ali RR (2006) Retinal repair by transplantation of photoreceptor precursors. Nature 444:203-207.
Martin PJ, Uberti JP, Soiffer RJ, Klingemann H, Waller EK, Daly A, Herrmann RP, Visani G, Bernardo ME, Schwerdtfeger R, Kebriaei P (2010) Prochymal (R) improves response rates in patients with steroid-refractory acute graft-versus-host disease involving the liver and gut: results of a randomized, placebo-controlled, multicentre phase III trial in GvHD. Bone Marrow Transpl 45:S17-S17.
Martino G, Pluchino S (2006) The therapeutic potential of neural stem cells. Nature Reviews Neuroscience 7:395-406.
McGill TJ, Cottam B, Lu B, Wang S, Girman S, Tian C, Huhn SL, Lund RD, Capela A (2012) Transplantation of human central nervous system stem cells - neuroprotection in retinal degeneration. The European Journal of Neuroscience 35:468-477.
Mead B, Berry M, Logan A, Scott RAH, Leadbeater W, Scheven BA (2015) Stem cell treatment of degenerative eye disease. Stem cell research 14:243-257.
Mead B and Scheven BA (2015) Mesenchymal stem cell therapy for retinal ganglion cell neuroprotection and axon regeneration. Neural Regen Res 10(3):373-373

Mellough CB, Cui Q, Spalding KL, Symons NA, Pollett MA, Snyder EY, Macklis JD, Harvey AR (2004) Fate of multipotent neural precursor cells transplanted into mouse retina selectively depleted of retinal ganglion cells. Experimental Neurology 186:6-19.

Mendel TA, Clabough EB, Kao DS, Demidova-Rice TN, Durham JT, Zotter BC, Seaman SA, Cronk SM, Rakoczy EP, Katz AJ, Herman IM, Peirce SM, Yates PA (2013) Pericytes derived from adipose-derived stem cells protect against retinal vasculopathy. Plos One 8:e65691.

Mesentier-Louro LA, Zaverucha-do-Valle C, da Silva-Junior AJ, Nascimento-Dos-Santos G, Gubert F, de Figueiredo AB, Torres AL, Paredes BD, Teixeira C, Tovar-Moll F, Mendez-Otero R, Santiago MF (2014) Distribution of mesenchymal stem cells and effects on neuronal survival and axon regeneration after optic nerve crush and cell therapy. Plos One 9:e110722.

Mey J, Thanos S (1993) Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. Brain Res 602:304-317.

Meyer JS, Katz ML, Maruniak JA, Kirk MD (2006) Embryonic stem cell-derived neural progenitors incorporate into degenerating retina and enhance survival of host photoreceptors. Stem Cells 24:274-283.

Meyer JS, Shearer RL, Capowski EE, Wright LS, Wallace KA, McMillan EL, Zhang SC, Gamm DM (2009) Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc Natl Acad Sci U S A 106:16698-16703.

Minguell JJ, Erices A, Conget P (2001) Mesenchymal stem cells. Exp Biol Med (Maywood) 226:507-520.

Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, Shi S (2003) SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 100:5807-5812.

Morgan-Warren PJ, Berry M, Ahmed Z, Scott RA, Logan A (2013) Exploiting mTOR signaling: a novel translatable treatment strategy for traumatic optic neuropathy? Invest Ophthalmol Vis Sci 54:6903-6916.

Nadal-Nicolas FM, Jimenez-Lopez M, Sobrado-Calvo P, Nieto-Lopez L, Canovas-Martinez I, Salinas-Navarro M, Vidal-Sanz M, Agudo M (2009) Brn3a as a Marker of Retinal Ganglion Cells: Qualitative and Quantitative Time Course Studies in Naive and Optic Nerve-Injured Retinas. Investigative Ophthalmology & Visual Science 50:3860-3868.

Nakano T, Ando S, Takata N, Kawada M, Muguruma K, Sekiguchi K, Saito K, Yonemura S, Eiraku M, Sasai Y (2012) Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell stem cell 10:771-785.

Neuhuber B, Gallo G, Howard L, Kostura L, Mackay A, Fischer I (2004) Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J Neurosci Res 77:192-204.

Nishida A, Takahashi M, Tanihara H, Nakano I, Takahashi JB, Mizoguchi A, Ide C, Honda Y (2000) Incorporation and differentiation of hippocampus-derived neural stem cells transplanted in injured adult rat retina. Invest Ophthalmol Vis Sci 41:4268-4274.

Nosrat CA, Fried K, Lindskog S, Olson L (1997) Cellular expression of neurotrophin mRNAs during tooth development. Cell Tissue Res 290:569-580.

Nosrat IV, Widenfalk J, Olson L, Nosrat CA (2001) Dental Pulp Cells Produce Neurotrophic Factors, Interact with Trigeminal Neurons in Vitro, and Rescue Motoneurons after Spinal Cord Injury. Developmental Biology 238:120-132.

Osakada F, Ikeda H, Mandai M, Wataya T, Watanabe K, Yoshimura N, Akaike A, Sasai Y, Takahashi M (2008) Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nature Biotechnology 26:215-224.

Osakada F, Ikeda H, Sasai Y, Takahashi M (2009a) Stepwise differentiation of pluripotent stem cells into retinal cells. Nat Protoc 4:811-824.

Osakada F, Jin ZB, Hirami Y, Ikeda H, Danjyo T, Watanabe K, Sasai Y, Takahashi M (2009b) In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. Journal of Cell Science 122:3169-3179.

Pakulska MM, Ballios BG, Shoichet MS (2012) Injectable hydrogels for central nervous system therapy. Biomed Mater 7:024101.

Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. Science 322:963-966.

Pearson RA, Barber AC, Rizzi M, Hippert C, Xue T, West EL, Duran Y, Smith AJ, Chuang JZ, Azam SA, Luhmann UF, Benucci A, Sung CH, Bainbridge JW, Carandini M, Yau KW, Sowden JC, Ali RR (2012) Restoration of vision after transplantation of photoreceptors. Nature 485:99-103.

Pera EM, Wessely O, Li SY, De Robertis EM (2001) Neural and head induction by insulin-like growth factor signals. Developmental Cell 1:655-665.

Phillips MJ, Wallace KA, Dickerson SJ, Miller MJ, Verhoeven AD, Martin JM, Wright LS, Shen W, Capowski EE, Percin EF, Perez ET, Zhong X, Canto-Soler MV, Gamm DM (2012) Blood-derived human iPS cells generate optic vesicle-like structures with the capacity to form retinal laminae and develop synapses. Invest Ophthalmol Vis Sci 53:2007-2019.

Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP (2005) Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 80:836-842.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143-147.

Puissant N, Barreau C, Bourin P, Clavel C, Corre J, Bousquet C, Taureau C, Cousin B, Abbal M, Laharrague P, Penicaud L, Casteilla L, Blancher A (2005) Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Brit J Haematol 129:118-129.

Pyle AD, Lock LF, Donovan PJ (2006) Neurotrophins mediate human embryonic stem cell survival. Nature Biotechnology 24:344-350.

Quigley HA, Broman AT (2006) The number of people with glaucoma worldwide in 2010 and 2020. The British journal of ophthalmology 90:262-267.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nature biotechnology 18:399-404.

Reynolds J, Lamba DA (2013) Human embryonic stem cell applications for retinal degenerations. Exp Eye Res.

Richardson PM, McGuinness UM, Aguayo AJ (1980) Axons from CNS neurons regenerate into PNS grafts. Nature 284:264-265.

Riess P, Zhang C, Saatman KE, Laurer HL, Longhi LG, Raghupathi R, Lenzlinger PM, Lifshitz J, Boockvar J, Neugebauer E, Snyder EY, McIntosh TK (2002) Transplanted neural stem cells survive, differentiate, and improve neurological motor function after experimental traumatic brain injury. Neurosurgery 51:1043-1052; Discussion 1052-1044.

Sakai K, Yamamoto A, Matsubara K, Nakamura S, Naruse M, Yamagata M, Sakamoto K, Tauchi R, Wakao N, Imagama S, Hibi H, Kadomatsu K, Ishiguro N, Ueda M (2012) Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. Journal of Clinical Investigation 122:80-90.

Sandvig A, Berry M, Barrett LB, Butt A, Logan A (2004) Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. Glia 46:225-251.

Sarkies N (2004) Traumatic optic neuropathy. Eye 18:1122-1125.

Schwartz SD, Hubschman JP, Heilwell G, Franco-Cardenas V, Pan CK, Ostrick RM, Mickunas E, Gay R, Klimanskaya I, Lanza R (2012) Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 379:713-720.

Singer NG, Caplan AI (2011) Mesenchymal Stem Cells: Mechanisms of Inflammation. Annual Review of Pathology: Mechanisms of Disease 6:457-478.

Singh T, Prabhakar S, Gupta A, Anand A (2012) Recruitment of Stem Cells into the Injured Retina After Laser Injury. Stem Cells and Development 21:448-454.

Sofroniew MV, Howe CL, Mobley WC (2001) NERVE GROWTH FACTOR SIGNALING, NEUROPROTECTION, AND NEURAL REPAIR. Annual Review of Neuroscience 24:1217-1281.

Sommerfeld MT, Schweigreiter R, Barde YA, Hoppe E (2000) Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. The Journal of biological chemistry 275:8982-8990.

Sonoyama W, Liu Y, Yamaza T, Tuan RS, Wang S, Shi S, Huang GTJ (2008) Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: A pilot study. Journal of Endodontics 34:166-171.

Strauss O (2005) The retinal pigment epithelium in visual function. Physiological Reviews 85:845-881.

Sugitani S, Tsuruma K, Ohno Y, Kuse Y, Yamauchi M, Egashira Y, Yoshimura S, Shimazawa M, Iwama T, Hara H (2013) The potential neuroprotective effect of human adipose stem cells conditioned medium against light-induced retinal damage. Experimental Eye Research 116:254-264.

Sun F, Park KK, Belin S, Wang D, Lu T, Chen G, Zhang K, Yeung C, Feng G, Yankner BA, He Z (2011) Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. Nature 480:372-375.

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676.

Tamaki Y, Nakahara T, Ishikawa H, Sato S (2012) In vitro analysis of mesenchymal stem cells derived from human teeth and bone marrow. Odontology 101(2):121-132.

Tan HB, Kang X, Lu SH, Liu L (2015) The therapeutic effects of bone marrow mesenchymal stem cells after optic nerve damage in the adult rat. Clin Interv Aging 10:487-490.

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145-1147.

Tsuruma K, Yamauchi M, Sugitani S, Otsuka T, Ohno Y, Nagahara Y, Ikegame Y, Shimazawa M, Yoshimura S, Iwama T, Hara H (2014) Progranulin, a Major Secreted Protein of Mouse Adipose-Derived Stem Cells, Inhibits Light-Induced Retinal Degeneration. Stem Cells Translational Medicine 3:42-53.

Tucker BA, Park IH, Qi SD, Klassen HJ, Jiang C, Yao J, Redenti S, Daley GQ, Young MJ (2011) Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. Plos One 6:e18992.

Tzameret A, Sher I, Belkin M, Treves AJ, Meir A, Nagler A, Levkovitch-Verbin H, Barshack I, Rosner M, Rotenstreich Y (2014) Transplantation of human bone marrow mesenchymal stem cells as a thin subretinal layer ameliorates retinal degeneration in a rat model of retinal dystrophy. Experimental Eye Research 118:135-144.

Vallières L, Sawchenko PE (2003) Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. The Journal of Neuroscience 23:5197-5207.

Vugler A, Carr AJ, Lawrence J, Chen LL, Burrell K, Wright A, Lundh P, Semo M, Ahmado A, Gias C, da Cruz L, Moore H, Andrews P, Walsh J, Coffey P (2008) Elucidating the phenomenon of HESC-derived RPE: anatomy of cell genesis, expansion and retinal transplantation. Exp Neurol 214:347-361.

Wang X, Sha XJ, Li GH, Yang FS, Ji K, Wen LY, Liu SY, Chen L, Ding Y, Xuan K (2012) Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. Arch Oral Biol 57:1231-1240.

West EL, Pearson RA, Tschernutter M, Sowden JC, MacLaren RE, Ali RR (2008) Pharmacological disruption of the outer limiting membrane leads to increased retinal integration of transplanted photoreceptor precursors. Experimental Eye Research 86:601-611.

Wilkins A, Kemp K, Ginty M, Hares K, Mallam E, Scolding N (2009) Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. Stem cell research 3:63-70.

Wilson SW, Houart C (2004) Early steps in the development of the forebrain. Developmental cell 6:167-181.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000) Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 61:364-370.

Yañez R, Lamana ML, García-Castro J, Colmenero I, Ramírez M, Bueren JA (2006) Adipose Tissue-Derived Mesenchymal Stem Cells Have In Vivo Immunosuppressive Properties Applicable for the Control of the Graft-Versus-Host Disease. Stem Cells 24:2582-2591.

Ye CQ, Yuan XD, Liu H, Cai YA, Ou Y (2010) Ultrastructure of neuronal-like cells differentiated from adult adipose-derived stromal cells. Neural Regen Res 5:1456-1463.

Yu S, Tanabe T, Dezawa M, Ishikawa H, Yoshimura N (2006) Effects of bone marrow stromal cell injection in an experimental glaucoma model. Biochemical and Biophysical Research Communications 344:1071-1079.

Zhang R, Zhang H, Xu L, Ma K, Wallrapp C, Jonas JB (2011) Neuroprotective effect of intravitreal cell-based glucagon-like peptide-1 production in the optic nerve crush model. Acta Ophthalmol 89:e320-326.

Zhang Y, Wang W (2010) Effects of bone marrow mesenchymal stem cell transplantation on light-damaged retina. Invest Ophthalmol Vis Sci 51:3742-3748.

Zhou Z, Chen Y, Zhang H, Min S, Yu B, He B, Jin A (2013) Comparison of mesenchymal stem cells from human bone marrow and adipose tissue for the treatment of spinal cord injury. Cytotherapy.

CHAPTER 2

Comparative Evaluation of Methods for Estimating Retinal Ganglion Cell Loss in Retinal Sections and Wholemounts

Ben Mead, Adam Thompson, Ben A. Scheven, Ann Logan, Martin Berry and Wendy Leadbeater

A version of this manuscript has been published in

PlosOne

Abstract

To investigate the reliability of different methods of quantifying retinal ganglion cells (RGCs) in rat retinal sections and wholemounts from eyes with either intact optic nerves or those axotomised after optic nerve crush (ONC). Adult rats received a unilateral ONC and after 21 days the numbers of $Brn3a^+$, $\beta III\text{-tubulin}^+$ and $Islet\text{-}1^+$ RGCs were quantified in either retinal radial sections or wholemounts in which FluoroGold (FG) was injected 48h before harvesting. Phenotypic antibody markers were used to distinguish RGCs from astrocytes, macrophages/microglia and amacrine cells. In wholemounted retinae, counts of FG^+ and $Brn3a^+$ RGCs were of similar magnitude in eyes with intact optic nerves and were similarly reduced after ONC. Larger differences in RGC number were detected between intact and ONC groups when images were taken closer to the optic nerve head. In radial sections, Brn3a did not stain astrocytes, macrophages/microglia or amacrine cells, whereas βIII-tubulin and Islet-1 did localize to amacrine cells as well as RGCs. The numbers of $\beta III\text{-tubulin}^+$ RGCs was greater than $Brn3a^+$ RGCs, both in retinae from eyes with intact optic nerves and eyes 21 days after ONC. Islet-1 staining also overestimated the number of RGCs compared to Brn3a, but only after ONC. Estimates of RGC loss were similar in Brn3a-stained radial retinal sections compared to both Brn3a-stained wholemounts and retinal wholemounts in which RGCs were backfilled with FG, with sections having the added advantage of reducing experimental animal usage.

Introduction

Retinal ganglion cells (RGCs) populate the ganglion cell layer (GCL) of the retina, although in some species a few are found in the inner nuclear layer (INL; displaced RGCs)[1]. All rat RGC axons become myelinated at the lamina cribrosa[2,3], project centripetally in the optic nerve, partially decussate in the chiasma and synapse in the lateral geniculate body, superior colliculus, hypothalamus and pretectal area[3]; a few non-/thinly-myelinated peptidergic axons course centrifugally in the optic nerve[4]. Accurate estimates of the total number of RGCs are compromised by counting errors engendered by: (1), the presence of astrocytes and displaced amacrine cells, estimated to contribute up to 50% of cells in the GCL[5,6] and which co-localize with many markers used to identify RGCs[7-9]; (2), RGCs displaced into the INL[1]; and (3), a progressive decrement in RGC density from the centre of the retina to the periphery[10].

Techniques aimed at overcoming these confounding issues include: (1), use of optimal detection and sampling methods; (2), counting myelinated RGC axons in the optic nerve; (3), using phenotypic antibody markers with exclusive affinity for RGCs; (4), transfecting RGCs with green fluorescent protein under control of the Thy-1 promoter; and (5), back filling exclusively RGCs with retrogradely transported axon tracers such as FluoroGold (FG) injected into an optic nerve or both superior colliculi, for which 98% of RGC project to[3,11-13]. The precision of estimates of total RGC numbers from either retinal sections or wholemounts relies on the sampling method used. In wholemounts, the location of standard sampling areas along superior, inferior, temporal and nasal radii is designed to account for declining RGC/mm^2 at increasing radial distances. In retinal sections, sampling of RGC/mm over a standard length of the GCL in matching sections between animals is required and consistency is best achieved by counting RGCs in radial retinal sections through the optic nerve head. For wholemounts, total absolute counts of RGC number is possible[10] yet may be erroneous if calculated from samples in which the spatial differences in RGC density are not taken into account when correcting for total retinal area, or if the retina is not dissected

accurately. Nonetheless, in RGC viability studies it is generally accepted that retinal wholemount and sectional quantitative data give acceptable estimates of percentage differences in RGC numbers between treatment and control groups.

Myelinated axon counts from the optic nerve yield good estimates of the total number of RGCs in the retina[14], but monoaminergic small diameter efferent axons with thin myelin sheaths[4] are difficult to differentiate from the axons of small RGCs and thus total myelinated axon counts overestimate RGC frequency. The use of antibody phenotypic markers to identify RGCs, such as βIII-tubulin[15,16], Islet-1[7], Thy-1[17] and Brain-Specific Homeobox/POU Domain Protein 3A (Brn3a)[18] is critically dependant on antibody specificity. Thy-1 is down-regulated in RGCs in the diseased retina[19] where this antibody underestimates RGC number and βIII-tubulin and Islet-1 antibodies both cross-react with ligands expressed by amacrine cells[7-9,15] which are less likely to die than RGCs after ONC[20], and thus both antibodies exaggerate RGC survival in cases of e.g. glaucoma and optic nerve neuritis. By contrast, Brn3a exclusively stains RGCs by binding to a RGC specific nuclear epitope[18] with some exceptions such as the intrinsically photosensitive RGC that are found to be $Brn3a^-$[21]. Transfection of RGCs with the green fluorescent protein gene (*gfp*), using the Thy-1 promoter is a function of transfection efficiency and the number of cells labelled is rarely absolute, e.g. intravitreal injected adeno-assosciated virus (AAV) vectors transfect < 85% RGCs[22].

Back filling all RGCs with FG requires either bilateral injection into the superior colliculi to capture both contralateral and ipsilateral RGC projections[11,23], or unilateral injection into the optic nerve[3,24]. The technique has the potential drawbacks of: (1), gap junctional transfer of tracer between coupled cells in the GCL[12] (although transfer takes significantly longer into amacrine cells than into RGCs); (2), incomplete uptake of tracer by RGC axons at the site of injection; (3), possible toxicity[25]; (4), erroneous counting of tracer-filled macrophages/microglia which have phagocytised dying/dead FG^+ RGC[26]; and (5), the lack of persistence of FG in neuronal cytoplasm[27], including RGCs 3 weeks after administration

to the superior colliculus[11]. Since 2.5-4.2% of albino/hooded rat RGC axons project ipsilaterally to the superior colliculus[21], respectively, FG retrograde transport after injection of one superior colliculus underestimates RGC numbers in the contralateral retina in this species[28].

Another disadvantage of labelling RGCs with FG in retinal whole mount analysis is that the entire retina and optic nerve are used to generate the labelled cells leaving no tissue for extending the analysis to include correlative axon counts in the optic nerve and wider cellular and molecular evaluations of treatments, making retinal section analysis more efficient both in terms of data acquisition and animal usage[7,9,24]. However, it is not clear how reliable counting RGCs in retinal sections is compared to wholemounts where a much larger sample of the retina is analysed.

The present study aimed to define and compare the reliability of RGC counting methods including FG RGC back-filling and immuno-staining with the commonly used phenotypic markers βIII-tubulin, Islet-1 and Brn3a in rat retinal wholemounts and radial sections to determine the most reliable estimates of RGC loss after induction of RGC death by ONC.

Materials and Methods

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

Experimental design

The left optic nerve in a total of 12 rats was crushed (ONC), the right optic nerve remained intact (controls). Animals were separated into 2 groups (Table 1), each of 6 rats, which were all euthanized on day 21 after ONC. In Group 1, FG was injected into the proximal segment of both optic nerves of each rat at day 19 and, 48h later, FG back-filled RGCs, along with Brn3a-stained RGCs were counted in retinal whole mounts. Group 1 comprised retinae from right eyes with an intact optic nerve (Group 1a) and retinae from left eyes that received an ONC (Group 1b). In Group 2, βIII-tubulin-, Islet-1- and Brn3a-stained RGCs were counted in

radial sections of the retinae through the optic disk and double stained for astrocytes using glial fibrillary acidic protein (GFAP) amacrine cells using syntaxin-1[29,30], and macrophages/microglia using ED1 (Table 2). Group 2 comprised retinae from right eyes with an intact optic nerve (Group 2a) and retinae from left eyes that received an ONC (Group 2b).

Group 1 (6 rats)	RGC counted in wholemounted retinae	Intact optic nerve	Group 1a (6 eyes)
		Optic nerve crush	Group 1b (6 eyes)
Group 2 (6 rats)	RGC counted in radial retinal sections	Intact optic nerve	Group 2a (6 eyes)
		Optic nerve crush	Group 2b (6 eyes)

Table 1: Animal grouping

Animals

All animal procedures were performed in strict accordance to the UK Home Office Animals Scientific Procedures Act, 1986 and approved by the University of Birmingham Ethical Review Sub-Committee. Twelve adult female Sprague Dawley rats weighing 150-200g (8-10 weeks; Charles River, Kent, UK) were housed in conditions of 21°C and 55% humidity under a 12h light and dark cycle, given food/water *ad libitum* and supervised constantly by trained staff. Anaesthesia was induced with 5% Isoflurane/1.5l per min O_2 (National Veterinary Supplies, Stoke, UK) and maintained at 3.5% during surgery.

Surgical procedures

After anaesthetic induction as described above and a subcutaneous injection of buprenorphine (0.1ml/100g; National Veterinary Supplies) animals were secured in a head-holding frame. Intraorbital left ONC was performed in Group 1b and 2b 8-10 week old rats as described previously[31]. Briefly, the optic nerve was exposed and crushed using forceps 1mm posterior to the lamina cribrosa, completely closed around the optic nerve for 5 seconds, without damaging the central retinal artery (confirmed by lack of ischemia in eyes 7-21 days after ONC). After surgery, animals were placed in warmed (30°C) recovery cages and closely monitored until the return of normal behaviour, when they were transferred to

home cages. Two days before tissue harvest, all Group 1 animals were re-anaesthetised and the optic nerves re-exposed as above and 2μl of 4% FG solution (Biotium, Hayward, CA) in sterile phosphate-buffered saline (PBS) was injected directly into the right and left nerves distal to the lamina cribrosa (proximal to the crush site in the left optic nerves in Group 1b), using a glass micropipette, produced in-house from a glass capillary rod (Harvard Apparatus, Kent, UK) using a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA). The injected FG is incorporated into axons and retrogradely transported axonally to RGC somata.

Tissue preparation

Group 1 rats were euthanized at 21 days by rising concentration of CO_2. After removal of the cornea and lens, the residual eye cups were immersion fixed in 4% paraformaldehyde (PFA; TAAB, Reading, UK) in PBS for 2h at 4^{o}C before the retinae were removed and flattened onto Superfrost glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA) facilitated by 4 equidistant radial cuts into the peripheral retina. Wholemounts were immunohistochemically stained immediately at room temperature.

Group 2 rats were euthanized at 21 days by rising concentration of CO_2 and perfused intracardially with 4% PFA in PBS. Eyes were dissected and immersion fixed in 4% PFA in PBS for 2h at 4°C and cryoprotected by sequential immersion in 10%, 20% and 30% sucrose solution in PBS, each for 24h with storage at 4°C. Eyes were orientated to permit radial sectioning and embedded using optimal cutting temperature embedding medium (Thermo Shandon, Runcorn, UK) in peel-away moulds (Agar Scientific, Essex, UK) by rapid freezing under crushed dry ice and stored at -80°C. Eyes were sectioned radially on a cryostat microtome (Bright, Huntingdon, UK) at -22°C at a thickness of 20μm and sections mounted on positively charged glass slides. Radial eye sections containing the optic disk and thus sectioned at a consistent axis were utilized for subsequent analysis. Sections were stored at -20C°.

Immunohistochemistry

Wholemounted retinae from Group 1 rats were permeabilized in 0.5% Triton x-100 in PBS for 15min at -70^{o}C before washing with room temperature 0.5% Triton x-100 for a further 15min. Retinae were incubated with primary antibodies diluted in wholemount antibody diluting buffer (wADB; 2% bovine serum albumin, 2% Triton x-100 in PBS) overnight at 4^{o}C and, the following day, were washed 3 x 10min in PBS and incubated with secondary antibodies in wADB for 2h at room temperature. After 2h, retinae were washed for 3 x 10min in PBS and mounted with the GCL uppermost on glass slides. Slides were allowed to air dry before mounting in Vectorshield medium (Vector Laboratories, Peterborough, UK) and applying cover slips. The antibodies used in this staining are detailed in Table 2.

Antigen	Specificity	Host species	Dilution	Supplier	Catalogue no.
βIII-tubulin (sections)	Monoclonal	Mouse	1:500	Sigma	#T8660
Brn3a (sections)	Polyclonal	Goat	1:200	Santa Cruz (Santa Cruz, CA)	#SC-31984
Islet-1 (sections & wholemounts)	Polyclonal	Rabbit	1:200	Abcam (Cambridge, UK)	#ab20670
Brn3a (wholemounts)	Polyclonal	Goat	1:100	Santa Cruz (Santa Cruz, CA)	#SC-31984
Syntaxin-1	Monoclonal	Mouse	1:200	Abcam	#ab3265
GFAP	Monoclonal	Mouse	1:200	Sigma	#G3893
ED1	Monoclonal	Mouse	1:200	Serotec (Oxford, UK)	#MCA341R
Mouse IgG (Fluor 488)	Polyclonal	Donkey	1:400	Molecular probes (Paisley, UK)	#A-21202
Rabbit IgG (Fluor 488)	Polyclonal	Donkey	1:400	Molecular Probes	#A-21206
Rabbit IgG (Fluor 594)	Polyclonal	Donkey	1:400	Molecular Probes	#A-21207
Goat IgG (Fluor 594)	Polyclonal	Donkey	1:400	Molecular Probes	#A-11058

Table 2: Antibodies used in immunohistochemistry

Mounted radial retinal sections through the optic nerve head from Group 2 rats 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 encircling with a hydrophobic PAP pen (Immedge pen; Vector Laboratories). Non-specific protein binding sites were blocked by incubating sections 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, drained and incubated with primary antibodies diluted in antibody diluting buffer (ADB; 0.5% bovine serum albumin, 0.3% Tween-20 in PBS) overnight at 4°C. The following day, retinal sections were washed for 3 X 5min in PBS and incubated with secondary antibodies diluted in ADB for 1h in a hydrated incubation chamber at room temperature and then washed for 3 X 5min in PBS, mounted in Vectorshield mounting medium containing DAPI (Vector Laboratories) and stored at 4°C before microscopic analysis. The antibodies used in this staining are detailed in Table 2. Omission of primary antibody was used as a staining control.

Microscopy and analysis

For Group 1 retinal wholemounts, images were photographed from 3 different regions from each retinal quadrant (as detailed in Fig. 1) using a Zeiss Axioplan-2 fluorescent microscope (Carl Zeiss, Ltd., Hertfordshire, UK). Images were captured at 200X magnification using an Axiocam HRc camera (Carl Zeiss, Ltd.) and all images equivalently contrast enhanced using Photoshop CS3 (Adobe Systems, Inc., San Jose, CA). The experimenter was blinded to treatment group and retinal region during counting of total numbers of $Brn3a^+$ and FG^+ cells (total= 3 counts/quadrant, 12 counts/retina and 6 retinae from 6 different animals per treatment group) observed in each $0.260mm^2$ imaged area. The mean number of RGCs/image was calculated from a mean count at each of the 3 radial distances as well as a mean composite count over all 3 distances.

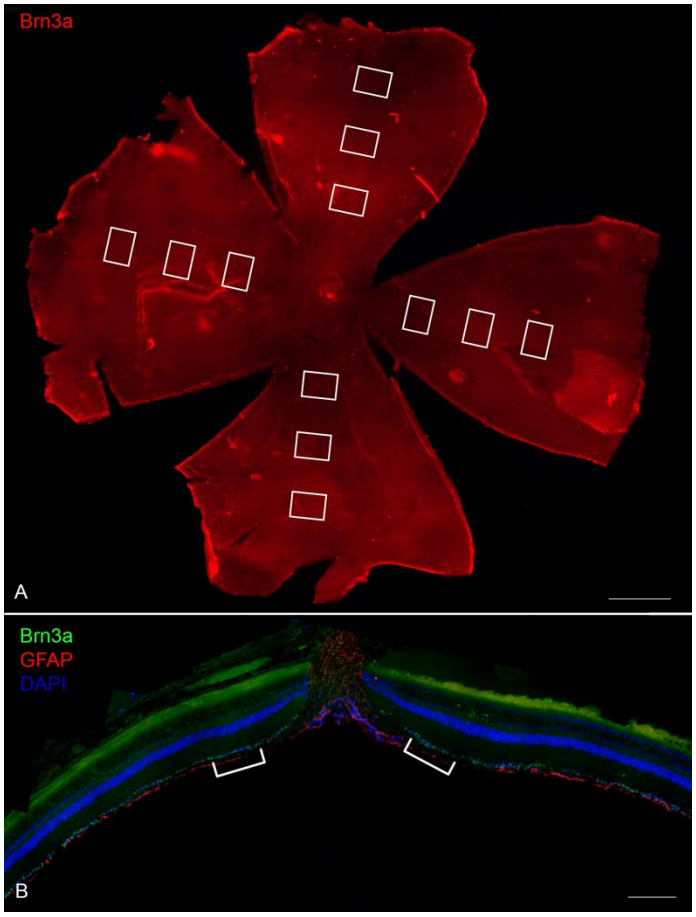


Figure 1: Sampling method for the counting of RGCs in Group 1 wholemounts (**A**) and Group 2 tissue sections (**B**). RGC counts in retinal wholemounts were made in sampling boxes (0.260mm^2) along quadrant radii at 1.5mm, 2.5mm and 3.5mm from the optic nerve head (white boxes; A). RGCs were counted in sections over 250µm length regions at a 200-300µm distance from the centre of the optic nerve in radial retinal sections (white lines; B). For panel A, Brn3a (red) was used to stain the retinal wholemount and RGCs (scale bar: 1mm). For panel B, Brn3a (*green*) was used to stain RGCs, GFAP (*red*) to stain glia and DAPI (*blue*) was used as a nucleus stain (scale bar: 250µm).

For Group 2 retinal sections, images were photographed as detailed above and analysed by an operator blinded to treatment groups. Brn3a$^+$, βIII-tubulin$^+$ and Islet-1$^+$ RGCs were counted in 20µm-thick radial sections of the retina, along a 250µm linear region of the GCL, either side of the optic nerve head (Fig. 1). Four sections/retina and 6 retinae from 6 different animals per treatment group were quantified. As sectioned tissue inevitably included split RGC nuclei, these were only included in the counts if the sizes of Brn3a$^+$//Islet-1$^+$ stained nuclei were similar to the co-localised DAPI$^+$ nuclei. Changes in soma/nuclear size have not

been reported after ONC[32], therefore, although this technique would be ineffective at calculating absolute RGC numbers, any inaccuracies would apply equally to both conditions and thus would not impede reliable comparisons of RGC loss between groups.

Statistics

All statistical tests were performed using SPSS 17.0 (IBMM SPSS, Inc., Chicago, IL) and data were presented as mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to ensure all data were normally distributed before applying a one-way analysis of variance (ANOVA) with a Tukey *post-hoc* test. Statistical differences were considered significant at p values < 0.05.

At the end of the study, a power calculation was performed to determine the required optimal animal numbers per treatment group for animal Group 1 and 2. Power calculations were performed using G*Power[33] with the following parameters: α error probability (P value) of 0.05, power (1 - β error probability) of 0.95 and effect size of 1.32, 2.77 and 7.64 which relates to 10%, 20% and 50% RGC death (as determined by the program) respectively compared to intact counts of 17.83 RGC/250μm of retina.

Results

Group 1 rats

$FG^+/Brn3a^+$ RGCs in retinal wholemounts

In the retinae of Group 1a eyes (intact optic nerve), mean numbers/mm^2 of $Brn3a^+$ and FG^+ RGCs were 869.7 ± 30.6 and 971.7 ± 67.0, respectively (Fig. 2). In the retinae of Group 1b eyes (21 days after ONC), mean numbers/mm^2 of $Brn3a^+$ and FG^+ RGCs were significantly reduced ($P<0.05$) compared to Group 1a values (83.8 ± 15.0 and 100.9 ± 13.8, respectively). In Group1a retinae, the density of RGCs decreased towards the periphery, reflected by a mean number/mm^2 of 1073.1 ± 21.9, 926.1 ± 39.5 and 644.7 ± 69.1 $Brn3a^+$ RGCs and 1209.9 ± 75.0, 1036.4 ± 73.3 and 629.0 ± 35.3 FG^+ RGCs at 1.5mm, 2.5mm and

3.5mm from the centre of the optic nerve, respectively. RGC number was significantly less ($P<0.05$) in Group 1b retinae compared to Group 1a retinae with mean counts of 92.9 ± 19.9, 79.3 ± 15.5, 79.2 ± 11.3 $Brn3a^+$ RGCs and 119.1 ± 17.3, 95.8 ± 15.6, 88.1 ± 10.3 FG^+ RGCs at 1.5mm, 2.5mm and 3.5mm from the centre of the retina, respectively.

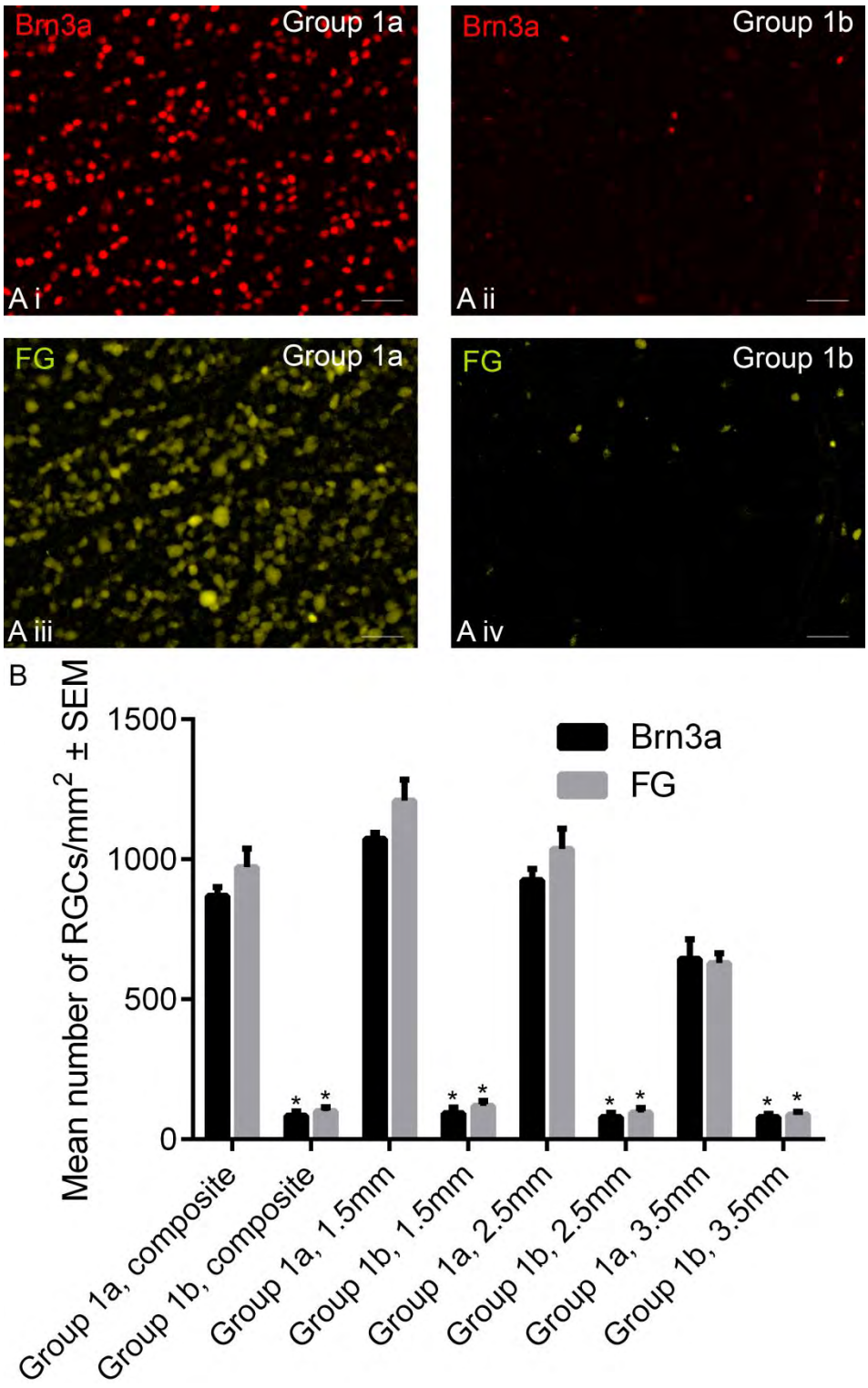


Figure 2: Brn3a$^+$ and FG$^+$ RGC counts in Group 1 wholemounted retinae. Immunohistochemically stained wholemounted retina, stained for Brn3a (*red*; **Ai** and **Aii**) and FG (*gold*; **Aiii** and **Aiv**; photographs taken in the same field, respectively), taken from Group 1a animals (intact optic nerve; **Ai** and **Aiii**) and Group 1b animals (21 days after ONC; **Aii** and **Aiv**). All images are representative of the 12 images taken per retina from 6 different animals (*scale bar*: 50μm). In (**B**), the mean number of Brn3a$^+$ and FG$^+$ RGCs in a 1mm^2 region, from Group 1a and Group 1b rats, calculated as an composite average of 12 images, or an average of 4 images taken at 1.5mm, 2.5mm and 3.5mm from the optic nerve head. *Asterisks* indicate significant difference at p<0.01 between the ONC counts and their respective Group1a controls.

FG/Brn3a double staining in retinal wholemounts of Group 1a

In the retinae of Group 1a eyes (intact optic nerve), quantification of RGCs double staining for Brn3a and FG revealed that 2.0 ± 0.15% of Brn3a$^+$ RGCs were FG$^-$ and 12.1 ± 0.7% of FG$^+$ RGCs were Brn3a$^-$. These data confirm that Brn3a is a specific marker of RGCs.

Group 2 rats

Brn3a$^+$, βIII-tubulin$^+$ and Islet-1$^+$ RGCs in radial retinal sections

The somata, dendrites and axons of RGCs were stained with βIII-tubulin in the GCL, inner plexiform layer and nerve fibre layer, respectively, and Islet-1-stained nuclei were present in the GCL as well as in the INL, whilst Brn3a exclusively stained RGC nuclei in the GCL.

In Group 2a retinae (intact optic nerve), the numbers/mm of Brn3a$^+$, Islet-1$^+$ and βIII-tubulin$^+$ RGCs in the GCL were 71.3 ± 2.2, 72.3 ± 1.8 and 83.6 ± 1.9, respectively (Fig. 3). In Group 2b retinae (21 days after ONC), the numbers/mm of Brn3a$^+$, Islet-1$^+$ and βIII-tubulin$^+$ RGCs in the GCL were 6.7 ± 0.7, 17.0 ± 1.3 and 27.4 ± 0.9, respectively; which were all significantly different (P<0.001) from the respective Group 2a counts (Fig. 3).

The number of βIII-tubulin$^+$ RGCs counted in the GCL was significantly reduced (P<0.001) in Group 2b retinae when compared to Group 2a retinae, but remained significantly higher (P<0.05) than the number of either Islet-1$^+$ or Brn3a$^+$ RGCs in the GCL of Group 2b retinae. Similarly, the number of Islet-1$^+$ RGCs in the GCL was significantly reduced 21 days after

ONC, but the number was significantly higher ($P<0.05$) than the number of Brn3a$^+$ RGCs counted in the GCL after ONC. These data demonstrate that βIII-tubulin and Islet-1 overestimate RGC numbers.

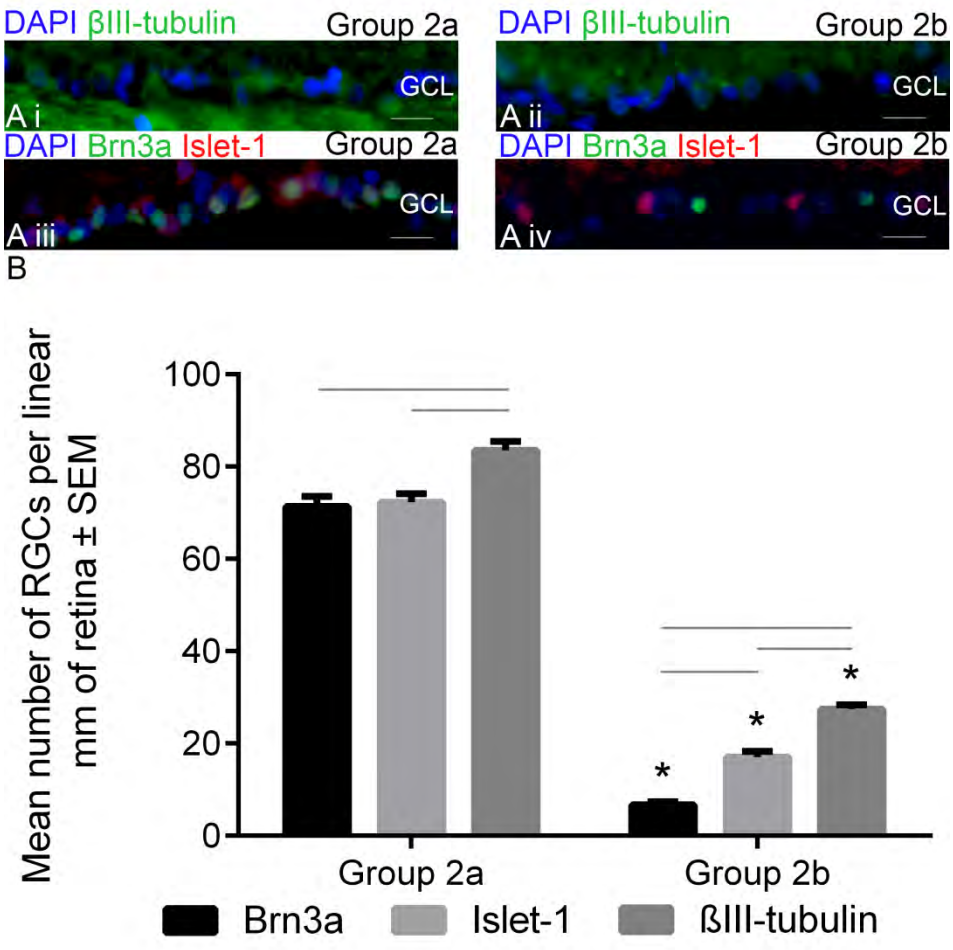


Figure 3: Brn3a$^+$, Islet-1$^+$ and βIII-tubulin$^+$ RGC counts in Group 2 radial retinal sections from Group 2a eyes (intact optic nerves) and Group 2b eyes (21 days after ONC). Immunohistochemically stained 20-µm-thick radial sections of retina through the optic nerve head, stained for βIII-tubulin (*green*; **Ai and Aii**) or Brn3a (*green*) and Islet-1 (*red;* **Aiii and Aiv**), taken from Group 2a eyes (**Ai** and **Aiii**) and Group 2b eyes (**Aii** and **Aiv**) with the ganglion cell layer (GCL) labelled. All images are representative of the two images/section, four sections/retina, and six retinae prepared from six different animals/group. DAPI was used as a nucleus stain (*scale bar*: 15µm). In (**B**), the number of Brn3a$^+$, Islet-1$^+$ and βIII-tubulin$^+$ cells, in a 1mm linear region of the GCL from Group 2a/2b eyes. *Asterisks* indicate significant difference at $p<0.05$ between groups (ONC and intact) and *black lines* indicate significant difference at $p<0.05$ within groups.

Co-localization of Brn3a$^+$, βIII-tubulin$^+$ and Islet-1$^+$ RGCs with GFAP, Syntaxin-1 and ED1 in radial retinal sections

GFAP$^+$ astrocytes were a distinct population of cells in the GCL that did not stain with Brn3a, Islet-1 or βIII-tubulin. Syntaxin-1$^+$ amacrine cells in the INL and GCL were Brn3a$^-$, although some were Islet-1$^+$ and βIII-tubulin$^+$ (Fig. 4). ED1$^+$ stained sparsely distributed macrophages/microglia throughout Group 2a retinae, which did not stain with Brn3a-, Islet-1- or βIII-tubulin. These data suggest that the overestimation of RGC numbers by βIII-tubulin and Islet-1 staining is due to the staining and subsequent counting of amacrine cells displaced into the GCL.

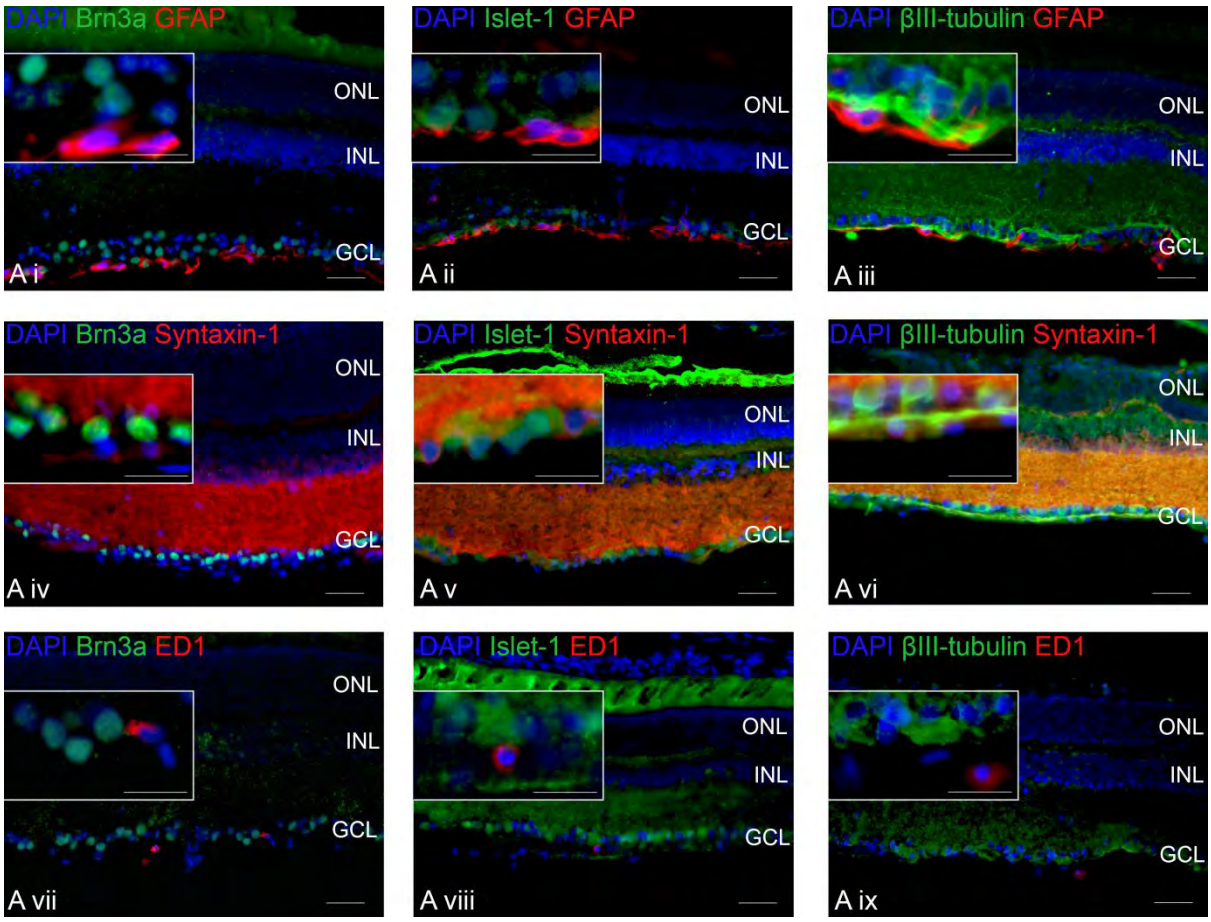


Figure 4: Expression of Brn3a, Islet-1 and βIII-tubulin in radial retinal sections and co-localisation of staining for the astrocyte marker GFAP, the amacrine cell marker Syntaxin-1 and the macrophage/microglia marker ED1. Immunohistochemically stained 20-μm-thick radial sections of retina, stained for Brn3a (*green;* ***Ai, Aiv and Avii***), Islet-1 (*green;* **Aii, Av and Aviii**), βIII-tubulin (*green*; **Aiii, Avi and Aix**), GFAP (*red;* **Ai, Aii and Aiii**), Syntaxin-1 (*red;* **Aiv, Av and Avi**) and ED1 (*red;* **Avii, Aviii and Aix**), taken from Group 2a eyes (intact

optic nerve), with the outer nuclear layer (ONL), inner nuclear layer (INL) and ganglion cell layer (GCL) labelled, insert showing higher power image of the GCL. All images are representative; DAPI (*blue*) was used as a nucleus stain (*scale bar*: 50μm; *inset scale bar*: 25μm).

Group 1 and 2 rats

Estimates of RGC death were similar in Group 1 Brn3a/FG-stained retinal wholemounts and Group 2 Brn3a-stained radial sections

In Group 2 radial retinal sections, the percentage death of Islet-1^{+}/βIII-tubulin^{+} RGC detected was 76.5 ± 1.5% and 67.0 ± 1.6%, respectively, which was significantly lower ($P < 0.05$) than that revealed by Brn3a staining (90.6 ± 0.9%) as well as that estimated after Brn3a/FG staining of retinal wholemounts (90.2 ± 1.8% and 89.3 ± 1.6%, respectively; Fig. 5). These data show that Brn3a-stained radial sections report the same percentage RGC death as Brn3a-/FG-stained wholemounts demonstrating that both techniques reliably quantify RGC loss. In contrast, Islet-1-/βIII-tubulin-stained radial sections overestimate the number of surviving RGCs after ONC and thus underestimate the percentage of RGC loss.

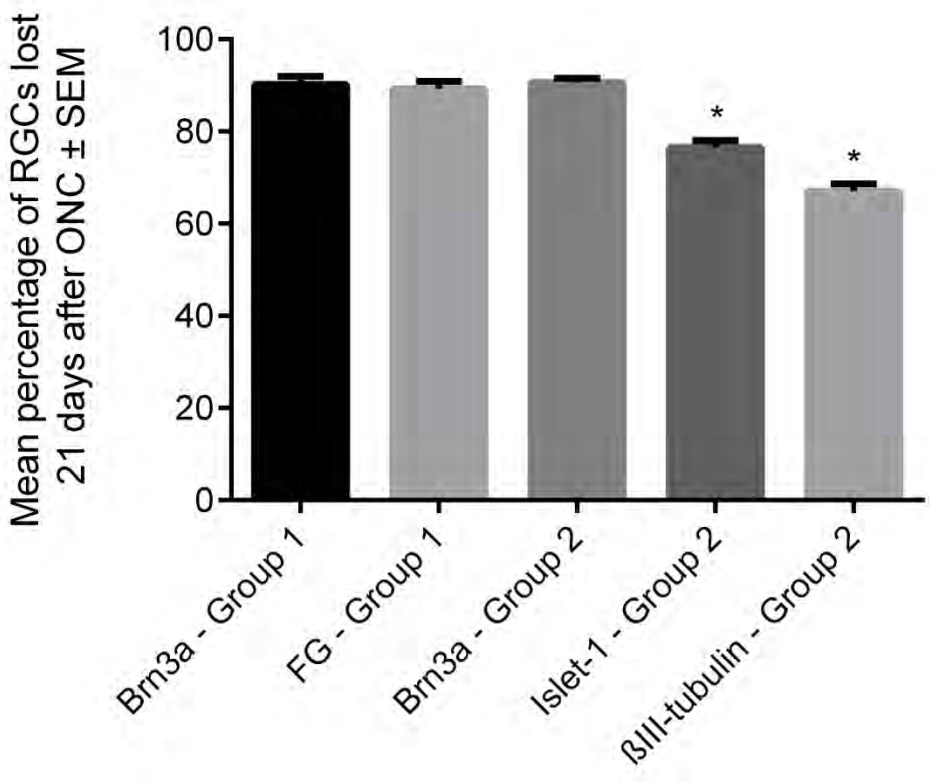


Figure 5: The mean percentage of RGC loss 21 days after ONC in Group 1 and Group 2 rats. *Asterisks* indicate significant difference at P<0.05 compared to $Brn3a^+$ RGCs in sections and $Brn3a^+/FG^+$ RGCs in wholemounts (n=6).

Brn3a-stained Group 1 wholemounts and Group 2 radial sections detect as low as 10% RGC death

Using data derived from this experiment, a power calculation was performed to determine that, in order to detect 50% RGC death at P<0.05, 3 animals/group are required, to detect 20% RGC death at P<0.05, 5 animals/group are required and to detect 10% RGC death at P<0.05, 16 animals/group are required. These data suggest that even as low as 20% RGC death can be detected in radial stained sections with acceptable animal numbers.

Discussion

Reliable measures of percentage RGC survival in experimental degenerative retinal conditions including glaucoma[34] and optic nerve neuropathies[35] are essential to evaluate

the efficacy of novel therapeutic agents designed to treat these disorders. Pertinent to this is the identification of good phenotypic markers for RGCs. This study provides evidence that, in determining percentage RGC loss in rats, estimates from radial sections of retinae are similar to estimates from wholemounted retinae. Furthermore, the data reinforce the notion that Brn3a antibodies are the most reliable phenotypic markers of RGCs both in wholemounts and sections, whereas βIII-tubulin and Islet-1 antibodies stain amacrine cells in the GCL, giving underestimates of RGC loss.

Our findings that Brn3a antibodies and FG tracer label statistically similar numbers of RGCs in wholemounted retinae, both before and after ONC-induced RGC death are consistent with the reported 95% co-localization of Brn3a and FG staining in RGCs[18], further emphasising the utility of Brn3a antibodies as a reliable RGC marker. The percentage of $Brn3a^+$ cells that were FG^- was 2.0%, which is comparable to the 4.4% previously reported[18]. FG, a weak base, freely diffuses into intracellular vesicles where it raises the pH, making the vesicle impermeable, trapping the FG within; the vesicle is then transported along the cytoskeleton of the axon[36]. A likely explanation for the presence of FG^-Brn3a^+ RGCs is that FG uptake into RGC axons is 98% efficient. In this study the percentage of FG^+ cells that were $Brn3a^-$ was 12.1%, slightly more than the 3.4% previously reported[18], a figure that is probably explained by the exclusion of $FG^+/Brn3a^-$ macrophages/microglia from the data in the Nadal-Nicolas study. By contrast, in the present study, all cells that were positive for their respective phenotypic markers were counted and included and thus we can postulate (but not be certain) that a small proportion of these FG^+ cells were macrophages/microglia. In Figure 2, FG^+ and $Brn3a^+$ images were taken in the same field but co-localization appears reduced after ONC, likely due to surviving $FG^+/Brn3a^-$ macrophages the frequency of which is increased after RGC death. Although RGC number decreased with increasing distance from the optic nerve head (1.5mm, 2.5mm and 3.5mm), the number of RGCs surviving after ONC did not change with distance (Fig. 2B.), and thus counts closest to the optic nerve head yielded the biggest relative differences. This is explained by the homogenous RGC death

occurring after axotomy/ONC[10] whereas the sectorial death seen in laser-induced ocular hypertension models of glaucoma[37-40] requires sampling from multiple distances to ensure accuracy in determining the extent of RGC loss. The counts made from 4 and 12 images had similar SEM, revealing the strength of the sampling method.

One limitation of this study is that eyes with intact optic nerves (Group 1a, 2a) contralateral to the ONC eyes (Group 1b, 2b) were used as controls. It has previously been shown that ocular hypertension induces microglia activation in eyes contralateral to the injury[41] as well as a decrease in astrocytes and activation of Müller cells[42], with these results partially replicated for unilateral ONC[43]. However, these changes have not been correlated with RGC number[42] and equally, the RGC loss observed in our study 21 days after ONC (when compared to contralateral eyes) is equivalent to losses reported in the published literature[3,35,44] leading us to assume that any contralateral effects instigated by the unilateral ONC had negligible effects on RGC numbers. An added value of contralateral eye counts is the resulting reduction in animal usage.

In wholemounted Group 1 retinae, Brn3a is preferred to FG as a RGC marker, since FG delivery into the optic nerve for retrograde transport to RGC somata requires further surgery under anaesthesia. Counts of RGCs with 4 sampling areas each 1.5mm from the optic nerve head gave reliable estimates of RGC number in which SEM values where similar to when 12 images/samples were used. In wholemounts, βIII-tubulin-stained axons and dendrites which obscured many RGCs, leading to their exclusion from the count (Fig. S1). In Islet-1-stained wholemounts, the high background staining of the INL (Fig. S1) made RGC counting difficult and excluded consistent 4-image sampling of some retinae.

Retinal wholemounts restricts the use of the retina to a limited number of antibody stains[45] and renders the tissue unusable for further analysis, such as evaluating the effects of treatments on other proteins and cells as well as retrieving morphological data such as retinal detachment or the location of grafted cells. By contrast, radial sections allow all of

these data to be obtained from multiple sections from the same eyes with no loss of fidelity in RGC quantification. Of note, the homogenous and diffuse loss of RGCs seen after ONC/axotomy[10] makes sampling around the optic nerve head in sections a reliable estimate of RGC loss, whereas in models such as glaucoma where the loss is sectorial[37-40], expanded sampling is required to include sections throughout the eye, counting in regions at multiple distances from the optic nerve head.

We tested Brn3a[18,24], Islet-1 and βIII-tubulin[7,9,24] as presumptive phenotypic RGC markers. Compared to Brn3a, βIII-tubulin-stained significantly more cells in the GCL both before and after ONC-induced RGC death, which we and others[8] interpret as being due to labelling of amacrine cells in addition to RGCs. Thus this antibody over estimates the numbers of RGCs both in Group 1a/2a animals (intact optic nerve) and even more so in Group 1b/2b animals (21 days after ONC) where the ratio of amacrine cells to RGCs is increased as RGCs are preferentially lost[20]. Compared to the Brn3a antibody, the Islet-1-stained similar numbers of cells before ONC but significantly more after ONC (although still significantly less than with βIII-tubulin staining after ONC). Since the Islet-1 antibody stains RGCs and amacrine cells[9], and more displaced amacrine cells survive relative to RGCs in the GCL 21 days after ONC[20], Islet-1 antibody will overestimate RGC numbers in comparison to Brn3a after ONC-induced RGC death.

To determine if counts from sections are as reliable as from wholemounts in quantifying RGC death, we compared the percentage loss of RGCs between all methods used in this study. The published figures of 90% RGC death 21 days after ONC[24,35,44] are similar to those seen in this study with Brn3a-/FG-stained wholemounts and with Brn3a-stained radial retinal sections, confirming that counts made in retinal sections are as reliable as those made in retinal wholemounts. Considering both the small SEM for each mean and the results from our power calculation, counts from sections and wholemounts have equal fidelity in determining significant differences between groups with as low as 20% difference

in RGC number, without the need for significant increases in animal numbers per treatment group.

Conclusions

We conclude that Brn3a antibody staining of radial retinal sections is a preferred method for estimating RGC frequency when compared to techniques using retinal wholemounts in rodent models of ocular disease. The method avoids both the additional surgery needed for injection of FG and the sacrifice of further molecular and morphological retinal analysis, potentially reducing animal usage and associated costs. We show that cell counts from retinal sections are as reliable as those from retinal wholemounts and have equivalent fidelity in determining RGC loss between control and experimental groups.

References

1. Buhl EH, Dann JF (1988) Morphological Diversity of Displaced Retinal Ganglion-Cells in the Rat - a Lucifer Yellow Study. Journal of Comparative Neurology 269: 210-218.
2. Wolburg H, Buerle C (1993) Astrocytes in the lamina cribrosa of the rat optic nerve: are their morphological peculiarities involved in an altered blood-brain barrier? J Hirnforsch 34: 445-459.
3. Berry M, Ahmed Z, Lorber B, Douglas M, Logan A (2008) Regeneration of axons in the visual system. Restorative Neurology and Neuroscience 26: 147-174.
4. Vereczki V, Koves K, Csaki A, Grosz K, Hoffman GE, et al. (2006) Distribution of hypothalamic, hippocampal and other limbic peptidergic neuronal cell bodies giving rise to retinopetal fibers: anterograde and retrograde tracing and neuropeptide immunohistochemical studies. Neuroscience 140: 1089-1100.
5. Bunt AH, Lund RD (1974) Vinblastine-Induced Blockage of Orthograde and Retrograde Axonal-Transport of Protein in Retinal Ganglion-Cells. Experimental Neurology 45: 288-297.
6. Perry VH (1981) Evidence for an Amacrine Cell System in the Ganglion-Cell Layer of the Rat Retina. Neuroscience 6: 931-944.
7. Johnson TV, Dekorver NW, Levasseur VA, Osborne A, Tassoni A, et al. (2013) Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain.
8. Sharma RK, Netland PA (2007) Early born lineage of retinal neurons express class III beta-tubulin isotype. Brain Res 1176: 11-17.
9. Lorber B, Tassoni A, Bull ND, Moschos MM, Martin KR (2012) Retinal ganglion cell survival and axon regeneration in Wld(S) transgenic rats after optic nerve crush and lens injury. Bmc Neuroscience 13.
10. Galindo-Romero C, Aviles-Trigueros M, Jimenez-Lopez M, Valiente-Soriano FJ, Salinas-Navarro M, et al. (2011) Axotomy-induced retinal ganglion cell death in adult mice: quantitative and topographic time course analyses. Exp Eye Res 92: 377-387.

11. Selles-Navarro I, Villegas-Perez MP, Salvador-Silva M, Ruiz-Gomez JM, Vidal-Sanz M (1996) Retinal ganglion cell death after different transient periods of pressure-induced ischemia and survival intervals. A quantitative in vivo study. Invest Ophthalmol Vis Sci 37: 2002-2014.
12. Abdel-Majid RM, Archibald ML, Tremblay F, Baldridge WH (2005) Tracer coupling of neurons in the rat retina inner nuclear layer labeled by Fluorogold. Brain Res 1063: 114-120.
13. Salinas-Navarro M, Mayor-Torroglosa S, Jimenez-Lopez M, Aviles-Trigueros M, Holmes TM, et al. (2009) A computerized analysis of the entire retinal ganglion cell population and its spatial distribution in adult rats. Vision Research 49: 115-126.
14. Vaney DI, Hughes A (1976) The rabbit optic nerve: fibre diameter spectrum, fibre count, and comparison with a retinal ganglion cell count. Journal of Comparative Neurology 170: 241-251.
15. Leibinger M, Andreadaki A, Diekmann H, Fischer D (2013) Neuronal STAT3 activation is essential for CNTF- and inflammatory stimulation-induced CNS axon regeneration. Cell Death & Disease 4.
16. Cui Q, Yip HK, Zhao RC, So KF, Harvey AR (2003) Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. Mol Cell Neurosci 22: 49-61.
17. Barnstable CJ, Drager UC (1984) Thy-1 Antigen - a Ganglion-Cell Specific Marker in Rodent Retina. Neuroscience 11: 847-&.
18. Nadal-Nicolas FM, Jimenez-Lopez M, Sobrado-Calvo P, Nieto-Lopez L, Canovas-Martinez I, et al. (2009) Brn3a as a Marker of Retinal Ganglion Cells: Qualitative and Quantitative Time Course Studies in Naive and Optic Nerve-Injured Retinas. Investigative Ophthalmology & Visual Science 50: 3860-3868.
19. Huang W, Fileta J, Guo Y, Grosskreutz CL (2006) Downregulation of Thy1 in retinal ganglion cells in experimental glaucoma. Current Eye Research 31: 265-271.
20. Kielczewski JL, Pease ME, Quigley HA (2005) The effect of experimental glaucoma and optic nerve transection on amacrine cells in the rat retina. Invest Ophthalmol Vis Sci 46: 3188-3196.
21. Nadal-Nicolas FM, Jimenez-Lopez M, Salinas-Navarro M, Sobrado-Calvo P, Alburquerque-Bejar JJ, et al. (2012) Whole number, distribution and co-expression of brn3 transcription factors in retinal ganglion cells of adult albino and pigmented rats. Plos One 7: e49830.
22. Martin KRG, Klein RL, Quigley HA (2002) Gene delivery to the eye using adeno-associated viral vectors. Methods 28: 267-275.
23. Ahmed Z, Kalinski H, Berry M, Almasieh M, Ashush H, et al. (2011) Ocular neuroprotection by siRNA targeting caspase-2. Cell Death & Disease 2.
24. Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2013) Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest Ophthalmol Vis Sci 54: 7544-7556.
25. Garrett WT, Mcbride RL, Williams JK, Feringa ER (1991) Fluoro-Golds Toxicity Makes It Inferior to True Blue for Long-Term Studies of Dorsal-Root Ganglion Neurons and Motoneurons. Neurosci Lett 128: 137-139.
26. Rinaman L, Milligan CE, Levitt P (1991) Persistence of Fluorogold Following Degeneration of Labeled Motoneurons Is Due to Phagocytosis by Microglia and Macrophages. Neuroscience 44: 765-776.
27. Gomez-Ramirez AM, Villegas-Perez MP, Miralles de Imperial J, Salvador-Silva M, Vidal-Sanz M (1999) Effects of intramuscular injection of botulinum toxin and doxorubicin on the survival of abducens motoneurons. Invest Ophthalmol Vis Sci 40: 414-424.
28. Dreher B, Sefton AJ, Ni SYK, Nisbett G (1985) The Morphology, Number, Distribution and Central Projections of Class-I Retinal Ganglion-Cells in Albino and Hooded Rats. Brain Behavior and Evolution 26: 10-48.

29. Bennett MK, Calakos N, Scheller RH (1992) Syntaxin - a Synaptic Protein Implicated in Docking of Synaptic Vesicles at Presynaptic Active Zones. Science 257: 255-259.
30. Kunzevitzky NJ, Almeida MV, Goldberg JL (2010) Amacrine Cell Gene Expression and Survival Signaling: Differences from Neighboring Retinal Ganglion Cells. Investigative Ophthalmology & Visual Science 51: 3800-3812.
31. Berry M, Carlile J, Hunter A (1996) Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. Journal of Neurocytology 25: 147-170.
32. Kalesnykas G, Oglesby EN, Zack DJ, Cone FE, Steinhart MR, et al. (2012) Retinal ganglion cell morphology after optic nerve crush and experimental glaucoma. Invest Ophthalmol Vis Sci 53: 3847-3857.
33. Faul F, Erdfelder E, Lang AG, Buchner A (2007) G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 39: 175-191.
34. Quigley HA (1999) Neuronal death in glaucoma. Progress in Retinal and Eye Research 18: 39-57.
35. Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ (1994) Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. J Neurosci 14: 4368-4374.
36. Wessendorf MW (1991) Fluoro-Gold: composition, and mechanism of uptake. Brain Res 553: 135-148.
37. Agudo-Barriuso M, Villegas-Perez MP, de Imperial JM, Vidal-Sanz M (2013) Anatomical and functional damage in experimental glaucoma. Curr Opin Pharmacol 13: 5-11.
38. Soto I, Pease ME, Son JL, Shi X, Quigley HA, et al. (2011) Retinal ganglion cell loss in a rat ocular hypertension model is sectorial and involves early optic nerve axon loss. Invest Ophthalmol Vis Sci 52: 434-441.
39. Vidal-Sanz M, Salinas-Navarro M, Nadal-Nicolas FM, Alarcon-Martinez L, Valiente-Soriano FJ, et al. (2012) Understanding glaucomatous damage: anatomical and functional data from ocular hypertensive rodent retinas. Prog Retin Eye Res 31: 1-27.
40. Salinas-Navarro M, Alarcon-Martinez L, Valiente-Soriano FJ, Jimenez-Lopez M, Mayor-Torroglosa S, et al. (2010) Ocular hypertension impairs optic nerve axonal transport leading to progressive retinal ganglion cell degeneration. Exp Eye Res 90: 168-183.
41. Rojas B, Gallego BI, Ramirez AI, Salazar JJ, de Hoz R, et al. (2014) Microglia in mouse retina contralateral to experimental glaucoma exhibit multiple signs of activation in all retinal layers. J Neuroinflammation 11: 133.
42. Ramirez AI, Salazar JJ, de Hoz R, Rojas B, Gallego BI, et al. (2010) Quantification of the effect of different levels of IOP in the astroglia of the rat retina ipsilateral and contralateral to experimental glaucoma. Invest Ophthalmol Vis Sci 51: 5690-5696.
43. Bodeutsch N, Siebert H, Dermon C, Thanos S (1999) Unilateral injury to the adult rat optic nerve causes multiple cellular responses in the contralateral site. J Neurobiol 38: 116-128.
44. Mey J, Thanos S (1993) Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. Brain Res 602: 304-317.
45. Ortin-Martinez A, Jimenez-Lopez M, Nadal-Nicolas FM, Salinas-Navarro M, Alarcon-Martinez L, et al. (2010) Automated quantification and topographical distribution of the whole population of S- and L-cones in adult albino and pigmented rats. Invest Ophthalmol Vis Sci 51: 3171-3183.

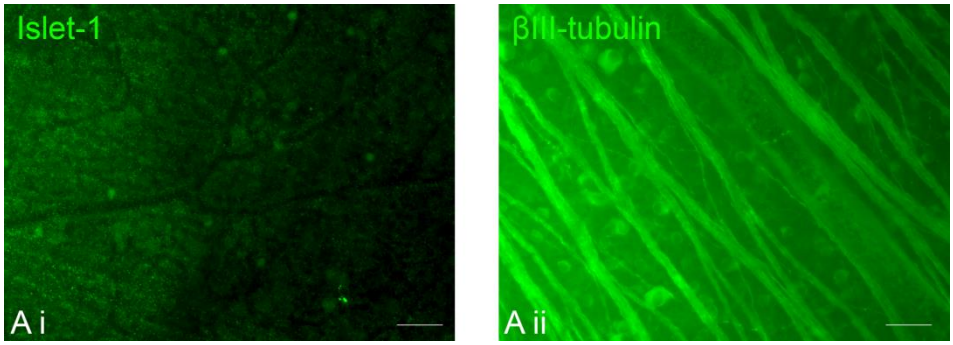


Figure S1: Islet-1- and βIII-tubulin-stained wholemounted retinae from Group 1a eyes (intact optic nerve). Immunohistochemically stained wholemounted retina stained for Islet-1 (*green*; **Ai**) or βIII-tubulin (*green*; **Aii**), taken from Group 1a eyes. All images are representative of the 12 images taken per retina from 6 different animals (*scale bar*: 50μm).

CHAPTER 3

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

Ben Mead, Ann Logan, Martin Berry, Wendy Leadbeater and Ben A. Scheven

A version of this manuscript has been published in

Investigative Ophthalmology & Visual Science

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.

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

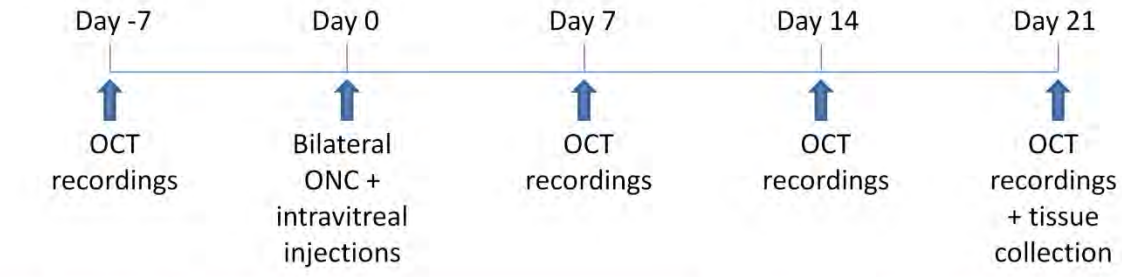
Cell culture 24-well plates (BD Biosciences) were coated for 60min with 100µg/ml poly-D-lysine and then for 30min with 20µg/ml laminin. After terminal anaesthesia, eyes were removed from 3 male Sprague-Dawley rats weighing 170-200g (Charles River) and the retinae minced in 1.25ml of papain (Worthington 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.



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**.** 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.

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]:

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

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).

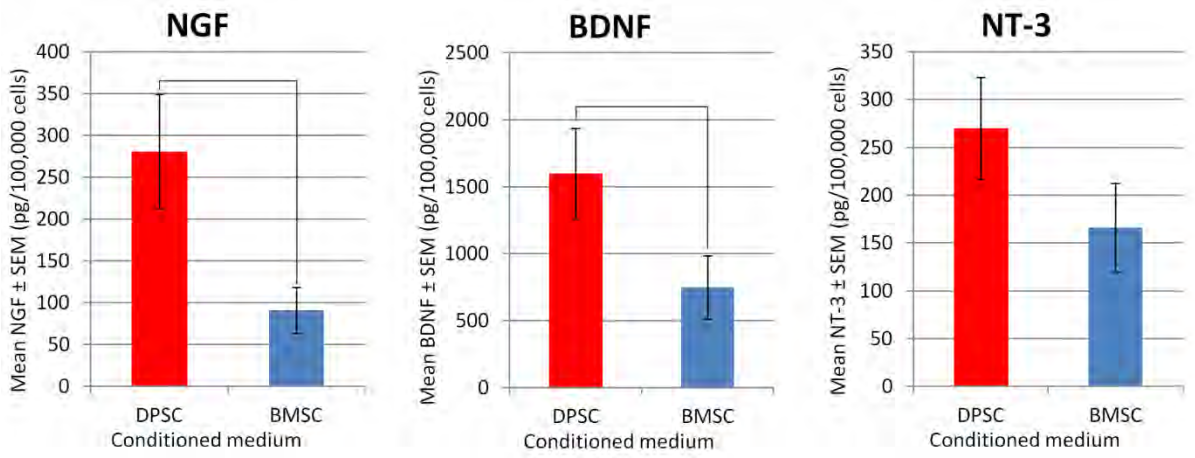


Figure 2: NGF, BDNF and NT-3 secretion from DPSC and BMSC. DPSC and BMSC conditioned medium, collected after 48h of cell culture, was assayed using specific ELISAs for rat NGF, BDNF and NT-3 (n = 3; Black lines indicate significant difference at $p<0.05$).

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 ($p>0.05$).

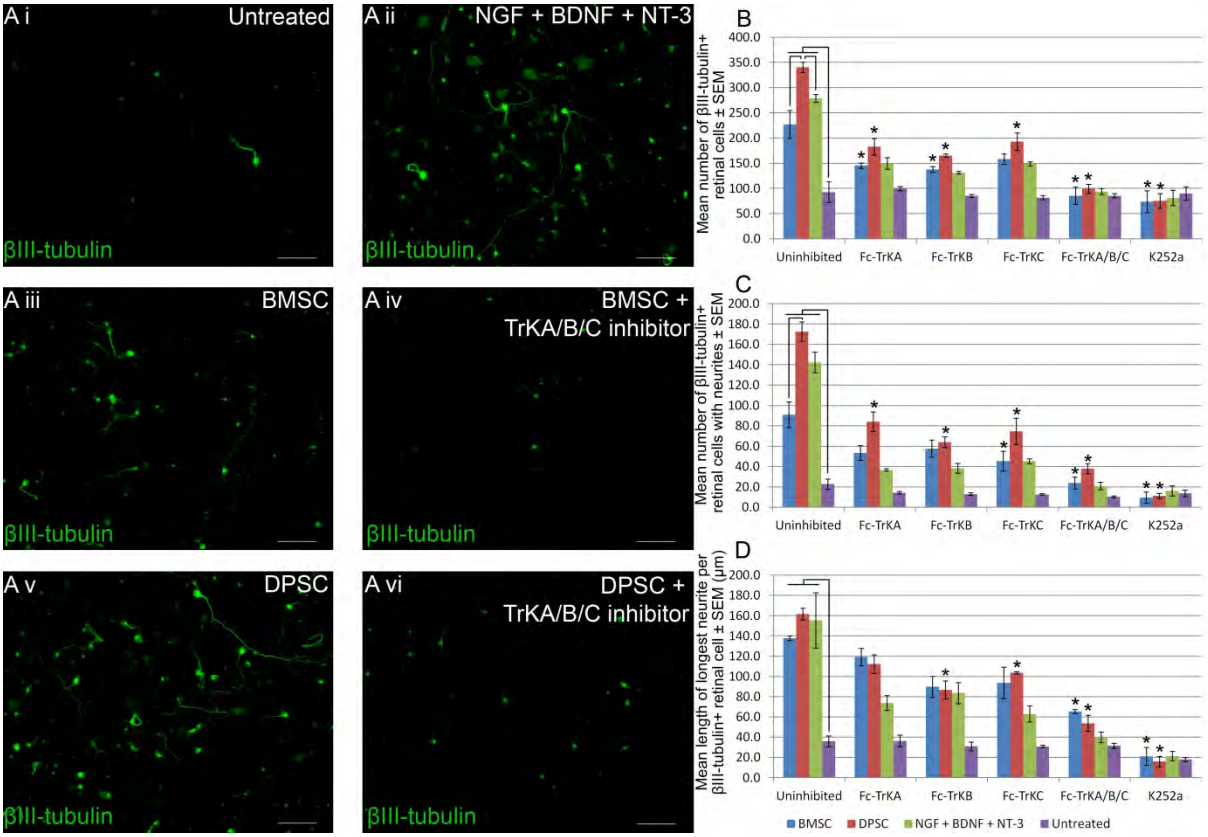


Figure 3: Effects of DPSC and BMSC on βIII-tubulin$^+$ retinal cells *in vitro.* βIII-tubulin$^+$ retinal cells, cultured either alone (A i), with exogenous neurotrophins (A ii), with BMSC (with or without TrK inhibitors, A iii and A iv, respectively) or with DPSC (with or without TrK inhibitors, A v 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 $p<0.05$).

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).

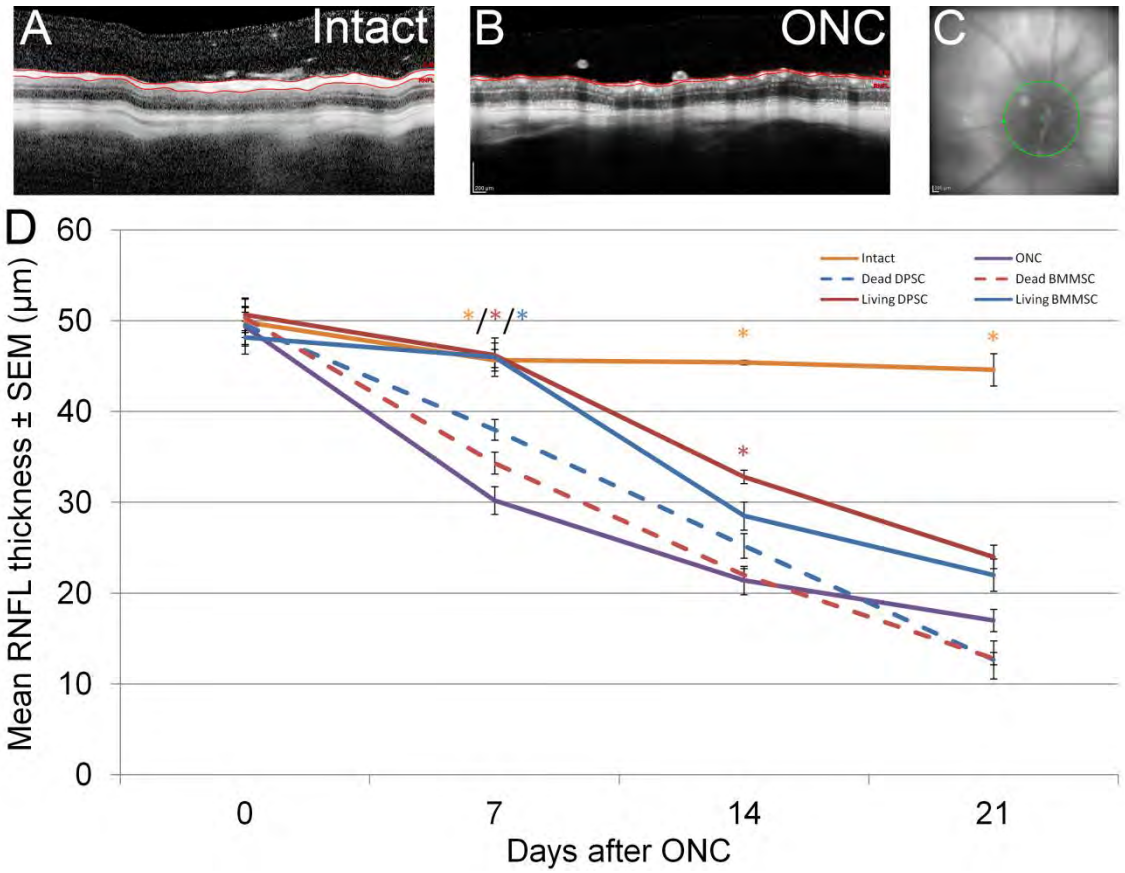


Figure 4: RNFL thickness after ONC. OCT images of retina from an uninjured rat (A) and a rat 21 days after ONC (B) are shown with red lines outlining the RNFL. OCT images were taken of the retinal section surrounding the optic nerve head, indicated by the green line (C). Images are representative of 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 $p<0.05$.

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).

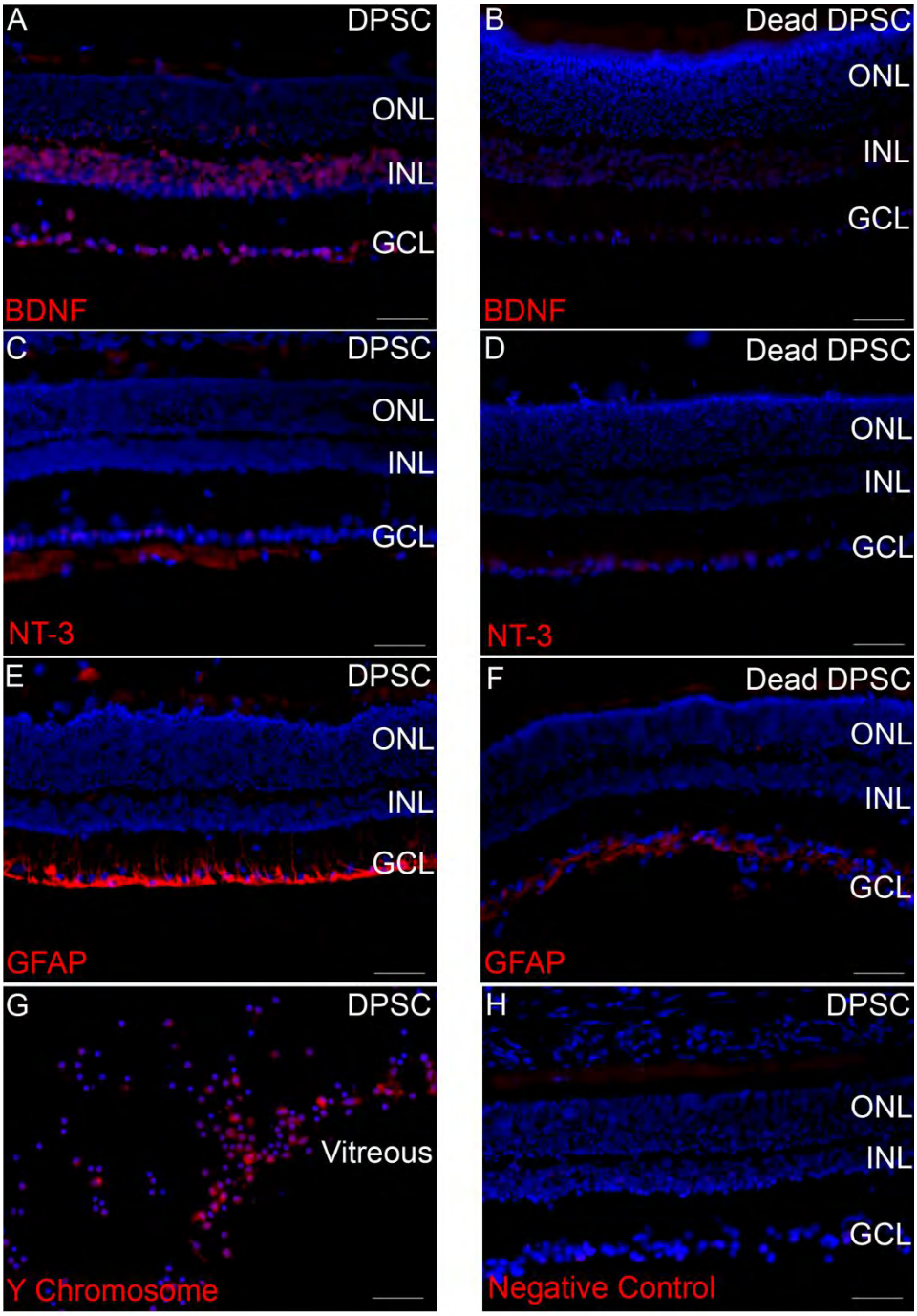


Figure 5: DPSC survival and trophic effects 21 days after ONC/cell transplantation. Immunohistochemically stained 20µm thick parasagittal sections of retina and vitreous, stained for BDNF (A and B), NT-3 (C and D), GFAP (E and F) and Y chromosome (G) 21 days after ONC and intravitreal transplantation 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).

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.

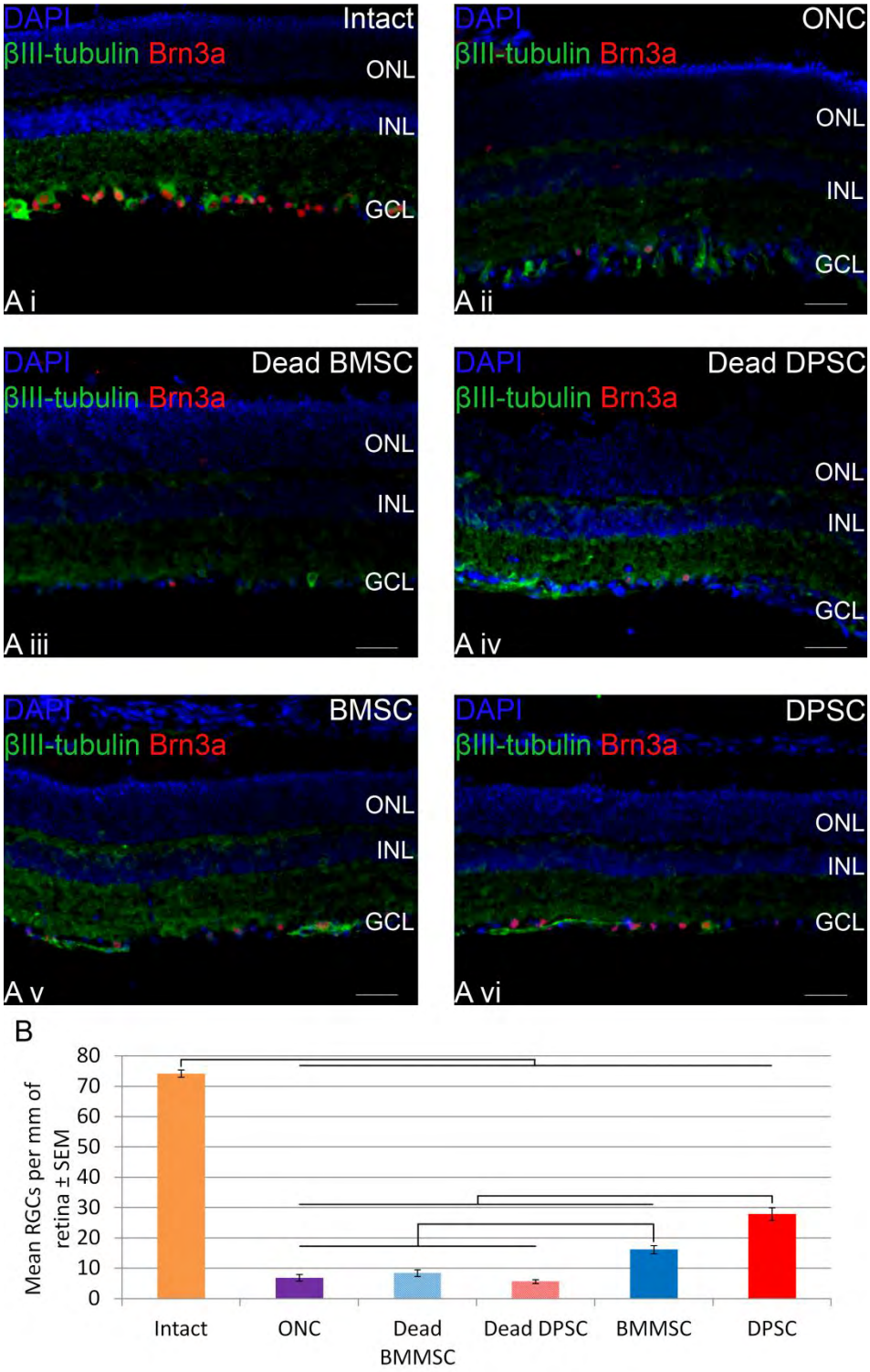


Figure 6: RGC survival 21 days after ONC/cell transplantation. Immunohistochemically stained 20µm thick parasagittal sections of retina, stained for βIII-tubulin (green) and Brn3a (red) in intact animals (A i) and 21 days after ONC (A ii) and intravitreal transplantation of dead BMSC (A iii), dead DPSC (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 $p<0.05$.

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.

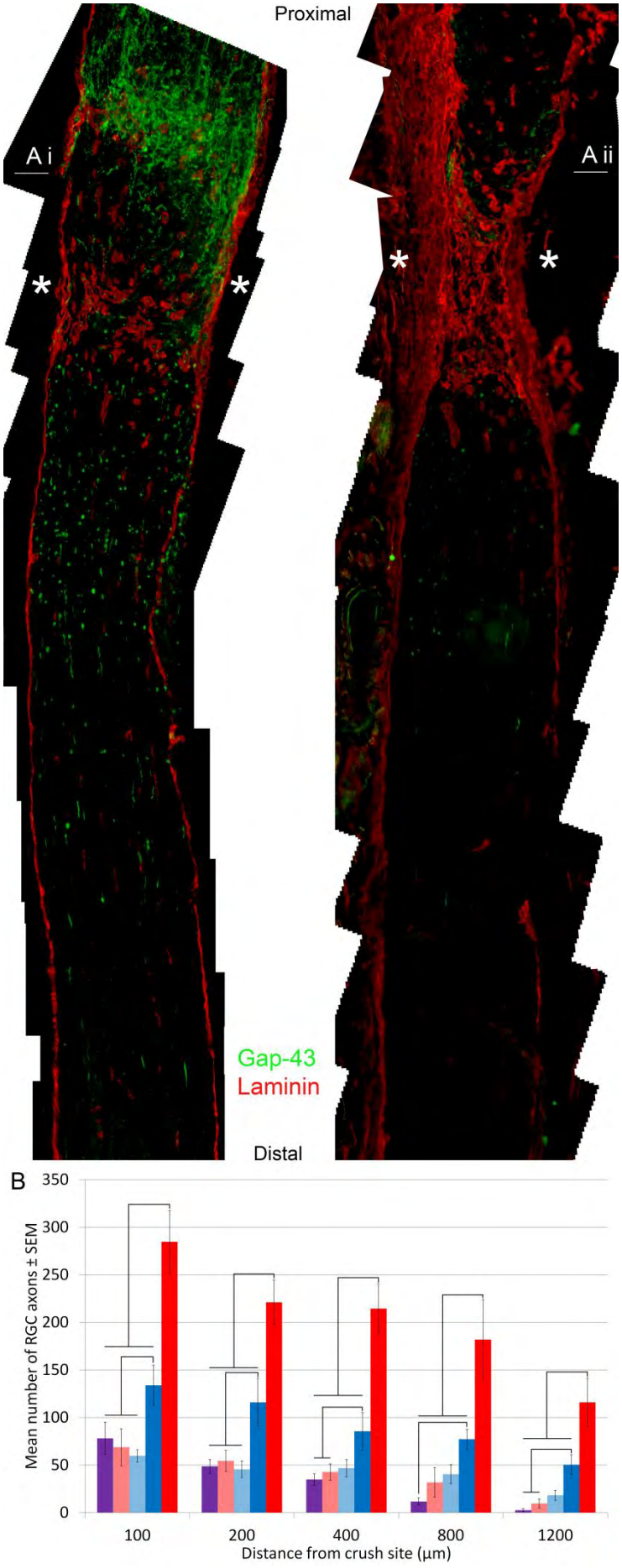

Proximal
A i
A ii
Gap-43
Laminin
Distal
B
Mean number of RGC axons ± SEM
350
300
250
200
150
100
50
0
100
200
400
800
1200
Distance from crush site (µm)

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.

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.

References

1. Wyndaele M, Wyndaele JJ. Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? *Spinal Cord* 2006;44:523-529.
2. Ghajar J. Traumatic brain injury. *The Lancet* 2000;356:923-929.
3. Sarkies N. Traumatic optic neuropathy. *Eye* 2004;18:1122-1125.
4. Stone EM, Fingert JH, Alward WLM, et al. Identification of a Gene That Causes Primary Open Angle Glaucoma. *Science* 1997;275:668-670.
5. Richardson PM, McGuinness UM, Aguayo AJ. Axons from CNS neurones regenerate into PNS grafts. *Nature* 1980;284:264-265.
6. Berry M, Ahmed Z, Lorber B, Douglas M, Logan A. Regeneration of axons in the visual system. *Restorative Neurology and Neuroscience* 2008;26:147-174.
7. Schwab M, Caroni P. Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *The Journal of Neuroscience* 1988;8:2381-2393.

8. Caroni P, Schwab ME. Antibody against myelin associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. *Neuron* 1988;1:85-96.
9. Benowitz LI, Yin Y. Combinatorial treatments for promoting axon regeneration in the CNS: Strategies for overcoming inhibitory signals and activating neurons' intrinsic growth state. *Developmental Neurobiology* 2007;67:1148-1165.
10. Mey J, Thanos S. Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. *Brain Res* 1993;602:304-317.
11. Ahmed Z, Kalinski H, Berry M, et al. Ocular neuroprotection by siRNA targeting caspase-2. *Cell Death & Disease* 2011;2.
12. Ko ML, Hu DN, Ritch R, Sharma SC. The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. *Invest Ophthalmol Vis Sci* 2000;41:2967-2971.
13. Ko ML, Hu DN, Ritch R, Sharma SC, Chen CF. Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. *Neuroscience letters* 2001;305:139-142.
14. Sommerfeld MT, Schweigreiter R, Barde YA, Hoppe E. Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. *The Journal of biological chemistry* 2000;275:8982-8990.
15. Chen H, Weber AJ. Brain-derived neurotrophic factor reduces TrkB protein and mRNA in the normal retina and following optic nerve crush in adult rats. *Brain Res* 2004;1011:99-106.
16. Logan A, Ahmed Z, Baird A, Gonzalez AM, Berry M. Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. *Brain* 2006;129:490-502.
17. Thomas KE, Moon LDF. Will stem cell therapies be safe and effective for treating spinal cord injuries? *British Medical Bulletin* 2011;98:127-142.
18. Abematsu M, Tsujimura K, Yamano M, et al. Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. *The Journal of Clinical Investigation* 2010;120:3255-3266.
19. Gu W, Zhang F, Xue Q, Ma Z, Lu P, Yu B. Transplantation of bone marrow mesenchymal stem cells reduces lesion volume and induces axonal regrowth of injured spinal cord. *Neuropathology* 2010;30:205-217.
20. Dormady SP, Bashayan O, Dougherty R, Zhang XM, Basch RS. Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. *Journal of hematotherapy & stem cell research* 2001;10:125-140.
21. Sakai K, Yamamoto A, Matsubara K, et al. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. *Journal of Clinical Investigation* 2012;122:80-90.
22. Wilkins A, Kemp K, Ginty M, Hares K, Mallam E, Scolding N. Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. *Stem cell research* 2009;3:63-70.
23. Levkovitch-Verbin H, Sadan O, Vander S, et al. Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. *Invest Ophthalmol Vis Sci* 2010;51:6394-6400.
24. Johnson TV, Bull ND, Hunt DP, Marina N, Tomarev SI, Martin KR. Neuroprotective Effects of Intravitreal Mesenchymal Stem Cell Transplantation in Experimental Glaucoma. *Investigative Ophthalmology & Visual Science* 2010;51:2051-2059.
25. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences* 2000;97:13625-13630.
26. Chai Y, Jiang X, Ito Y, et al. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 2000;127:1671-1679.

27. Kiraly M, Porcsalmy B, Pataki A, et al. Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. *Neurochemistry International* 2009;55:323-332.
28. Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. Adult Human Dental Pulp Stem Cells Differentiate Toward Functionally Active Neurons Under Appropriate Environmental Cues. *STEM CELLS* 2008;26:1787-1795.
29. Kiraly M, Kadar K, Horvathy DB, et al. Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo. *Neurochemistry International* 2011;59:371-381.
30. Gale Z, Cooper PR, Scheven BAA. Effects of Glial Cell Line-derived Neurotrophic Factor on Dental Pulp Cells. *Journal of Dental Research* 2011;90:1240-1245.
31. Nosrat IV, Widenfalk J, Olson L, Nosrat CA. Dental Pulp Cells Produce Neurotrophic Factors, Interact with Trigeminal Neurons in Vitro, and Rescue Motoneurons after Spinal Cord Injury. *Developmental Biology* 2001;238:120-132.
32. Nosrat CA, Fried K, Lindskog S, Olson L. Cellular expression of neurotrophin mRNAs during tooth development. *Cell Tissue Res* 1997;290:569-580.
33. Huang AH-C, Snyder BR, Cheng P-H, Chan AWS. Putative Dental Pulp-Derived Stem/Stromal Cells Promote Proliferation and Differentiation of Endogenous Neural Cells in the Hippocampus of Mice. *STEM CELLS* 2008;26:2654-2663.
34. Basso DM, Beattie MS, Bresnahan JC. A Sensitive and Reliable Locomotor Rating-Scale for Open-Field Testing in Rats. *Journal of Neurotrauma* 1995;12:1-21.
35. Leong WK, Henshall TL, Arthur A, et al. Human Adult Dental Pulp Stem Cells Enhance Poststroke Functional Recovery Through Non-Neural Replacement Mechanisms. *Stem Cells Translational Medicine* 2012;1:177-187.
36. Douglas MR, Morrison KC, Jacques SJ, et al. Off-target effects of epidermal growth factor receptor antagonists mediate retinal ganglion cell disinhibited axon growth. *Brain* 2009;132:3102-3121.
37. Berry M, Carlile J, Hunter A, Tsang WL, Rosustrel P, Sievers J. Optic nerve regeneration after intravitreal peripheral nerve implants: trajectories of axons regrowing through the optic chiasm into the optic tracts. *Journal of Neurocytology* 1999;28:721-741.
38. Sullivan KF. Structure and Utilization of Tubulin Isotypes. *Annual Review of Cell Biology* 1988;4:687-716.
39. Nadal-Nicolas FM, Jimenez-Lopez M, Sobrado-Calvo P, et al. Brn3a as a Marker of Retinal Ganglion Cells: Qualitative and Quantitative Time Course Studies in Naive and Optic Nerve-Injured Retinas. *Investigative Ophthalmology & Visual Science* 2009;50:3860-3868.
40. Berry M, Carlile J, Hunter A. Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. *J Neurocytol* 1996;25:147-170.
41. Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 1994;14:4368-4374.
42. Parrilla-Reverter G, Agudo M, Sobrado-Calvo P, Salinas-Navarro M, Villegas-Perez MP, Vidal-Sanz M. Effects of different neurotrophic factors on the survival of retinal ganglion cells after a complete intraorbital nerve crush injury: a quantitative in vivo study. *Exp Eye Res* 2009;89:32-41.
43. Logan A, Ahmed Z, Baird A, Gonzalez AM, Berry M. Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. *Brain* 2006;129:490-502.
44. Ahmed Z, Dent RG, Leadbeater WE, Smith C, Berry M, Logan A. Matrix metalloproteases: degradation of the inhibitory environment of the transected optic nerve and the scar by regenerating axons. *Molecular and cellular neurosciences* 2005;28:64-78.
45. Ahmed Z, Suggate EL, Brown ER, et al. Schwann cell-derived factor-induced modulation of the NgR/p75NTR/EGFR axis disinhibits axon growth through CNS myelin in vivo and in vitro. *Brain* 2006;129:1517-1533.

46. Fischer D. CNTF, a key factor mediating the beneficial effects of inflammatory reactions in the eye. *Brain* 2008;131.
47. Muller A, Hauk TG, Leibinger M, Marienfeld R, Fischer D. Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF. *Molecular and Cellular Neuroscience* 2009;41:233-246.
48. Tao W, Wen R, Goddard MB, et al. Encapsulated cell-based delivery of CNTF reduces photoreceptor degeneration in animal models of retinitis pigmentosa. *Invest Ophthalmol Vis Sci* 2002;43:3292-3298.
49. Sieving PA, Caruso RC, Tao W, et al. Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. *Proc Natl Acad Sci U S A* 2006;103:3896-3901.

CHAPTER 4

Paracrine-Mediated Neuroprotection and Neuritogenesis of Axotomised Retinal Ganglion Cells by Human Dental Pulp Stem Cells: Comparison with Human Bone Marrow and Adipose-Derived Mesenchymal Stem Cells

Ben Mead, Ann Logan, Martin Berry, Wendy Leadbeater and Ben A. Scheven

A version of this manuscript has been published in

PlosOne

ABSTRACT

We have investigated and compared the neurotrophic activity of human dental pulp stem cells (hDPSC), human bone marrow-derived mesenchymal stem cells (hBMSC) and human adipose-derived stem cells (hADSC) on axotomised adult rat retinal ganglion cells (RGC) *in vitro* in order to evaluate their therapeutic potential for neurodegenerative conditions of RGC.

Using the transwell system, RGC survival and length/number of neurites were quantified in coculture with stem cells in the presence or absence of specific Fc-receptor inhibitors to determine the role of NGF, BDNF, NT-3, VEGF, GDNF, PDGF-AA and PDGF-AB/BB in stem cell-mediated RGC neuroprotection and neuritogenesis. Conditioned media, collected from cultured hDPSC/hBMSC/hAMSC, were assayed for the secreted growth factors detailed above using ELISA. PCR array determined the hDPSC, hBMSC and hAMSC expression of genes encoding 84 growth factors and receptors.

The results demonstrated that hDPSC promoted significantly more neuroprotection and neuritogenesis of axotomised RGC than either hBMSC or hAMSC, an effect that was neutralized after the addition of specific Fc-receptor inhibitors. hDPSC secreted greater levels of various growth factors including NGF, BDNF and VEGF compared with hBMSC/hAMSC. The PCR array confirmed these findings and identified VGF as a novel potentially therapeutic hDPSC-derived neurotrophic factor (NTF) with significant RGC neuroprotective properties after coculture with axotomised RGC.

In conclusion, hDPSC promoted significant multi-factorial paracrine-mediated RGC survival and neurite outgrowth and may be considered a potent and advantageous cell therapy for retinal nerve repair.

INTRODUCTION

The axons of retinal ganglion cells (RGC) transmit action potentials along the optic nerve to the superior colliculus (SC) and lateral geniculate nucleus (LGN) that are relayed onwards to the visual cortex. Axotomised RGC die[1,2] so that blindness ensues after traumatic (crush or transection)[3] optic nerve injury. RGC loss is caused by a failure in the supply of neurotrophic factors (NTF; including neurotrophins), retrogradely transported from the SC/LGN neurons, that act as survival signals, ensuring the functional integrity of RGC connections[4-6].

As well as protecting RGC from death, NTF have the potential to promote the regeneration of transected axons and establish re-connection with their targets. The paucity of NTF in the central nervous system (CNS) is one explanation for the lack of axon regeneration compared to the peripheral nervous system (PNS)[2,7] in which successful and functional axon regeneration occurs, largely promoted by Schwann cell-derived NTF[8]. Attempts to promote long distance axon regeneration by the transplantation of peripheral nerve grafts into the CNS have met with some success[9]. For example, grafting a peripheral nerve into the vitreous body after optic nerve crush[8] promotes more RGC axon regeneration in the transected optic nerve than occurs after the removal of Schwann cells before transplantation, suggesting that axotomised RGC regenerate their axons when provided with a constant supply of NTF. However, single NTF supplementation[7], or single dose treatments of NTF such as BDNF[10,11], have proven unsuccessful and sustained delivery of multiple NTF to RGC over extended periods of time is difficult to achieve.

The vitreous is a relatively accessible immunoprivileged transplantation site[12] that lies directly adjacent to the RGC layer of the retina, allowing diffusion or transport of NTF across the inner limiting membrane to the RGC. Previously, we used intravitreally transplanted genetically modified fibroblasts expressing FGF-2, BDNF and NT-3 to promote RGC survival and axon regeneration after optic nerve crush[7]. Since the translational potential of

genetically modified cells is limited, mesenchymal stem cells (MSC), which secrete a large array of NTF, have gained credence as a potential cell therapy for diseased and injured CNS neurons. Human bone marrow-derived mesenchymal stem cells (hBMSC) protect RGC from death in both optic nerve crush[13] and glaucoma experimental models[14-16] through the production of NTF (e.g. platelet-derived growth factor (PDGF)[15]), without differentiation of hBMSC into replacement RGC/RGC-like cells.

We recently demonstrated that rat dental pulp stem cells (DPSC) protected adult rat RGC from death in an optic nerve crush model[17,18]. This effect was significantly greater than that achieved by rat BMSC and mediated through nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) via, TrKA, B and C receptor signalling. Our findings were consistent with previous studies showing significant expression[19,20] and secretion of NGF, BDNF and NT-3 by hDPSC[21]. The neuroprotective and axogenic properties of DPSC[17,18] and BMSC[14,15,22] can also be found in other MSC types, in particular adipose-derived mesenchymal stem cells (AMSC) that also secrete multiple NTF[22,23] and promote functional recovery after CNS trauma including spinal cord injury[22,24], stroke[25] and light induced photoreceptor damage[26,27]. However, AMSC have not been tested in a model of RGC death. Comparative analyses of different human MSC is still lacking although important for the determination of the most efficacious paracrine-mediated therapy for the injured retina. Thus, the aim of this study was to evaluate and compare the neuroprotective and neuritogenic effects of hDPSC, hBMSC and hAMSC and to define the stem cell NTF secretome using ELISA and PCR microarray analysis.

MATERIALS AND METHODS

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

DPSC/BMSC/AMSC cultures

hDPSC were purchased from AllCell LLC (Berkeley, CA) and both hBMSC and hAMSC from Lonza (Slough, UK), and each represented pooled samples from 3 donors. The $CD29^+/CD44^+/CD73^+/CD90^+/CD45^-$ (confirmed by supplier) stem cells were seeded into T25/T75 flasks (Corning, Amsterdam, NL) in both a total volume of 5ml/15ml DMEM containing 1% penicillin/streptomycin and 10% foetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and at a density of $1x10^6$ cells/$2x10^6$ cells, respectively. Cultures were maintained at 37°C in 5% CO_2, the supplemented medium was changed every 3d and the cells were passaged when 80% confluent using 0.05% trypsin/EDTA to lift them from their surface attachment.

Animals

Nine adult male Sprague-Dawley rats weighing 170-200g (Charles River, Kent, UK) were housed under Home Office guidelines 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. Animals were killed by "Schedule 1 Methods" i.e. rising concentrations of CO_2 before extraction of retinae. Ethical approval by the University of Birmingham ethical review Sub-Committee for this study was not required due to the *in vitro* nature of the experiment.

Retinal cell coculture

Cell culture 24-well plates (BD Biosciences, Oxford, UK) were pre-coated with 100µg/ml poly-D-lysine for 60min and then with 20µg/ml laminin for 30min. After culling and ocular dissection, the retinae of nine male Sprague-Dawley were minced in 1.25ml of papain (20U/ml; Worthington Biochem, Lakewood, NJ; as per manufacturer's instructions) containing 50µg/ml of DNase I (62.5µl; Worthington Biochem) and incubated for 90min at 37°C. The retinal cell suspension was centrifuged at 300xg for 5min and the pellet resuspended in 1.575ml of Earle's balanced salt solution (Worthington Biochem) containing 1.1 mg/ml of reconstituted albumin ovomucoid inhibitor (150µl; Worthington Biochem) and

56μg/ml of DNase I (75μl). After adding to the top of 2.5ml of albumin ovomucoid inhibitor (10mg/ml) to form a discontinuous density gradient, the retinal cell suspension was centrifuged at 70xg for 6min and the cell pellet resuspended in 1ml of supplemented Neurobasal-A (25ml Neurobasal-A (Life Technologies, Gibco, Paisley, UK), 1X concentration of B27 supplement (Life Technologies, Invitrogen, Paisley, UK), 0.5mM of L-glutamine (62.5μl; Invitrogen) and 50μg/ml of gentamycin (125μl; Invitrogen)) and seeded at a density of 125,000 cells/800μl/well in a 24 well plate. Previous immunocytochemical analysis of these cultures in our lab demonstrates that 60% of these retinal cells are neurons (neurofilament$^+$/βIII-tubulin$^+$), of which 10% are Thy1$^+$ RGC[28].

hDPSC, hBMSC and hAMSC were used at passage 2-4 and plated at a density of 50,000 cells/200μl onto a 0.4μm porous cell culture insert (Millipore, Watford, UK) that was placed into each of the 24 wells containing retinal cells to give a total volume of 1ml of medium/well. Particular wells containing retinal cell cultures were treated either singly or in combination with 5μg/ml Fc-TrKA, Fc-TrKB and/or Fc-TrKC (R&D Systems, Abingdon, UK) as well as Fc-VEGFr, Fc-GDNFr, Fc-PDGFAr and Fc-PDGFBr fusion protein inhibitors[29] (R&D Systems) which are highly specific inhibitors for the corresponding cognate neurotrophic factor receptors (NTFR). A combination of recombinant human NGF, BDNF and NT-3 was also added to some retinal cell cultures at 60ng/ml as a positive control. Particular wells containing retinal cells were treated with 0.1μm, 1μm and 10μm of VGF (R&D Systems) instead of hDPSC/hBMSC/hAMSC.

Cocultures were incubated for 3d at 37°C before immunocytochemical staining of RGC with βIII-tubulin. For this study, large spherical βIII-tubulin$^+$ retinal cells[30] cultured after neuronal isolation from retinae, are referred to as RGC. βIII-tubulin is a reliable and relatively specific marker for RGC[30], although cross reactivity with amacrine cells has been suggested[31]. However, it should be emphasised, that the isolation procedure and growth medium used preferentially selects and yields populations of neuronal cells, we are confident that our findings accurately reflect RGC numbers. 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.

Immunocytochemistry

Retinal cells in 24 well plates (BD Biosciences) were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10min, washed for 3 X 10min of PBS, blocked in blocking solution (0.5% bovine serum albumin (g/ml), 0.3% Tween-20, 15% normal goat serum (Vector Laboratories, Peterborough, UK) in PBS) for 20min and incubated with primary antibody (anti-rat βIII-tubulin, raised in mouse, #T8660 diluted at 1:500 in antibody diluting buffer (ADB; 0.5% bovine serum albumin, 0.3% Tween-20 in PBS) for 1h at room temperature. Cells were then washed for 3 X 10min in PBS, incubated with the secondary antibody (anti-mouse IgG Fluor 488, raised in goat, 1:400, #A-11001; Life Technologies, Molecular Probes, Paisley, UK) diluted in ADB for 1h at room temperature, washed for 3 X 10min in PBS, mounted in Vectorshield mounting medium containing DAPI (Vector Laboratories) and stored at 4°C.

Microscopy and analysis

For immunocytochemistry, all βIII-tubulin^{+} RGC, with or without neurites, were counted in each well of the 24 well plates, recording the total number of RGC and the number of RGC with neurites. Neurite outgrowth was measured in images taken at 20X magnification using an Axiocam HRc camera (Carl Zeiss Ltd, Hertfordshire, UK). Each well was divided into 9 equal sectors and the length of the longest neurite of each RGC in each sector was measured using Axiovision software (Carl Zeiss Ltd). Fluorescently stained cells were analysed by an operator blinded to treatment groups, using a Zeiss Axiovert fluorescent microscope (Carl Zeiss Ltd).

NTF ELISA

To quantify the growth factors and NTF produced by hDPSC, hBMSC and hAMSC, conditioned medium was collected from cultures at passage 2 and 5 after 48h in culture and assayed using a duoset ELISA kit for human NGF, BDNF and NT-3, VEGF, GDNF, platelet-derived growth factor (PDGF-AA) and PDGF-AB/BB according to the manufacturer's instructions (R&D Systems). Briefly, a standard curve was constructed using NTF standards and test samples of conditioned medium at increasing dilutions, run in duplicate with NTF concentrations extrapolated from the standard curve. Values were normalized to the number of cells in the flask (manually counted *via* haemocytometer) and the volume of medium, and corrected for analyte found in the medium/serum.

Human NTF and NTFR PCR array

The expression of 84 NTF and NTFR genes by hDPSC, hBMSC and hAMSC (passage 2) was assayed using quantitative RT-PCR profiler arrays (PAMM-031) by a commercially run service (Sabiosciences, Qiagen, Hilden, Germany). Housekeeping genes (*b2m* and *hprt1*) were used to normalize the data and the $^{-\Delta Ct}$ compared between cell types and expressed as + or - fold changes. Samples contained one million cells and were run in triplicate.

Statistics

All statistical tests were performed using SPSS 17.0 (IBM SPSS, Inc., Chicago, IL) and data presented as mean ± standard error of the mean (SEM). The Shapiro-Wilkes 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 differences were considered significant at p values <0.05. For the qRT-PCR, data were compared by a Student's t test and statistical significance set at $p<0.001$.

RESULTS

hDPSC promoted significantly greater paracrine-mediated neuroprotection and neuritogenesis than hBMSC/hAMSC

hDPSC, hBMSC and hAMSC all promoted a significant increase ($p<0.05$) in the survival of cocultured RGC (282.7 ± 17.1 cells/well; 219 ± 28.4 cells/well; 200.0 ± 10.2 cells/well; respectively) compared with retinal cells cultured alone (100.7 ± 9.5 cells/well); hDPSC, hBMSC and hAMSC neuroprotection was similar to the level obtained after retinal cell culture with recombinant NGF/BDNF/NT-3 (239.7 ± 15.4 cells/well; Figs. 1, 2.). The increase in survival of RGC in hDPSC-treated retinal cultures was significantly greater ($p<0.05$) than that achieved in cocultures with hAMSC ($p<0.05$) but not significantly greater than that seen in cocultures with hBMSC.

Coculture of retinal cells with hDPSC, hBMSC and hAMSC significantly increased ($p<0.05$) the percentage of surviving RGC bearing tubulin^{+} neurites (64.8 ± 4.0%, 51.1 ± 2.1%, 42.2 ± 4.3%, respectively), as well as the length of neurites (236.7μm ± 27.6μm, 150.7μm ± 24.1μm, 101.1μm ± 12.1μm, respectively) compared with retinal cells cultured alone (18.2 ± 4.6%; 32.8μm ± 1.4μm; Fig. 1, 2.). Coculture with hDPSC promoted a significant increase ($p<0.05$) in the number of neurite-bearing RGC as well as neurite length when compared with cocultures with hBMSC and hAMSC, or with retinal cultures exposed to recombinant NGF, BDNF and NT-3. These data confirm that hDPSC, hBMSC and hAMSC have paracrine-mediated neuroprotective and neuritogenic properties, with hDPSC promoting the most significant effects.

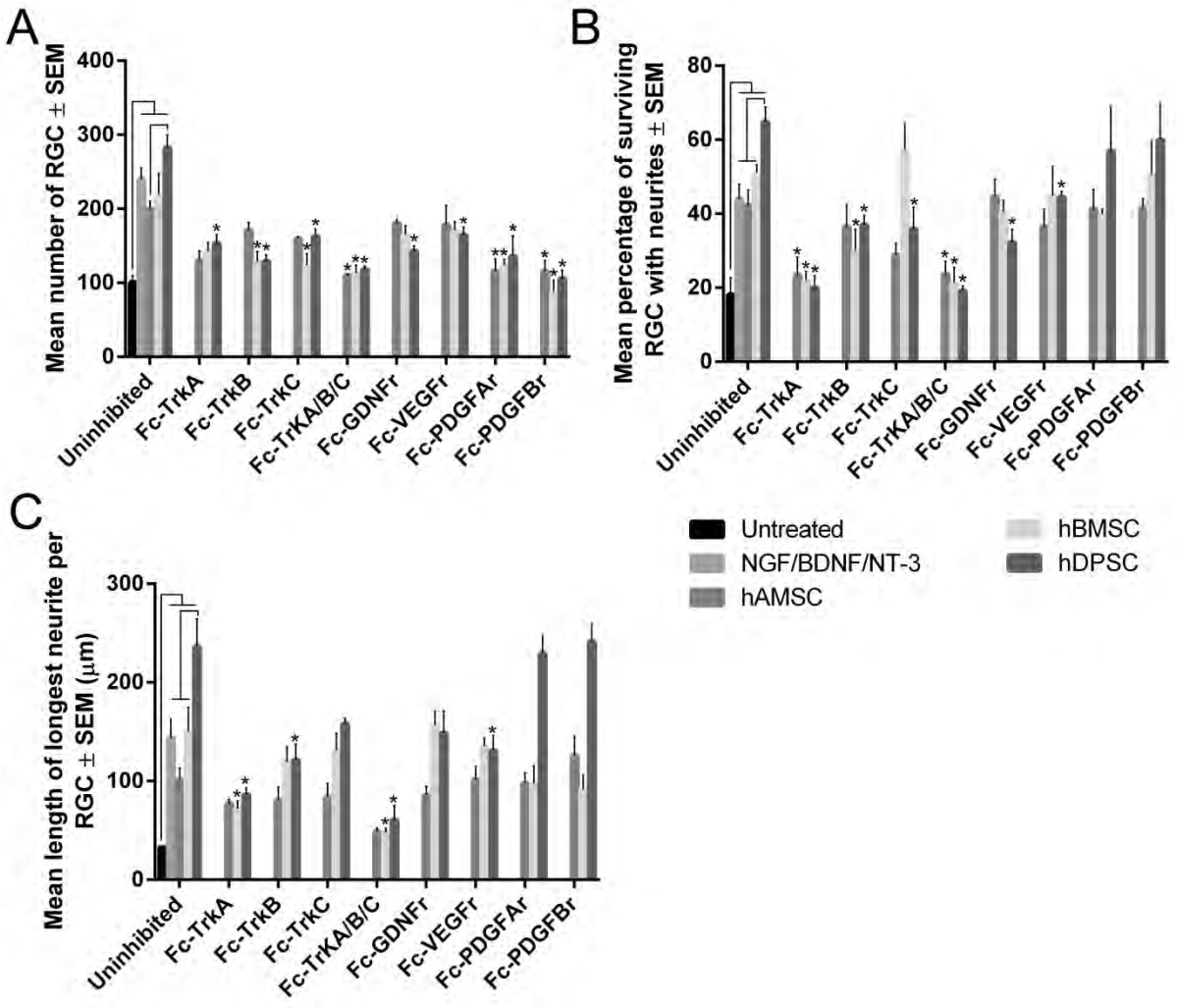


Figure 1: Effects of hDPSC, hBMSC and hAMSC on axotomised RGC in culture. The number of RGC (**A**), percentage of surviving RGC bearing neurites (**B**) and the length of the longest RGC neurite (**C**) in both untreated retinal cultures and after coculture with hDPSC, hBMSC, hAMSC, with or without added exogenous neurotrophins. *Black lines* indicate significant difference at $P < 0.05$. The effects of Fc-TrKA, -B, -C, Fc-GDNFr, Fc-VEGFr, Fc-PDGFAr and Fc-PDGFBr inhibitors on RGC survival (**A**) and neuritogenesis (**B, C**) in hDPSC, hBMSC and hAMSC cocultures are also shown (values marked with an *asterisk* indicate significant difference from uninhibited cultures at $P < 0.05$).

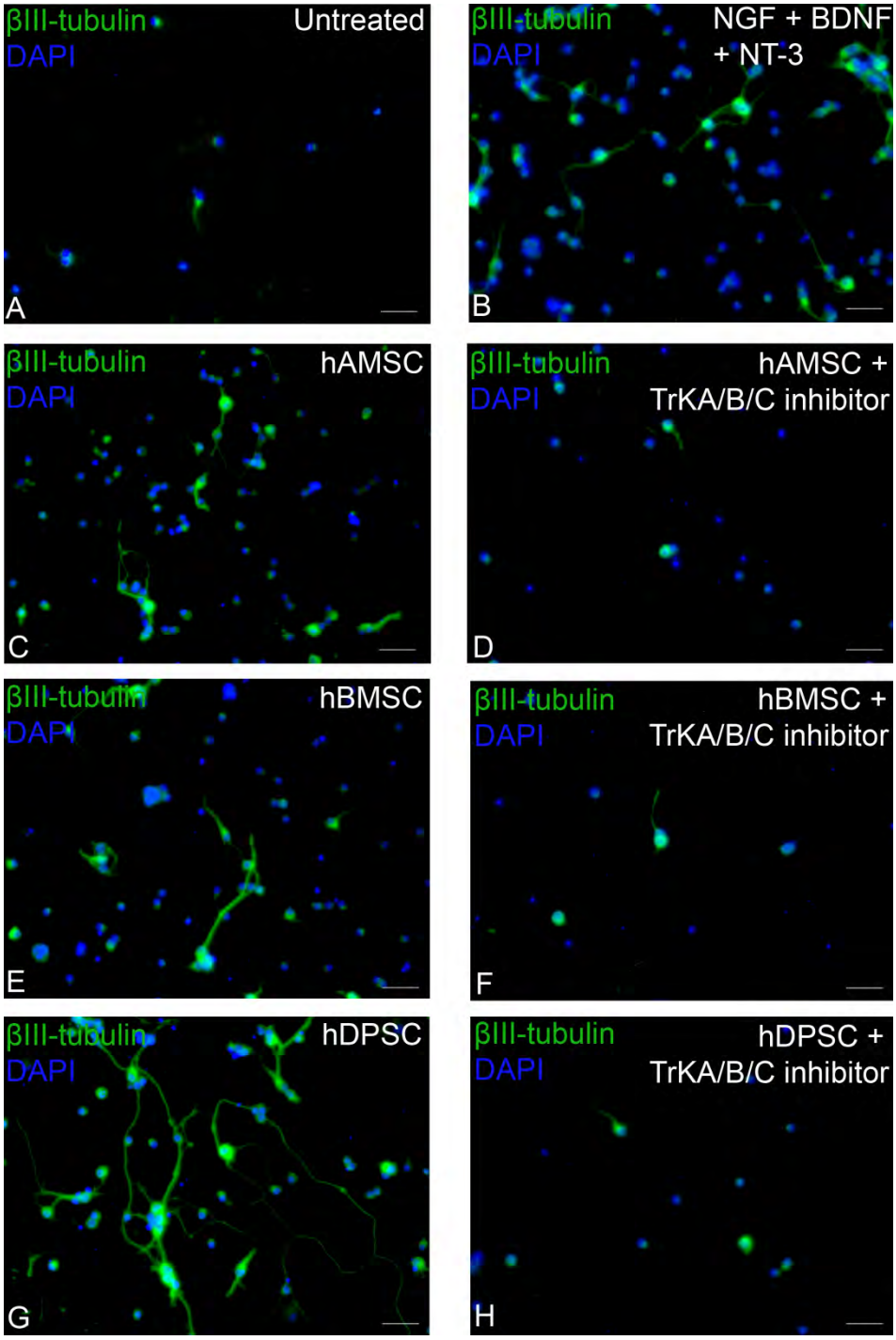


Figure 2: Immunocytochemical staining of RGC after retinal coculture with hDPSC, hBMSC and hAMSC in transwell inserts. *In vitro* RGC cultured either alone (**A**), with exogenous neurotrophins (**B**), with hAMSC (with or without TrK inhibitors (**C**, **D**, respectively)), with hBMSC (with or without TrK inhibitors (**E**, **F**, respectively)) or with hDPSC (with or without TrK inhibitors (**G**, **H**, respectively). All images are representative of the entire culture, nine separate culture wells/treatment, with every three wells using a different animal (*scale bars*: 50μm), cell nuclei were stained with DAPI.

NTFR Fc-receptor blockers for multiple NTFR attenuated the neuroprotective and neuritogenic effect of hDPSC/hBMSC/hAMSC

The NTFR blockers Fc-TrKA, Fc-TrKB, Fc-TrKC, Fc-TrKA/B/C, Fc-GDNFr, Fc-VEGFr, Fc-PDGFAr and Fc-PDGFBr significantly attenuated hDPSC mediated neuroprotection and/or neuritogenesis of cocultured RGC (Table. 1; Fig. 1, 2.) compared to uninhibited hDPSC/retinal cell cocultures. Fc-TrKA, Fc-TrKB, Fc-TrKC, Fc-TrKA/B/C, Fc-PDGFAr and Fc-PDGFBr significantly attenuated hBMSC mediated neuroprotection and/or neuritogenesis of cocultured RGC compared to uninhibited hBMSC/retinal cell cocultures. Fc-TrKA/B/C, Fc-PDGFAr and Fc-PDGFBr significantly attenuated hAMSC mediated neuroprotection and/or neuritogenesis of cocultured RGC compared to uninhibited hAMSC/retinal cell cocultures. These data demonstrate that the neuroprotective and neuritogenic effects afforded by each of the stem cell types are mediated through a variety of different NTF.

Treatment		Without inhibition	Fc-TrKA	Fc-TrKB	Fc-TrKC	Fc-TrKA/B/C	Fc-GDNFr	Fc-VEGFr	Fc-PDGFAr	Fc-PDGFBr
hDPSC	RGC/well	282.7 ± 17.1	**153.0 ± 12.1**	**129.3 ± 8.5**	**163.3 ± 9.4**	**118.3 ± 4.3**	**143.3 ± 6.6**	**164.7 ± 10.7**	**136.3 ± 27.1**	**106.0 ± 11.3**
	% surviving RGC with neurites	64.8 ± 4.0	**20.0 ± 3.2**	**37.1 ± 2.4**	**35.9 ± 5.8**	**19.2 ± 1.3**	**32.3 ± 3.4**	**44.5 ± 1.4**	57.0 ± 12.1	60.1 ± 9.7
	Length of longest neurite (μm)	236.7 ± 27.6	**86.4 ± 7.0**	**121.6 ± 16.1**	158.2 ± 5.1	**60.6 ± 14.8**	148.9 ± 22.2	**130.8 ± 15.4**	229.5 ± 18.4	241.6 ± 17.6
hBMSC	RGC/well	219 ± 28.4	143.0 ± 11.4	**128.7 ± 13.6**	**124.0 ± 15.4**	**112.7 ± 11.0**	165.3 ± 11.9	171.0 ± 11.6	**123.7 ± 8.0**	**87.7 ± 16.1**
	% surviving RGC with neurites	51.1 ± 2.1	**21.9 ± 2.5**	**30.1 ± 5.7**	57.3 ± 7.0	**21.4 ± 4.1**	40.5 ± 3.0	44.83 ± 8.0	39.9 ± 1.4	50.6 ± 9.0
	Length of longest neurite (μm)	150.7 ± 24.1	**72.0 ± 7.8**	119.9 ± 15.3	131.1 ± 17.6	**47.9 ± 4.2**	156.7 ± 14.2	135.0 ± 8.9	97.4 ± 18.2	91.3 ± 15.0
hAMSC	RGC/well	200.0 ± 10.2	130.0 ± 13.1	171.3 ± 10.4	160.0 ± 2.6	**110.3 ± 1.2**	165.3 ± 11.9	171.0 ± 11.6	**116.3 ± 15.9**	**115.7 ± 14.5**
	% surviving RGC with neurites	42.2 ± 4.3	**23.5 ± 4.8**	36.6 ± 6.0	29.0 ± 3.0	**23.7 ± 3.5**	44.6 ± 4.7	36.6 ± 4.7	41.3 ± 5.3	41.6 ± 2.4
	Length of longest neurite (μm)	101.1 ± 12.1	77.1 ± 4.3	80.8 ± 13.1	83.6 ± 14.2	49.1 ± 3.6	156.7 ± 14.2	135.0 ± 8.9	97.6 ± 10.5	125 ± 19.5

Table 1: The number of surviving RGC (cells/well), percentage of surviving RGC growing neurites (%) and the length of the longest neurite (μm) after coculture of RGC with hDPSC,

hBMSC or hAMSC and inhibition with Fc-TrKA, Fc-TrKB, Fc-TrKC, Fc-TrKA/B/C, Fc-GDNFr, Fc-VEGFr, Fc-PDGFAr and Fc-PDGFBr; values significantly ($p<0.05$) different from coculture without inhibition are given in bold (Mean +/- SEM; n = 3).

hDPSC, hBMSC and hAMSC have distinct NTF expression profiles

The PCR array detected 84 NTF and NTFR genes differentially expressed by hDPSC, hBMSC and hAMSC. For example, hDPSC express ≥4 fold higher *cd40, crhbp, grpr, il1r1, ntrk1, ptger2* and *vgf* than hBMSC and ≥4 fold higher *bdnf, gdnf, grpr, nt-3, ptger2, tacr1* and *vgf* than hAMSC (Fig. 3.). hDPSC express ≥4 fold lower *cckar, fgf9, gfra1, hspb1, il1b, il6, ngfr, ntrk2, ntsr1, stat1, stat4 and tgfa* than hBMSC and ≥4 fold lower *adcyap1r1, bcl2, cxcr4, fgf9, gfra1, il1b, il6, ntrk2, ppyr1, stat1, tgfa* than hAMSC. Significant differences ($p<0.001$) are highlighted in the graph (Fig. 3). These data confirm that, despite hDPSC, hBMSC and hAMSC all being designated as mesenchymal stem cells, the NTF secretome is distinct between each stem cell phenotype.

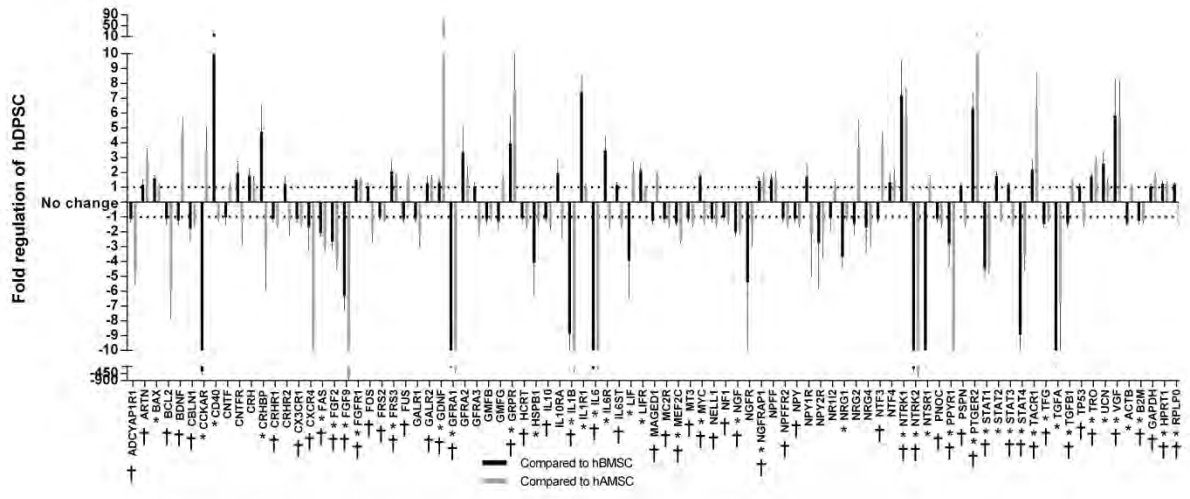


Figure 3: Expression of NTF and NTFR genes by hDPSC, hBMSC and hAMSC. RT-PCR array analysis of 84 genes encoding NTF and NTFR expressed by hDPSC, displayed as fold regulation in comparison to either hBMSC (black bars) or hAMSC (grey bars). The horizontal dotted lines represent fold-changes of ±1 (no difference). Significant differences between hDPSC and hBMSC (*) and hDPSC and hAMSC (†) at $p < 0.05$ are labelled on the x axis.

hDPSC secrete multiple NTF at higher levels than hBMSC/hAMSC

The levels of secretion by hDPSC, hBMSC and hAMSC of NGF, BDNF, NT-3, VEGF, GDNF, PDGF-AA, PDGF-AB/BB and CNTF, at passage 2 and 5, are detailed in Table 2, and presented as pg/100,000 cells/48h. CNTF and PDGF-AB/BB were undetectable in hDPSC/hBMSC/hAMSC conditioned media, while BDNF and NT-3 were undetectable in hAMSC conditioned medium. The hDPSC secreted significantly greater ($p<0.05$) titres of NGF, BDNF and VEGF than hBMSC/hAMSC (Table. 2; Fig. 4.). These data confirm that the hBMSC, hAMSC, and in particular, hDPSC secrete a variety of different NTF.

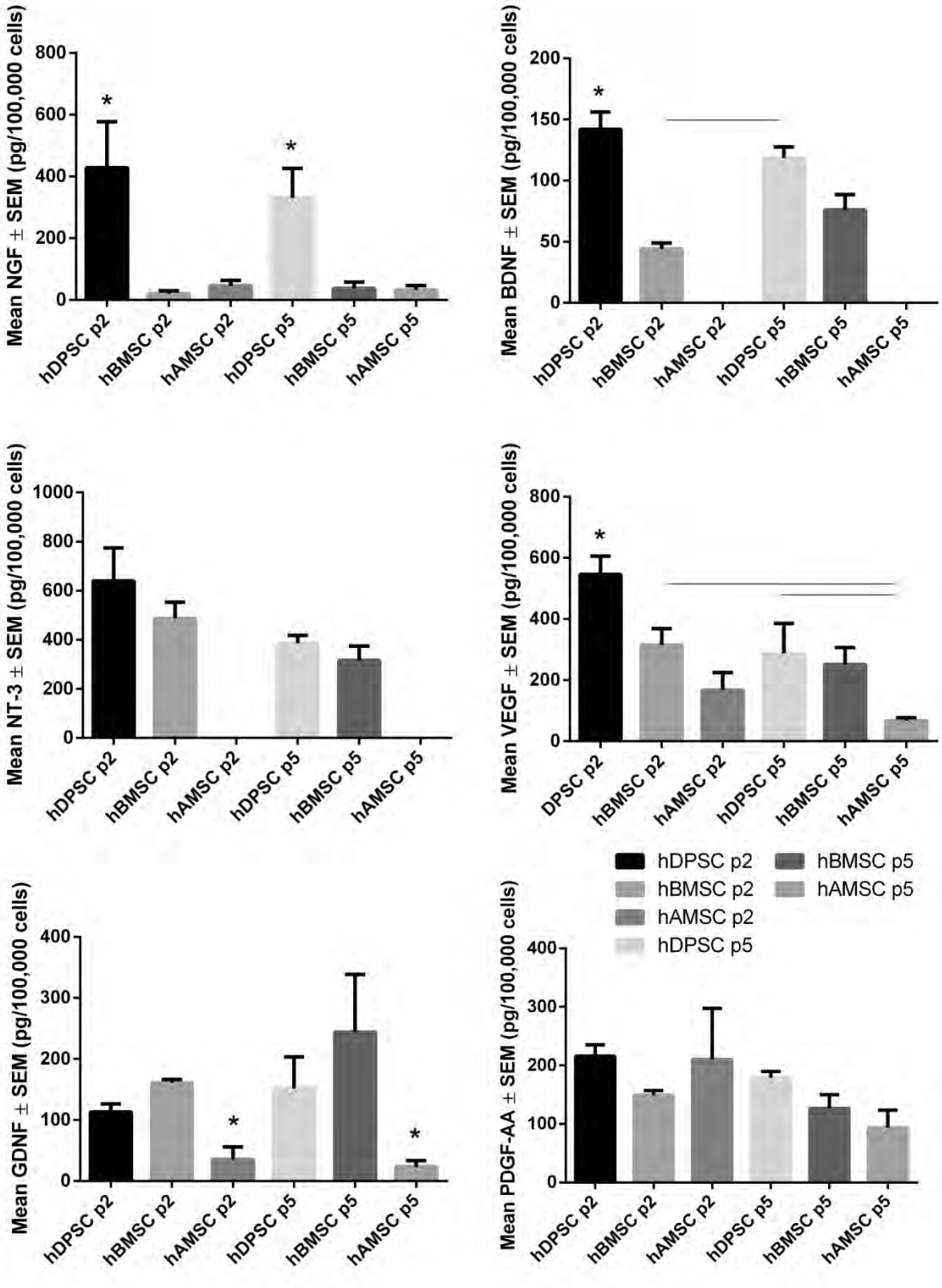


Figure 4: NGF, BDNF, NT-3, CNTF, VEGF, GDNF, PDGF-AA and PDGF-AB/BB titres by ELISA in hDPSC, hBMSC and hAMSC. hDPSC-, hBMSC- and hAMSC-conditioned media (passage 2 and 5) collected after 48h of cell culture (n = 3; *asterisks* indicate significant

differences from all other samples/*black lines* indicate significant difference between specific samples at $p < 0.05$). CNTF and PDGF-AB/BB were undetectable in all samples.

		NGF	BDNF	NT-3	VEGF	GDNF	PDGF-AA	PDGF-AB/BB	CNTF
hDPSC	Passage 2	428.0 ± 149.0	141.9 ± 14.4	639.8 ± 134.9	314.7 ± 53.9	113.2 ± 13.1	216.0 ± 19.0	0	0
	Passage 5	331.2 ± 94.8	118.3 ± 9.2	385.3 ± 32.6	287.3 ± 98.4	151.7 ± 51.6	178.9 ± 11.0	0	0
hBMSC	Passage 2	20.4 ± 9.5	44.1 ± 4.8	487.4 ± 65.4	314.7 ± 53.9	161.2 ± 5.2	149.2 ± 7.9	0	0
	Passage 5	37.7 ± 20.7	76.0 ± 12.6	316.2 ± 58.3	251.2 ± 54.9	244.3 ± 94.2	126.8 ± 23.2	0	0
hAMSC	Passage 2	46.1 ± 17.5	0	0	167.6 ± 57.2	35.5 ± 20.2	210.3 ± 87.1	0	0
	Passage 5	33.2 ± 14.0	0	0	66.7 ± 10.5	23.6 ± 10.0	93.9 ± 29.8	0	0

Table 2: The titre of secreted NGF, BDNF, NT-3, VEGF, GDNF, PDGF-AA, PDGF-AB/BB and CNTF in conditioned media from hDPSC, hBMSC and hAMSC at passage 2 and 5, as determined by ELISA. Data are presented as pg/100,000 cells/48h (Mean +/- SEM; n=3).

VGF was neuroprotective for RGC

The differentially and significantly greater transcription of *vgf* in hDPSC compared to hBMSC/hAMSC (Fig. 3.) led to investigation of the neuroprotective and/or proregenerative properties of VGF. VGF promoted a significant increase ($p<0.05$) in the survival of cultured RGC at concentrations of 1μM (255.5 ± 29.4 cells/well) and 10μm (263.5 ± 24.4 cells/well), but not at 0.1μM (148.3 ± 33.1 cells/well), compared to untreated controls (118.7 ± 18.7 cells/well; Fig. 5.).

By contrast, the percentage of surviving RGC bearing neurites in cultures of axotomised retinal neurons did not significantly change after treatment with 0.1μM (15.8 ± 1.4%), 1μM (10.0 ± 1.9%) or 10μM (10.9 ± 1.9%) when compared to untreated controls (17.3 ± 1.3%). The data suggest that VGF is a novel DPSC-derived neuroprotective, but not neuritogenic, factor for RGC at the optimal dose of 1μm.

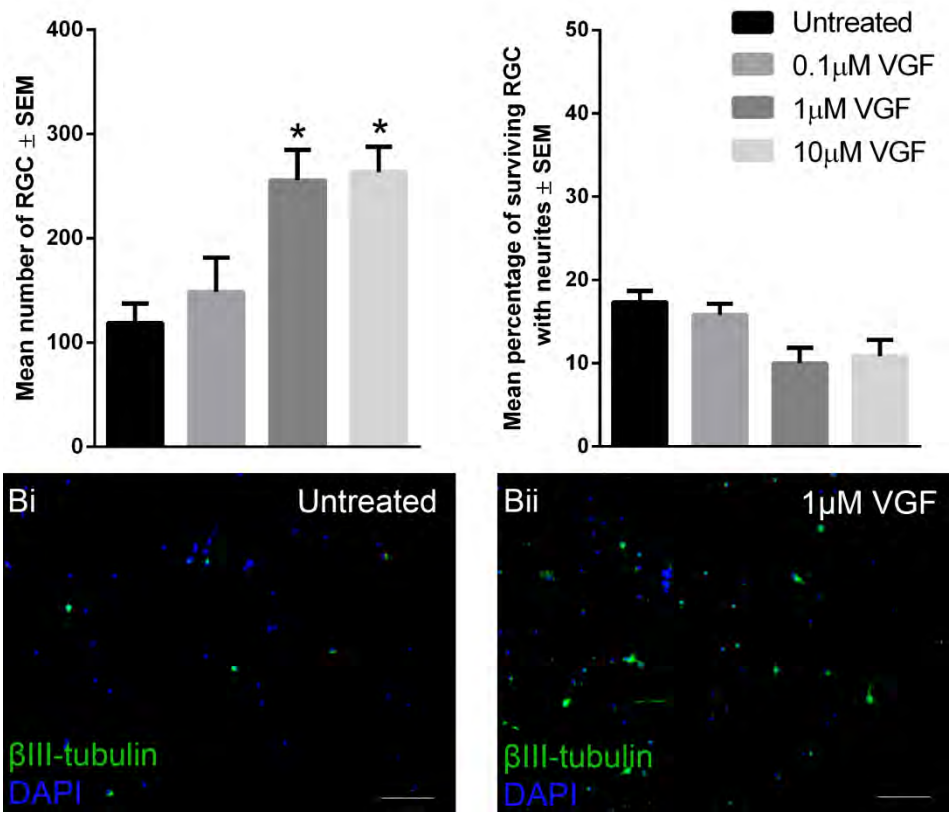


Figure 5: The effects of VGF on RGC in retinal cultures. The total number of surviving RGC as well as the percentage of RGC bearing neurites either cultured alone, or with 0.1μM, 1μM and 10μM VGF (**A**). *Asterisks* indicate significant difference from other treatment groups at $p < 0.05$. RGC cultured either alone (**Bi**) or with 1μm VGF (**Bii**). All images are representative of the entire culture, nine separate culture wells/treatment with every three wells using different animals (*scale bars*: 100μm), cell nuclei were stained with DAPI.

DISCUSSION

We have previously shown that rat DPSC promoted neurotrophin-mediated neuroprotection and neuritogenesis/axogenesis of axotomised RGC, both *in vitro* and *in vivo*[17]. Interestingly, rat DPSC promoted significantly greater neuroprotection and axon regeneration than rat BMSC, which reportedly protect RGC from death in models of optic nerve crush and glaucoma[14,17]. We aimed to determine if the more potent neurotrophic properties were replicated by hDPSC, when compared to hBMSC and hAMSC and confirm that, like rat DPSC, hDPSC secreted multiple neuroprotective and axogenic NTF that protected RGC from death and promoted the growth of their neurites to a significantly

greater extent than either hAMSC or hBMSC, which both have distinct NTF profiles as determined by RT-PCR and ELISA. Thus, we conclude that hDPSC may constitute an effective paracrine-mediated cellular therapy for retinal, and potentially other CNS, injuries[17].

To determine the neuroprotective and neuritogenic effects of hDPSC-, hBMSC- and hAMSC-derived NTF, we cultured these human-derived stem cells with RGC. Similar to our previous results using rat stem cells[17], hDPSC and hBMSC stimulated RGC survival and neuritogenesis to levels greater than those seen in untreated control retinal cultures. Moreover, hDPSC were more neuroprotective and neuritogenic than hBMSC (although the former measure did not reach statistical significance), which corroborates our previous findings[17] as well as those of others showing greater functional restoration when hDPSC, as opposed to hBMSC, were transplanted into spinal cord lesion sites[32]. The neuroprotective and neuritogenic properties of hAMSC were significantly less than hDPSC and this was correlated with lower secreted levels of NGF, BDNF, NT-3, VEGF and GDNF observed by ELISA.

We used Fc-NTFR fusion protein blockers to examine the mechanism of the hDPSC-, hBMSC- and hAMSC-mediated neuroprotection and neuritogenesis. The neuroprotective effect of hDPSC was significantly reduced after the addition of each individual Fc-NTFR, confirming the contribution of stem cell-derived NGF, BDNF, NT-3, GDNF, VEGF and PDGF-AA/AB/BB. Similar observations were seen with hBMSC, suggesting that the mechanisms for neuroprotection/neuritogenesis are similar and that the reduced neuroprotective effect of hBMSC compared to hDPSC is explained by the reduced neurotrophic profile. Moreover, hAMSC had a similar RGC protective/regenerative potency to hBMSC yet, owing to a lack of BDNF secretion, Fc-TrKB had no effect on hAMSC-mediated RGC survival or neuritogenesis. Interestingly, Fc-PDGFBr reduced hDPSC-/hBMSC-/hAMSC-mediated neuroprotection despite no PDGF-AB/BB being detected in conditioned media by ELISA. This might be explained by the previous observation that

PDGF-AB/BB was secreted at very low levels by hBMSC (40-fold < PDGF-AA)[15] and therefore expected to fall below the detectable range for the ELISA used. Thus, the efficacy of Fc-PDGFBr could be attributable either to the potency of low levels of PDGF-AB/BB or to PDGF-AB/BB secreted by glia present in the retinal cultures in response to hDPSC-/hBMSC-/hAMSC-derived growth factor stimulation.

The secretion of multiple NTF by hDPSC confirms previous findings showing NTF gene expression by hDPSC[19,20,32] as well as NTF secretion by rat-derived DPSC[17,33] and neurotrophins by hDPSC[21]. In concert with our findings using rat DPSC[17], hDPSC secreted significantly more NGF and BDNF than hBMSC. Interestingly, compared to previous data on rat DPSC, hDPSC secreted higher titres of NGF but lower titres of BDNF[17]. Our results confirmed those in the literature on the extensive NTF secretory profile of hBMSC including NGF, BDNF, NT-3 and GDNF[17,34-36], adding VEGF to the list of known secreted factors but failed to detect CNTF[32]. hAMSC secrete NGF, BDNF, NT-3, GDNF and VEGF[22,23]. However in the present study, we have not detected the secretion of BDNF and NT-3, possibly because the titre was below the detectable range of the ELISA or that none was secreted in this study. Our results also support the findings of recent studies by others showing that hBMSC secrete PDGF-AA[15] and extend this observation to show that hDPSC and hAMSC share this property.

To further elucidate the relative neurotrophic activities of hDPSC, hBMSC and hAMSC, we conducted a RT-PCR array of 84 NTF/NTFR genes. The data showed that all three stem cell types have distinct NTF gene expression profiles. In particular, we found that hDPSC expressed prostaglandin E2 receptor (*ptger2*) at 6 and 10 fold higher than both hBMSC and hAMSC, respectively. Ptger2 stimulates the synthesis and release of neurotrophins from multiple cell types[37-39]. hDPSC also expressed over 100-fold lower interleukin-6 (*il6*) than hBMSC and hAMSC. The cytokine IL6 is neuroprotective after binding to the gp130 receptor[40] and promotes axon regeneration after activating the JAK/STAT3 pathway[41]. As a pro-inflammatory cytokine it is likely there are other IL6-mediated effects not fully

realised in our *in vitro* paradigm. Finally, hBMSC express 6-fold higher fibroblast growth factor-9 (*fgf9*) than hDPSC, whereas hAMSC express over 700-fold higher *fgf9* than hDPSC. FGF9, unlike other FGF isoforms, stimulates the survival of RGC by binding to FGF receptor-3[42], possibly highlighting a distinct mechanism for hAMSC-induced RGC neuroprotection. These data reinforce the notion that the stem cell origin is critical in determining their selection and application as cellular therapies for the treatment of particular neurological conditions.

NTF analyses by ELISA corresponded well with the RT-PCR microarray data, a point particularly well illustrated by the correlative BDNF and GDNF protein and mRNA data. Interestingly NGF values for protein and mRNA were paradoxical; demonstrating lower levels of NGF gene expression in hDPSC compared to hBMSC/hAMSC, while the titres of NGF protein in the conditioned medium from hDPSC cultures was significantly higher than that in the medium from hBMSC/hAMSC. These findings underline some discrepancies between gene expression and NTF protein secretion, which may be explained by differences in either the timing of sampling (PCR reflecting a snap-shot event while protein levels are cumulative) or an abundance of pre-existing stores of NGF in hDPSC.

The results presented here support the assertion that the RGC survival effects of the MSC are mediated in part by PDGF-AA[15]. Interestingly, inhibitors to the PDGFr did not significantly reduce RGC neuritogenesis, suggesting that MSC-derived PDGF is important for neuroprotection but not neuritogenesis in RGC.

RGC neurite outgrowth appeared to be particularly dependent on hDPSC-, hBMSC- and hAMSC-derived NGF. Thus, considering the enhanced secretion of NGF by hDPSC, may explain why hDPSC are more neuritogenic than hBMSC and hAMSC. BDNF and NT-3 are also neuritogenic for RGC, since neurite outgrowth was suppressed by the cognate Fc-TrK inhibitors. Noteworthy, hAMSC promoted very little neuritogenesis, although the response was significantly reduced when all three TrK receptors were simultaneously blocked.

Finally, this study is the first to identify VGF as a novel factor expressed at higher levels (>4 fold) in hDPSC than in hBMSC or hAMSC. VGF is a peptide present in the CNS and PNS[43] that protects motor neurons in animal models of amyotrophic lateral sclerosis[44] and increases the survival of cerebellar granule cells after serum deprivation[45]. We also showed that VGF was active in the retinal culture model at a concentration similar to that previously reported as active in other models of CNS injury[45]. In particular, VGF was significantly RGC neuroprotective, but not neuritogenic, suggesting that VGF may be involved in hDPSC-mediated RGC neuroprotection. Thus we propose that VGF may be a novel therapeutic NTF for RGC neuroprotection.

In conclusion, our results show that hDPSC is a more potent stem cell type for paracrine-mediated neuroprotection and regeneration of RGC than either hBMSC or hAMSC. The hDPSC-mediated neuroprotection and neuritogenesis is achieved through the paracrine effect of multiple secreted NTF, including PDGF (neuroprotection) and NGF (axon regeneration). Moreover, VGF is identified as a novel RGC neuroprotective factor expressed by hDPSC. hDPSC may represent an effective and advantageous cellular therapy for retinal nerve protection and repair.

REFERENCES

1. Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ (1994) Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. J Neurosci 14: 4368-4374.
2. Berry M, Ahmed Z, Lorber B, Douglas M, Logan A (2008) Regeneration of axons in the visual system. Restorative Neurology and Neuroscience 26: 147-174.
3. Sarkies N (2004) Traumatic optic neuropathy. Eye 18: 1122-1125.
4. Quigley HA (1999) Neuronal death in glaucoma. Progress in Retinal and Eye Research 18: 39-57.
5. Quigley HA, McKinnon SJ, Zack DJ, Pease ME, Kerrigan-Baumrind LA, et al. (2000) Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats. Invest Ophthalmol Vis Sci 41: 3460-3466.
6. Dawbarn D, Allen SJ (2003) Neurotrophins and neurodegeneration. Neuropathol Appl Neurobiol 29: 211-230.
7. Logan A, Ahmed Z, Baird A, Gonzalez AM, Berry M (2006) Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. Brain 129: 490-502.
8. Berry M, Carlile J, Hunter A (1996) Peripheral nerve explants grafted into the vitreous body of the eye promote the regeneration of retinal ganglion cell axons severed in the optic nerve. Journal of Neurocytology 25: 147-170.

9. Richardson PM, McGuinness UM, Aguayo AJ (1980) Axons from CNS neurons regenerate into PNS grafts. Nature 284: 264-265.
10. Ko ML, Hu DN, Ritch R, Sharma SC, Chen CF (2001) Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. Neurosci Lett 305: 139-142.
11. Ko ML, Hu DN, Ritch R, Sharma SC (2000) The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. Invest Ophthalmol Vis Sci 41: 2967-2971.
12. Jiang LQ, Streilein JW (1991) Immune privilege extended to allogeneic tumor cells in the vitreous cavity. Invest Ophthalmol Vis Sci 32: 224-228.
13. Levkovitch-Verbin H, Sadan O, Vander S, Rosner M, Barhum Y, et al. (2010) Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. Invest Ophthalmol Vis Sci 51: 6394-6400.
14. Johnson TV, Bull ND, Hunt DP, Marina N, Tomarev SI, et al. (2010) Neuroprotective Effects of Intravitreal Mesenchymal Stem Cell Transplantation in Experimental Glaucoma. Investigative Ophthalmology & Visual Science 51: 2051-2059.
15. Johnson TV, Dekorver NW, Levasseur VA, Osborne A, Tassoni A, et al. (2013) Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain.
16. Johnson TV, Martin KR (2012) Cell transplantation approaches to retinal ganglion cell neuroprotection in glaucoma. Curr Opin Pharmacol.
17. Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2013) Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest Ophthalmol Vis Sci 54: 7544-7556.
18. Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2014) Dental pulp stem cells, a paracrine-mediated therapy for the retina. Neural Regeneration Research 9: 577-578.
19. Nosrat CA, Fried K, Lindskog S, Olson L (1997) Cellular expression of neurotrophin mRNAs during tooth development. Cell and Tissue Research 290: 569-580.
20. Nosrat IV, Widenfalk J, Olson L, Nosrat CA (2001) Dental Pulp Cells Produce Neurotrophic Factors, Interact with Trigeminal Neurons in Vitro, and Rescue Motoneurons after Spinal Cord Injury. Developmental Biology 238: 120-132.
21. Martens W, Sanen K, Georgiou M, Struys T, Bronckaers A, et al. (2013) Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro. The FASEB Journal.
22. Zhou Z, Chen Y, Zhang H, Min S, Yu B, et al. (2013) Comparison of mesenchymal stem cells from human bone marrow and adipose tissue for the treatment of spinal cord injury. Cytotherapy.
23. Kalbermatten DF, Schaakxs D, Kingham PJ, Wiberg M (2011) Neurotrophic activity of human adipose stem cells isolated from deep and superficial layers of abdominal fat. Cell and Tissue Research 344: 251-260.
24. Arboleda D, Forostyak S, Jendelova P, Marekova D, Amemori T, et al. (2011) Transplantation of Predifferentiated Adipose-Derived Stromal Cells for the Treatment of Spinal Cord Injury. Cellular and Molecular Neurobiology 31: 1113-1122.
25. Kang SK, Lee DH, Bae YC, Kim HK, Baik SY, et al. (2003) Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Experimental Neurology 183: 355-366.
26. Sugitani S, Tsuruma K, Ohno Y, Kuse Y, Yamauchi M, et al. (2013) The potential neuroprotective effect of human adipose stem cells conditioned medium against light-induced retinal damage. Experimental Eye Research 116: 254-264.
27. Tsuruma K, Yamauchi M, Sugitani S, Otsuka T, Ohno Y, et al. (2014) Progranulin, a Major Secreted Protein of Mouse Adipose-Derived Stem Cells, Inhibits Light-Induced Retinal Degeneration. Stem Cells Translational Medicine 3: 42-53.

28. Suggate EL, Ahmed Z, Read ML, Eaton-Charnock K, Douglas MR, et al. (2009) Optimisation of siRNA-mediated RhoA silencing in neuronal cultures. Molecular and Cellular Neuroscience 40: 451-462.
29. Douglas MR, Morrison KC, Jacques SJ, Leadbeater WE, Gonzalez AM, et al. (2009) Off-target effects of epidermal growth factor receptor antagonists mediate retinal ganglion cell disinhibited axon growth. Brain 132: 3102-3121.
30. Sullivan KF (1988) Structure and Utilization of Tubulin Isotypes. Annual Review of Cell Biology 4: 687-716.
31. Lorber B, Tassoni A, Bull ND, Moschos MM, Martin KR (2012) Retinal ganglion cell survival and axon regeneration in Wld(S) transgenic rats after optic nerve crush and lens injury. Bmc Neuroscience 13.
32. Sakai K, Yamamoto A, Matsubara K, Nakamura S, Naruse M, et al. (2012) Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. Journal of Clinical Investigation 122: 80-90.
33. Gale Z, Cooper PR, Scheven BAA (2011) Effects of Glial Cell Line-derived Neurotrophic Factor on Dental Pulp Cells. Journal of Dental Research 90: 1240-1245.
34. Dormady SP, Bashayan O, Dougherty R, Zhang XM, Basch RS (2001) Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. J Hematother Stem Cell Res 10: 125-140.
35. Wilkins A, Kemp K, Ginty M, Hares K, Mallam E, et al. (2009) Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. Stem Cell Res 3: 63-70.
36. Chen Q, Long Y, Yuan X, Zou L, Sun J, et al. (2005) Protective effects of bone marrow stromal cell transplantation in injured rodent brain: synthesis of neurotrophic factors. J Neurosci Res 80: 611-619.
37. Cruz Duarte P, St-Jacques B, Ma W (2012) Prostaglandin E2 contributes to the synthesis of brain-derived neurotrophic factor in primary sensory neuron in ganglion explant cultures and in a neuropathic pain model. Exp Neurol 234: 466-481.
38. Kanda N, Koike S, Watanabe S (2005) Prostaglandin E2 enhances neurotrophin-4 production via EP3 receptor in human keratinocytes. J Pharmacol Exp Ther 315: 796-804.
39. Hutchinson AJ, Chou CL, Israel DD, Xu W, Regan JW (2009) Activation of EP2 prostanoid receptors in human glial cell lines stimulates the secretion of BDNF. Neurochem Int 54: 439-446.
40. Jung JE, Kim GS, Chan PH (2011) Neuroprotection by interleukin-6 is mediated by signal transducer and activator of transcription 3 and antioxidative signaling in ischemic stroke. Stroke 42: 3574-3579.
41. Leibinger M, Muller A, Gobrecht P, Diekmann H, Andreadaki A, et al. (2013) Interleukin-6 contributes to CNS axon regeneration upon inflammatory stimulation. Cell Death Dis 4: e609.
42. Kinkl N, Ruiz J, Vecino E, Frasson M, Sahel J, et al. (2003) Possible involvement of a fibroblast growth factor 9 (FGF9)-FGF receptor-3-mediated pathway in adult pig retinal ganglion cell survival in vitro. Mol Cell Neurosci 23: 39-53.
43. Salton SRJ, Ferri GL, Hahm S, Snyder SE, Wilson AJ, et al. (2000) VGF: A novel role for this neuronal and neuroendocrine polypeptide in the regulation of energy balance. Frontiers in Neuroendocrinology 21: 199-219.
44. Shimazawa M, Tanaka H, Ito Y, Morimoto N, Tsuruma K, et al. (2010) An Inducer of VGF Protects Cells against ER Stress-Induced Cell Death and Prolongs Survival in the Mutant SOD1 Animal Models of Familial ALS. Plos One 5.
45. Severini C, Ciotti MT, Biondini L, Quaresima S, Rinaldi AM, et al. (2008) TLQP-21, a neuroendocrine VGF-derived peptide, prevents cerebellar granule cells death induced by serum and potassium deprivation. Journal of Neurochemistry 104: 534-544.

CHAPTER 5

Stem Cell-Mediated Neuroprotection and Functional Preservation of Retinal Ganglion Cells in a Rodent Model of Glaucoma

Ben Mead, Lisa J Hill, Richard J Blanch, Kelly Ward, Ann Logan, Martin Berry,

Wendy Leadbeater, Ben A Scheven

A version of the manuscript has been submitted to

Stem Cell Research

Abstract

Glaucoma is a leading cause of irreversible blindness involving loss of retinal ganglion cells (RGC). Mesenchymal stem cells (MSC) have shown promise as a paracrine-mediated therapy for compromised neurons. It is however unknown if dental pulp stem cells (DPSC) are effective as a cellular therapy in glaucoma and how their hypothesised influence compares to other more widely researched MSC sources. The present study aimed to compare the efficacy of adipose-derived stem cells, bone marrow-derived mesenchymal stem cells (BMSC) and DPSC in preventing the loss of RGC and visual function when transplanted into the vitreous of glaucomatous rodent eyes. Thirty five days after raised intraocular pressure (IOP) and intravitreal stem cell transplantation, $Brn3a^+$ RGC numbers, retinal nerve fibre layer thickness (RNFL) and RGC function were evaluated by immunohistochemistry (IHC), optical coherence tomography (OCT) and electroretinography (ERG), respectively. Control glaucomatous eyes that were sham-treated with heat killed DPSC had a significant loss of RGC numbers, RNFL thickness and function compared with Intact eyes. BMSC and, to a greater extent, DPSC provided significant protection from RGC loss, RNFL thinning and preserved RGC function. The study supports the use of DPSC as a neuroprotective cellular therapy in retinal degenerative disease such as glaucoma.

1 Introduction

Glaucoma is a common cause of irreversible blindness and is characterised by a degenerative loss of retinal ganglion cells (RGC) and their axons, leading to optic disc cupping and reduced visual acuity(1). Current treatments are designed to reduce intraocular pressure (IOP) to slow disease progression whereas neuroprotective treatments that directly target the injured RGC are still in their infancy. Neurotrophic factors (NTF), in particular neurotrophins, are neuronal survival factors that are retrogradely transported along a functionally connected axon to the soma maintaining the survival of connected neurons, but unconnected neurons die by apoptosis(2). Elevation of IOP significantly inhibits retrograde transport of NTF (3) and is one of the mechanisms involved in RGC death(4). NTF, especially when delivered in combinations, promote the survival of injured RGC *in vitro* (5, 6). However their neuroprotective efficacy *in vitro* is not easily translatable to *in vivo* models as a constant delivery of multiple NTF is required for maintaining therapeutic effect.(7, 8)

Mesenchymal stem cells (MSC) are multipotent self-replicating stromal cells are being evaluated as a cellular therapy for treating glaucoma based on their secretion of a wide array of NTF(9-12). MSC-derived NTF protect injured RGC, protecting them from death and ultimately preserving vision(13) and several clinical trials evaluating their neuroprotective efficacy are ongoing(14). MSC can be isolated from a variety of adult tissues such as bone marrow (BMSC) and adipose tissue (ADSC) but here we have focused on the use of MSC-like cells from the dental pulp (DPSC). DPSC are multipotent cranial neural crest-derived stem cells(15, 16) that secrete significantly more NTF, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) than ADSC and BMSC(9, 10). We and others have demonstrated significant RGC neuroprotection by DPSC(9) and BMSC(17, 18) after traumatic optic neuropathy and shown *in vitro* that this neuroprotective effect is most likely due to NTF, including neurotrophins(9, 10) and platelet-derived growth factor (PDGF)(12). These findings indicated that MSC, and in particular

DPSC, may be more effective as a treatment for neurodegenerative conditions such as glaucoma.

We have demonstrated a significantly greater neuroprotective effect of DPSC compared to BMSC/ADSC in an *in vitro* model of RGC injury, with multiple secreted NTF being the mechanism behind the effect(10). We now hypothesise that DPSC may also be a candidate cellular therapy for protecting RGC from loss in glaucoma and determined to test this using an *in vivo* model. Accordingly, three widely researched stem cells for ocular repair(14), BMSC, ADSC and DPSC were transplanted into the vitreous body of rats in which ocular hypertension was induced using exogenously administered transforming growth factor-β1 (TGF-β). Administration of TGF- β1(19) or TGF- β2(20, 21) are both accepted models that induce sustained elevations in IOP leading to significant RGC loss. In this study RGC survival was assessed using immunohistochemical quantification of $Brn3^+$ RGC and retinal imaging using optical coherence tomography (OCT) of the retinal nerve fibre layer (RNFL) thickness. Changes in retinal function were measured using electroretinography (ERG).

2 Materials and Methods

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

2.1 Human (h)DPSC/BMSC/ADSC cultures

hDPSC were obtained from AllCell LLC (Berkeley, CA) and both hBMSC and hADSC from Lonza (Slough, UK), Each MSC batch represented pooled samples from 3 donors. The MSC were characterised by $CD29^+/CD44^+/CD73^+/CD90^+/CD45^-$ profile confirmed by the supplier) and demonstrated multi-differentiation (osteogenic, adipogenic and chondrogenic) capability. The stem cells were cultured into T25/T75 flasks (Corning, Amsterdam, NL) in both a total volume of 5ml/15ml DMEM containing 1% penicillin/streptomycin and 10% foetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) and at a density of $1x10^6$ cells/$2x10^6$ cells, respectively. Cultures were maintained at 37°C in 5% CO_2, the supplemented medium was changed every 3d and the cells were passaged when 80% confluent using 0.05%

trypsin/EDTA. One week before transplantation, cells were transfected with a *gfp* plasmid using lipofectamine 3000 (Life Technologies, Invitrogen, Paisley, UK) according to the manufacturer's protocol.

2.2 Experimental design

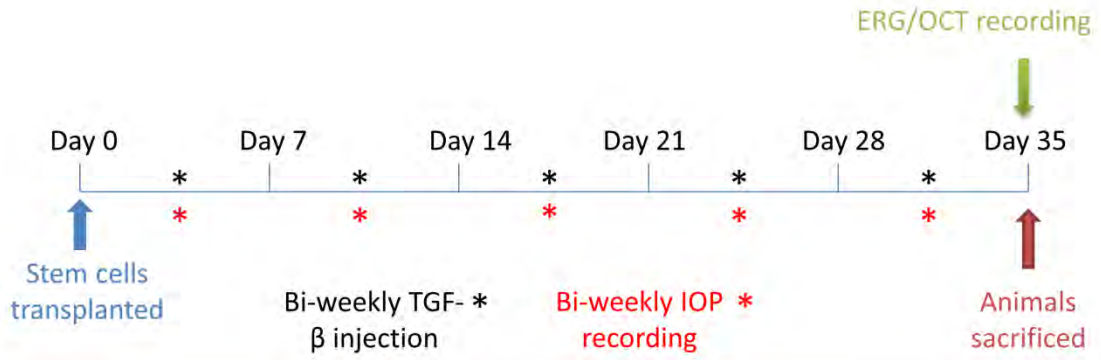
Twelve rats (24 eyes) received bi-weekly (twice a week) bilateral intracameral (IC) injections of TGF-$\beta_{0\text{-}35d}$ (for 5 weeks) and were separated into 2 groups of 6. On 0d, Group 1 received an *ivit* transplantation of DPSC into one eye and dead DPSC (sham control) into the other eye (left and right eyes, respectively, in 3 rats, and *vice versa* in the remaining 3 rats). On 0d, Group 2 received an *ivit* transplantation of BMSC into one eye and ADSC into the other eye (left and right eyes, respectively, in 3 rats, and *vice versa* in the remaining 3 rats). All rats in Group 1 and 2 received ERG and OCT recordings on 35d, before culling and tissue processing for immunohistochemistry. Animal numbers in each group were determined using a previously published power calculation(22, 23). A separate group of 6 rats received bi-weekly unilateral IC injections of $PBS_{0\text{-}35d}$ and these rats are referred to as the Intact Group.

2.3 Animals

All animal procedures were performed in strict accordance to the UK Home Office Animals Scientific Procedures Act, 1986, and approved by the University of Birmingham Ethical Review Sub-Committee. Eighteen adult female Sprague Dawley rats weighing 150-200g (Charles River, Margate, UK) were housed in conditions of 21°C and 55% humidity under a 12h light/dark cycle with a daytime luminance of 80 lux, given food/water *ad libitum* and were monitored by welfare staff. Gaseous anaesthesia was induced with 5% Isoflurane/1.5L per minute O_2 (National Veterinary Supplies, Stoke, UK) and maintained at 3.5% during surgery and 2% during ERG recording.

2.4 Surgery for IC injections to induce ocular hypertension and *ivit* transplantation of MSC

Following anaesthetic induction, IOP were recorded for all rats using an icare tonometer (Tonolab, Helsinki, Finland). Rats were then secured in a head-holding frame for IC injections of TGF-β1 (Peprotech, London, UK) through a single corneal incision, 2mm anterior to the limbus using a 15° blade (BD Ophthalmic System, Warwickshire, UK). Using the same incision site a glass micropipette, produced in-house from a glass capillary rod (Harvard Apparatus, Kent, UK) using a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA) was used to inject 3.5µl of 5µg/ml activated TGF-β1 IC into all 12 rats. Contemporaneously, while the animals were still anaesthetised, a glass micropipette preloaded with 150,000 MSC suspended in 5µl of PBS, was used to inject living or dead cells (killed by heating for 30min at 80°C), into the vitreous of the eye (Fig. 1). After surgery, animals were placed in warm recovery cages and monitored for recovery of normal behaviour before being returned to their home cages. IOP recordings and IC injections of TGF-β were repeated bi-weekly$_{0\text{-}35d}$ throughout the study. A separate 6 rats (Intact Group) received bi-weekly$_{0\text{-}35d}$ IC injections of PBS alone.



Group number	Number of animals	Injury	Treatment Left eye (1-3) Right eye (4-6)	Right eye (1-3) Left eye (4-6)
1	6	TGF-β-induced glaucoma	Intravitreal DPSC transplant	Intravitreal dead DPSC transplant
2	6	TGF-β-induced glaucoma	Intravitreal BMSC transplant	Intravitreal AMSC transplant
3	6	PBS control injection	No treatment	No treatment

Figure 1: Experimental design for the study. Time line of the *in vivo* experiment detailing when the stem cells were transplanted, glaucoma induction using bi-weekly injections of TGF-β and control animals using bi-weekly injections of PBS, ERG/OCT recording and day of animal culling. The group numbers and treatment regime for each eye are also given.

2.5 Optical coherence tomography (OCT) of retinal nerve fibre layer

OCT retinal nerve fibre layer analysis was performed at 35d on all rats while under inhalation anaesthesia 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 images and quantify the RNFL thickness.

2.6 Electroretinography

ERG (HMsERG; Ocuscience, Kansas City, MO) were recorded at 35d. Rats were dark adapted for 12 hours overnight and prepared for ERG recording under dim red light (>630nm). Scotopic flash ERG were recorded from -5.5 to +1 log units with respect to standard flash in half log-unit steps, using DTL fiber (Unimed Electrode Supplies, Farnham, UK) corneal electrodes with pressure-molded Aclar contact lenses and needle skin recording electrodes (Unimed Electrode Supplies).

2.7 ERG analysis

ERG traces were analysed using ERGVIEW (Ocuscience). Oscillatory potentials were removed by filtering waveforms above 20Hz from the trace before analysis. Traces at a light intensity of 1 and 3 mcd/s were chosen for analysis as they gave a clean, unambiguous positive scotopic threshold response (pSTR) with a mean latency of 100ms. The amplitude of the pSTR was measured from baseline. A representative, unfiltered trace is shown in Figure 5C.

2.8 Tissue preparation

Rats were given an intraperitoneal injection of 1ml sodium pentobarbital (National Veterinary Supplies) at 35d and perfused intracardially with 4% paraformaldehyde (PFA; TAAB, Reading, UK) in PBS while under terminal anaesthesia. Eyes 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 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 stored at -80°C. After embedding, eyes were sectioned on a cryostat microtome (Bright, Huntingdon, UK) at -22°C at a thickness of 20 µm and mounted on positively charged glass slides (Superfrost Plus, Fisher Scientific, Pittsburgh, PA). Radial eye sections were left to dry on

slides overnight at 37°C before storage at -20°C. Standard eye sections containing the optic nerve head were used to account for variation in RGC density.

2.9 Immunohistochemistry

Mounted tissue sections were equilibrated to room temperature (RT), hydrated in PBS for 2 X 5min, permeabilized in 0.1% triton x-100 in PBS for 20min at RT 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 RT and then sections were drained and incubated with either Brn3a primary antibody (1:200; Santa Cruz, CA, #SC-31984) or Stro1 primary antibody (1:100; R&D Systems, MN, #MAB1038) 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 Goat IgG Alexa 594 secondary antibody (1:400; Molecular Probes, Paisley, UK; #A-11058) diluted in ADB for 1h in a hydrated incubation chamber at RT. 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.

2.10 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 immunohistochemistry, $Brn3a^+$ RGC were counted in 20µm thick standard sections of the retina, along a 250µm linear region of the ganglion cell layer either side of the optic nerve, as described previously(22). Four sections per retina and 6 retinae from 6 different animals per treatment group were quantified.

2.11 Statistics

All statistical tests were performed using SPSS 17.0 (IBMM SPSS, Inc., Chicago, IL) and data were presented as mean ± standard error of the mean (SEM). The Shapiro-Wilk test was used to ensure all data were normally distributed before parametric testing. IOP data were measured using the generalised estimated equations (autoregressive correlation matrix). RGC survival and function data were tested for significance using 1-way ANOVA for >2 group comparisons ± SEM and Tukey *post-hoc* test. Statistical difference was considered significant at p values < 0.05.

3 Results

3.1 IC injections of TGF-β significantly raised IOP over 35d

IC injections of TGF-β induced a significant increase in IOP by 7d (16.9 ± 2.3mmHg) compared to Intact (PBS injected) eyes (10.2 ± 0.7mmHg) which was maintained between 14d (13.8 ± 0.7mmHg) and 35d (13.9 ± 0.9mmHg) compared to PBS (9.3 ± 0.2mmHg and 11.4 ± 1.1mmHg, respectively; Fig. 2). No significance differences were seen at 3d and 10d. IOP measurements between the four separate MSC treatment groups IC injected with TGF-β were not significantly different from each other at any time point (not shown). These data show that IC TGF-β injections raised IOP and that IOP was not affected by the IC injection of stem cells.

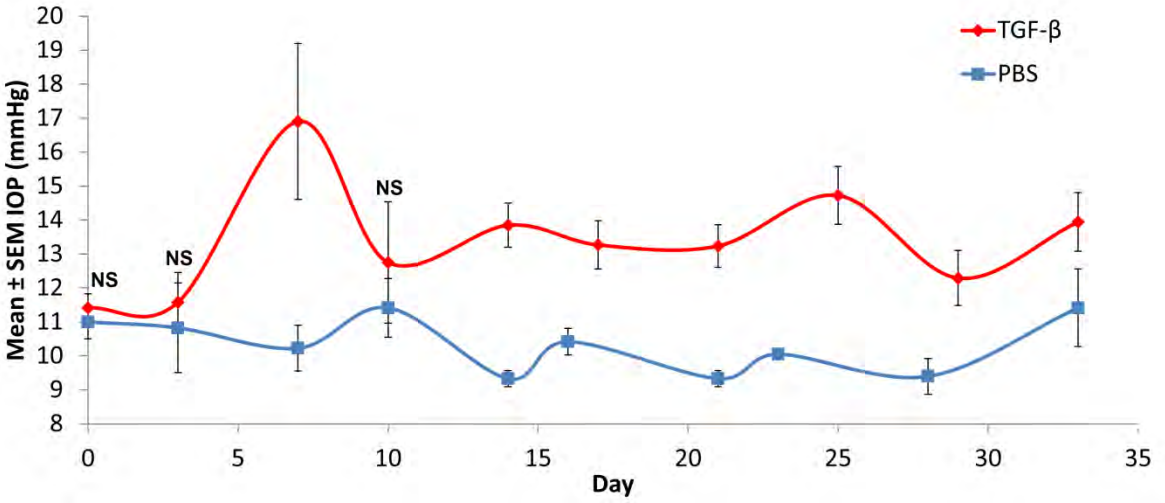


Figure 2: IOP in rats injected with either TGF-β or PBS. IOP measurements (mmHg) from rats IC injected with bi-weekly TGF-β (*red line*) or PBS (*blue line*) and *ivit* injected with stem cells (see Figure 1.) Note the acute peak rise in IOP at 7d and the chronically maintained elevation from 14d and beyond, all time points are significantly different from each other ($p<0.05$) unless otherwise stated (NS; Not Significant), as determined using the generalised estimated equations (autoregressive correlation matrix). The PBS data is from 6 eyes in 6 rats, whereas the TGF-β data is consolidated from the 4 treatment groups (12 rats, 24 eyes). Note that no significant differences in IOP were seen between the TGF-β injected groups transplanted with different MSC (not shown).

3.2 MSC protected eyes from elevated IOP-induced RNFL thinning

The RNFL comprises axons belonging to the RGC and is lost concomitantly with the loss of the RGC cell body. TGF-β-induced elevations in IOP led to a significant RNFL thinning by 35d (27.0 ± 1.8μm) compared to Intact controls (55.5 ± 0.6μm; Fig. 3). Intravitreal (*ivit*) transplantation of both BMSC and, to a greater degree, DPSC, preserved RNFL thickness (37.3 ± 0.6μm and 41.3 ± 1.3μm, respectively) compared to sham-treated (dead DPSC transplanted) eyes (27.0 ± 1.8μm). *Ivit* transplantation of ADSC did not affect IOP-induced loss of RNFL thickness (29 ± 0.8μm) compared to sham-treated eyes (27.0 ± 1.8μm). RNFL thickness in Intact eyes was significantly greater than the RNFL thickness in all treated/sham-treated eyes.

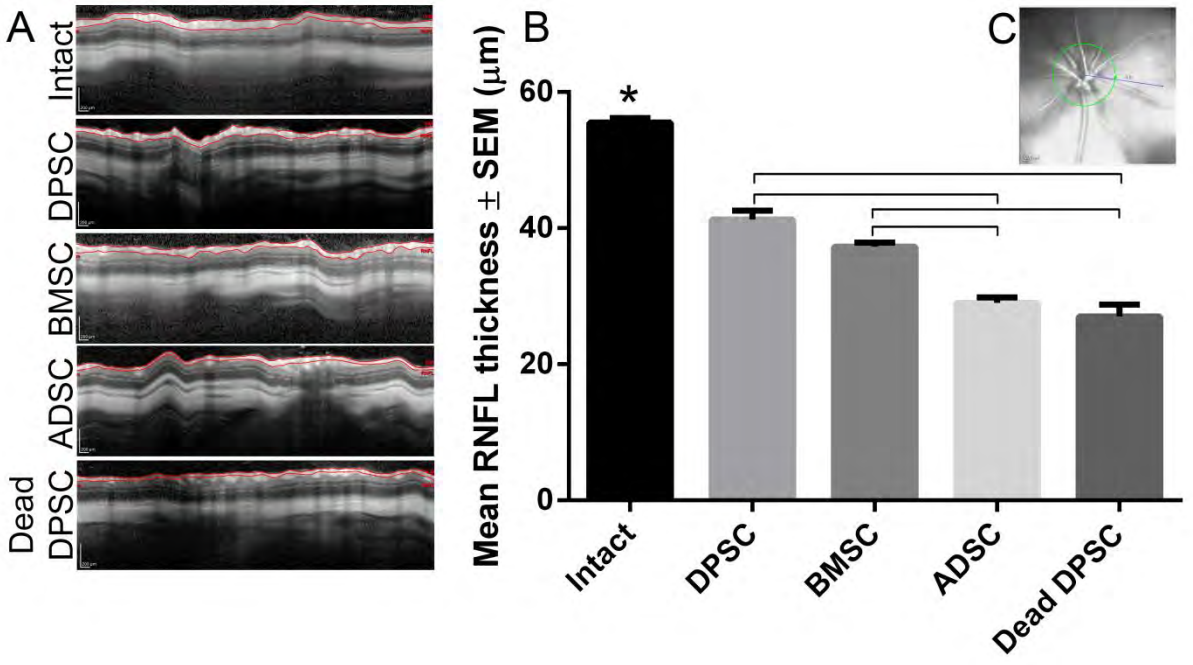


Figure 3: RNFL thickness after TGF-β-induced glaucoma in rats. OCT images of retina from an uninjured eye and a TGF-β-induced glaucomatous eye treated with *ivit* DPSC, BMSC, ADSC and dead DPSC (sham-treated) are shown (**A**) with *red lines* outlining the RNFL. The graph (**B**) depicts the RNFL thickness (µm) at 35d after glaucoma induction for the above treatment groups as well as for uninjured eyes. *Asterisks* indicate significant difference from all groups where as *black lines* indicate significant difference between particular groups ($p<0.05$), as determined using a 1-way ANOVA and Tukey *post-hoc* test. Measurements were taken from images of sections containing the optic nerve head, indicated by the *green line* (**C**). Images and data representative from 5 animals/eyes per treatment group (*scale bar*: 200µm).

3.3 MSC protected eyes from elevated IOP-induced loss of RGC

TGF-β-mediated elevations in IOP induced a significant 33% loss of RGC by 35d (49.0 ± 1.6/mm of retina; sham-treated group) compared to Intact controls (73.2 ± 2.5/mm of retina; Fig. 4). *Ivit* transplantation of both BMSC and to a greater degree, DPSC, protected against this RGC loss and thus exhibited greater numbers of RGC (60.2 ± 1.3/mm of retina and 69.7 ± 1.9/mm of retina, respectively) compared with sham-treated eyes (49.0 ± 1.6/mm of retina). DPSC-induced RGC survival was significantly greater than that achieved by BMSC. Notably, RGC numbers in the DPSC treatment group was not different from RGC numbers in Intact healthy eyes. Interestingly, *Ivit* transplantation of ADSC did not affect RGC survival

(52.8 ± 1.9/mm of retina) compared with sham-treated eyes (49.0 ± 1.6μm/mm of retina). $GFP^+/Stro1^+$ MSC were detectable on the vitreal side of the inner limiting membrane in all animals at select regions throughout the eye (Fig. 4B). No evidence of migration into the retina was seen.

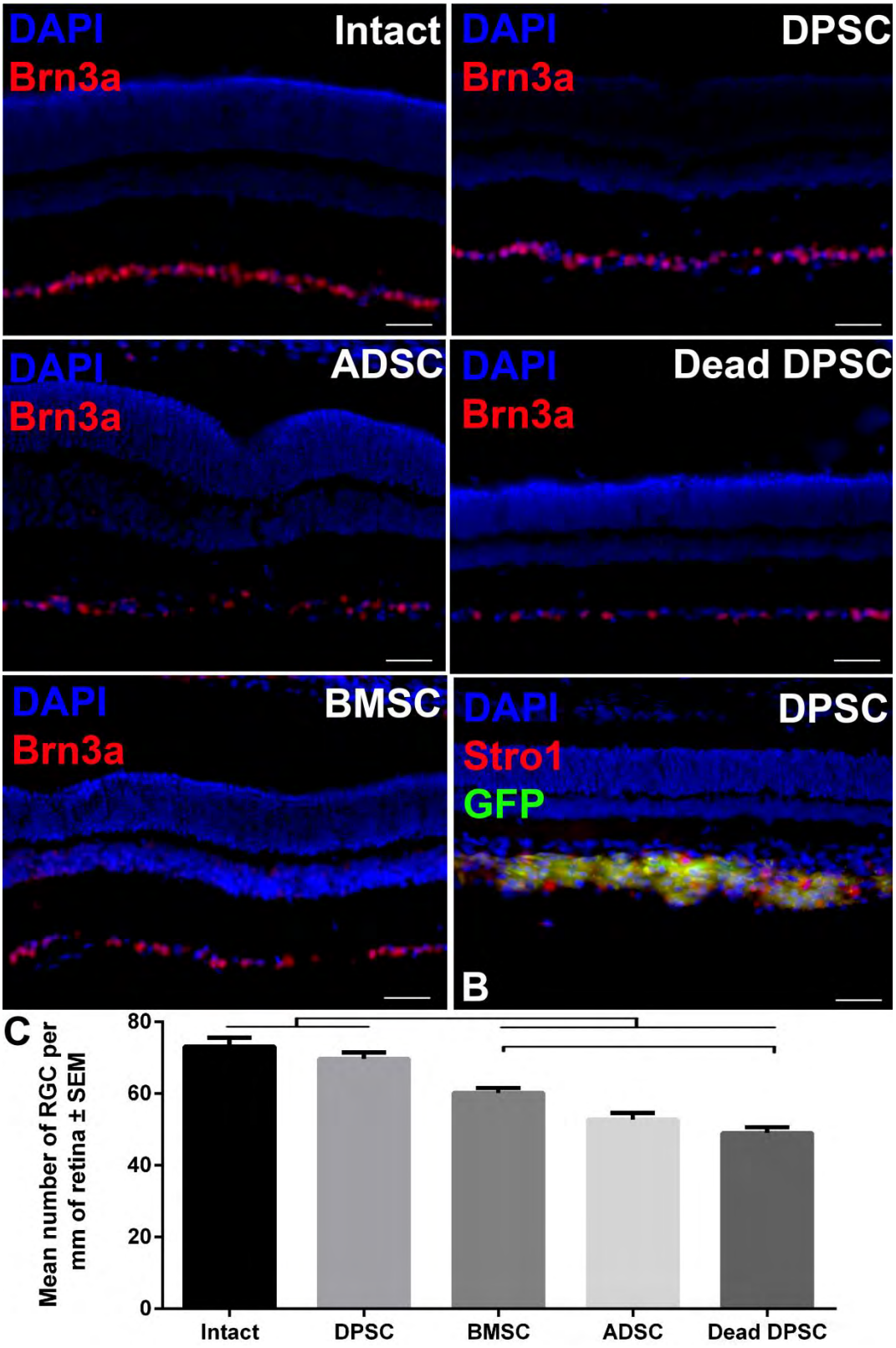


Figure 4: Brn3a^{+} RGC counts in standard radial eye sections. Immunohistochemically stained radially sectioned retina, stained for Brn3a (*red*), from Intact rats and rats IC injected with TGF-β for 35d and also *ivit* transplanted with DPSC, BMSC, ADSC and dead DPSC (sham-treated). All images are representative of the 8 images taken per retina from 6 different animals (*scale bar*: 50μm). All images show tissues counterstained with the nuclear

marker DAPI (*blue*). In (**B**), GFP^+ MSC stained for the MSC marker Stro1 identified in the vitreous, adhered to the inner limiting membrane. In (**C**), the mean number of $Brn3a^+$ RGC in a 1mm region of retina either side of the optic nerve head is shown from each of the above groups. *Black lines* indicate significant difference between groups ($p<0.01$), as determined using a 1-way ANOVA and Tukey *post-hoc* test.

3.4 MSC protected eyes from elevated IOP-induced loss of RGC function

The pSTR represents the compound action potentials of RGC in response to the light dependant electrochemical signal originating from photoreceptors and thus its amplitude provides a read out of RGC function. TGF-β-mediated elevations in IOP induced a significant 85% loss of pSTR amplitude by 35d (21.2 ± 11.8μv) compared to Intact controls (140.8 ± 6.3μv; Fig. 5A). *Ivit* transplantation of both BMSC and to a greater degree, DPSC, protected against the RGC dysfunction associated with raised IOP and thus promoted greater preservation of pSTR (60.5 ± 18.3μv and 75.5 ± 17.4μv, respectively) compared to ADSC/sham-treated eyes (25.3 ± 7.8μv/21.2 ± 11.8μv, respectively), although this was only statistically different for DPSC treated eyes.

Recordings at a light intensity stimulus of 1000mcd/s as opposed to 3000mcd/s gave lower recordings of amplitude but the same relative differences (Fig. 5B)

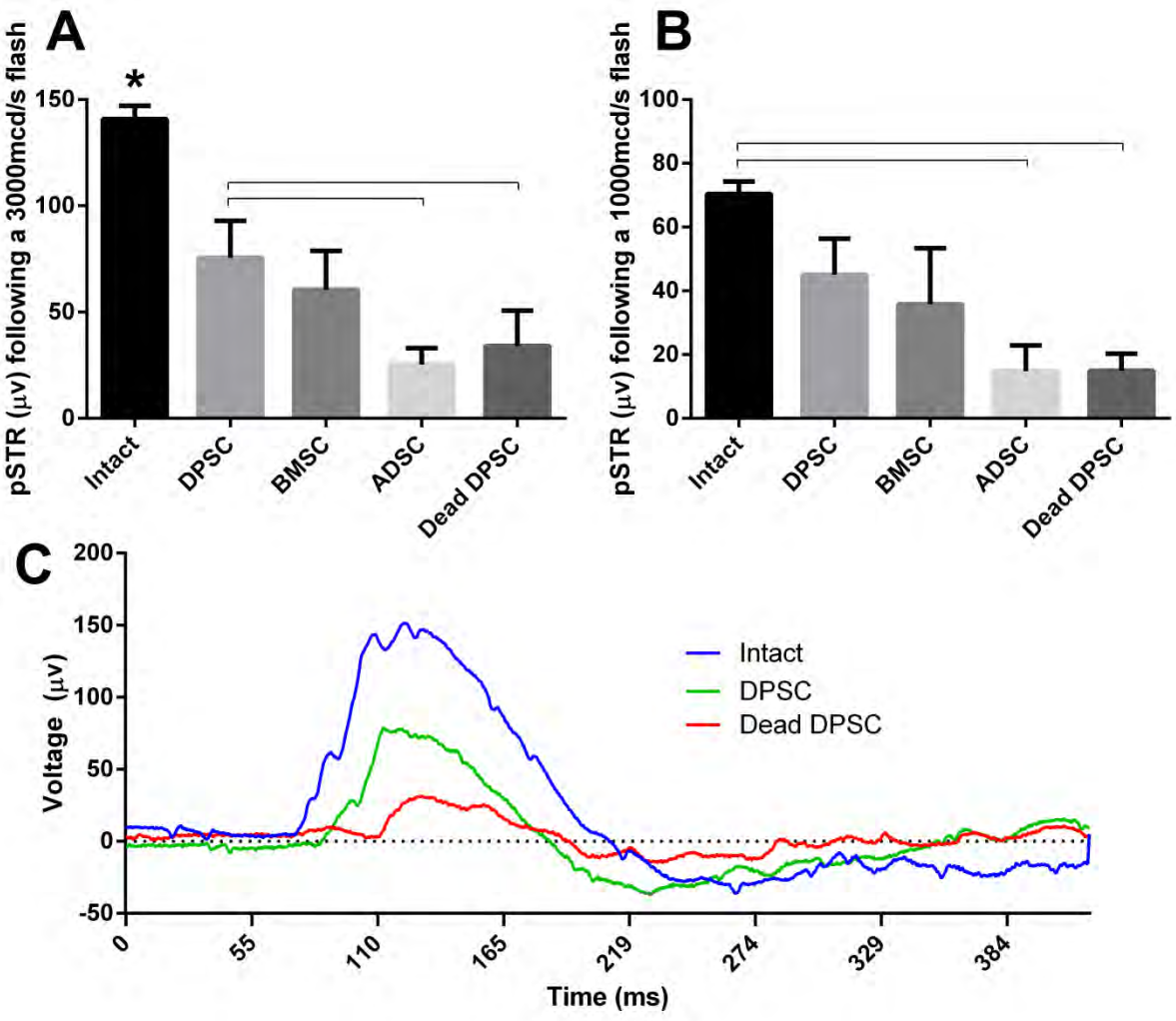


Figure 5: pSTR response after TGF-β-induced glaucoma. The mean amplitude of the pSTR response from the eyes of Intact rats and rats IC injected with TGF-β for 35d and also *ivit* transplanted with DPSC, BMSC, ADSC and dead DPSC (sham-treated) after a receiving a flash intensity of 3000mcd/s (**A**) and 1000mcd/s (**B**). *Asterisks* indicate significant differences between all groups whereas *black lines* indicate significant difference between groups ($p<0.05$), as determined using a 1-way ANOVA and Tukey *post-hoc* test. In (**C**), a representative image is shown of the ERG traces with an observable pSTR response from Intact rats and from rats IC injected with TGF-β for 35d with *ivit* transplantation of DPSC or dead DPSC, 6 different animals per group.

4 Discussion

This study demonstrates for the first time that in a model of open angle glaucoma, DPSC promotes neuroprotection of injured RGC following *ivit* transplantation, preserving both their $Brn3a^+$ cell body within the ganglion cell layer, axons within the RNFL and electrical function

as measured using ERG. Of note DPSC had the greatest therapeutic effect for RGC in this model of glaucoma whereas ADSC had the least. DPSC thus represent a potential effective cellular neuroprotective therapy for glaucoma patients.

Open angle glaucoma is the most common form of glaucoma and is characterised by elevated IOP related to trabecular meshwork fibrosis that perturbs the aqueous outflow pathway, as opposed to physical obstruction of the pathway by the iris i.e. angle closure glaucoma. The TGF-β model of glaucoma used in this study is based on the observation that patients with open angle glaucoma have elevated levels of TGF-β in the aqueous humour(24). This, coupled with the observations that TGF-β is a potent pro-fibrotic cytokine and that fibrosis of the trabecular meshwork is a pathology that underpins the elevation in IOP in open angle glaucoma, makes this model a suitable representation of the human pathology. As seen in previous studies both from our laboratory and others(20, 21), TGF-β induced a reliable chronic rise in IOP and thus may be considered a suitable model to test neuroprotective cellular therapies relevant to glaucomatous RGC loss.

Our findings are consistent with our previous study that showed DPSC to be RGC neuroprotective in an optic nerve crush injury model(9). Optic nerve crush causes a rapid loss of RGC with approximately 90% dead by 3 weeks. DPSC promoted some neuroprotection of RGC when *ivit* transplanted, however, there was still approximately 60% RGC death after complete RGC axotomy. We postulated that the slow consistent release of NTF from MSC, would be better suited as a therapy for a chronic neurodegenerative condition, such as glaucoma where the retrogradle NTF supply is attenuated but still present, as opposed to an acute traumatic condition such as traumatic optic neuropathy, where the retrogradle NTF supply is completely ablated.

The present study is the first to demonstrate both the potential of DPSC to treat glaucomatous eyes and also to show that their therapeutic neuroprotective effect is significantly greater than BMSC and ADSC transplantation. RGC survival increased from

67% after treatment with dead DPSC to 95% after treatment with living DPSC; notably a RGC count not significantly different from that in Intact animals. This result corroborates the neuroprotective effects observed after optic nerve crush(9), as well as in transplantation studies after spinal cord injury in the rat(25), both of which demonstrated that DPSC promoted greater neuronal survival than BMSC. Similar to the studies described above, we also found that the transplanted GFP^+ MSC (stained for the MSC marker Stro1) survived within the vitreous space for the full length of the study with no evidence of migration into retinal tissue (Fig. 4B).

Previous studies by Johnson *et al,* have explored the use of BMSC as a treatment for glaucoma(13, 26). In their earliest study, BMSC were *ivit* transplanted into a laser-induced ocular hypertensive glaucoma model. The model yielded a 40% loss of RGC axons 28d after induction of glaucoma, which was reduced to a 10% after *ivit* BMSC transplantation. In the present study, our model yielded a $Brn3a^+$ RGC loss of 33% which was reduced to an 18% loss when eyes received *ivit* transplantation of BMSC. Possible explanations for why BMSC-mediated neuroprotection was 30% in a previous study(13) and only 15% in the present study may be that: A), the present study used a different model of glaucoma which results in less RGC death and thus less potential for neuroprotection, and B), the present study counted $Brn3a^+$ RGC whereas the Johnson *et al,* counted axons within the optic nerve, whose death precedes the loss of the RGC soma and will therefore yield greater disparities between treatment and control groups. A more recent study(27) has explored the use of BMSC as a treatment for open angle glaucoma and demonstrated a 17% increase in the survival of RGC, similar to the 15% neuroprotection observed in this study. However, the authors injected hyaluronic acid IC to elevate IOP instead of TGF-β (and thus this was a model of acute rather than chronic open angle glaucoma) and used animal-derived BMSC instead of human-derived as was used in the present study.

As well as counting RGC somata we used OCT to measure the thickness of the RNFL which reflects RGC axon loss. The RNFL is comprised of axons of the RGC as they course over

the inner surface of the retina and towards the optic disc. Although axonal loss precedes RGC loss, the resolution of OCT is not as high as direct fluorescent microscopy making direct comparisons of the two measurements difficult. In addition, the RNFL contains astrocytes and retinal blood vessels and thus is not an absolute measure of RGC axon numbers. Despite this, we have previously shown that RNFL thickness is a reliable measure of moderate axonal loss but becomes inaccurate when the RNFL is substantially thinned (9). In the present study, RNFL thickness was reduced by 51% in the sham-treated glaucomatous eyes when compared to Intact controls. This is higher than the 33% loss in RGC reported, and can be explained by fact that in glaucoma, RGC axonal loss precedes RGC loss and will thus is expected to be higher(28, 29). Indeed, although the pattern of neuroprotection as inferred from the RNFL thickness (i.e. DPSC being the most efficacious followed by BMSC and ADSC showing no neuroprotection) is the same as determined from $Brn3a^+$ RGC counts, there is more retinal cell "death" recorded by RNFL thickness measurements than $Brn3a^+$ RGC counts of the same group for the above reason.

The present study did not explore the mechanisms by which MSC elicit neuroprotection of RGC in glaucomatous eyes, or why DPSC have greater therapeutic efficacy than BMSC/ADSC. However, previous published studies by us and others provided strong evidence for paracrine-dependent effects. Perturbations in retrograde axonal transport of NTF have been implicated in the pathology of glaucoma(3, 4) and explains the success of NTF supplementation as a neuroprotective strategy(9-13). We have previously shown that DPSC, BMSC and ADSC secrete multiple NTF including NGF, BDNF, NT-3, glial cell line-derived neurotrophic factor, vascular endothelial growth factor and PDGF(10), whereas a recent paper demonstrated that PDGF(12) was a significant contributor to the neuroprotective effects elicited by BMSC on injured RGC. Our results corroborate this, demonstrating a significant ablation of the neuroprotective effects of DPSC, BMSC and ADSC when NTF receptors are blocked in culture(9, 10). As after optic nerve crush, the present study found that DPSC were the most RGC neuroprotective stem cells out of the

tested MSC in our model of glaucoma which can be explained by their enhanced NTF profile in comparison to BMSC and ADSC(10).

Our study did not find a neuroprotective effect of ADSC in this model of glaucoma. This is unsurprising given the reduced neurotrophic profile of ADSC in comparison to BMSC/DPSC, particularly since the neurotrophins BDNF and NT-3 showing no detectable expression(10). However, a recent previous study demonstrated a 19% RGC neuroprotective effect of ADSC after transplantation into the vitreous of an animal model of glaucoma(27). The disparity between the finding of this study and ours could be explained by: A), the use of a different model of glaucoma focusing on closed angle glaucoma rather than open angle glaucoma; B), study duration of only 4 weeks in comparison to our 5 weeks; and C), the study used ADSC derived from rats whereas the present study used ADSC derived from humans.

Finally, we measured the amplitude of the pSTR after ERG recordings on rats, using this as a measure of RGC function. A previous study(30) induced ocular hypertension in rats by injecting hypertonic saline into the episcleral vein, 5 times per week for 5 weeks. They demonstrated a 50% decrease in pSTR amplitude which correlated with both raised IOP and optic nerve damage. Our study demonstrated an 80-85% decrease in RGC function in sham-treated glaucomatous eyes compared to Intact eyes, a more profound effect than the previous study, which could be due to the difference in the animal model used. Interestingly, pSTR amplitude showed a similar pattern to the Brn3a survival and OCT RNFL thickness data, with DPSC and BMSC promoting the most significant RGC survival/functional preservation and ADSC/sham-treated eyes showing the least. There is a profound neuroprotective effect of DPSC on RGC, with RGC numbers no different from that of an Intact eye, but less functional preservation, with pSTR amplitude in the DPSC group significantly lower than in Intact eyes. This observation suggests that DPSC significantly protect RGC from death and dysfunction in glaucoma, however a portion of surviving RGC may still be dysfunctional or destined to die at a later time point, likely because of the underlying raised IOP. Indeed, a previous study(31) demonstrated a significant 50%

decrease in pSTR after only 75min of acute ocular hypertension (from 12mmHg to 60mmHg) suggesting, together with this study, that RGC function is more sensitive to raised IOP than is RGC survival. The data highlights the fact that IOP lowering drugs may still be a useful adjunct to a cellular neuroprotective therapy.

Future work will focus on unravelling the precise mechanism for the paracrine-mediated neuroprotection of RGC and ensuring the safety of DPSC as a cellular therapy, for example, through the encapsulation of cells to prevent unwanted migration/proliferation(32), or the genetic integration of a "suicide gene" to ensure cells can be selectively removed with ganciclovir after transplantation(33).

5 Conclusions

We demonstrate here for the first time the potential of DPSC to act as a cellular therapy for glaucomatous visual loss by protecting RGC and their axons from death and preserving RGC function. In comparison to BMSC and ADSC, DPSC were the most efficacious and thus may potentially represent an "ideal" cell to be trialled as a neuroprotective treatment for glaucoma.

References

1. Quigley, H.A. 1996. Number of people with glaucoma worldwide. *British Journal of Ophthalmology* 80:389-393.
2. Berry, M., Ahmed, Z., Lorber, B., Douglas, M., and Logan, A. 2008. Regeneration of axons in the visual system. *Restorative Neurology and Neuroscience* 26:147-174.
3. Quigley, H.A., McKinnon, S.J., Zack, D.J., Pease, M.E., Kerrigan-Baumrind, L.A., Kerrigan, D.F., and Mitchell, R.S. 2000. Retrograde axonal transport of BDNF in retinal ganglion cells is blocked by acute IOP elevation in rats. *Invest Ophthalmol Vis Sci* 41:3460-3466.
4. Quigley, H.A. 1999. Neuronal death in glaucoma. *Progress in Retinal and Eye Research* 18:39-57.
5. Mey, J., and Thanos, S. 1993. Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. *Brain Res* 602:304-317.
6. Logan, A., Ahmed, Z., Baird, A., Gonzalez, A.M., and Berry, M. 2006. Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. *Brain* 129:490-502.
7. Ko, M.L., Hu, D.N., Ritch, R., Sharma, S.C., and Chen, C.F. 2001. Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. *Neurosci Lett* 305:139-142.

8. Ko, M.L., Hu, D.N., Ritch, R., and Sharma, S.C. 2000. The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. *Invest Ophthalmol Vis Sci* 41:2967-2971.
9. Mead, B., Logan, A., Berry, M., Leadbeater, W., and Scheven, B.A. 2013. Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. *Invest Ophthalmol Vis Sci* 54:7544-7556.
10. Mead, B., Logan, A., Berry, M., Leadbeater, W., and Scheven, B.A. 2014. Paracrine-Mediated Neuroprotection and Neuritogenesis of Axotomised Retinal Ganglion Cells by Human Dental Pulp Stem Cells: Comparison with Human Bone Marrow and Adipose-Derived Mesenchymal Stem Cells. *Plos One* 9:e109305.
11. Mead, B., Logan, A., Berry, M., Leadbeater, W., and Scheven, B.A. 2014. Dental pulp stem cells, a paracrine-mediated therapy for the retina. *Neural Regeneration Research* 9:577-578.
12. Johnson, T.V., Dekorver, N.W., Levasseur, V.A., Osborne, A., Tassoni, A., Lorber, B., Heller, J.P., Villasmil, R., Bull, N.D., Martin, K.R., et al. 2013. Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. *Brain*.
13. Johnson, T.V., Bull, N.D., Hunt, D.P., Marina, N., Tomarev, S.I., and Martin, K.R. 2010. Neuroprotective Effects of Intravitreal Mesenchymal Stem Cell Transplantation in Experimental Glaucoma. *Investigative Ophthalmology & Visual Science* 51:2051-2059.
14. Mead, B., Berry, M., Logan, A., Scott, R.A.H., Leadbeater, W., and Scheven, B.A. 2015. Stem cell treatment of degenerative eye disease. *Stem Cell Res* 14:243-257.
15. Gronthos, S., Mankani, M., Brahim, J., Robey, P.G., and Shi, S. 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 97:13625-13630.
16. Chai, Y., Jiang, X., Ito, Y., Bringas, P., Han, J., Rowitch, D.H., Soriano, P., McMahon, A.P., and Sucov, H.M. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* 127:1671-1679.
17. Levkovitch-Verbin, H., Sadan, O., Vander, S., Rosner, M., Barhum, Y., Melamed, E., Offen, D., and Melamed, S. 2010. Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. *Invest Ophthalmol Vis Sci* 51:6394-6400.
18. Mesentier-Louro, L.A., Zaverucha-do-Valle, C., da Silva-Junior, A.J., Nascimento-Dos-Santos, G., Gubert, F., de Figueiredo, A.B., Torres, A.L., Paredes, B.D., Teixeira, C., Tovar-Moll, F., et al. 2014. Distribution of mesenchymal stem cells and effects on neuronal survival and axon regeneration after optic nerve crush and cell therapy. *Plos One* 9:e110722.
19. Robertson, J.V., Siwakoti, A., and West-Mays, J.A. 2013. Altered expression of transforming growth factor beta 1 and matrix metalloproteinase-9 results in elevated intraocular pressure in mice. *Mol Vis* 19:684-695.
20. Swaminathan, S.S., Oh, D.-J., Kang, M.H., Shepard, A.R., Pang, I.-H., and Rhee, D.J. 2014. TGF-β2–Mediated Ocular Hypertension Is Attenuated in SPARC-Null Mice. *Investigative Ophthalmology & Visual Science* 55:4084-4097.
21. Shepard, A.R., Millar, J.C., Pang, I.-H., Jacobson, N., Wang, W.-H., and Clark, A.F. 2010. Adenoviral Gene Transfer of Active Human Transforming Growth Factor-β2 Elevates Intraocular Pressure and Reduces Outflow Facility in Rodent Eyes. *Investigative Ophthalmology & Visual Science* 51:2067-2076.
22. Mead, B., Thompson, A., Scheven, B.A., Logan, A., Berry, M., and Leadbeater, W. 2014. Comparative evaluation of methods for estimating retinal ganglion cell loss in retinal sections and wholemounts. *Plos One* 9:e110612.
23. Faul, F., Erdfelder, E., Lang, A.G., and Buchner, A. 2007. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav Res Methods* 39:175-191.

24. Ozcan, A., Ozdemir, N., and Canataroglu, A. 2004. The Aqueous Levels of TGF-β2 in Patients with Glaucoma. *International Ophthalmology* 25:19-22.
25. Sakai, K., Yamamoto, A., Matsubara, K., Nakamura, S., Naruse, M., Yamagata, M., Sakamoto, K., Tauchi, R., Wakao, N., Imagama, S., et al. 2012. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. *Journal of Clinical Investigation* 122:80-90.
26. Johnson, T.V., and Martin, K.R. 2012. Cell transplantation approaches to retinal ganglion cell neuroprotection in glaucoma. *Curr Opin Pharmacol*.
27. Emre, E., Yuksel, N., Duruksu, G., Pirhan, D., Subasi, C., Erman, G., and Karaoz, E. 2015. Neuroprotective effects of intravitreally transplanted adipose tissue and bone marrow-derived mesenchymal stem cells in an experimental ocular hypertension model. *Cytotherapy*.
28. Buckingham, B.P., Inman, D.M., Lambert, W., Oglesby, E., Calkins, D.J., Steele, M.R., Vetter, M.L., Marsh-Armstrong, N., and Horner, P.J. 2008. Progressive ganglion cell degeneration precedes neuronal loss in a mouse model of glaucoma. *J Neurosci* 28:2735-2744.
29. Agudo-Barriuso, M., Villegas-Perez, M.P., de Imperial, J.M., and Vidal-Sanz, M. 2013. Anatomical and functional damage in experimental glaucoma. *Curr Opin Pharmacol* 13:5-11.
30. Fortune, B., Bui, B.V., Morrison, J.C., Johnson, E.C., Dong, J., Cepurna, W.O., Jia, L., Barber, S., and Cioffi, G.A. 2004. Selective ganglion cell functional loss in rats with experimental glaucoma. *Invest Ophthalmol Vis Sci* 45:1854-1862.
31. Bui, B.V., Edmunds, B., Cioffi, G.A., and Fortune, B. 2005. The gradient of retinal functional changes during acute intraocular pressure elevation. *Invest Ophthalmol Vis Sci* 46:202-213.
32. Sieving, P.A., Caruso, R.C., Tao, W., Coleman, H.R., Thompson, D.J., Fullmer, K.R., and Bush, R.A. 2006. Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. *Proc Natl Acad Sci U S A* 103:3896-3901.
33. Schuldiner, M., Itskovitz-Eldor, J., and Benvenisty, N. 2003. Selective ablation of human embryonic stem cells expressing a "suicide" gene. *Stem Cells* 21:257-265.

CHAPTER 6

GENERAL DISCUSSION

6.1 **Main findings and future work**

Traumatic and degenerative eye disease leads to permanent blindness due both to an irreplaceable loss of retinal neurons and an irreparable degeneration of their axons. This research project attempted to protect injured RGC and regenerate their axons by exploiting DPSC as a neuroprotective/neuroregenerative cellular therapy, in comparison to BMSC and ADSC. The rat optic nerve crush model was used to model TON and transforming growth factor (TGF)-β-induced ocular hypertension was used to model glaucoma. As an adjunct to this hypothesis, the reliability of the preferred method of quantifying RGC (counting in radial sections) was tested and compared to the current gold standard of counting RGC in flat mounted whole retina.

The first finding of this research is relevant to all ocular research and details how the quantification of $Brn3a^+$ RGC for the determination of cell death and to evaluate the efficacy of neuroprotective treatments can be done just as reliably in radial sections of retina as the current gold standard for RGC quantitation, retinal wholemounts (Chapter 2; Mead *et al*., 2014b). Since retinal wholemounts consume the whole tissue, whereas large numbers of radial retinal sections can be probed for multiple proteins, this allows for a greater degree of analysis while saving on animal costs. Moreover, the work validates the results generated from previous studies in the literature that have used radial sections to measure RGC death.

The main focus of this research was to determine the most suitable stem cell therapy for traumatic and degenerative eye disease by testing the hypothesis that DPSC, a neural crest-derived stem cell, would be the most efficacious stem cell phenotype. This hypothesis was supported partially by work done in the spinal cord (Sakai *et al*., 2012) that demonstrated a greater degree of functional improvement following DPSC transplantation when compared with BMSC. Using a model of TON, it was demonstrated that DPSC promoted a significant increase in the survival of RGC and regeneration of their axons compared to BMSC (Chapter 3; Mead *et al*., 2013). In an effort to determine the mechanism behind this effect, it

was decided to co-culture MSC with RGC and block specific NTF receptors. This experiment utilized human-derived MSC and also included ADSC, giving the research its clinical relevance and impact (Chapter 4; Mead *et al*., 2014a). The findings were that A), each of the three types of MSC (DPSC, BMSC and ADSC) had distinct neurotrophic secretory profiles; B), the neuroprotective and neuroregenerative efficacy of rat-derived DPSC was greater when compared to other rat-derived MSC (Mead *et al*., 2013) and was also greater than human-derived MSC and C), the mechanism of RGC neuroprotection, as well as axon/neurite regeneration, was attributable to multiple stem cell-derived NTF, including NGF, BDNF, NT-3 and PDGF, based on the use of permeable filters separating the DPSC from the RGC and the use of specific blockers of NTF receptors.

Finally, the suitability of DPSC as a treatment for glaucoma was determined. The slow progressive release of NTF by DPSC suggested that DPSC are a more suitable treatment for slow degenerative diseases rather than for acute traumatic injuries, like TON. This hypothesis was confirmed with an almost complete neuroprotective effect of transplanted DPSC on RGC in a TGF-β-mediated rodent model of glaucoma (Hill *et al*., 2015), accompanied by an appreciable preservation in visual function (Chapter 5; Mead *et al*., 2015; in submission). The fact that DPSC performed with greater efficacy than BMSC or ADSC makes this finding particularly pertinent to the on-going clinical trials utilizing BMSC as a therapy for ocular diseases, including glaucoma (Mead *et al*., 2015) and makes the findings particularly impactful.

The next logical steps for this research are to: (1), test the efficacy of DPSC in a more widely used model of glaucoma, such as photocoagulation of the trabecular meshwork (Johnson *et al*., 2010) and (2), test MSC as a therapy for glaucoma in a different species, such as rabbit, to corroborate the conclusions.

Since glaucoma is a long term condition, it is ideal that cells remain within the eye for extended periods, to avoid repeat injection. It has been demonstrated that MSC survive

within the vitreous for over 3-5 weeks (Johnson *et al*., 2010, Levkovitch-Verbin *et al*., 2010, Mead *et al*., 2013, Haddad-Mashadrizeh *et al*., 2013) but a more long term study would be necessary to accurately predict their longevity in humans. Equally, the safety of stem cell transplantation for the recipient is under researched although stem cell tumorigenicity and uncontrolled proliferation is unlikely based on the numerous studies in rodents (Johnson *et al*., 2010, Singh *et al*., 2012, Mendel *et al*., 2013, Mesentier-Louro *et al*., 2014, Tzameret *et al*., 2014, Tan *et al*., 2015) and the on-going clinical trials (Mead *et al*., 2015) will further corroborate this. Removal of the stem cells from the eye could be achieved by transfecting the stem cells with a suicide gene to render them susceptible to selective loss by ganciclovir acts as a failsafe (Zhang *et al*., 2011), whereas encapsulating the cells in a permeable membrane prevents unbridled proliferation and migration (Sieving *et al*., 2006) and allows physical removal at the end of the treatment.

Finally, this project concentrated on comparisons between DPSC, BMSC and ADSC, however, other stem cells are good candidates for the treatment of traumatic and degenerative eye disease. For example, umbilical cord-derived MSC are RGC protective and promote regeneration of their axons after optic nerve transection (Zwart *et al*., 2009). Equally, NSC have paracrine potential in models of SCI (Lu *et al*., 2003) and their use in the eye for RGC survival would be worth future study.

6.2 **Conclusions**

The findings described in this thesis confirm the reliability and accuracy of counting RGC in radial sections of retina as a method for quantifying RGC survival and neuroprotection in rats. Using this method and others, a neuroprotective and axogenic effect of DPSC on injured RGC in a model TON was demonstrated. The observation that DPSC were significantly more neuroprotective/axogenic than BMSC was likely due to the enhanced NTF secretion by DPSC. Finally it was demonstrated that DPSC, in comparison to both BMSC

and ADSC, significantly protected RGC from death and preserved visual function in a rat model of glaucoma.

6.3 References

Haddad-Mashadrizeh A, Bahrami AR, Matin MM, Edalatmanesh MA, Zomorodipour A, Gardaneh M, Farshchian M, Momeni-Moghaddam M (2013) Human adipose-derived mesenchymal stem cells can survive and integrate into the adult rat eye following xenotransplantation. Xenotransplantation 20:165-176.

Hill LJ, Mead B, Blanch RJ, et al. Decorin Reduces Intraocular Pressure and Retinal Ganglion Cell Loss in Rodents Through Fibrolysis of the Scarred Trabecular Meshwork. *Invest Ophthalmol Vis Sci* 2015;56:3743-3757.

Johnson TV, Bull ND, Hunt DP, Marina N, Tomarev SI, Martin KR (2010) Neuroprotective Effects of Intravitreal Mesenchymal Stem Cell Transplantation in Experimental Glaucoma. Investigative Ophthalmology & Visual Science 51:2051-2059.

Levkovitch-Verbin H, Sadan O, Vander S, Rosner M, Barhum Y, Melamed E, Offen D, Melamed S (2010) Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. Invest Ophthalmol Vis Sci 51:6394-6400.

Lu P, Jones LL, Snyder EY, Tuszynski MH (2003) Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp Neurol 181:115-129.

Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2013) Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest Ophthalmol Vis Sci 54:7544-7556.

Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2014a) Paracrine-Mediated Neuroprotection and Neuritogenesis of Axotomised Retinal Ganglion Cells by Human Dental Pulp Stem Cells: Comparison with Human Bone Marrow and Adipose-Derived Mesenchymal Stem Cells. Plos One 9:e109305.

Mead B, Thompson A, Scheven BA, Logan A, Berry M, Leadbeater W (2014b) Comparative evaluation of methods for estimating retinal ganglion cell loss in retinal sections and wholemounts. Plos One 9:e110612.

Mead B, Berry M, Logan A, Scott RAH, Leadbeater W, Scheven BA (2015) Stem cell treatment of degenerative eye disease. Stem cell research 14:243-257.

Mendel TA, Clabough EB, Kao DS, Demidova-Rice TN, Durham JT, Zotter BC, Seaman SA, Cronk SM, Rakoczy EP, Katz AJ, Herman IM, Peirce SM, Yates PA (2013) Pericytes derived from adipose-derived stem cells protect against retinal vasculopathy. Plos One 8:e65691.

Mesentier-Louro LA, Zaverucha-do-Valle C, da Silva-Junior AJ, Nascimento-Dos-Santos G, Gubert F, de Figueiredo AB, Torres AL, Paredes BD, Teixeira C, Tovar-Moll F, Mendez-Otero R, Santiago MF (2014) Distribution of mesenchymal stem cells and effects on neuronal survival and axon regeneration after optic nerve crush and cell therapy. Plos One 9:e110722.

Sakai K, Yamamoto A, Matsubara K, Nakamura S, Naruse M, Yamagata M, Sakamoto K, Tauchi R, Wakao N, Imagama S, Hibi H, Kadomatsu K, Ishiguro N, Ueda M (2012) Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. Journal of Clinical Investigation 122:80-90.

Sieving PA, Caruso RC, Tao W, Coleman HR, Thompson DJ, Fullmer KR, Bush RA (2006) Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. Proc Natl Acad Sci U S A 103:3896-3901.

Singh T, Prabhakar S, Gupta A, Anand A (2012) Recruitment of Stem Cells into the Injured Retina After Laser Injury. Stem Cells and Development 21:448-454.

Tan HB, Kang X, Lu SH, Liu L (2015) The therapeutic effects of bone marrow mesenchymal stem cells after optic nerve damage in the adult rat. Clin Interv Aging 10:487-490.

Tzameret A, Sher I, Belkin M, Treves AJ, Meir A, Nagler A, Levkovitch-Verbin H, Barshack I, Rosner M, Rotenstreich Y (2014) Transplantation of human bone marrow mesenchymal stem cells as a thin subretinal layer ameliorates retinal degeneration in a rat model of retinal dystrophy. Experimental Eye Research 118:135-144.

Zhang R, Zhang H, Xu L, Ma K, Wallrapp C, Jonas JB (2011) Neuroprotective effect of intravitreal cell-based glucagon-like peptide-1 production in the optic nerve crush model. Acta Ophthalmol 89:e320-326.

Zwart I, Hill AJ, Al-Allaf F, Shah M, Girdlestone J, Sanusi AB, Mehmet H, Navarrete R, Navarrete C, Jen LS (2009) Umbilical cord blood mesenchymal stromal cells are neuroprotective and promote regeneration in a rat optic tract model. Exp Neurol 216:439-448.

APPENDICES

This Appendix contains all publications derived from work done during this PhD but not used in the results chapters (2-5)

APPENDIX 1

NEURAL REGENERATION RESEARCH
March 2014, Volume 9, Issue 6
www.nrronline.org

PERSPECTIVES

Dental pulp stem cells, a paracrine-mediated therapy for the retina

The functional loss that occurs after retinal/optic nerve injury is permanent and can arise through trauma or neurodegenerative conditions such as glaucoma. Neurotrophic factors (NTFs) promote survival of injured retinal ganglion cells (RGCs) and regeneration of their axons, suggesting their clinical utility to prevent further damage and restore lost function. Delivery of optimal concentrations of NTFs to RGCs is difficult to achieve by injection but single implants of stem cells which naturally secrete multiple NTFs for sustained periods better addresses this problem. This review discusses a relatively new source of adult stem cells, the dental pulp stem cells, and compares their efficacy and feasibility with other stem cells, such as the well-studied bone marrow-derived mesenchymal stem cells (BMSCs), in the context of cellular therapy for the retina.

Retinal and/or optic nerve damage after trauma or degenerative diseases leads to partial or complete blindness. Like other parts of the central nervous system (CNS), RGC axons in the optic nerve fail to regenerate after injury and RGCs are not replaced after death (reviewed in Berry et al., 2008). Unlike the rest of the CNS, death of RGCs is rapid after optic nerve injury with over 90% lost after 21 days (Berkelaar et al., 1994), as there are no collateral axons to maintain the retrograde delivery of neuroprotective NTFs to RGC somata. Thus, a major therapeutic goal is to develop new strategies to promote both RGC neuroprotection and regeneration of their axons after trauma and in degenerative retinal disease.

A requirement for neuroprotection and axon regeneration is the delivery of an effective titre of NTFs to neuronal cell bodies. However, effective delivery of exogenous NTFs to the eye is limited by disadvantages such as the need for repeated intravitrealed injections (Ko et al., 2000, 2001), the down-regulation of TrK receptors after bolus administration (Sommerfeld et al., 2000; Chen and Weber, 2004) and the lack of efficacy when single NTFs are used (Logan et al., 2006).

We have previously explored the application of cellular therapy to address these problems by intravitreal transplantation of cells which, due to their placement at close proximity to the retina, will continuously supply multiple NTFs directly to the RGC somata, promoting both RGC survival and the regeneration of their axons after optic nerve injury. Intravitreal rather than intranerve transplantation of NTF secreting cells avoids the formation of a sink of high NTF concentration that traps axons within transplant sites, preventing their forward growth along the optic nerve. In our first study (Logan et al., 2006), we achieved this by intravitreal grafting of fibroblasts engineered to express fibroblast growth factor-2 (FGF-2), neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) which led to survival and axon regeneration of approximately 1.25% of RGCs (compared to intact retina) with axons extending 2 mm distal to the crush site.

Genetic modification of autologously transplanted cells however adds costs and time required to prepare the therapy, making translation to the clinic more challenging.

An emerging new therapeutic theme is the use of stem cells as a sustained source of multiple NTFs for CNS injury. For example, various studies have attributed functional recovery after transplantation in models of spinal cord injury to stem cell-derived NTFs (reviewed in Li and Lepski, 2013). These studies have demonstrated improvements in locomotion, but few stem cell-derived neurons are formed, causing us and others to speculate that a paracrine mechanism, rather than neuronal differentiation is responsible for the restoration of function (Burdon et al., 2011). Studies from other laboratories in ocular models have explored this phenomenon by transplanting BMSCs into the rat vitreous body after either optic nerve transection (Levkovitch-Verbin et al., 2010) or episcleral vein ligation/trabecular meshwork laser photocoagulation induced-glaucoma (Yu et al., 2006; Johnson et al., 2010). Following BMSC transplantation, these authors demonstrated significant RGC neuroprotection with the number of surviving RGCs increased by 10–20% in animal models of glaucoma (Yu et al., 2006; Johnson et al., 2010) and by 20% at 8 days after optic nerve transection (Levkovitch-Verbin et al., 2010) compared to survival of RGCs in untreated animals. Notably, GFP^+ BMSCs survived in the eye for at least 4 weeks post-transplantation (Yu et al., 2006; Johnson et al., 2010, Levkovitch-Verbin et al., 2010). The failure of BMSCs to differentiate into neurons and/or migrate and integrate into the retina, along with the positive expression of NTFs by the transplanted BMSCs (Yu et al., 2006; Levkovitch-Verbin et al., 2010) strongly suggests that their neuroprotective effects are paracrine-mediated.

We have recently explored the alternative use of dental pulp stem cells (DPSCs) for neural protection and regeneration in the eye. The use of DPSCs is a relatively recent development in the field of neuroregenerative medicine and is of particular interest since they are neural crest-derived cells that can be isolated from exfoliated or extracted adult teeth, making them an easily accessible stem cell from patients of all ages (Gronthos et al., 2000). We hypothesized that their neural crest origin and neural characteristics make DPSCs more suited than other mesenchymal stem cell sources, such as BMSCs, in the treatment of CNS injuries. Conflicting evidence exists for the successful differentiation of DPSCs into neurons *in vitro* with evidence for (Arthur et al., 2008; Kiraly et al., 2009) and against, both from us (our unpublished data) and others (Aanismaa et al., 2012). The evidence for a paracrine mechanism of DPSC action in neural support (Nosrat et al., 2001) has recently been strengthened by the results of a study using a rodent model of spinal cord injury (Sakai et al., 2012) in which, compared to BMSCs, DPSCs significantly restored locomotory function. Recovery was attributed to paracrine mechanisms, with the gene expression of many NTFs, such as nerve growth factor (NGF), BDNF and NT-3, being greater in DPSCs than BMSCs. However, other mechanisms of action such as their neuronal and oligodendrocyte differentiation could not be ruled out in this spinal cord injury study.

We addressed this possibility in our latest study in which we studied the paracrine-mediated neuroprotective and pro-regenerative properties of DPSCs compared to BMSCs for axotomised RGCs both *in vitro* and in an optic nerve crush model after intravitreal transplantation (Mead et al., 2013). *In vitro*, we showed not only that DPSCs are more neuroprotective and neuritogenic than BMSCs, but that these effects were abolished after Fc-TrK blockade. This paracrine-mediated effect was corroborated by ELISA showing greater titres of NGF, BDNF and NT-3 in the DPSC's secretome compared to that of BMSCs. Finally, we transplanted DPSCs into the rat vitreous body and observed a 27% increase in the number of surviving RGCs 21 days after optic nerve crush compared to the survival of RGCs in untreated/dead cell transplanted animals. This survival was significantly greater than was seen after intravitreal BMSC transplants which yielded an 11% increase in the number of surviving RGCs 21 days after optic nerve crush compared to the survival of RGCs in untreated/dead cell transplanted animals. Compared to BMSCs, DPSC transplantation promoted over twice the number of regenerating RGC axons which grew through the optic nerve and lesion scar and over 1.2 mm into the distal optic nerve segment. Duplication of the *in vitro* experiments using human-derived stem cells have now yielded similar results (Figure 1; unpublished data).

The paracrine basis of neuroprotection and axogenesis is the subject of our current study and, although DPSC-derived NTFs have been clearly implicated, the large and diverse secretomes of BMSCs and DPSCs suggest other secreted molecules may also be involved in the stem cell-mediated neuroprotection/axogenesis, such as platelet-derived growth factor (Johnson et al., 2013). One candidate neurotrophic signalling cascade is the mTOR pathway which, when activated, promotes pronounced regeneration of axons in the optic nerve (Park et al., 2008), although it is currently unknown if stem cells significantly activate the mTOR pathway. It is also unknown if secreted growth factors directly interact with their cognate receptors on the injured neurons or activate glia to signal RGC protection and axon regeneration indirectly (Muller et al., 2009). Glia are activated after DPSC transplantation (Mead et al., 2013) and this juxtacrine mechanism could supplement the local NTF supply.

Although we have shown DPSCs perform better as a cell therapy than BMSCs in our models of retinal/optic nerve injury, comparisons with other stem cells are necessary to ensure the correct stem cell type is taken forward into clinical trials. Adipose-derived stem cells have proven efficacy in promoting neuritogenesis (Kalbermatten et al., 2011), yet have not been tested in the eye. Similarly neural stem cells (NSCs), isolated from foetal spinal cord and transplanted with growth factors have promoted some of the most significant axon regeneration seen to date after transplantation in spinal cord injury sites (Lu et al., 2012), probably explained by NSC differentiation into neurons which directly integrate into the host circuitry, and not by paracrine mechanisms. However, intravitreal transplantation of undifferentiated NSCs has not yet been explored and would determine any paracrine properties. Despite their efficacy, their foetal and cadaveric source poses both ethical issues and a significant challenge in obtaining adequate numbers of cells for transplantation, particular if the therapy was to translate to the clinic. Secondly, unlike DPSCs, for which autologous transplantation is feasible, patients receiving NSCs would re-

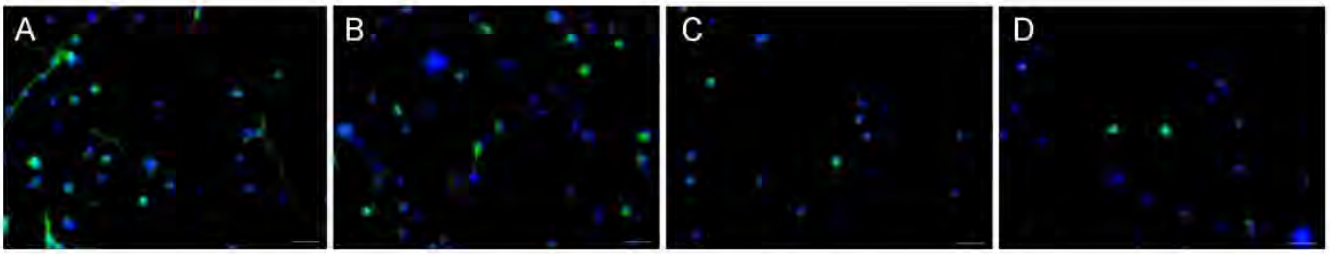


Figure 1 Neuroprotection and neuritogenesis of axotomized/injured adult βIII-tubulin-positive (green) retinal neurons after culture with human-derived dental pulp stem cells (DPSCs; A) and human-derived BMSCs (B). DPSCs more significantly promoted neuroprotection of βIII-tubulin-positive retinal neurons and regeneration of their neurites compared to untreated βIII-tubulin-positive retinal neurons (C) or those treated with bone marrow-derived mesenchymal stem cells (BMSCs). These effects are neurotrophin-dependent as emphasized by the response abolition after addition of TrKA/B/C inhibitors (D), which block nerve growth factor, brain-derived neurotrophic factor and NT-3 receptor binding. Representative images shown with DAPI (blue) used as a nuclear counterstain, scale bars represent 50 μm.

quire lifelong immunosuppressive treatment.

With BMSCs already being used in clinical trials for retinal and optic nerve damage (www.clinicaltrials.gov/show/NCT01920867), the future for stem cell therapy in treating traumatic and degenerative ocular conditions is fast becoming a reality. We have evidence that DPSCs may be a more appropriate cell type than BMSCs for retinal therapy (although NSCs may also be concluded as a strong alternative candidate)(Mead et al., 2013) and we are engaged in further work to substantiate this claim. Thus, an in depth comparison with other available stem cells is necessary as well as research into the exact mechanism behind DPSC-mediated RGC neuroprotection and axon regeneration to support the preclinical and translational development of this cellular therapy.

Ben Mead[1, 2], Ann Logan[1], Martin Berry[1], Wendy Leadbeater[1], Ben A. Scheven[2]
1 Neurotrauma and Neurodegeneration Section, School of Clinical and Experimental Medicine, University of Birmingham, Birmingham B15 2TT, United Kingdom
2 School of Dentistry, University of Birmingham, Birmingham B4 6NN, United Kingdom

Author contributions: *Mead B was responsible for study conception and design, collection and/or assembly of data, data analysis and interpretation and manuscript writing. Logan A participated in study conception and design, data analysis and interpretation and manuscript writing. Berry M was responsible for manuscript writing. Scheven BA was in charge of study conception and design, data analysis and interpretation and manuscript writing. Leadbeater W participated in study conception and design, data analysis and interpretation and manuscript writing. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*
Wendy Leadbeater and Ben A. Scheven contributed equally and were joint senior authors.
Corresponding author: *Ben Mead, Molecular Neuroscience Group, Neurotrauma and Neurodegeneration Section, School of Clinical and Experimental Medicine, University of Birmingham, B15 2TT, United Kingdom, BXM813@bham.ac.uk.*
doi: *10.4103/1673-5374.130089* ***http:*** *//www.nrronline.org/*
Accepted: *2014-02-26*
Funding: *The studentship of Ben Mead was funded by the BBSRC (grant number BB/F017553/1) and the Rosetrees Trust.*
Mead B, Logan A, Berry M, Leadbeater W, Scheven BA. Dental pulp stem cells, a paracrine-mediated therapy for the retina. Neural Regen Res. 2014;9(6):577-578.

References

Aanismaa R, Hautala J, Vuorinen A, Miettinen S, Narkilahti S (2012) Human dental pulp stem cells differentiate into neural precursors but not into mature functional neurons. Stem Cell Discovery 2:85-91.
Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S (2008) Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 26:1787-1795.
Berkelaar M, Clarke DB, Wang YC, Bray GM, Aguayo AJ (1994) Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. J Neurosci 14:4368-4374.
Berry M, Ahmed Z, Lorber B, Douglas M, Logan A (2008) Regeneration of axons in the visual system. Restor Neurol Neurosci 26:147-174.
Burdon TJ, Paul A, Noiseux N, Prakash S, Shum-Tim D (2011) Bone marrow stem cell derived paracrine factors for regenerative medicine: current perspectives and therapeutic potential. Bone Marrow Res 2011.
Chen H, Weber AJ (2004) Brain-derived neurotrophic factor reduces TrkB protein and mRNA in the normal retina and following optic nerve crush in adult rats. Brain Res 1011:99-106.
Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A 97:13625-13630.
Johnson TV, Bull ND, Hunt DP, Marina N, Tomarev SI, Martin KR (2010) Neuroprotective effects of intravitreal mesenchymal stem cell transplantation in experimental glaucoma. Invest Ophthalmol Vis Sci 51:2051-2059.
Johnson TV, Dekorver NW, Levasseur VA, Osborne A, Tassoni A, Lorber B, Heller JP, Villasmil R, Bull ND, Martin KR, Tomarev SI (2014) Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain137(Pt 2):503-519.
Kalbermatten DF, Schaakxs D, Kingham PJ, Wiberg M (2011) Neurotrophic activity of human adipose stem cells isolated from deep and superficial layers of abdominal fat. Cell Tissue Res 344:251-260.
Kiraly M, Porcsalmy B, Pataki A, Kadar K, Jelitai M, Molnar B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G (2009) Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochem Int 55:323-332.
Ko ML, Hu DN, Ritch R, Sharma SC (2000) The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. Invest Ophthalmol Vis Sci 41:2967-2971.
Ko ML, Hu DN, Ritch R, Sharma SC, Chen CF (2001) Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. Neurosci Lett 305:139-142.
Levkovitch-Verbin H, Sadan O, Vander S, Rosner M, Barhum Y, Melamed E, Offen D, Melamed S (2010) Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. Invest Ophthalmol Vis Sci 51:6394-6400.
Li J, Lepski G (2013) Cell transplantation for spinal cord injury: a systematic review. Biomed Res Int 2013:786475.
Logan A, Ahmed Z, Baird A, Gonzalez AM, Berry M (2006) Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. Brain 129:490-502.
Lu P, Wang Y, Graham L, McHale K, Gao M, Wu D, Brock J, Blesch A, Rosenzweig ES, Havton LA, Zheng B, Conner JM, Marsala M, Tuszynski MH (2012) Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell 150:1264-1273.
Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2013) Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest Ophthalmol Vis Sci 54:7544-7556.
Muller A, Hauk TG, Leibinger M, Marienfeld R, Fischer D (2009) Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF. Mol Cell Neurosci 41:233-246.
Nosrat IV, Widenfalk J, Olson L, Nosrat CA (2001) Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury. Dev Biol 238:120-132.
Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting Axon Regeneration in the Adult CNS by Modulation of the PTEN/mTOR Pathway. Science 322:963-966.
Sakai K, Yamamoto A, Matsubara K, Nakamura S, Naruse M, Yamagata M, Sakamoto K, Tauchi R, Wakao N, Imagama S, Hibi H, Kadomatsu K, Ishiguro N, Ueda M (2012) Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. J Clin Invest 122:80-90.
Sommerfeld MT, Schweigreiter R, Barde YA, Hoppe E (2000) Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. J Biol Chem 275:8982-8990.
Yu S, Tanabe T, Dezawa M, Ishikawa H, Yoshimura N (2006) Effects of bone marrow stromal cell injection in an experimental glaucoma model. Biochem Biophys Res Commun 344:1071-1079.

APPENDIX 2

Send Orders for Reprints to reprints@benthamscience.ae

Current Tissue Engineering, **2014**, *3*, **93-101**

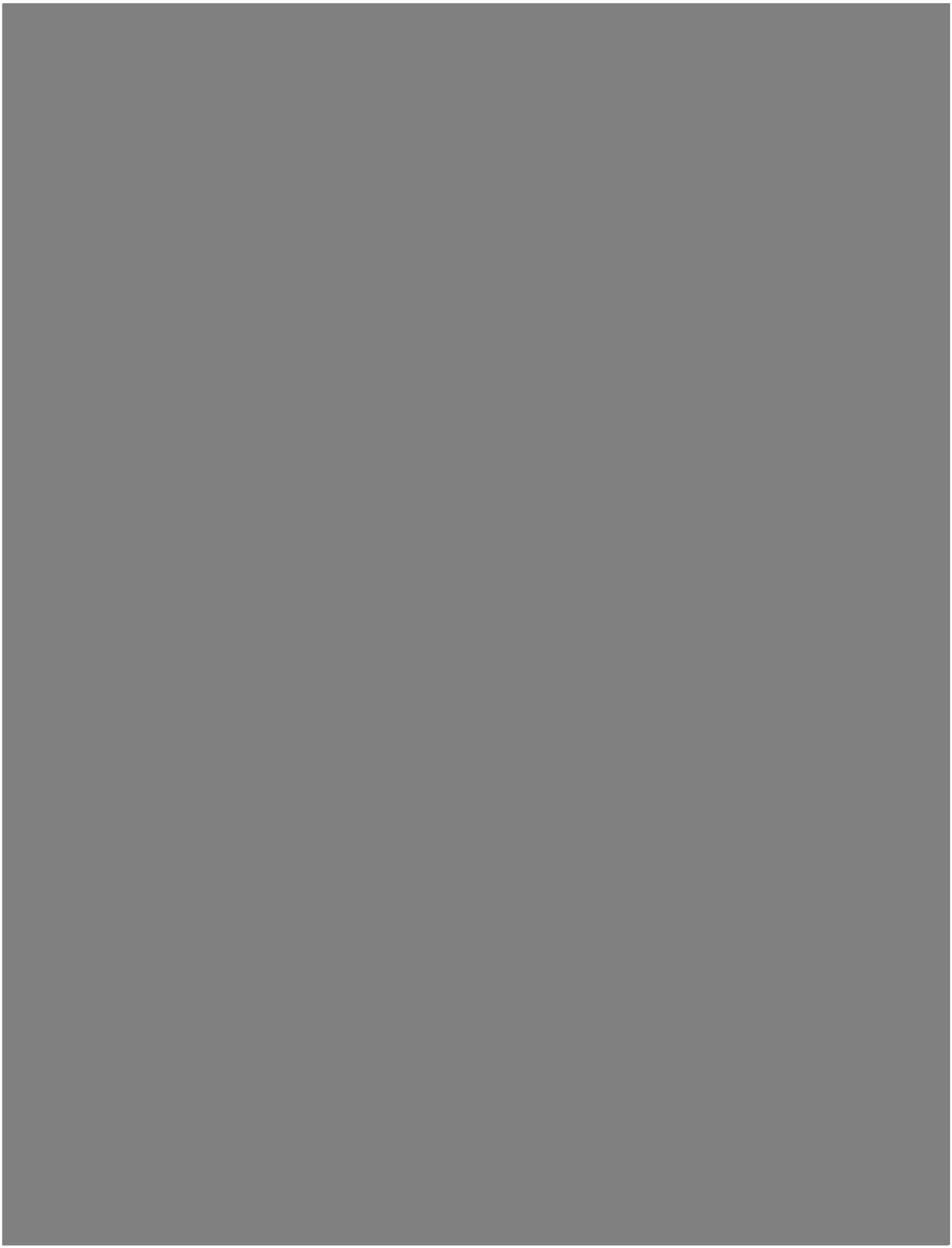
Adult Stem Cell Treatment for Central Nervous System Injury

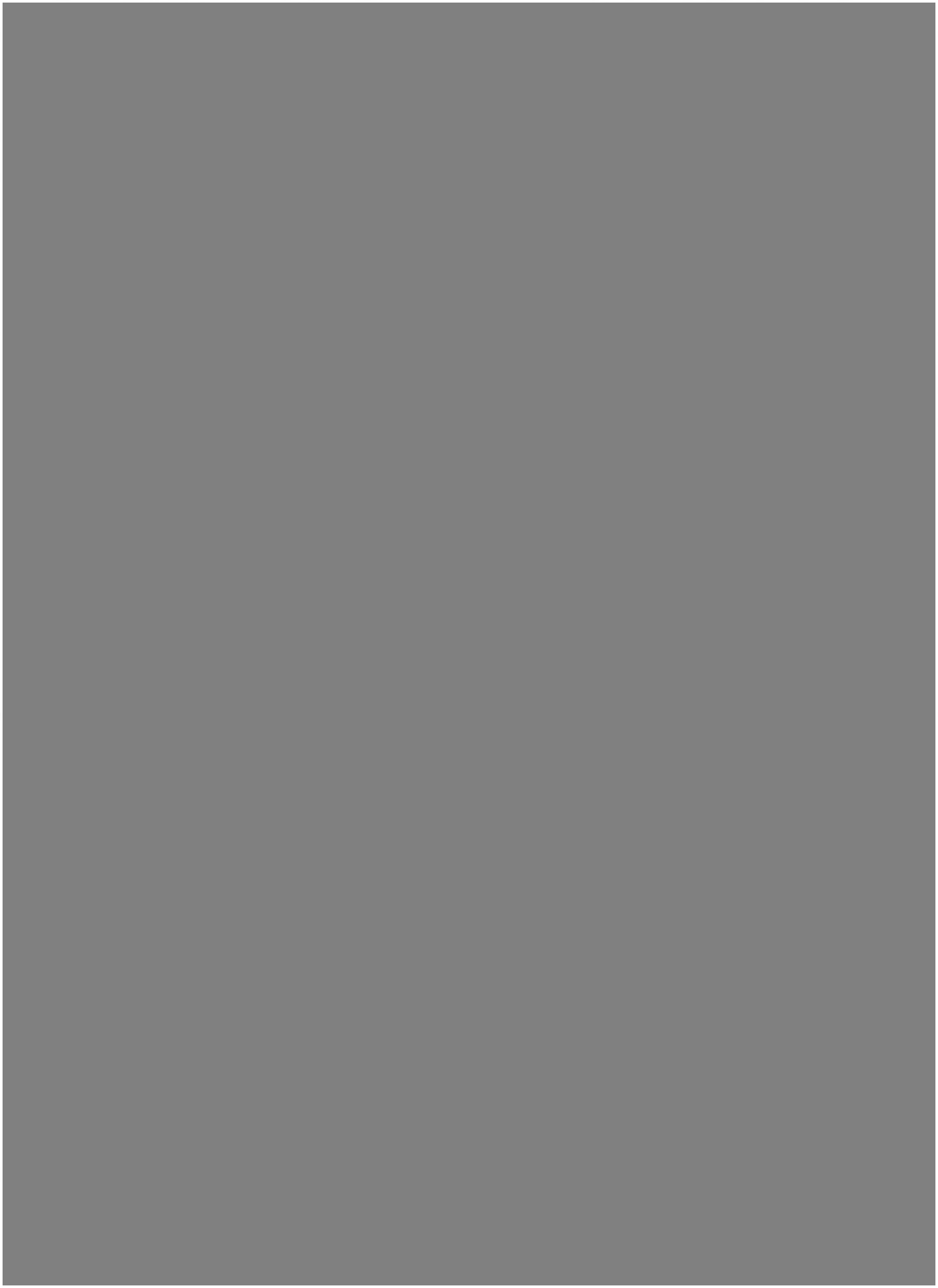
Ben Mead*[1,2], Ann Logan[1#], Martin Berry[1], Wendy Leadbeater[1#] and Ben A. Scheven[2#]

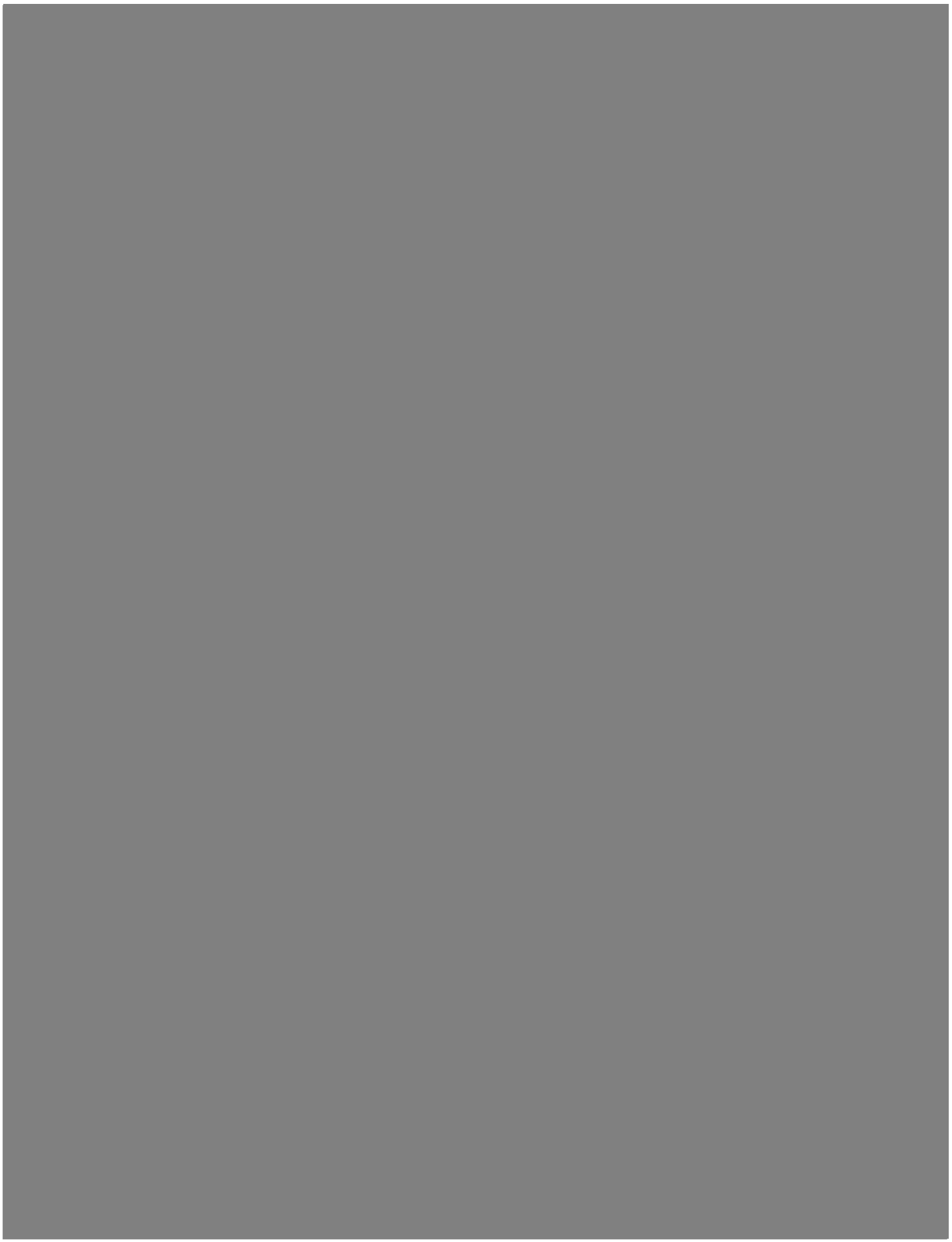
[1]*Neurotrauma and Neurodegeneration Section, School of Clinical and Experimental Medicine, University of Birmingham, B15 2TT, United Kingdom;* [2]*School of Dentistry, University of Birmingham, B4 6NN, United Kingdom*

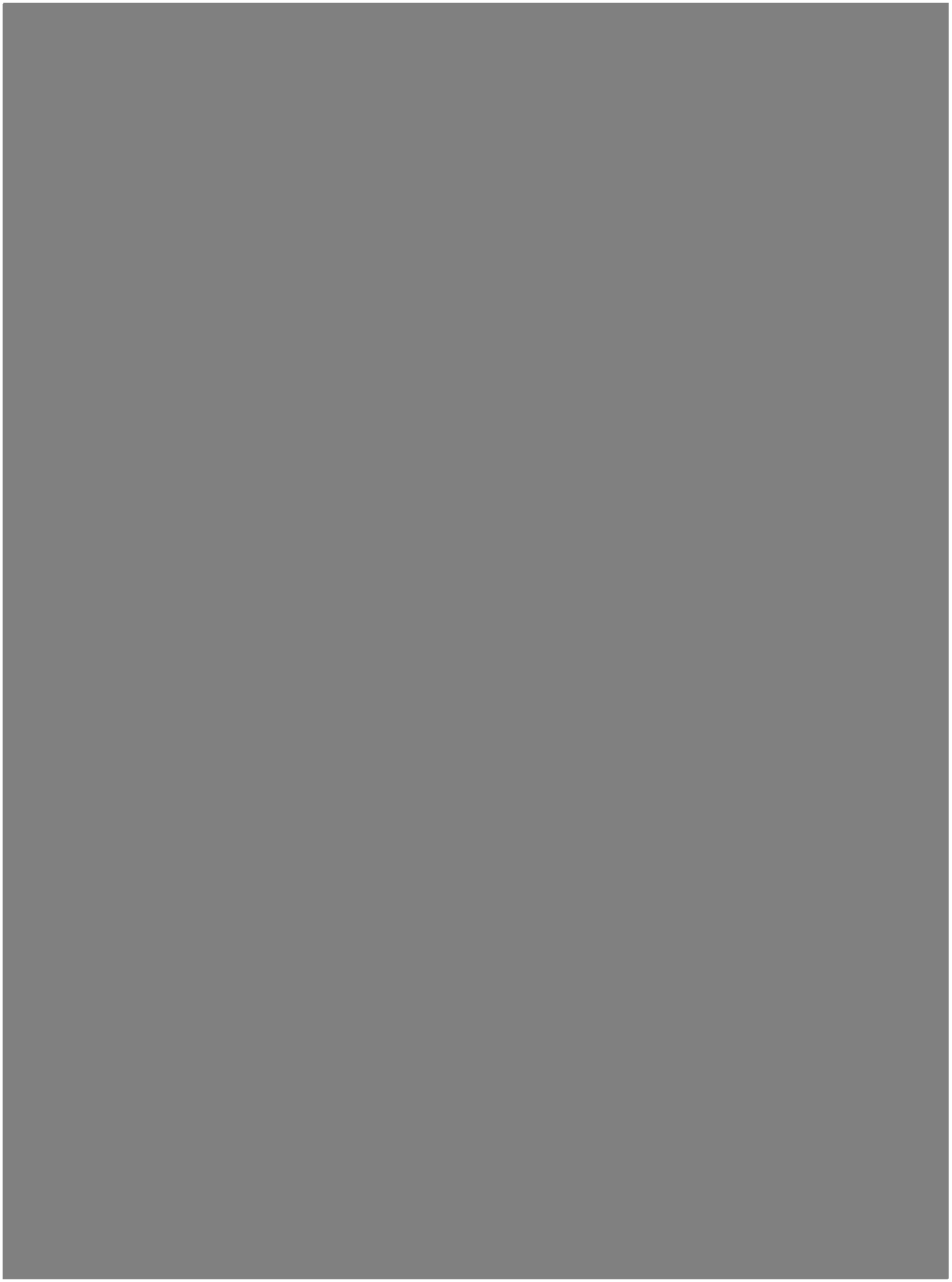
Abstract: Stem cells possess both self-renewing and multi-lineage differentiation properties and are being explored extensively for use as a cellular therapy for regenerative medicine. Historically, replacement of lost neurons and restoration of neural circuits was primarily considered as the main mechanism by which stem cells restore function in the injured central nervous system (CNS). However, evidence is accumulating that implicates stem cell-derived trophic factors in the neuroprotection of compromised endogenous neurons and regeneration of their axons and dendrites. In this concise review, we summarise the potential of bone marrow-derived stem cells (BMSC), adipose-derived mesenchymal stem cells (AMSC), dental pulp stem cells (DPSC) and neural stem cells (NSC) to repair the injured CNS, with particular reference to spinal cord injury and optic nerve/retinal injury.

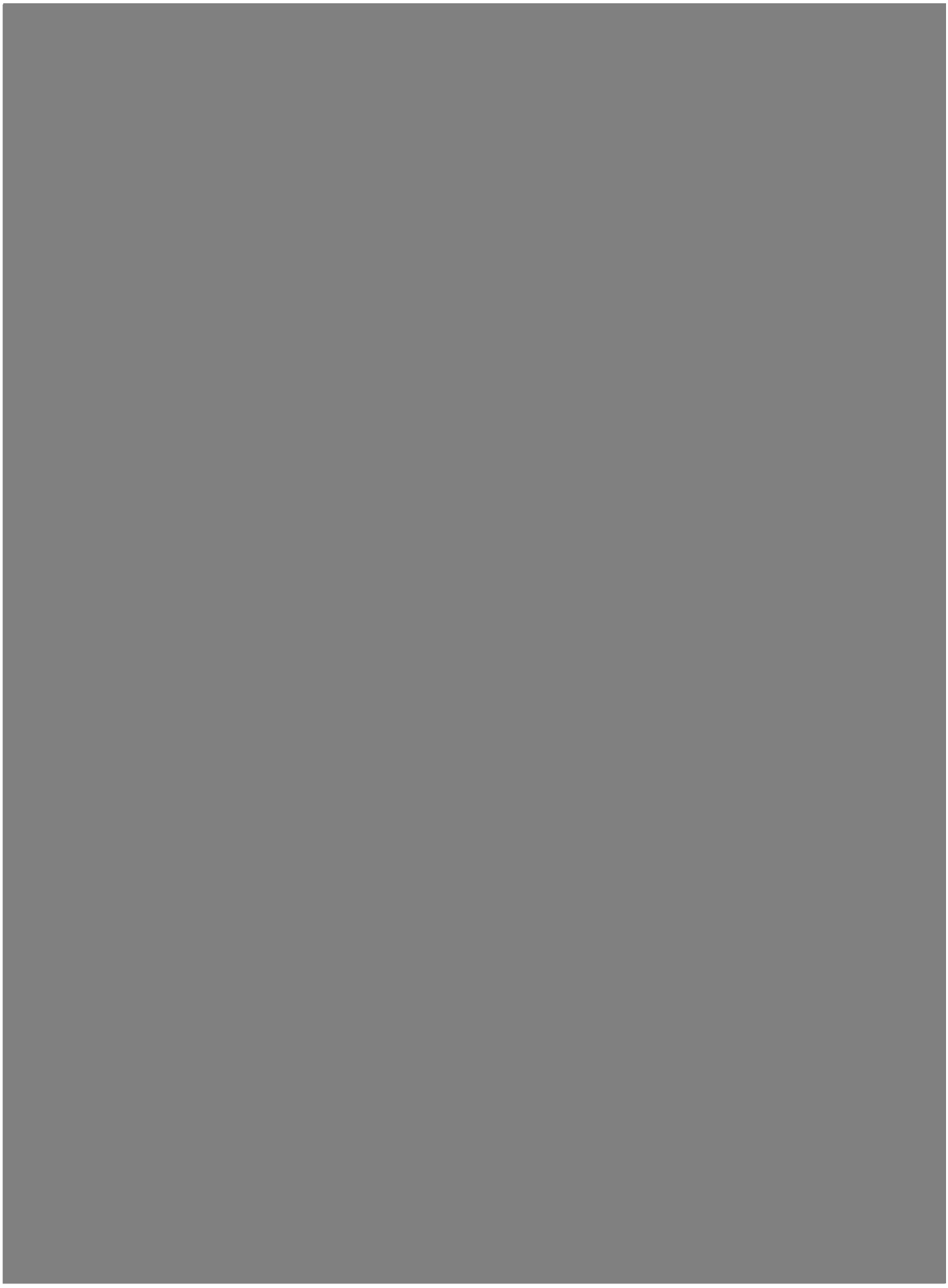
Keywords: Cell transplantation, mesenchymal stem cells, medicine, neural stem cells, regenerative spinal cord injury, retina.

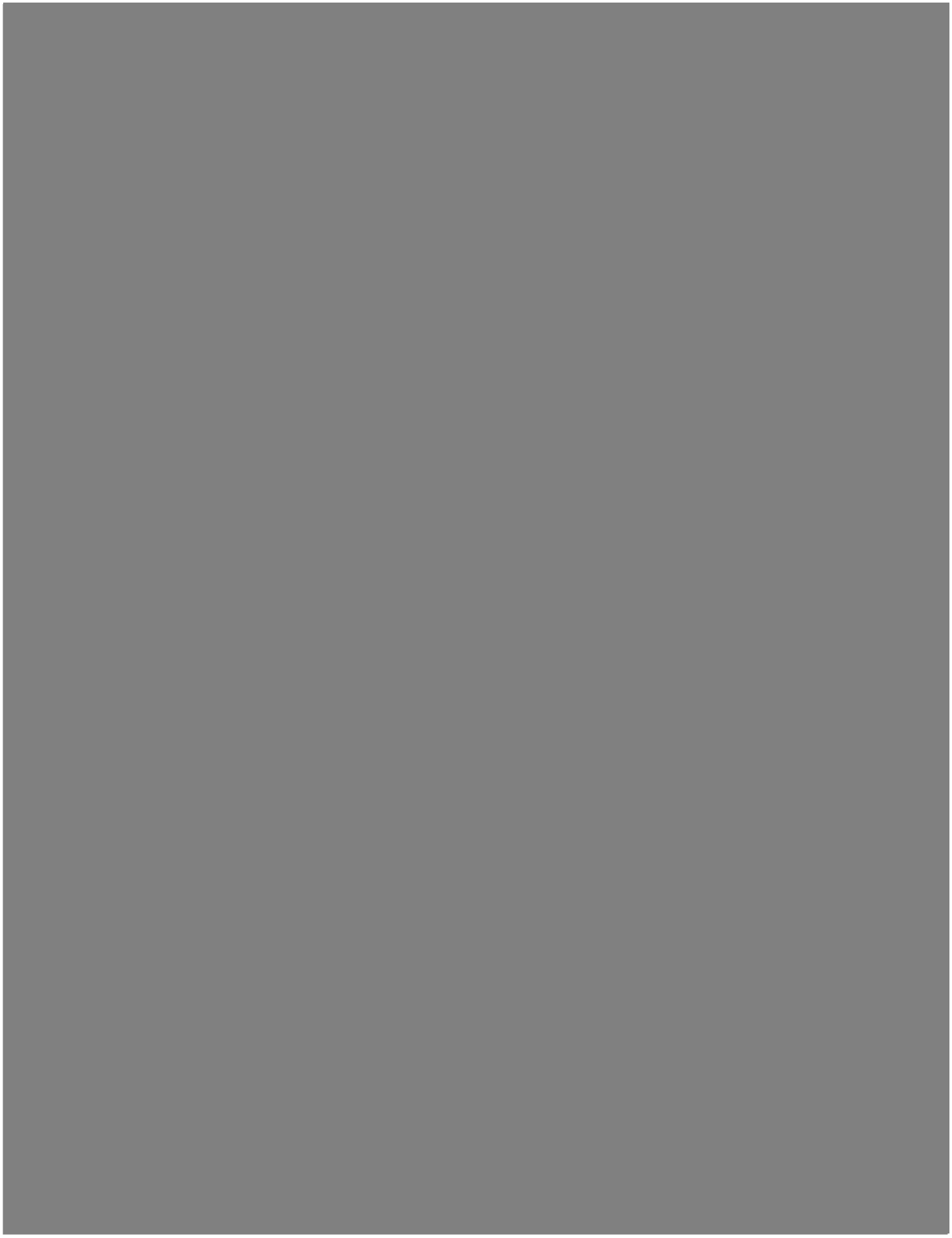


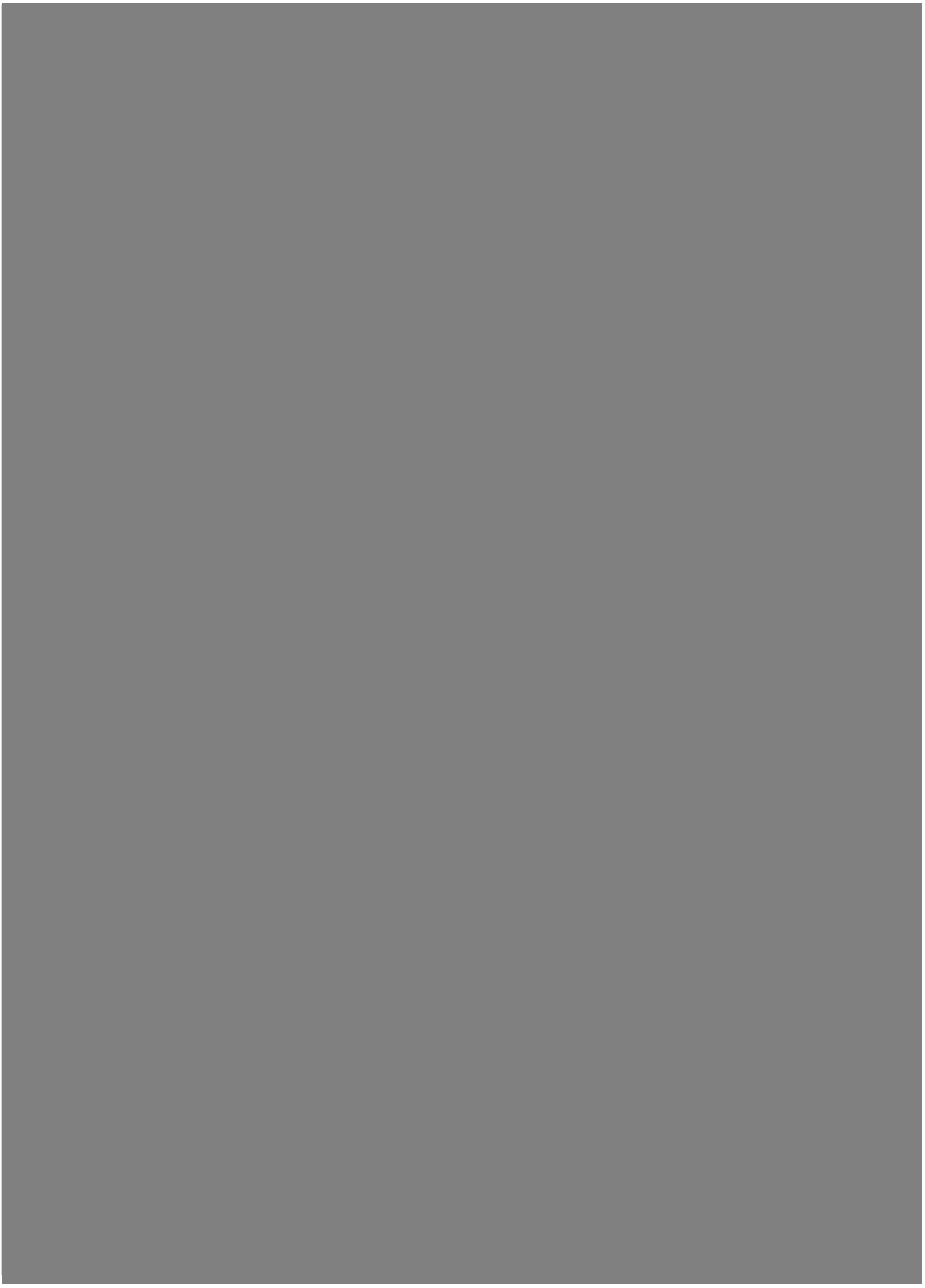












APPENDIX 3

Stem Cell Research (2015) 14, 243–257

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/scr

REVIEW

Stem cell treatment of degenerative eye disease☆

Ben Mead[a,b,*], Martin Berry[a], Ann Logan[a], Robert A.H. Scott[a], Wendy Leadbeater[a,1], Ben A. Scheven[b,1]

[a] *Neurotrauma Research Group, Neurobiology Section, School of Clinical and Experimental Medicine, University of Birmingham, B15 2TT, UK*
[b] *School of Dentistry, University of Birmingham, B4 6NN, UK*

Received 24 September 2014; received in revised form 12 February 2015; accepted 14 February 2015

Abstract
Stem cell therapies are being explored extensively as treatments for degenerative eye disease, either for replacing lost neurons, restoring neural circuits or, based on more recent evidence, as paracrine-mediated therapies in which stem cell-derived trophic factors protect compromised endogenous retinal neurons from death and induce the growth of new connections. Retinal progenitor phenotypes induced from embryonic stem cells/induced pluripotent stem cells (ESCs/iPSCs) and endogenous retinal stem cells may replace lost photoreceptors and retinal pigment epithelial (RPE) cells and restore vision in the diseased eye, whereas treatment of injured retinal ganglion cells (RGCs) has so far been reliant on mesenchymal stem cells (MSC). Here, we review the properties of non-retinal-derived adult stem cells, in particular neural stem cells (NSCs), MSC derived from bone marrow (BMSC), adipose tissues (ADSC) and dental pulp (DPSC), together with ESC/iPSC and discuss and compare their potential advantages as therapies designed to provide trophic support, repair and replacement of retinal neurons, RPE and glia in degenerative retinal diseases. We conclude that ESCs/iPSCs have the potential to replace lost retinal cells, whereas MSC may be a useful source of paracrine factors that protect RGC and stimulate regeneration of their axons in the optic nerve in degenerate eye disease. NSC may have potential as both a source of replacement cells and also as mediators of paracrine treatment.

Abbreviations: ADSCs, adipose-derived stem cells; AMD, age-related macular degeneration; BDNF, brain-derived neurotrophic factor; BMSCs, bone marrow-derived stem cells; CNS, central nervous system; CNTF, ciliary neurotrophic factor; DPSC, dental pulp stem cells; EGF, epidermal growth factor; ERG, electroretinogram; ESCs, embryonic stem cells; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; iPSCs, induced pluripotent stem cells; *ivit*, intravitreal; MSC, mesenchymal stem cells; mTOR, mammalian target of rapamycin; NGF, nerve growth factor; NSCs, neural stem cells; NT-3, neurotrophin-3; NTFs, neurotrophic factors; ONL, outer nuclear layer; RCS, Royal College of Surgeons rats; RGC, retinal ganglion cell; RPE, retinal pigment epithelial cells; SCI, spinal cord injury; TBI, traumatic brain injury; TrK, tropomyosin related kinase; VEGF, vascular endothelial growth factor.

☆ Grant information: Rosetrees Trust and BBSRC studentship, grant number BB/F017553/1.

* Corresponding author at: Neurotrauma Research Group, Neurobiology Section, School of Clinical and Experimental Medicine, University of Birmingham, B15 2TT, UK.
E-mail address: BXM813@bham.ac.uk (B. Mead).

[1] Contributed equally and were joint senior authors.

http://dx.doi.org/10.1016/j.scr.2015.02.003

Contents

Introduction

The loss of retinal neurons, their connections and supporting glia in ocular degenerative diseases causes permanent blindness, principally because lost photoreceptors and retinal ganglion cells (RGCs) are not replaced and RGC axons fail to regenerate (Berry et al., 2008). Clinically, there are neither neuroprotective nor axogenic therapies available that restore lost visual system connectivity in retinal degenerative disease and translatable techniques for the replacement of lost RGC and photoreceptors are in their infancy. The retina is classified as central nervous system (CNS) tissue and the characteristics of its regenerative response are shared by other CNS tissues, including the brain and spinal cord.

Stem cell treatments developed as therapies for retinal degeneration fall into two broad categories: stem cells from (1), sources exogenous to the retina including mesenchymal stem cells (MSC) neural stem cells (NSCs) and embryonic/induced pluripotent stem cells (ESCs/iPSCs); and (2), endogenous retinal stem cells such as Müller glia (Ooto et al., 2004; Reichenbach and Bringmann, 2013), ciliary epithelia-derived stem cells (Ahmad et al., 2000; Tropepe et al., 2000) and retinal pigment epithelial (RPE) stem cells.

Potential non-retinal-derived adult stem cell based strategies being developed to treat retinal degeneration include NSC (McGill et al., 2012; Lu et al., 2013) and MSC derived from either bone marrow (BMSC) (Yu et al., 2006; Johnson et al., 2010; Levkovitch-Verbin et al., 2010), adipose tissues (ADSC) (Tsuruma et al., 2014) or dental pulp (DPSC) (Mead et al., 2013). MSC predominantly provide trophic support for the neuroprotection and axon regeneration of damaged retinal cells either directly through the secretion of neurotrophic factors (NTFs) (Johnson et al., 2010; Johnson et al., 2013; Mead et al., 2013) or possibly indirectly after stimulation of endogenous retinal cells (Lee et al., 2012) which, when activated, could provide additional paracrine support and/or effect cell replacement. There is no evidence that ESCs/iPSCs provide substantial paracrine support, but they do seem to be able to replace degenerating photoreceptors and RPE cells (Carr et al., 2009b; Lamba et al., 2009). NSCs directly differentiate into neural and glial phenotypes after transplantation into spinal cord injury (SCI) and traumatic brain injury (TBI) sites (Jeong et al., 2003; Lu et al., 2012). They also secrete trophic factors (Lu et al., 2003) and, although limited work has been performed in the eye with NSC, may have potential for both the neuroprotection and replacement of retinal neurons, including RGC. The differential efficiency of NSC/MSC/ESC/iPSC to perform these disparate tasks is the key to identifying the phenotype most fitted to provide the optimal safe therapy for retinal disease.

Of the endogenous retinal stem cells, Müller glia have been induced to dedifferentiate into retinal progenitors which can then transform into multiple retinal phenotypes including photoreceptors in the photoreceptor-damaged eye (Osakada et al., 2007; Liu et al., 2013). Ciliary epithelial-derived stem cells are self-renewing, multipotential retinal progenitor cells found in the pigmented ciliary epithelium of the retina (Xu et al., 2007), some of which differentiate in vitro into rhodopsin$^+$ photoreceptors (Ballios et al., 2012; Clarke et al., 2012; Del Debbio et al., 2013). The RPE layer generates new retina in some animals (Fischer, 2005) and, in humans, contains a small population of stem cells that can mature into new RPE cells as well as cells with a neuronal phenotype (Salero et al., 2012). Whilst manipulation (Yu et al., 2014) and transplantation (Chacko et al., 2003; Canola et al., 2007) of endogenous retinal stem cells have the potential to treat retinal degeneration, their mechanism of action is largely restricted to RPE and

Table 1 Current clinical trials that test the safety and efficacy of stem cell transplantation for the treatment of degenerative eye disease. Further details found at www.clinicaltrials.gov.

Treatment	Disease	Stage	No. of subjects	Estimated completion date	Outcome	Clinicaltrials.gov identifier
Intravitreal BMSC	AMD, glaucoma	Recruiting participants	300	Aug 2017	Visual acuity, visual field	NCT01920867
Intravitreal BMSC	AMD, diabetic retinopathy, retinitis pigmentosa	Recruiting participants (Park et al., 2015)	15	Dec 2015	Incidence and severity of adverse events	NCT01736059
Intravitreal BMSC	Retinitis pigmentosa	Recruiting participants	10	Aug 2016	Visual acuity, quality of life, visual field, ERG, VEP, colour vision, contrast sensitivity	NCT02280135
Intravitreal BMSC	Glaucoma	Recruiting participants	10	Dec 2016	Incidence and severity of adverse events, visual acuity, visual field, OCT, ERG	NCT02330978
Intravitreal BMSC	Retinitis pigmentosa	Completed (Siqueira et al., 2011)	50	June 2013	Visual acuity	NCT01560715
Intravitreal BMSC	Ischemic retinopathy	Recruiting participants	30	Jan 2014	Size of foveal avascular zone	NCT01518842
Intravitreal BMSC	AMD	Recruiting participants	1	June 2015	Incidence and severity of adverse events	NCT02016508
Intravitreal BMSC	AMD, Stargardt's macular dystrophy	Recruiting participants	10	Dec 2015	Visual acuity	NCT01518127
Intravenous bone marrow mononuclear cells	Optic atrophy	Recruiting participants	24	July 2016	Visual function, reduction in optic nerve degeneration	NCT01834079
Intravitreal AMSC	Dry AMD	Recruiting participants	100	June 2016	Incidence and severity of adverse events, visual acuity	NCT02024269
Subretinal ESC-derived RPE	Dry AMD	Recruiting participants	12	April 2016	Visual acuity, ERG, OCT	NCT01674829
Subretinal ESC-derived RPE	AMD	Pre-recruitment	10	June 2017	Incidence and severity of adverse events, visual acuity	NCT01691261
Subretinal ESC-derived RPE	Stargardt's macular dystrophy	Recruiting participants (Schwartz et al., 2012; Schwartz et al., 2014)	16	Dec 2014	Incidence and severity of adverse events	NCT01345006
Subretinal ESC-derived RPE	Dry AMD	Recruiting participants Schwartz et al. (2014)	16	Dec 2014	Incidence and severity of adverse events	NCT01344993

photoreceptor replacement with RGC replacement proving more refractory to such strategies.

There are currently many clinical trials ongoing which aim to test the safety and efficacy of stem cell transplantation in the eye (Table 1). This review focuses on the potential of non retinal-derived stem cells, in particular NSC, BMSC, ADSC, DPSC and ESC/iPSC for the treatment of traumatic and degenerative eye disease and, where relevant, inter-relates some findings from stem cell research in the spinal cord and brain. Much overlap exists regarding the mechanisms and efficacy of ESC and iPSC and is therefore discussed together, readers are directed towards the following reviews for specific discussion on ESC (Reynolds and Lamba, 2013) and iPSC (Wright et al., 2014) for the treatment of the retina. Readers are also referred to the following articles discussing treatments using MSC not discussed in this review, such as umbilical blood-derived MSC (Zwart et al., 2009; Chen et al., 2013), as well as endogenous retinal stem cells (Yu et al., 2014).

Ntf-mediated effects of stem cells

Retinal cell degeneration

The extensive literature on NTF-mediated neuroprotection has been reviewed by Barde (1989), Sofroniew et al. (2001), Jones et al. (2001) and Morgan-Warren et al. (2013). After uptake by axons innervating distant neuronal targets, NTFs are retrogradely transported to somata (Dawbarn and Allen, 2003) where they are neuroprotective. During development, neurons that fail to innervate their targets are starved of these survival signals and die by apoptosis (Butowt and von Bartheld, 2003). Many adult axotomised neurons also atrophy and die after disconnection from target-derived NTF, but the viability of neurons with collaterals proximal to the transection site is protected by a supply of NTF from spared innervated targets and from local glia (Dougherty et al., 2000; Faulkner et al., 2004). Since axon collaterals are absent in the optic nerve, RGCs are exquisitely sensitive to optic nerve damage, so that approximately 40% die within 7 days (Ahmed et al., 2011) and 90% are lost by 14–21 days (Mey and Thanos, 1993; Berkelaar et al., 1994). RGC loss detailed above is of relevance to diseases such as glaucoma and traumatic optic neuropathy.

The failure of adult CNS neurons to regenerate damaged axons is attributed to suppression of intrinsic axogenic machinery, the paucity of NTF essential for axon growth cone advance (Berry et al., 2008) and the presence of axon growth inhibitory factors (Richardson et al., 1980; Sandvig et al., 2004) mediating growth cone collapse although the relative importance of these differing factors is debatable. The neurotrophins nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) phosphorylate tyrosine residues (Dawbarn and Allen, 2003) after binding to the tropomyosin related kinase (TrK) receptor and promote RGC survival and axon growth (Berry et al., 2008) by activating intracellular signalling pathways (MAPK/PI3K/PKC; Fig. 1), whilst ciliary neurotrophic factor (CNTF) activates the JAK pathway after binding to the heterotrimeric gp130 receptor complex and signal through phosphoinositide-3-kinase (PI3K)/protein kinase B (Akt) to activate the serine–threonine kinase mammalian target of rapamycin (mTOR), to promote axogenic protein synthesis and inhibit glycogen synthase kinase-3β (GSK3β) which, amongst other roles, regulates growth cone dynamics (Morgan-Warren et al., 2013). Experimental activation of mTOR signalling in adult mice promotes RGC survival and axon regeneration after optic nerve transection (Park et al., 2008; Morgan-Warren et al., 2013). Promoting axon regeneration is relevant to scenarios in which either the optic nerve is injured, or RGCs are transplanted into the ganglion cell layer (Hertz et al., 2014) which subsequently require long distance regeneration of their axons.

Photoreceptor outer segments are damaged by light and approximately 10% of the outer segments are recycled by RPE-mediated phagocytosis each day. The digestion of internalised phagosomes is not 100% efficient and toxic lysosomal proteins such as lipofuscin, build up leading to RPE degeneration (Bharti et al., 2011). The thickening of the outer limiting membrane and successive reduction in the supply of diffusible factors to the RPE also contribute to the degeneration. The subsequent failure in photoreceptor outer segment phagocytosis by the degenerating RPE is the primary pathology in age related macular degeneration (AMD) and retinitis pigmentosa (Bharti et al., 2011).

NTF treatment strategies

Treatments for long term RGC neuroprotection and axon regeneration are limited and delivery of individual NTF promotes incomplete and unsustained axon regeneration in the transected rat optic nerve (Logan et al., 2006) and spinal cord (Lu et al., 2004b). For example, intravitreal (*ivit*) injection of recombinant BDNF and CNTF rescues axotomised RGC from death for up to 7 days (Mey and Thanos, 1993; Ahmed et al., 2011). Long term trophic support requires repeated low dose NTF injections (Ko et al., 2000; Ko et al., 2001) since transient high peak bolus delivery of NTF down-regulates TrK receptors (Sommerfeld et al., 2000; Chen and Weber, 2004). Injectable hydrogel formulations composed of collagen, alginate or chitosan are being developed (Pakulska et al., 2012) that continuously and slowly release low titres of NTF in vivo over several weeks. However, drug loading of hydrogels is limited and thus, for chronic neurodegenerative diseases like glaucoma, sustained delivery requires repeated hydrogel implantation, making FDA approval a significant challenge. Alternative treatments, such as the transplantation of cells with extended longevity engineered to continuously produce low levels of specific NTF combinations, remove the need for repeated injections and overcome the problems with bolus NTF delivery regimes. For example, *ivit* transplantation of genetically engineered fibroblasts that overexpress fibroblast growth factor-2 (FGF-2), NT-3 and BDNF significantly increases RGC survival and axon regeneration after optic nerve crush (Logan et al., 2006).

Stem cells and NTF treatment

Stem cells, transfected with *ntf* genes or induced to secrete NTF using epidermal growth factor (EGF)/FGF have been grafted into the retina to treat retinal degeneration e.g. : (1), BMSC secreting BDNF, glial cell line-derived neurotrophic factor (GDNF) and neurotrophin-4 are RGC neuroprotective and improve visual function in cases of traumatic optic neuropathy (Levkovitch-Verbin et al., 2010), sodium iodate-induced

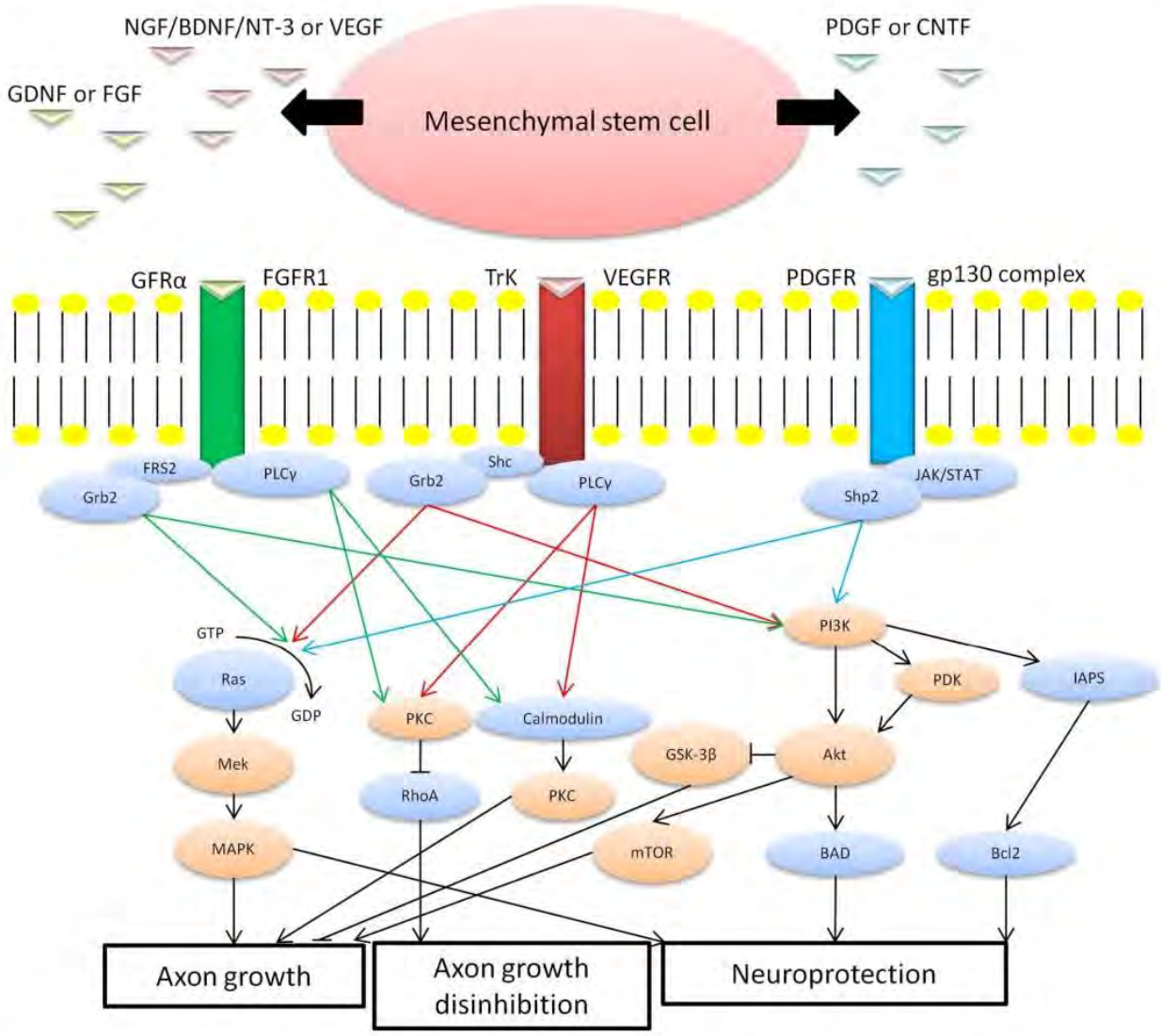


Figure 1 A schematic diagram showing the proposed mechanism by which MSCs exert their neurotrophic effects on the injured CNS, including the retina, through secretion of NGF, BDNF, NT-3, CNTF GDNF, VEGF, FGF and PDGF which engage TrK A, B and C, CNTFα, GFRα, VEGFR, FGFR1 and PDGFR receptors, respectively, leading to the activation of intracellular pathways for axon growth, axon growth disinhibition and neuroprotection, accounting for the functional recovery seen in animals receiving MSC transplants after CNS/retinal injury. Some ligand-receptor interactions lead to the activation of the same signalling pathways (abbreviations: BAD, bcl-2-associated death promoter; Bcl2, B-cell lymphoma 2; FGFR1, fibroblasts growth factor receptor 1; FRS2, fibroblast growth factor receptor substrate 2; GFRα, GDNF family receptor alpha; Grb2, growth factor receptor-bound protein 2; GSK-3β, glycogen synthase kinase-3β; IAPS, inhibitor of apoptosis; MAPK, mitogen-activated protein kinase; Mek, mitogen-activated protein kinase; PDK, phosphoinositide-dependant kinase; PKC, protein kinase C; PLCγ, phospholipase C-gamma).

damage of the retina (Machalińska et al., 2013) and chronic ocular hypertension (Harper et al., 2011); (2), NSCs engineered to secrete CNTF attenuate photoreceptor death in mouse models of retinitis pigmentosa (Jung et al., 2013); (3), ESC-derived neural progenitor cells transfected with crystallin-β-b2 promote both RGC and photoreceptor survival (Bohm et al., 2012); and (4), a glucagon-like peptide-1-secreting cell line promotes RGC survival after optic nerve crush (Zhang et al., 2011). Despite possible adverse effects, cell transplantation "mono-therapies" offer the potential advantages of continuous secretion of multiple NTFs for the duration of the viability of the transplant. In the eye, BMSC/ADSC/DPSC survive for at least 3 to 5 weeks (Johnson et al., 2010; Levkovitch-Verbin et al., 2010; Haddad-Mashadrizeh et al., 2013; Mead et al., 2013) and *ivit* delivery of cell suspensions and transplantation of a retrievable permeable capsule loaded with stem cells (Zhang et al., 2011) are also viable options for patients with retinal degenerative disease (Sieving et al., 2006).

Ivit/subretinal stem cell implantation

The fate of transplanted stem cells in the eye remains undetermined and thus the incidence of immune rejection, differentiation into unpredicted phenotypes and unbridled migration within CNS neuropil, together with possible

oncogenesis, all remain poorly defined. Safeguards against these outcomes include encapsulation of the stem implant (Zhang et al., 2011) and genetic modification so that the cells carry inducible suicide genes, such as viral-derived thymidine kinase allowing selective destruction of the transplanted cells when treated with the toxic drug ganciclovir (Zhang et al., 2011). However, the potential risks of transplanting stem cells in the eye may have been exaggerated where cell movement is restrained and immune reactions muted. For example, after *ivit* injection, MSC cluster in the vitreous body (Johnson et al., 2010; Haddad-Mashadrizeh et al., 2013; Mead et al., 2013, 2013), although a small number do migrate into the retina they are neither tumorigenic nor exhibit uncontrolled growth (Johnson et al., 2010; Mendel et al., 2013; Tzameret et al., 2014). In laser-induced glaucoma and retinal injury, *ivit* BMSCs also migrate into the retina (Singh et al., 2012) where they continue to proliferate (Wang et al., 2010). After subretinal transplantation, NSCs remain immature for at least 7 months, barely proliferate and neither exhibit uncontrolled growth nor oncogenesis, but they do migrate from the injection site within the subretinal space (McGill et al., 2012; Lu et al., 2013). By contrast, after *ivit* transplantation, NSCs either attach to the retina and lens where they remain (Jung et al., 2013), or integrate into the inner retinal layers (Grozdanic et al., 2006). ESC-derived RPE cells transplanted into the subretinal space of Royal College of Surgeon (RCS) rats (which spontaneously undergo RPE and subsequent photoreceptor degeneration) survive for over 200 days, preserve visual function with evidence of neither teratoma formation (Lu et al., 2009) nor proliferation (Vugler et al., 2008). Reactive retinal gliosis rather than penetration of the internal limiting membrane is proposed as a major limitation to retinal integration of ESC after *ivit* implantation (Banin et al., 2006); whilst after subretinal grafting cell migration is more extensive (Banin et al., 2006; Lamba et al., 2009) yet still hindered by the outer limiting membrane (West et al., 2008).

Immunological acceptance of stem cells transplanted into the eye

The vitreous cavity, like the anterior chamber of the eye, is an immunoprivileged environment (Jiang and Streilein, 1991) and thus amenable to cell transplantation. MSC fail to trigger an immune response when challenged with allogeneic lymphocytes and MSC-derived factors inhibit the proliferation of immunological cells (Kode et al., 2009; Singer and Caplan, 2011). These immunosuppressive/immunomodulatory actions of BMSC have led to Phase I (Le Blanc et al., 2004), Phase II (Le Blanc et al., 2008) and Phase III (Martin et al., 2010) clinical trials for the treatment of steroid refractory graft-versus-host disease. ADSCs suppress the immune system with the same efficacy as BMSC in vitro (Puissant et al., 2005) and increase the survival rate of transplants in animal models of graft versus host disease (Yañez et al., 2006), whereas DPSC are as efficient as BMSC in the suppression of T cell proliferation in vitro (Pierdomenico et al., 2005). Thus, the failure of the host to launch immune reactions after *ivit*/subretinal implantation of MSC is probably explained by both the immune privileged status of these sites and the immunosuppressive properties of MSC. For example, immunosuppression is not required and adverse effects are not recorded after human BMSC (Johnson et al., 2010; Levkovitch-Verbin et al., 2010; Tzameret et al., 2014)/ADSC (Haddad-Mashadrizeh et al., 2013)/rodent DPSC (Mead et al., 2013) transplantation into the eye. Equally, although not immunosuppressive, iPSC derived from the somatic cells of the recipient carry the same histocompatibility antigens and do not require immunosuppression after transplantation. By contrast, ESCs/NSCs require immunosuppression when transplanted into the CNS in animals and, since autologous transplantation is not possible, immunosuppression is required in NSC-based treatment (Cummings et al., 2005; Lu et al., 2012; Schwartz et al., 2012; Lu et al., 2013). Indeed, NSC transplantation into the subretinal space requires daily immunosuppressive treatment with cyclosporine A and dexamethasone (McGill et al., 2012). When transplanted into the vitreous without immunosuppression, NSCs are detected in just 50% of transplanted eyes 32 days after grafting (Grozdanic et al., 2006) suggesting that the immunoprivileged environment of the vitreous does not sustain survival of NSC. ESC-derived RPE cells are one of the first ESC based therapies to be used in humans and early reports of subretinal transplantation as a treatment for AMD confirm their safety, although patients require immunosuppression throughout (Schwartz et al., 2012).

Therapeutic potential of stem cell replacement therapies

NSC

NSC transplantation is beneficial to recovery in a range of CNS injury models, including retinal degeneration (McGill et al., 2012), SCI (Lu et al., 2003; Abematsu et al., 2010; Lu et al., 2012), stroke (Jeong et al., 2003) and TBI (Riess et al., 2002), although in many cases, it is unclear if the improved functional recovery observed is attributable to replacement of lost cells and/or trophic support of surviving cells.

For example, when transplanted into injured CNS sites such as those of SCI and TBI, NSCs differentiate into neurons and glia (Riess et al., 2002; Jeong et al., 2003; Cummings et al., 2005; Martino and Pluchino, 2006; Abematsu et al., 2010; Lu et al., 2012), replacing lost cells and providing trophic support for damaged endogenous neurons (Lu et al., 2003). NSC differentiation is greatly enhanced by containment in a matrix loaded with multiple growth factors (Lu et al., 2012) and treatment with specific differentiation factors (Cao et al., 2001). In these instances, functional recovery is attributed to the generation of new NSC-derived neurons that directly integrate into the host neuronal circuitry (Martino and Pluchino, 2006; Abematsu et al., 2010) and not to the paracrine mediated axon regeneration and neuroprotection characteristic of MSC treatment.

Despite recent success with NSC in other CNS injury models, few studies have shown the same effect in the eye. In rats, *ivit* transplantation of NSC after optic neuropathy induced by elevated intraocular pressure does not improve retinal function, despite neuronal differentiation and integration into inner retinal layers (Grozdanic et al., 2006). A similar study using mice lacking RGC (induced by removal of the superior colliculus) showed that NSCs integrate into the

retina but sparsely form βIII-tubulin$^+$ mature neurons and do not form functional RGC (Mellough et al., 2004). Re-innervation of central targets by the axons of replacement RGC is not yet possible and there is no evidence to suggest that regeneration of stem cell-derived RGC axons along the optic nerve occurs. Indeed, more success has been seen in RCS rats in which the retinal degeneration is of the photoreceptors rather than the RGC (McGill et al., 2012). Subretinal transplantation of NSC protects photoreceptors from death in RCS rats (McGill et al., 2012) by their phagocytosis of photoreceptor outer segments, a role usually restricted to RPE cells which, in RCS rats, are dysfunctional (Cuenca et al., 2013). Although a paracrine effect (i.e. secretion of NTF) has been suggested to mediate the effects of NSC in the retina, studies have only demonstrated this when NSCs are genetically modified (e.g. to secrete CNTF (Jung et al., 2013)). The limited number of studies published on NSC in the eye suggests that this stem cell is currently not useful for replacement of RGC, however, functional replacement of photoreceptors by NSC is more plausible because of their short synaptic distances. Despite this, integration of NSC into the outer nuclear layer (ONL) is not followed by differentiation into calbindin$^+$/rhodopsin$^+$ mature photoreceptors (Nishida et al., 2000) and subretinal transplanted NSCs protect, rather than replace photoreceptors (McGill et al., 2012; Jung et al., 2013).

BMSC

In vitro neuronal differentiation and neuritogenesis of BMSC are probably artefacts resulting from cell shrinkage and toxicity yielding morphologies characteristic of neurons (Lu et al., 2004a; Neuhuber et al., 2004). Undifferentiated BMSCs co-express many functional ion channels (Li et al., 2006) as well as mature neuronal and glial markers, such as βIII-tubulin and GFAP, respectively (Karaoz et al., 2011; Tamaki et al., 2012) making successful phenotypic differentiation difficult to detect. The ability of BMSC to differentiate into neurons and replace those lost from injury is rarely reported in vivo (Vallières and Sawchenko, 2003). Their transplantation into the injury site after SCI promotes functional recovery without any evidence of neuronal replacement by BMSC differentiation (Kang et al., 2012).

In the eye, transplantation of BMSC into the vitreous after experimentally-induced glaucoma and optic nerve transection shows no evidence of their differentiation into mature retinal cells, despite some integration into the retina (Yu et al., 2006; Johnson et al., 2010; Levkovitch-Verbin et al., 2010). After transplantation into the subretinal space in RCS rats and mouse models of retinitis pigmentosa, BMSCs sparsely differentiate into cells with neuron and glia characteristics, but not mature photoreceptors or RPE cells (Zhang and Wang, 2010; Tzameret et al., 2014) and a protective effect on endogenous photoreceptors and RPE cells is observed (Arnhold et al., 2007; Lu et al., 2010).

ADSC

There is conflicting evidence for the differentiation of ADSC into neurons in vivo and in vitro (Anghileri et al., 2008; Ye et al., 2010). BDNF/retinoic acid treatments induce the differentiation of ADSC into functional neurons, confirmed by patch clamp analysis and the expression of phenotypic neuronal markers (Anghileri et al., 2008). The ADSC-derived neuronal phenotypes demonstrated in this and other studies (Ye et al., 2010) is only transient with de-differentiation occurring after withdrawal of the differentiation-inducing medium (Ye et al., 2010), explaining why ADSC-derived neurons are rarely seen in vivo after transplantation in animal models of stroke (Kang et al., 2003). Both studies (Kang et al., 2003; Ye et al., 2010) concluded that cerebrospinal fluid and CNS neuropil do not sustain neuronal differentiation of ADSC. By contrast, ADSCs pre-differentiated into NG2$^+$/S100$^+$ glia survive for up to 8 weeks after transplantation into rodent SCI sites (Arboleda et al., 2011) and thus, like other MSC, probably differentiate preferentially into glia in vivo (Cao et al., 2001; Cho et al., 2009; Leong et al., 2012).

ADSCs survive for up to 90 days in the vitreous cavity after transplantation although their fate has not been studied (Haddad-Mashadrizeh et al., 2013). Interestingly, ADSC transplanted into the vitreous cavity of mouse models of diabetic retinopathy preferentially differentiate into pericytes, associating with and conserving the retinal vasculature, suggesting a unique role for ADSC in treating diabetic retinopathy (Mendel et al., 2013). The failure of ADSC to integrate into the retinal layers diminishes their potential for RGC and photoreceptor replacement (Mendel et al., 2013).

DPSC

DPSC differentiate into functionally active neurons in vitro (Arthur et al., 2008; Kiraly et al., 2009) and, when transplanted, integrate and survive in injured rat brain tissue for at least 4 weeks (Kiraly et al., 2011; Fang et al., 2013). Other studies demonstrate that, although DPSC-derived neurons express neuronal phenotypic markers, they neither generate action potentials nor form functional neuronal networks (Aanismaa et al., 2012). Like BMSC, they constitutively express mature neuronal and glial phenotypic markers even in an undifferentiated state and this may explain the contradictions in the literature if these characteristics are taken as a read out of successful differentiation (Karaoz et al., 2011; Tamaki et al., 2012). In vivo, transplantation of DPSC into rat SCI lesion sites leads to functional recovery yet only glial, not neuronal, differentiation is observed (Sakai et al., 2012), suggesting that differentiation of DPSC into neurons is possible in vitro but currently has not yet been realised in vivo. After transplantation into the vitreous, DPSC do not differentiate into neurons and fail to integrate into the retina (Mead et al., 2013), limiting their potential as a cell replacement therapy.

ESC/iPSC

The greatest potential for cell replacement has been seen with ESC/iPSC, which can be successfully predifferentiated prior to transplantation in the eye, with the most success demonstrated in RPE/photoreceptor replacement for AMD (Fig. 2).

ESC can be directed towards a retinal phenotype with developmental induction signals including bone morphogenetic protein (BMP) antagonists (Lamb et al., 1993), Wnt inhibition (Wilson and Houart, 2004) and insulin-like growth

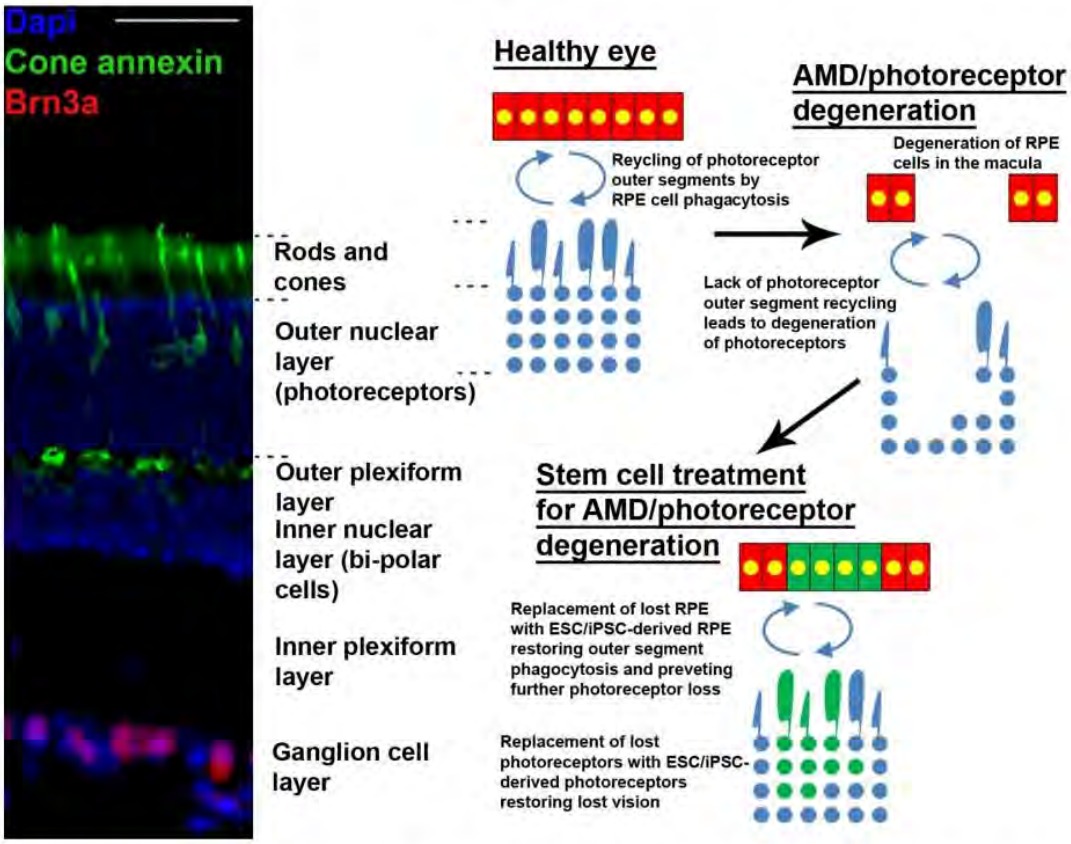


Figure 2 A diagram showing the proposed way in which ESC/iPSC-derived RPE and photoreceptors can be used to treat AMD/photoreceptor degeneration. The left panel shows a rat retina immunohistochemically stained for cone annexin (cone photoreceptor marker; green), Brn3a (RGC marker; red) and DAPI (nuclear marker; blue) with the individual layers labelled (scale bar: 100 μm). On the right, RPE is represented together with photoreceptor loss in AMD and the potential for cell replacement in preventing visual decline and restoring vision.

factor (IGF) treatment (Pera et al., 2001). Accordingly, 30% of ESC/iPSC differentiate into retinal progenitors (Ikeda et al., 2005), a number that increases to 80% for both ESC (Lamba et al., 2006) and iPSC (Tucker et al., 2011) by incorporating BMP/Wnt inhibition with IGF and FGF treatments. These ESC/iPSC-derived retinal progenitors successfully mature into photoreceptors as well as RPE cells and integrate into retinal explants after co-culture with adult retina/retinal neurons (Osakada et al., 2008) or after the addition of a cocktail of small molecules (Osakada et al., 2009b). Comparisons of the gene expression profiles of ESC-derived retinal cells with primary developing foetal retinal cells using microarray analysis show them to be highly conserved between the two cell sources throughout development (Lamba and Reh, 2011).

ESC-derived retinal progenitors, primed to form neuronal retina rather than RPE using FGF, successfully differentiate into photoreceptors (Hambright et al., 2012), integrate into the ONL (Lamba et al., 2009) and survive for over 3 months in the subretinal space of non-immunosuppressed mice with an intact blood–retinal barrier, with integration more significant when the retina is injured (Hambright et al., 2012). iPSC-derived photoreceptors transplanted into the subretinal space integrate into the ONL and increase retinal function as determined by electroretinogram (ERG) (Tucker et al., 2011). Transplantation of ESC-derived photoreceptors into the vitreous of newborn mice leads to their correct topographic integration into all the layers of the retina, i.e. ESC-derived photoreceptors move to the ONL, whereas ESC-derived amacrine cells and RGC-like cells migrate to the inner nuclear layer/ganglion cell layer (Lamba et al., 2009; Reynolds and Lamba, 2013). However, integration is only possible up to 48 h after birth, corroborating reports that in adult rats, *ivit* ESC-derived cells fail to integrate into the retina (Banin et al., 2006).

Both mouse (Eiraku et al., 2011) and human (Nakano et al., 2012) ESC can be induced to form a complete topographically organized retina, including the RPE. Developing photoreceptors, isolated from ESC-derived ex vivo retina, integrate after transplantation into mouse models of retinal degeneration (Gonzalez-Cordero et al., 2013). These findings have been replicated using iPSC showing the formation of a synaptically connected stratified retina (Phillips et al., 2012).

ESC/iPSC can be induced to predominantly differentiate into RPE cells using similar protocols as above, but with the omission/antagonism of FGF to bias the generation of RPE cells over neural retina (Meyer et al., 2009; Osakada et al., 2009a). These ESC/iPSC-derived RPE cells phagocytise photoreceptor outer segments (Carr et al., 2009a) and preserve retinal function in the RCS rats (Vugler et al., 2008; Carr et al.,

Table 2 NTF known to be secreted by NSC, BMSC, ADSC and DPSC. NTF secretion by ESC/iPSC is currently unreported.

Stem cells	Neurotrophic factor secretion profile
NSC	NGF, BDNF, NT-3, GDNF (Lu et al., 2003); (Gu et al., 2012); (He et al., 2012)
BMSC	NGF, BDNF, NT-3, NT-4/5, CNTF, GDNF, PDGF (Dormady et al., 2001); (Chen et al., 2005); (Wilkins et al., 2009); (Ghorbanian et al., 2012); (Sakai et al., 2012); (Johnson et al., 2013); (Mead et al., 2013, 2014)
ADSC	NGF, BDNF, NT-3, GDNF, VEGF, Progranulin, SPARC (Kalbermatten et al., 2011); (Sugitani et al., 2013); (Zhou et al., 2013); (Tsuruma et al., 2014); (Mead et al., 2014)
DPSC	NGF, BDNF, NT-3, CNTF, GDNF, VEGF, FGF-2 (Nosrat et al., 1997, 2001); (Huang et al., 2008); (Gale et al., 2011); (Sakai et al., 2012); (Mead et al., 2013, 2014)

2009b). A study comparing adult human ESC-derived RPE with foetal human RPE demonstrated a strong correlation in their gene expression profiles. However, iPSC-derived RPE have a distinct gene expression profile, indicating potential differences between ESC-derived retinal cells and iPSC-derived retinal cells (Liao et al., 2010).

Subretinal transplantation of ESC/iPSC-derived RPE in cases of AMD requires approximately 60,000 cells (Bharti et al., 2011) to restore RPE-mediated recycling of photoreceptor outer segments. In contrast to photoreceptor replacement, in this instance significant migration, integration and synaptogenesis is not required to achieve functional efficacy. Its effectiveness is already proven by the fact that current surgical intervention relies on the same principles i.e. translocating the macula to an adjacent, healthy portion of RPE (da Cruz et al., 2007). These attributes have led to the first clinical trial transplanting ESC-derived RPE cells in patients with AMD (Schwartz et al., 2012).

ESCs/iPSCs are able to differentiate into RGC and, during the formation of ESC-/iPSC-derived retina ex vivo, RGCs are the first cells to develop which mimic normal retinal development (Eiraku et al., 2011; Nakano et al., 2012; Phillips et al., 2012). The yield of RGC is enhanced by transfection of the stem cells with genes regulating RGC development, namely *math5* and *sox4* (Jiang et al., 2013). Similar to ESC/iPSC-derived photoreceptors integrating into the ONL, transplanted adult rat RGCs integrate and survive in the ganglion cell layer (Hertz et al., 2014) but, unlike photoreceptors, the long distances over which RGC axons must regenerate to re-innervate central targets is unachievable (Sun et al., 2011).

Therapeutic potential of stem cell trophic support (Fig. 1; Table 2)

NSC

When transplanted into SCI lesion sites, NSCs increase the expression of NGF, BDNF, NT-3 and GDNF within the lesion site (Gu et al., 2012; He et al., 2012) and promote axonal sprouting (Lu et al., 2003). However, the trophic support provided by undifferentiated NSC only minimally restores function compared to when they are induced to differentiate down a neuronal lineage before or after transplantation into SCI sites (Cao et al., 2001; Abematsu et al., 2010; Gu et al., 2012; He et al., 2012). In the eye, as stated above, *ivit* NSCs transplanted into the vitreous fail to improve function in models of elevated intraocular pressure-induced RGC loss (Grozdanic et al., 2006) and axotomy (Flachsbarth et al., 2014) and only show neuroprotective efficacy when transfected to secrete CNTF. However, transplantation was made four weeks post-injury, so that it cannot be ruled out that NSC may be able to have a paracrine-mediated neuroprotective effect on RGC if they were transplanted at the time of injury when injured RGCs are most amenable to neuroprotective strategies. Nonetheless, after subretinal transplantation of NSC into RCS rats, rather than replaced, photoreceptors are protected against death by NSC-directed phagocytosis of photoreceptor outer segments (Cuenca et al., 2013) and induction of CNTF expression by Müller glia (Lu et al., 2013).

BMSC

The neurotrophic secretome of BMSC, which includes NGF, BDNF, NT-3, NT4/5, CNTF, GDNF and PDGF is widely documented (Dormady et al., 2001; Chen et al., 2005; Wilkins et al., 2009; Ghorbanian et al., 2012; Sakai et al., 2012; Johnson et al., 2013; Mead et al., 2013; Mead et al., 2014) and places them as a candidate cellular therapy to combat ocular neurodegeneration. BMSC-mediated neuroprotection of RGC is reported to be mediated by PDGF (Johnson et al., 2013), whilst other studies have shown that BMSC-induced RGC neuroprotection and axon/neurite growth is mediated by NGF, BDNF and NT-3 (Mead et al., 2013). The importance of BMSC-derived NTF for retinal neuron survival is confirmed by using TrK and PDGFR inhibitors which significantly diminish the RGC neuroprotection and/or neurite growth effects elicited by BMSC (Johnson et al., 2013; Mead et al., 2013; Mead et al., 2014). The vitreous does not permit the differentiation of BMSC into neurons (Hill et al., 2009). Nonetheless, *ivit* transplanted BMSCs secrete diffusible NTF, BDNF and NT-3 (Mead et al., 2013), directly protecting RGC from death in animal models of glaucoma (Yu et al., 2006; Johnson et al., 2010) and optic nerve transection (Levkovitch-Verbin et al., 2010; Mead et al., 2013), and can also be indirectly effective by inducing Müller cell NTF production (Lee et al., 2012). Interestingly, BMSC also promote the regeneration of RGC axons after optic nerve crush (Mead et al., 2013), probably through the same NTF-mediated mechanisms (Berry et al., 2008). Subretinal and *ivit* BMSC transplantation in RCS rats and mouse models of retinitis pigmentosa significantly improves retinal function by preserving photoreceptor and RPE cell viability (Arnhold et al., 2007; Lu et al., 2010; Tzameret et al., 2014) and, although the underlying observations remain equivocal, a role for the NTF secretome in promoting cell survival is a likely explanation.

ADSC

ADSCs express NGF, BDNF, NT-3, GDNF, VEGF and PDGF (Kalbermatten et al., 2011; Zhou et al., 2013; Mead et al., 2014), with titres of BDNF and vascular endothelial growth factor (VEGF) being significantly higher than those secreted by BMSC (Zhou et al., 2013). Despite this, ADSCs are relatively untested in the eye but have efficacy as a paracrine-mediated therapy in other CNS animal injury models like SCI (Arboleda et al., 2011; Zhou et al., 2013) and stroke (Kang et al., 2003). In co-culture, ADSC-derived NTF promote neuroprotection and neuritogenesis of injured RGC, although the effects are not as pronounced as those achieved with BMSC/DPSC (Mead et al., 2014). In a mouse model of light induced photoreceptor damage, both *ivit* ADSC and ADSC-conditioned medium preserve ONL thickness and the amplitude of the a-wave of the ERG (Sugitani et al., 2013; Tsuruma et al., 2014). Progranulin, tissue inhibitor of metalloproteinases-1 (TIMP1) and the secreted protein rich in cysteine (SPARC) are the active agents produced by ADSC in vitro and, after *ivit* transplantation, have similar effects to *ivit* ADSC/ADSC conditioned medium. Together, these data suggest that ADSC have therapeutic potential for neurodegenerative conditions through NTF production, with many of the active factors different from those produced by BMSC and DPSC.

DPSC

Like other MSCs, DPSCs have an extensive neurotrophic secretome which includes NGF, BDNF, NT-3, GDNF, VEGF and PDGF (Nosrat et al., 1997, 2001; Gale et al., 2011; Sakai et al., 2012; Mead et al., 2013, 2014). Interestingly, DPSCs express significantly greater amounts of *ngf*, *bdnf* and *nt-3* mRNA than BMSC (Sakai et al., 2012) and this is true also for the secreted proteins NGF, BDNF and NT-3 (Mead et al., 2013). DPSC-conditioned medium containing the above factors promotes neurite outgrowth of cortical neurons (Sakai et al., 2012), a neuroblastoma cell line (Ishizaka et al., 2013) and primary RGC (Mead et al., 2013, 2014) with significantly greater efficacy than BMSC and ADSC-conditioned medium. DPSCs transplanted into mouse hippocampus increase the basal expression levels of many NTF such as CNTF, VEGF, FGF-2 and NGF (Huang et al., 2008), although it is unknown if the transplanted DPSCs directly express these NTFs and/or indirectly promote the expression of NTFs by neighbouring cells in the surrounding neuropil. DPSC transplantation into rat SCI lesion sites leads to greater functional improvement than BMSC transplantation and, with a lack of observable neuronal differentiation, the evidence strongly suggests a paracrine-mediated mechanism (Sakai et al., 2012). Following either *ivit* transplantation or co-culture with injured RGC, DPSCs secrete NGF, BDNF and NT-3 and promote RGC survival and axon/neurite regeneration; effects which are attenuated by Fc-TrK blockers (Mead et al., 2013, 2014). These neuroprotective/pro-regenerative effects are significantly greater in DPSC transplanted animals compared to BMSC transplanted animals and are correlated with a more favourable neurotrophic secretome by DPSC compared to BMSC (Mead et al., 2013, 2014). Currently, no evidence exists for DPSC-mediated protection of photoreceptors whilst further research into the mechanisms of DPSC-mediated RGC neuroprotection is required.

ESC/iPSC

Unlike MSC, the paracrine potential of ESC/iPSC for treating the injured retina/CNS is as yet unknown. Addition of TrK receptor blockers to ESC cultures perturbs their survival, indicating that neurotrophins are released and active in an autocrine fashion, but further analysis on the secretome is required (Pyle et al., 2006). *ivit* transplantation of ESC-derived photoreceptors promotes the survival of nearby endogenous photoreceptors (Meyer et al., 2006). Similarly it is known that RPE cells secrete VEGF and PEDF, which may further explain how ESC-derived RPE cells protect photoreceptors from death (Strauss, 2005).

Conclusions

The use of stem cells has proven potential as a cellular therapy for retinal degenerative conditions through replacement of lost cells in the eye and/or the release of growth factors into damaged neuropil. However, the mechanism of action as well as the efficacy of the cellular therapy vary between different stem cells and can contrast greatly with what is seen in other models of CNS injury. ESCs/iPSCs have shown potential as a source of retinal cells for replacement of particularly photoreceptors and RPE, but their possible paracrine action is currently not known. Although the potential trophic properties are still not fully understood, NSCs have proven impressive cell replacement properties in other CNS regions and these faculties may be enhanced, optimised and refined by pre-treatment with selected growth/inducible factors leading to their formulation as an effective cell replacement therapy in the retina. By contrast the dominant mechanism by which MSCs restore lost retinal function appears to be paracrine-mediated, which offers the potential for their use to provide continuous delivery of multiple growth factors to provide direct trophic support for neurons in the degenerate retina and to stimulate glia to indirectly help effect neural repair. The non-invasive, non-tumorigenic, immunosuppressive and trophic characteristics of MSC, along with the relatively ease of access from their diverse adult tissue sources, circumvent moral and ethical dilemmas and make the autologous and allogeneic intra-ocular implantation of MSC a promising paracrine-mediated therapy for the diseased eye.

Author contributions

Ben Mead: conception and design; collection and/or assembly of data; data analysis and interpretation; manuscript writing.

Martin Berry: manuscript writing; final approval of manuscript.

Ann Logan: conception and design; data analysis and interpretation; manuscript writing; final approval of manuscript.

Robert Scott: final approval of manuscript.

Wendy Leadbeater: conception and design; data analysis and interpretation; manuscript writing; final approval of manuscript.

Ben A. Scheven: conception and design; data analysis and interpretation; manuscript writing; final approval of manuscript.

References

Aanismaa, R., Hautala, J., Vuorinen, A., Miettinen, S., Narkilahti, S., 2012. Human dental pulp stem cells differentiate into neural precursors but not into mature functional neurons. Stem Cell Discov. 2, 85–91.

Abematsu, M., Tsujimura, K., Yamano, M., Saito, M., Kohno, K., Kohyama, J., Namihira, M., Komiya, S., Nakashima, K., 2010. Neurons derived from transplanted neural stem cells restore disrupted neuronal circuitry in a mouse model of spinal cord injury. J. Clin. Invest. 120, 3255–3266.

Ahmad, I., Tang, L., Pham, H., 2000. Identification of neural progenitors in the adult mammalian eye. Biochem. Biophys. Res. Commun. 270, 517–521.

Ahmed, Z., Kalinski, H., Berry, M., Almasieh, M., Ashush, H., Slager, N., Brafman, A., Spivak, I., Prasad, N., Mett, I., Shalom, E., Alpert, E., Di Polo, A., Feinstein, E., Logan, A., 2011. Ocular neuroprotection by siRNA targeting caspase-2. Cell Death Dis. 2, e173.

Anghileri, E., Marconi, S., Pignatelli, A., Cifelli, P., Galie, M., Sbarbati, A., Krampera, M., Belluzzi, O., Bonetti, B., 2008. Neuronal differentiation potential of human adipose-derived mesenchymal stem cells. Stem Cells Dev. 17, 909–916.

Arboleda, D., Forostyak, S., Jendelova, P., Marekova, D., Amemori, T., Pivonkova, H., Masinova, K., Sykova, E., 2011. Transplantation of predifferentiated adipose-derived stromal cells for the treatment of spinal cord injury. Cell. Mol. Neurobiol. 31, 1113–1122.

Arnhold, S., Absenger, Y., Klein, H., Addicks, K., Schraermeyer, U., 2007. Transplantation of bone marrow-derived mesenchymal stem cells rescue photoreceptor cells in the dystrophic retina of the rhodopsin knockout mouse. Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle. Ophthalmologie 245, 414–422.

Arthur, A., Rychkov, G., Shi, S., Koblar, S.A., Gronthos, S., 2008. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 26, 1787–1795.

Ballios, B.G., Clarke, L., Coles, B.L.K., Shoichet, M.S., Van Der Kooy, D., 2012. The adult retinal stem cell is a rare cell in the ciliary epithelium whose progeny can differentiate into photoreceptors. Biol. Open 1 (3), 237–246.

Banin, E., Obolensky, A., Idelson, M., Hemo, I., Reinhardtz, E., Pikarsky, E., Ben-Hur, T., Reubinoff, B., 2006. Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells. Stem Cells 24, 246–257.

Barde, Y.-A., 1989. Trophic factors and neuronal survival. Neuron 2, 1525–1534.

Berkelaar, M., Clarke, D.B., Wang, Y.C., Bray, G.M., Aguayo, A.J., 1994. Axotomy results in delayed death and apoptosis of retinal ganglion cells in adult rats. J. Neurosci. 14, 4368–4374.

Berry, M., Ahmed, Z., Lorber, B., Douglas, M., Logan, A., 2008. Regeneration of axons in the visual system. Restor. Neurol. Neurosci. 26, 147–174.

Bharti, K., Miller, S.S., Arnheiter, H., 2011. The new paradigm: retinal pigment epithelium cells generated from embryonic or induced pluripotent stem cells. Pigment Cell Melanoma Res. 24, 21–34.

Bohm, M.R.R., Pfrommer, S., Chiwitt, C., Bruckner, M., Melkonyan, H., Thanos, S., 2012. Crystallin-beta-b2-overexpressing NPCs support the survival of injured retinal ganglion cells and photoreceptors in rats. Invest. Ophthalmol. Vis. Sci. 53, 8265–8279.

Butowt, R., von Bartheld, C.S., 2003. Connecting the dots: trafficking of neurotrophins, lectins and diverse pathogens by binding to the neurotrophin receptor p75(NTR). Eur. J. Neurosci. 17, 673–680.

Canola, K., Angénieux, B., Tekaya, M., Quiambao, A., Naash, M.I., Munier, F.L., Schorderet, D.F., Arsenijevic, Y., 2007. Retinal stem cells transplanted into models of late stages of retinitis pigmentosa preferentially adopt a glial or a retinal ganglion cell fate. Invest. Ophthalmol. Vis. Sci. 48, 446–454.

Cao, Q.L., Zhang, Y.P., Howard, R.M., Walters, W.M., Tsoulfas, P., Whittemore, S.R., 2001. Pluripotent stem cells engrafted into the normal or lesioned adult rat spinal cord are restricted to a glial lineage. Exp. Neurol. 167, 48–58.

Carr, A.J., Vugler, A., Lawrence, J., Chen, L.L., Ahmado, A., Chen, F.K., Semo, M., Gias, C., da Cruz, L., Moore, H.D., Walsh, J., Coffey, P.J., 2009a. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. Mol. Vis. 15, 283–295.

Carr, A.J., Vugler, A.A., Hikita, S.T., Lawrence, J.M., Gias, C., Chen, L.L., Buchholz, D.E., Ahmado, A., Semo, M., Smart, M.J., Hasan, S., da Cruz, L., Johnson, L.V., Clegg, D.O., Coffey, P.J., 2009b. Protective effects of human iPS-derived retinal pigment epithelium cell transplantation in the retinal dystrophic rat. Plos One 4, e8152.

Chacko, D.M., Das, A.V., Zhao, X., James, J., Bhattacharya, S., Ahmad, I., 2003. Transplantation of ocular stem cells: the role of injury in incorporation and differentiation of grafted cells in the retina. Vis. Res. 43, 937–946.

Chen, H., Weber, A.J., 2004. Brain-derived neurotrophic factor reduces TrkB protein and mRNA in the normal retina and following optic nerve crush in adult rats. Brain Res. 1011, 99–106.

Chen, Q., Long, Y., Yuan, X., Zou, L., Sun, J., Chen, S., Perez-Polo, J.R., Yang, K., 2005. Protective effects of bone marrow stromal cell transplantation in injured rodent brain: synthesis of neurotrophic factors. J. Neurosci. Res. 80, 611–619.

Chen, M., Xiang, Z., Cai, J., 2013. The anti-apoptotic and neuroprotective effects of human umbilical cord blood mesenchymal stem cells (hUCB-MSCs) on acute optic nerve injury is transient. Brain Res. 1532, 63–75.

Cho, S.-R., Kim, Y.R., Kang, H.-S., Yim, S.H., C-i, Park, Min, Y.H., Lee, B.H., Shin, J.C., Lim, J.-B., 2009. Functional recovery after the transplantation of neurally differentiated mesenchymal stem cells derived from bone barrow in a rat model of spinal cord injury. Cell Transplant. 18, 1359–1368.

Clarke, L., Ballios, B.G., van der Kooy, D., 2012. Generation and clonal isolation of retinal stem cells from human embryonic stem cells. Eur. J. Neurosci. 36, 1951–1959.

Cuenca, N., Fernandez-Sanchez, L., McGill, T.J., Lu, B., Wang, S.M., Lund, R., Huhn, S., Capela, A., 2013. Phagocytosis of photoreceptor outer segments by transplanted human neural stem cells as a neuroprotective mechanism in retinal degeneration. Invest. Ophthalmol. Vis. Sci. 54, 6745–6756.

Cummings, B.J., Uchida, N., Tamaki, S.J., Salazar, D.L., Hooshmand, M., Summers, R., Gage, F.H., Anderson, A.J., 2005. Human neural stem cells differentiate and promote locomotor recovery in spinal cord-injured mice. Proc. Natl. Acad. Sci. U. S. A. 102, 14069–14074.

da Cruz, L., Chen, F.K., Ahmado, A., Greenwood, J., Coffey, P., 2007. RPE transplantation and its role in retinal disease. Prog. Retin. Eye Res. 26, 598–635.

Dawbarn, D., Allen, S.J., 2003. Neurotrophins and neurodegeneration. Neuropathol. Appl. Neurobiol. 29, 211–230.

Del Debbio, C.B., Peng, X., Xiong, H., Ahmad, I., 2013. Adult ciliary epithelial stem cells generate functional neurons and differentiate into both early and late born retinal neurons under non-cell autonomous influences. BMC Neurosci. 14, 130.

Dormady, S.P., Bashayan, O., Dougherty, R., Zhang, X.M., Basch, R.S., 2001. Immortalized multipotential mesenchymal cells and the hematopoietic microenvironment. J. Hematother. Stem Cell Res. 10, 125–140.

Dougherty, K.D., Dreyfus, C.F., Black, I.B., 2000. Brain-derived neurotrophic factor in astrocytes, oligodendrocytes, and microglia/macrophages after spinal cord injury. Neurobiol. Dis. 7, 574–585.

Eiraku, M., Takata, N., Ishibashi, H., Kawada, M., Sakakura, E., Okuda, S., Sekiguchi, K., Adachi, T., Sasai, Y., 2011. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472, 51–56.

Fang, C.-z., Yang, Y.-j., Wang, Q.-h., Yao, Y., Zhang, X.-y., He, X.-h., 2013. Intraventricular injection of human dental pulp stem cells improves hypoxic–ischemic brain damage in neonatal rats. Plos One 8, e66748.

Faulkner, J.R., Herrmann, J.E., Woo, M.J., Tansey, K.E., Doan, N.B., Sofroniew, M.V., 2004. Reactive astrocytes protect tissue and preserve function after spinal cord injury. J. Neurosci. 24, 2143–2155.

Fischer, A.J., 2005. Neural regeneration in the chick retina. Prog. Retin. Eye Res. 24, 161–182.

Flachsbarth, K., Kruszewski, K., Jung, G., Jankwiak, W., Riecken, K., Wagenfeld, L., Richard, G., Fehse, B., Bartsch, U., 2014. Neural stem cell-based intraocular administration of ciliary neurotrophic factor attentuates the loss of axotomized ganglion cells in adult mice. Invest. Ophthalmol. Vis. Sci. 55, 7029–7039.

Gale, Z., Cooper, P.R., Scheven, B.A.A., 2011. Effects of glial cell line-derived neurotrophic factor on dental pulp cells. J. Dent. Res. 90, 1240–1245.

Ghorbanian, M.T., Haji Ghasem Kashani, M., Lashkarblouki, T., Hosseinpour, L., Mirzaiyan, L., 2012. Characterization of in vitro cultured bone marrow and adipose tissue-derived mesenchymal stem cells and their ability to express neurotrophic factors. Cell Biol. Int. 36 (12), 1239–1249.

Gonzalez-Cordero, A., West, E.L., Pearson, R.A., Duran, Y., Carvalho, L.S., Chu, C.J., Naeem, A., Blackford, S.J.I., Georgiadis, A., Lakowski, J., Hubank, M., Smith, A.J., Bainbridge, J.W.B., Sowden, J.C., Ali, R.R., 2013. Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures integrate and mature within adult degenerate retina. Nat. Biotechnol. 31, 741–747.

Grozdanic, S.D., Ast, A.M., Lazic, T., Kwon, Y.H., Kardon, R.H., Sonea, I.M., Sakaguchi, D.S., 2006. Morphological integration and functional assessment of transplanted neural progenitor cells in healthy and acute ischemic rat eyes. Exp. Eye Res. 82, 597–607.

Gu, Y.L., Yin, L.W., Zhang, Z., Liu, J., Liu, S.J., Zhang, L.F., Wang, T.H., 2012. Neurotrophin expressions in neural stem cells grafted acutely to transected spinal cord of adult rats linked to functional improvement. Cell. Mol. Neurobiol. 32, 1089–1097.

Haddad-Mashadrizeh, A., Bahrami, A.R., Matin, M.M., Edalatmanesh, M.A., Zomorodipour, A., Gardaneh, M., Farshchian, M., Momeni-Moghaddam, M., 2013. Human adipose-derived mesenchymal stem cells can survive and integrate into the adult rat eye following xenotransplantation. Xenotransplantation 20, 165–176.

Hambright, D., Park, K.Y., Brooks, M., McKay, R., Swaroop, A., Nasonkin, I.O., 2012. Long-term survival and differentiation of retinal neurons derived from human embryonic stem cell lines in un-immunosuppressed mouse retina. Mol. Vis. 18, 920–936.

Harper, M.M., Grozdanic, S.D., Blits, B., Kuehn, M.H., Zamzow, D., Buss, J.E., Kardon, R.H., Sakaguchi, D.S., 2011. Transplantation of BDNF-secreting mesenchymal stem cells provides neuroprotection in chronically hypertensive rat eyes. Invest. Ophthalmol. Vis. Sci. 52, 4506–4515.

He, B.-L., Ba, Y.-c., Wang, X.-y., Liu, S.-j., Ou, S., Liu, G.-d., Gu, Y.-l., Pan, X.-h., Wang, T.-H., 2012. BDNF expression with functional improvement in transected spinal cord treated with neural stem cells in adult rats. Neuropeptides 47 (1), 1–7.

Hertz, J., Qu, B., Hu, Y., Patel, R.D., Valenzuela, D.A., Goldberg, J.L., 2014. Survival and integration of developing and progenitor-derived retinal ganglion cells following transplantation. Cell Transplant. 23, 855–872.

Hill, A.J., Zwart, I., Tam, H.H., Chan, J., Navarrete, C., Jen, L.S., Navarrete, R., 2009. Human umbilical cord blood-derived mesenchymal stem cells do not differentiate into neural cell types or integrate into the retina after intravitreal grafting in neonatal rats. Stem Cells Dev. 18, 399–409.

Huang, A.H.-C., Snyder, B.R., Cheng, P.-H., Chan, A.W.S., 2008. Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice. Stem Cells 26, 2654–2663.

Ikeda, H., Osakada, F., Watanabe, K., Mizuseki, K., Haraguchi, T., Miyoshi, H., Kamiya, D., Honda, Y., Sasai, N., Yoshimura, N., Takahashi, M., Sasai, Y., 2005. Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 102, 11331–11336.

Ishizaka, R., Hayashi, Y., Iohara, K., Sugiyama, M., Murakami, M., Yamamoto, T., Fukuta, O., Nakashima, M., 2013. Stimulation of angiogenesis, neurogenesis and regeneration by side population cells from dental pulp. Biomaterials 34, 1888–1897.

Jeong, S.W., Chu, K., Jung, K.H., Kim, S.U., Kim, M., Roh, J.K., 2003. Human neural stem cell transplantation promotes functional recovery in rats with experimental intracerebral hemorrhage. Stroke 34, 2258–2263.

Jiang, L.Q., Streilein, J.W., 1991. Immune privilege extended to allogeneic tumor cells in the vitreous cavity. Invest. Ophthalmol. Vis. Sci. 32, 224–228.

Jiang, Y., Ding, Q., Xie, X., Libby, R.T., Lefebvre, V., Gan, L., 2013. Transcription factors SOX4 and SOX11 function redundantly to regulate the development of mouse retinal ganglion cells. J. Biol. Chem. 288, 18429–18438.

Johnson, T.V., Bull, N.D., Hunt, D.P., Marina, N., Tomarev, S.I., Martin, K.R., 2010. Neuroprotective effects of intravitreal mesenchymal stem cell transplantation in experimental glaucoma. Invest. Ophthalmol. Vis. Sci. 51, 2051–2059.

Johnson, T.V., Dekorver, N.W., Levasseur, V.A., Osborne, A., Tassoni, A., Lorber, B., Heller, J.P., Villasmil, R., Bull, N.D., Martin, K.R., Tomarev, S.I., 2013. Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain 137, 503–519.

Jones, L.L., Oudega, M., Bunge, M.B., Tuszynski, M.H., 2001. Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. J. Physiol. 533, 83–89.

Jung, G.L., Sun, J., Petrowitz, B., Riecken, K., Kruszewski, K., Jankowiak, W., Kunst, F., Skevas, C., Richard, G., Fehse, B., Bartsch, U., 2013. Genetically modified neural stem cells for a local and sustained delivery of neuroprotective factors to the dystrophic mouse retina. Stem Cells Transl. Med. 2, 1001–1010.

Kalbermatten, D.F., Schaakxs, D., Kingham, P.J., Wiberg, M., 2011. Neurotrophic activity of human adipose stem cells isolated from deep and superficial layers of abdominal fat. Cell Tissue Res. 344, 251–260.

Kang, S.K., Lee, D.H., Bae, Y.C., Kim, H.K., Baik, S.Y., Jung, J.S., 2003. Improvement of neurological deficits by intracerebral transplantation of human adipose tissue-derived stromal cells after cerebral ischemia in rats. Exp. Neurol. 183, 355–366.

Kang, K.N., Kim da, Y., Yoon, S.M., Lee, J.Y., Lee, B.N., Kwon, J.S., Seo, H.W., Lee, I.W., Shin, H.C., Kim, Y.M., Kim, H.S., Kim, J.H., Min, B.H., Lee, H.B., Kim, M.S., 2012. Tissue engineered regeneration of completely transected spinal cord using human mesenchymal stem cells. Biomaterials 33, 4828–4835.

Karaoz, E., Demircan, P.C., Saglam, O., Aksoy, A., Kaymaz, F., Duruksu, G., 2011. Human dental pulp stem cells demonstrate better neural and epithelial stem cell properties than bone marrow-derived mesenchymal stem cells. Histochem. Cell Biol. 136, 455–473.

Kiraly, M., Porcsalmy, B., Pataki, A., Kadar, K., Jelitai, M., Molnar, B., Hermann, P., Gera, I., Grimm, W.D., Ganss, B., Zsembery, A., Varga, G., 2009. Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochem. Int. 55, 323–332.

Kiraly, M., Kadar, K., Horvathy, D.B., Nardai, P., Racz, G.Z., Lacza, Z., Varga, G., Gerber, G., 2011. Integration of neuronally

predifferentiated human dental pulp stem cells into rat brain in vivo. Neurochem. Int. 59, 371–381.

Ko, M.L., Hu, D.N., Ritch, R., Sharma, S.C., 2000. The combined effect of brain-derived neurotrophic factor and a free radical scavenger in experimental glaucoma. Invest. Ophthalmol. Vis. Sci. 41, 2967–2971.

Ko, M.L., Hu, D.N., Ritch, R., Sharma, S.C., Chen, C.F., 2001. Patterns of retinal ganglion cell survival after brain-derived neurotrophic factor administration in hypertensive eyes of rats. Neurosci. Lett. 305, 139–142.

Kode, J.A., Mukherjee, S., Joglekar, M.V., Hardikar, A.A., 2009. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. Cytotherapy 11, 377–391.

Lamb, T.M., Knecht, A.K., Smith, W.C., Stachel, S.E., Economides, A.N., Stahl, N., Yancopolous, G.D., Harland, R.M., 1993. Neural induction by the secreted polypeptide noggin. Science 262, 713–718.

Lamba, D.A., Reh, T.A., 2011. Microarray characterization of human embryonic stem cell–derived retinal cultures. Invest. Ophthalmol. Vis. Sci. 52, 4897–4906.

Lamba, D.A., Karl, M.O., Ware, C.B., Reh, T.A., 2006. Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc. Natl. Acad. Sci. U. S. A. 103, 12769–12774.

Lamba, D.A., Gust, J., Reh, T.A., 2009. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 4, 73–79.

Le Blanc, K., Rasmusson, I., Sundberg, B., Gotherstrom, C., Hassan, M., Uzunel, M., Ringden, O., 2004. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. Lancet 363, 1439–1441.

Le Blanc, K., Frassoni, F., Ball, L., Locatelli, F., Roelofs, H., Lewis, I., Lanino, E., Sundberg, B., Bernardo, M.E., Remberger, M., Dini, G., Egeler, R.M., Bacigalupo, A., Fibbe, W., Ringden, O., DCEGB, M., 2008. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371, 1579–1586.

Lee, J.Y., Shin, J.M., Yeum, C.E., Chae, G.T., Chun, M.H., Oh, S.J., 2012. Intravitreal delivery of mesenchymal stem cells loaded onto hydrogel affects the regulatory expression of endogenous NGF and BDNF in ischemic rat retina. Tissue Eng. Regen. Med. 9, 249–258.

Leong, W.K., Henshall, T.L., Arthur, A., Kremer, K.L., Lewis, M.D., Helps, S.C., Field, J., Hamilton-Bruce, M.A., Warming, S., Manavis, J., Vink, R., Gronthos, S., Koblar, S.A., 2012. Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms. Stem Cells Transl. Med. 1, 177–187.

Levkovitch-Verbin, H., Sadan, O., Vander, S., Rosner, M., Barhum, Y., Melamed, E., Offen, D., Melamed, S., 2010. Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. Invest. Ophthalmol. Vis. Sci. 51, 6394–6400.

Li, G.R., Deng, X.L., Sun, H., Chung, S.S., Tse, H.F., Lau, C.P., 2006. Ion channels in mesenchymal stem cells from rat bone marrow. Stem Cells 24, 1519–1528.

Liao, J.L., Yu, J., Huang, K., Hu, J., Diemer, T., Ma, Z., Dvash, T., Yang, X.J., Travis, G.H., Williams, D.S., Bok, D., Fan, G., 2010. Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. Hum. Mol. Genet. 19, 4229–4238.

Liu, B., Hunter, D.J., Rooker, S., Chan, A., Paulus, Y.M., Leucht, P., Nusse, Y., Nomoto, H., Helms, J.A., 2013. Wnt signaling promotes Muller cell proliferation and survival after injury. Invest. Ophthalmol. Vis. Sci. 54, 444–453.

Logan, A., Ahmed, Z., Baird, A., Gonzalez, A.M., Berry, M., 2006. Neurotrophic factor synergy is required for neuronal survival and disinhibited axon regeneration after CNS injury. Brain 129, 490–502.

Lu, P., Jones, L.L., Snyder, E.Y., Tuszynski, M.H., 2003. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. Exp. Neurol. 181, 115–129.

Lu, P., Blesch, A., Tuszynski, M.H., 2004a. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? J. Neurosci. Res. 77, 174–191.

Lu, P., Yang, H., Jones, L.L., Filbin, M.T., Tuszynski, M.H., 2004b. Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. J. Neurosci. 24, 6402–6409.

Lu, B., Malcuit, C., Wang, S., Girman, S., Francis, P., Lemieux, L., Lanza, R., Lund, R., 2009. Long-term safety and function of RPE from human embryonic stem cells in preclinical models of macular degeneration. Stem Cells 27, 2126–2135.

Lu, B., Wang, S., Girman, S., McGill, T., Ragaglia, V., Lund, R., 2010. Human adult bone marrow-derived somatic cells rescue vision in a rodent model of retinal degeneration. Exp. Eye Res. 91, 449–455.

Lu, P., Wang, Y., Graham, L., McHale, K., Gao, M., Wu, D., Brock, J., Blesch, A., Rosenzweig, E.S., Havton, L.A., Zheng, B., Conner, J.M., Marsala, M., Tuszynski, M.H., 2012. Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell 150, 1264–1273.

Lu, B., Morgans, C.W., Girman, S., Luo, J., Zhao, J., Du, H., Lim, S., Ding, S., Svendsen, C., Zhang, K., Wang, S., 2013. Neural stem cells derived by small molecules preserve vision. Transl. Vis. Sci. Technol. 2, 1.

Machalińska, A., Kawa, M., Pius-Sadowska, E., Stępniewski, J., Nowak, W., Rogińska, D., Kaczyńska, K., Baumert, B., Wiszniewska, B., Józkowicz, A., Dulak, J., Machaliński, B., 2013. Long-term neuroprotective effects of NT-4-engineered mesenchymal stem cells injected intravitreally in a mouse model of acute retinal injury. Invest. Ophthalmol. Vis. Sci. 54, 8292–8305.

Martin, P.J., Uberti, J.P., Soiffer, R.J., Klingemann, H., Waller, E.K., Daly, A., Herrmann, R.P., Visani, G., Bernardo, M.E., Schwerdtfeger, R., Kebriaei, P., 2010. Prochymal (R) improves response rates in patients with steroid-refractory acute graft-versus-host disease involving the liver and gut: results of a randomized, placebo-controlled, multicentre phase III trial in GvHD. Bone Marrow Transplant. 45, S17.

Martino, G., Pluchino, S., 2006. The therapeutic potential of neural stem cells. Nat. Rev. Neurosci. 7, 395–406.

McGill, T.J., Cottam, B., Lu, B., Wang, S., Girman, S., Tian, C., Huhn, S.L., Lund, R.D., Capela, A., 2012. Transplantation of human central nervous system stem cells – neuroprotection in retinal degeneration. Eur. J. Neurosci. 35, 468–477.

Mead, B., Logan, A., Berry, M., Leadbeater, W., Scheven, B.A., 2013. Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest. Ophthalmol. Vis. Sci. 54, 7544–7556.

Mead, B., Logan, A., Berry, M., Leadbeater, W., Scheven, B.A., 2014. Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells. Plos One 9, e109305.

Mellough, C.B., Cui, Q., Spalding, K.L., Symons, N.A., Pollett, M.A., Snyder, E.Y., Macklis, J.D., Harvey, A.R., 2004. Fate of multipotent neural precursor cells transplanted into mouse retina selectively depleted of retinal ganglion cells. Exp. Neurol. 186, 6–19.

Mendel, T.A., Clabough, E.B., Kao, D.S., Demidova-Rice, T.N., Durham, J.T., Zotter, B.C., Seaman, S.A., Cronk, S.M., Rakoczy, E.P., Katz, A.J., Herman, I.M., Peirce, S.M., Yates, P.A., 2013. Pericytes derived from adipose-derived stem cells protect against retinal vasculopathy. Plos One 8, e65691.

Mey, J., Thanos, S., 1993. Intravitreal injections of neurotrophic factors support the survival of axotomized retinal ganglion cells in adult rats in vivo. Brain Res. 602, 304–317.

Meyer, J.S., Katz, M.L., Maruniak, J.A., Kirk, M.D., 2006. Embryonic stem cell-derived neural progenitors incorporate into degenerating retina and enhance survival of host photoreceptors. Stem Cells 24, 274–283.

Meyer, J.S., Shearer, R.L., Capowski, E.E., Wright, L.S., Wallace, K.A., McMillan, E.L., Zhang, S.C., Gamm, D.M., 2009. Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc. Natl. Acad. Sci. U. S. A. 106, 16698–16703.

Morgan-Warren, P.J., Berry, M., Ahmed, Z., Scott, R.A., Logan, A., 2013. Exploiting mTOR signaling: a novel translatable treatment strategy for traumatic optic neuropathy? Invest. Ophthalmol. Vis. Sci. 54, 6903–6916.

Nakano, T., Ando, S., Takata, N., Kawada, M., Muguruma, K., Sekiguchi, K., Saito, K., Yonemura, S., Eiraku, M., Sasai, Y., 2012. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10, 771–785.

Neuhuber, B., Gallo, G., Howard, L., Kostura, L., Mackay, A., Fischer, I., 2004. Reevaluation of in vitro differentiation protocols for bone marrow stromal cells: disruption of actin cytoskeleton induces rapid morphological changes and mimics neuronal phenotype. J. Neurosci. Res. 77, 192–204.

Nishida, A., Takahashi, M., Tanihara, H., Nakano, I., Takahashi, J.B., Mizoguchi, A., Ide, C., Honda, Y., 2000. Incorporation and differentiation of hippocampus-derived neural stem cells transplanted in injured adult rat retina. Invest. Ophthalmol. Vis. Sci. 41, 4268–4274.

Nosrat, C.A., Fried, K., Lindskog, S., Olson, L., 1997. Cellular expression of neurotrophin mRNAs during tooth development. Cell Tissue Res. 290, 569–580.

Nosrat, I.V., Widenfalk, J., Olson, L., Nosrat, C.A., 2001. Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury. Dev. Biol. 238, 120–132.

Ooto, S., Akagi, T., Kageyama, R., Akita, J., Mandai, M., Honda, Y., Takahashi, M., 2004. Potential for neural regeneration after neurotoxic injury in the adult mammalian retina. Proc. Natl. Acad. Sci. U. S. A. 101, 13654–13659.

Osakada, F., Ooto, S., Akagi, T., Mandai, M., Akaike, A., Takahashi, M., 2007. Wnt signaling promotes regeneration in the retina of adult mammals. J. Neurosci. 27, 4210–4219.

Osakada, F., Ikeda, H., Mandai, M., Wataya, T., Watanabe, K., Yoshimura, N., Akaike, A., Sasai, Y., Takahashi, M., 2008. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat. Biotechnol. 26, 215–224.

Osakada, F., Ikeda, H., Sasai, Y., Takahashi, M., 2009a. Stepwise differentiation of pluripotent stem cells into retinal cells. Nat. Protoc. 4, 811–824.

Osakada, F., Jin, Z.B., Hirami, Y., Ikeda, H., Danjyo, T., Watanabe, K., Sasai, Y., Takahashi, M., 2009b. In vitro differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. J. Cell Sci. 122, 3169–3179.

Pakulska, M.M., Ballios, B.G., Shoichet, M.S., 2012. Injectable hydrogels for central nervous system therapy. Biomed. Mater. 7, 024101.

Park, K.K., Liu, K., Hu, Y., Smith, P.D., Wang, C., Cai, B., Xu, B., Connolly, L., Kramvis, I., Sahin, M., He, Z., 2008. Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. Science 322, 963–966.

Park, S.S., Bauer, G., Abedi, M., Pontow, S., Panorgias, A., Jonnal, R., Zawadzki, R.J., Werner, J.S., Nolta, J., 2015. Intravitreal autologous bone marrow CD34+ cell therapy for ischemic and degenerative retinal disorders: preliminary phase 1 clinical trial findings. Invest. Ophthalmol. Vis. Sci. 56, 81–89.

Pera, E.M., Wessely, O., Li, S.Y., De Robertis, E.M., 2001. Neural and head induction by insulin-like growth factor signals. Dev. Cell 1, 655–665.

Phillips, M.J., Wallace, K.A., Dickerson, S.J., Miller, M.J., Verhoeven, A.D., Martin, J.M., Wright, L.S., Shen, W., Capowski, E.E., Percin, E.F., Perez, E.T., Zhong, X., Canto-Soler, M.V., Gamm, D.M., 2012. Blood-derived human iPS cells generate optic vesicle-like structures with the capacity to form retinal laminae and develop synapses. Invest. Ophthalmol. Vis. Sci. 53, 2007–2019.

Pierdomenico, L., Bonsi, L., Calvitti, M., Rondelli, D., Arpinati, M., Chirumbolo, G., Becchetti, E., Marchionni, C., Alviano, F., Fossati, V., Staffolani, N., Franchina, M., Grossi, A., Bagnara, G.P., 2005. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 80, 836–842.

Puissant, N., Barreau, C., Bourin, P., Clavel, C., Corre, J., Bousquet, C., Taureau, C., Cousin, B., Abbal, M., Laharrague, P., Penicaud, L., Casteilla, L., Blancher, A., 2005. Immunomodulatory effect of human adipose tissue-derived adult stem cells: comparison with bone marrow mesenchymal stem cells. Brit. J. Haematol. 129, 118–129.

Pyle, A.D., Lock, L.F., Donovan, P.J., 2006. Neurotrophins mediate human embryonic stem cell survival. Nat. Biotechnol. 24, 344–350.

Reichenbach, A., Bringmann, A., 2013. New functions of Muller cells. Glia 61, 651–678.

Reynolds, J., Lamba, D.A., 2013. Human embryonic stem cell applications for retinal degenerations. Exp. Eye Res. 123, 151–160.

Richardson, P.M., McGuinness, U.M., Aguayo, A.J., 1980. Axons from CNS neurons regenerate into PNS grafts. Nature 284, 264–265.

Riess, P., Zhang, C., Saatman, K.E., Laurer, H.L., Longhi, L.G., Raghupathi, R., Lenzlinger, P.M., Lifshitz, J., Boockvar, J., Neugebauer, E., Snyder, E.Y., McIntosh, T.K., 2002. Transplanted neural stem cells survive, differentiate, and improve neurological motor function after experimental traumatic brain injury. Neurosurgery 51, 1043–1052 (Discussion 1052–1044).

Sakai, K., Yamamoto, A., Matsubara, K., Nakamura, S., Naruse, M., Yamagata, M., Sakamoto, K., Tauchi, R., Wakao, N., Imagama, S., Hibi, H., Kadomatsu, K., Ishiguro, N., Ueda, M., 2012. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. J. Clin. Investig. 122, 80–90.

Salero, E., Blenkinsop, T.A., Corneo, B., Harris, A., Rabin, D., Stern, J.H., Temple, S., 2012. Adult human RPE can be activated into a multipotent stem cell that produces mesenchymal derivatives. Cell Stem Cell 10, 88–95.

Sandvig, A., Berry, M., Barrett, L.B., Butt, A., Logan, A., 2004. Myelin-, reactive glia-, and scar-derived CNS axon growth inhibitors: expression, receptor signaling, and correlation with axon regeneration. Glia 46, 225–251.

Schwartz, S.D., Hubschman, J.P., Heilwell, G., Franco-Cardenas, V., Pan, C.K., Ostrick, R.M., Mickunas, E., Gay, R., Klimanskaya, I., Lanza, R., 2012. Embryonic stem cell trials for macular degeneration: a preliminary report. Lancet 379, 713–720.

Schwartz, S.D., Regillo, C.D., Lam, B.L., Eliott, D., Rosenfeld, P.J., Gregori, N.Z., Hubschman, J.P., Davis, J.L., Heilwell, G., Spirn, M., Maguire, J., Gay, R., Bateman, J., Ostrick, R.M., Morris, D., Vincent, M., Anglade, E., Del Priore, L.V., Lanza, R., 2014. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. Lancet http://dx.doi.org/10.1016/S0140-6736(08)61345-8.

Sieving, P.A., Caruso, R.C., Tao, W., Coleman, H.R., Thompson, D.J., Fullmer, K.R., Bush, R.A., 2006. Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. Proc. Natl. Acad. Sci. U. S. A. 103, 3896–3901.

Singer, N.G., Caplan, A.I., 2011. Mesenchymal stem cells: mechanisms of inflammation. Annu. Rev. Pathol. 6, 457–478.

Singh, T., Prabhakar, S., Gupta, A., Anand, A., 2012. Recruitment of stem cells into the injured retina after laser injury. Stem Cells Dev. 21, 448–454.

Siqueira, R.C., Messias, A., Voltarelli, J.C., Scott, I.U., Jorge, R., 2011. Intravitreal injection of autologous bone marrow-derived mononuclear cells for hereditary retinaldystrophy: a phase I trial. Retina 31, 1207–1214.

Sofroniew, M.V., Howe, C.L., Mobley, W.C., 2001. Nerve growth factor signaling, neuroprotection, and neural repair. Annu. Rev. Neurosci. 24, 1217–1281.

Sommerfeld, M.T., Schweigreiter, R., Barde, Y.A., Hoppe, E., 2000. Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. J. Biol. Chem. 275, 8982–8990.

Strauss, O., 2005. The retinal pigment epithelium in visual function. Physiol. Rev. 85, 845–881.

Sugitani, S., Tsuruma, K., Ohno, Y., Kuse, Y., Yamauchi, M., Egashira, Y., Yoshimura, S., Shimazawa, M., Iwama, T., Hara, H., 2013. The potential neuroprotective effect of human adipose stem cells conditioned medium against light-induced retinal damage. Exp. Eye Res. 116, 254–264.

Sun, F., Park, K.K., Belin, S., Wang, D., Lu, T., Chen, G., Zhang, K., Yeung, C., Feng, G., Yankner, B.A., He, Z., 2011. Sustained axon regeneration induced by co-deletion of PTEN and SOCS3. Nature 480, 372–375.

Tamaki, Y., Nakahara, T., Ishikawa, H., Sato, S., 2012. In vitro analysis of mesenchymal stem cells derived from human teeth and bone marrow. Odontology 101 (2), 121–132.

Tropepe, V., Coles, B.L., Chiasson, B.J., Horsford, D.J., Elia, A.J., McInnes, R.R., van der Kooy, D., 2000. Retinal stem cells in the adult mammalian eye. Science 287, 2032–2036.

Tsuruma, K., Yamauchi, M., Sugitani, S., Otsuka, T., Ohno, Y., Nagahara, Y., Ikegame, Y., Shimazawa, M., Yoshimura, S., Iwama, T., Hara, H., 2014. Progranulin, a major secreted protein of mouse adipose-derived stem cells, inhibits light-induced retinal degeneration. Stem Cells Transl. Med. 3, 42–53.

Tucker, B.A., Park, I.H., Qi, S.D., Klassen, H.J., Jiang, C., Yao, J., Redenti, S., Daley, G.Q., Young, M.J., 2011. Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. Plos One 6, e18992.

Tzameret, A., Sher, I., Belkin, M., Treves, A.J., Meir, A., Nagler, A., Levkovitch-Verbin, H., Barshack, I., Rosner, M., Rotenstreich, Y., 2014. Transplantation of human bone marrow mesenchymal stem cells as a thin subretinal layer ameliorates retinal degeneration in a rat model of retinal dystrophy. Exp. Eye Res. 118, 135–144.

Vallières, L., Sawchenko, P.E., 2003. Bone marrow-derived cells that populate the adult mouse brain preserve their hematopoietic identity. J. Neurosci. 23, 5197–5207.

Vugler, A., Carr, A.J., Lawrence, J., Chen, L.L., Burrell, K., Wright, A., Lundh, P., Semo, M., Ahmado, A., Gias, C., da Cruz, L., Moore, H., Andrews, P., Walsh, J., Coffey, P., 2008. Elucidating the phenomenon of HESC-derived RPE: anatomy of cell genesis, expansion and retinal transplantation. Exp. Neurol. 214, 347–361.

Wang, H.C., Brown, J., Alayon, H., Stuck, B.E., 2010. Transplantation of quantum dot-labelled bone marrow-derived stem cells into the vitreous of mice with laser-induced retinal injury: survival, integration and differentiation. Vis. Res. 50, 665–673.

West, E.L., Pearson, R.A., Tschernutter, M., Sowden, J.C., MacLaren, R.E., Ali, R.R., 2008. Pharmacological disruption of the outer limiting membrane leads to increased retinal integration of transplanted photoreceptor precursors. Exp. Eye Res. 86, 601–611.

Wilkins, A., Kemp, K., Ginty, M., Hares, K., Mallam, E., Scolding, N., 2009. Human bone marrow-derived mesenchymal stem cells secrete brain-derived neurotrophic factor which promotes neuronal survival in vitro. Stem Cell Res. 3, 63–70.

Wilson, S.W., Houart, C., 2004. Early steps in the development of the forebrain. Dev. Cell 6, 167–181.

Wright, L.S., Phillips, M.J., Pinilla, I., Hei, D., Gamm, D.M., 2014. Induced pluripotent stem cells as custom therapeutics for retinal repair: progress and rationale. Exp. Eye Res. 123, 161–172.

Xu, H., Sta Iglesia, D.D., Kielczewski, J.L., Valenta, D.F., Pease, M.E., Zack, D.J., Quigley, H.A., 2007. Characteristics of progenitor cells derived from adult ciliary body in mouse, rat, and human eyes. Invest. Ophthalmol. Vis. Sci. 48, 1674–1682.

Yañez, R., Lamana, M.L., García-Castro, J., Colmenero, I., Ramírez, M., Bueren, J.A., 2006. Adipose tissue-derived mesenchymal stem cells have in vivo immunosuppressive properties applicable for the control of the graft-versus-host disease. Stem Cells 24, 2582–2591.

Ye, C.Q., Yuan, X.D., Liu, H., Cai, Y.A., Ou, Y., 2010. Ultrastructure of neuronal-like cells differentiated from adult adipose-derived stromal cells. Neural. Regen. Res. 5, 1456–1463.

Yu, S., Tanabe, T., Dezawa, M., Ishikawa, H., Yoshimura, N., 2006. Effects of bone marrow stromal cell injection in an experimental glaucoma model. Biochem. Biophys. Res. Commun. 344, 1071–1079.

Yu, H., Vu, T.H., Cho, K.S., Guo, C., Chen, D.F., 2014. Mobilizing endogenous stem cells for retinal repair. Transl. Res. 163, 387–398.

Zhang, Y., Wang, W., 2010. Effects of bone marrow mesenchymal stem cell transplantation on light-damaged retina. Invest. Ophthalmol. Vis. Sci. 51, 3742–3748.

Zhang, R., Zhang, H., Xu, L., Ma, K., Wallrapp, C., Jonas, J.B., 2011. Neuroprotective effect of intravitreal cell-based glucagon-like peptide-1 production in the optic nerve crush model. Acta Ophthalmol. 89, e320–e326.

Zhou, Z., Chen, Y., Zhang, H., Min, S., Yu, B., He, B., Jin, A., 2013. Comparison of mesenchymal stem cells from human bone marrow and adipose tissue for the treatment of spinal cord injury. Cytotherapy 15 (4), 434–448.

Zwart, I., Hill, A.J., Al-Allaf, F., Shah, M., Girdlestone, J., Sanusi, A.B., Mehmet, H., Navarrete, R., Navarrete, C., Jen, L.S., 2009. Umbilical cord blood mesenchymal stromal cells are neuroprotective and promote regeneration in a rat optic tract model. Exp. Neurol. 216, 439–448.

APPENDIX 4

● PERSPECTIVE

Mesenchymal stem cell therapy for retinal ganglion cell neuroprotection and axon regeneration

Retinal ganglion cells (RGCs) are responsible for propagating signals derived from visual stimuli in the eye to the brain, along their axons within the optic nerve to the superior colliculus, lateral geniculate nucleus and visual cortex of the brain. Damage to the optic nerve either through trauma, such as head injury, or degenerative disease, such as glaucoma causes irreversible loss of function through degeneration of non-regenerating RGC axons and death of irreplaceable RGCs, ultimately leading to blindness (Berry et al., 2008). The degeneration of RGCs and their axons is due to the loss of the necessary source of retrogradely transported neurotrophic factors (NTFs) being hindered by axonal injury. NTFs are survival factors for neurons and play a pivotal part in axon regeneration. Stem cells particularly mesenchymal stem cells (MSCs) have been shown to possess a natural intrinsic capacity for paracrine support, releasing multiple signalling molecules including NTFs. By transplanting MSCs into the vitreous, they are positioned adjacent to the injured retina to provide paracrine-mediated therapy for the retinal neuronal cells (Johnson et al., 2010a; Mead et al., 2013). Additionally, MSCs may be pre-differentiated into supportive glial-like cells, such as Schwann cells, which could further increase their potential for paracrine support of injured neurons (Martens et al., 2013). Thus, MSCs have received considerable attention as a new cellular therapy for both traumatic and degenerative eye disease, acting as an alternative source of NTFs, protecting injured RGCs and promoting regeneration of their axons (**Figure 1**).

Bone marrow mesenchymal stem cells: Bone marrow mesenchymal stem cells (BMSCs) were the first MSCs to gather interest as a cellular therapy for ocular disease. Following transplantation into the vitreous of a rat model of glaucoma, BMSCs increased the number of surviving RGCs by 10–20% (Yu et al., 2006; Johnson et al., 2010a). In a model of traumatic optic nerve injury, BMSCs increased the survival of RGCs by 15–20% 8–28 days after transection/crush of the optic nerve (Levkovitch-Verbin et al., 2010; Mead et al., 2013; Mesentier-Louro et al., 2014) and increased the number of regenerating axons found at distances 100–1,200 μm distal to the lesion site by 2-fold compared to control animals receiving dead cells (Mead et al., 2013; Mesentier-Louro et al., 2014). In both models, the BMSCs survived but showed no sign of differentiating into neuronal or glial phenotypes, thus leading to the conclusion that the neuroprotective effects elicited were through paracrine-mediated effects, either direct signalling between the grafted stem cells and the injured RGCs, or activation of retinal glia by the stem cells and glia-mediated neuroprotection/axogenesis.

Dental pulp stem cells: We are interested in exploring the use of dental pulp stem cells (DPSCs) as an alternative source of stem cells for cellular therapy for the eye (Mead et al., 2013, 2014). DPSCs are neural crest-derived cells that can be isolated from adult teeth, an easily accessible source. Previous PCR-based gene expression studies suggested that, like BMSCs, DPSCs secrete multiple NTFs. In our most recent study using an *in vitro* co-culture system using axotomised RGC, we compared human-derived DPSCs, BMSCs and adipose-derived mesenchymal stem cells (ADSCs) for their potential to protect and regenerate injured RGCs (Mead et al., 2014). Like BMSCs and DPSCs ADSCs secrete multiple different NTFs; however, their efficacy as a treatment for the eye is unknown. We cultured human-derived MSCs with injured rat retinal cells and assessed their neuroprotective and neuritogenic potential, and the role of specific NTFs including platelet-derived growth factor (PDGF) which was recognised as an important BMSC-derived factor for RGC neuroprotection (Johnson et al., 2013). In co-culture, we administered a variety of different Fc-fusion protein inhibitors to selectively block particular receptors and assess the changes in neuroprotective and neuritogenic effects elicited by the MSCs. This study highlighted several important points: firstly, human-derived DPSCs were the most neuroprotective and neuritogenic, followed by BMSCs and ADSCs, respectively; secondly, a variety of NTFs were identified to play a significant role in the neuroprotection/neuritogenesis seen, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), as well as other NTFs such as glial cell line-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF) and PDGF-AA/AB/BB; thirdly, the neuritogenic properties of the MSCs were strongly inhibited by Fc-TrkAr, suggesting NGF plays an important role in MSC-mediated axon regeneration. Finally, using Fc-PDGFA/Br inhibitors, our study underscored the important role of DPSC/MSC-derived PDGF-AA and PDGF-AB/BB in retinal neuroprotection confirming a previous study using BMSCs (Johnson et al., 2013). We substantiated our findings using ELISA analyses on conditioned media from MSCs, confirming the secretion of NTFs by the MSCs with significantly higher quantities from DPSCs (Mead et al., 2014). We also performed a PCR array on the MSCs which indicated a diverse NTF profile of the three MSC populations. The distinct NTF profiles of DPSCs, BMSCs and ADSCs underlined the fact that the source of MSC is critical for determining the effectiveness of a planned cellular therapy. The PCR array data also revealed a previously unconsidered, and relatively unknown, factor, VGF-neuropeptide, which was expressed at considerably higher titres in DPSCs than BMSCs or ADSCs. At the time of our studies, very little was known about the neuroprotective/neuroregenerative properties of VGF. Thus, we ventured to investigate the

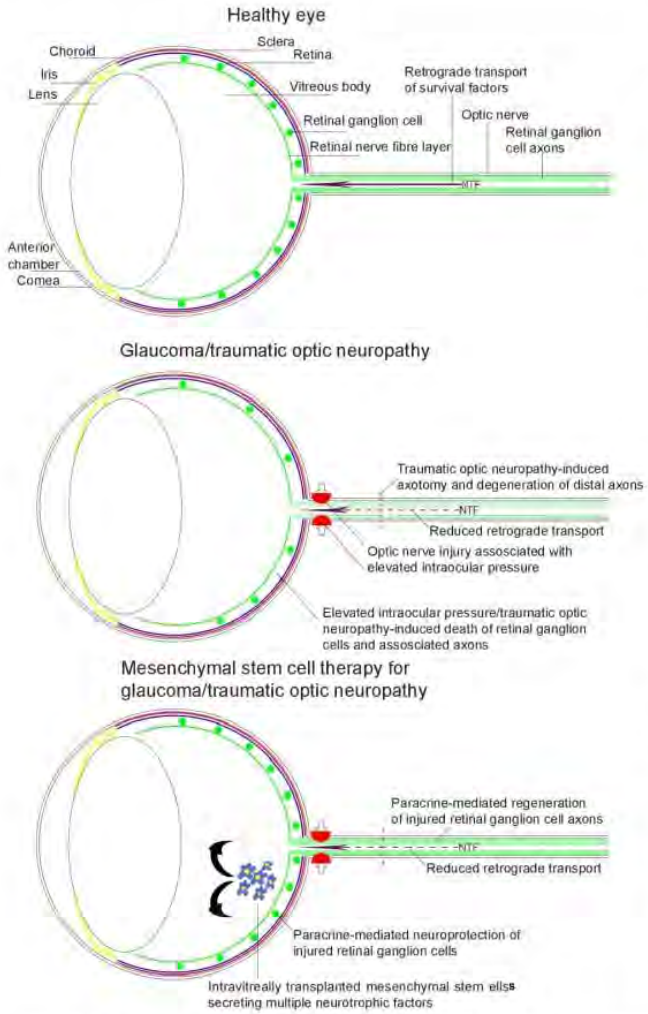


Figure 1 Schematic diagram demonstrating the effects of glaucoma and traumatic optic neuropathy on the eye and the potential of mesenchymal stem cells as a therapy.

effects of the recombinant VGF-neuropeptide on injured retinal cultures and elucidated that this new factor presented a potent neuroprotective effect (Mead et al., 2014). Considering this novel finding as well as the recently demonstrated importance of FGF-2 in BMSC-mediated neuroprotection of RGCs (Mesentier-Louro et al., 2014), it is very plausible that other neuroprotective/axogenic trophic molecules may be residing in the cocktail of the MSC secretome. Our study using primary human-derived MSCs corroborate our previous findings using rat primary cells that DPSCs were more potent in their *in vitro* RGC neuroprotection and RGC neuritogenesis which corresponded with their secretion of significantly higher levels of NGF, BDNF and NT-3 than BMSCs (Mead et al., 2013). DPSCs were also more effective in an *in vivo* model of optic nerve/RGC injury whereby DPSCs promoted a significantly greater increase in RGC survival and a further 2-fold increase in the number of regenerating axons found at distances 100–1,200 μm distal to the lesion site after intravitreal transplantation compared with BMSCs (Mead et al., 2013).This remarkable ability of DPSCs/MSCs to promote axon regeneration of RGCs after intravitreal transplantation has recently been corroborated by another study (Mesentier-Louro et al., 2014).

The question is whether it is possible to further enhance the neurotrophic property of DPSCs/MSCs, and hence their therapeutic potential for nerve repair. In a recent study DPSCs that were differentiated into Schwann cells, a supportive glial cell of the peripheral nervous system, were shown to have significantly higher levels of secreted NTFs (Martens et al., 2013) compared to undifferentiated cells. The effectiveness of differentiating stem cells into glia prior to treating the injured nervous system was evaluated by culturing the cells with injured dorsal root ganglion cells, a neuron found in the peripheral nervous system of the spinal cord. The authors demonstrated a significant increase in survival and neuritogenesis of dorsal root ganglion cells and also showed myelination of the growing neurites by DPSC-derived Schwann cells, in comparison to undifferentiated DPSCs. Although this was only an *in vitro* study in the peripheral nervous system, it is tempting to speculate that the elevated NTF secretion and subsequent neuroprotection of differentiated DPSC-derived Schwann cells may represent a more efficacious therapy for traumatic and degenerative eye disease and nerve repair.

Engraftment of stem cells in the retina: One interesting observation is the surprising ability for MSCs to survive *in vivo* when transplanted in the eye, with multiple studies detecting cells months after transplantation (Johnson et al., 2010a; Mead et al., 2013), which may be attributed to the immunoprivileged environment of the eye. However, despite this survival, MSCs were restricted to the vitreous, failing to engraft into the retina. A previous study identified that the barrier to engraftment is the activated glia which may prevent the injected stem cells migrating into the retina (Johnson et al., 2010b). It may be argued that the NTF-secreting MSCs would be more efficacious if in the same retinal microenvironment as the RGCs and even that the MSC survival following transplantation would be more pronounced if embedded in the cellular retina rather than clustered in the vitreous. Therefore, as well as enhancing the neurotrophic profile of MSCs by potentially differentiating them into glia, increasing the propensity for MSCs to engraft within the retina may possibly increase the neuroprotective and axogenic effects further. Further studies are warranted to clarify the most suitable stem cell injection site for retinal neural therapy.

Conclusions: Although we have performed an in depth comparison of three common human-derived MSC types and identified DPSCs as the most efficacious cell type for RGC neuroprotection and axon regeneration, further studies are required to confirm the relative (pre)clinical efficacy of the different human-derived stem cells *in vivo* and therefore the most "advantageous" MSCs for ocular repair. Noteworthy, early clinical trials have recently started to test

the safety of BMSCs for retinal and optic nerve damage (www.clinicaltrials.gov/show/NCT01920867). Based on our recent findings, we propose DPSCs as a novel and advantageous MSC type for retinal neuroprotection and repair (Mead et al., 2013, 2014).

Ben Mead was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) (No. BB/F017553/1) and the Rosetrees Trust.

Ann Logan, Martin Berry and Wendy Leadbeater were co-authors on the original paper.

Ben Mead *, Ben A. Scheven

Neurotrauma Research Group, Neurobiology Section, School of Clinical and Experimental Medicine, University of Birmingham, Birmingham, B15 2TT, United Kingdom (Mead B) School of Dentistry, University of Birmingham, B4 6NN, United Kingdom (Mead B, Scheven BA)

***Correspondence to:** Ben Mead, Ph.D., BXM813@bham.ac.uk.*
***Accepted:** 2015-02-12*
***doi:**10.* ***http**://www.nrronline.org/*
Mead B, Scheven BA (2015) Mesenchymal stem cell therapy for retinal ganglion cell neuroprotection and axon regeneration. Neural Regen Res 10(00):000.

References

Berry M, Ahmed Z, Lorber B, Douglas M, Logan A (2008) Regeneration of axons in the visual system. Restor Neurol Neurosci 26:147-174.

Johnson TV, Bull ND, Hunt DP, Marina N, Tomarev SI, Martin KR (2010a) Neuroprotective effects of intravitreal mesenchymal stem cell transplantation in experimental glaucoma. Invest Ophthalmol Vis Sci 51:2051-2059.

Johnson TV, Bull ND, Martin KR (2010b) Identification of barriers to retinal engraftment of transplanted stem cells. Invest Ophthalmol Vis Sci 51:960-970.

Johnson TV, Dekorver NW, Levasseur VA, Osborne A, Tassoni A, Lorber B, Heller JP, Villasmil R, Bull ND, Martin KR, Tomarev SI (2013) Identification of retinal ganglion cell neuroprotection conferred by platelet-derived growth factor through analysis of the mesenchymal stem cell secretome. Brain 137:503-519.

Levkovitch-Verbin H, Sadan O, Vander S, Rosner M, Barhum Y, Melamed E, Offen D, Melamed S (2010) Intravitreal injections of neurotrophic factors secreting mesenchymal stem cells are neuroprotective in rat eyes following optic nerve transection. Invest Ophthalmol Vis Sci 51:6394-6400.

Martens W, Sanen K, Georgiou M, Struys T, Bronckaers A, Ameloot M, Phillips J, Lambrichts I (2013) Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro. FASEB. 28:1634-1643.

Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2013) Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury. Invest Ophthalmol Vis Sci 54:7544-7556.

Mead B, Logan A, Berry M, Leadbeater W, Scheven BA (2014) Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells. PLoS One 9:e109305.

Mesentier-Louro LA, Zaverucha-do-Valle C, da Silva-Junior AJ, Nascimento-Dos-Santos G, Gubert F, de Figueiredo AB, Torres AL, Paredes BD, Teixeira C, Tovar-Moll F, Mendez-Otero R, Santiago MF (2014) Distribution of mesenchymal stem cells and effects on neuronal survival and axon regeneration after optic nerve crush and cell therapy. PLoS One 9:e110722.

Yu S, Tanabe T, Dezawa M, Ishikawa H, Yoshimura N (2006) Effects of bone marrow stromal cell injection in an experimental glaucoma model. Biochem Biophys Res Commun 344:1071-1079.