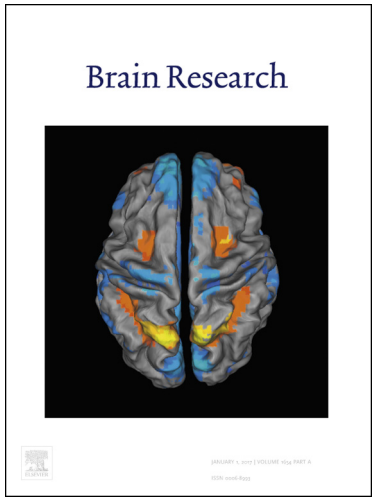

Research report

Transplantation of cultured olfactory mucosal cells rescues optic nerve axons in a rat glaucoma model

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Transplantation of cultured olfactory mucosal cells rescues optic nerve axons in a rat glaucoma model

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Abstract

Purpose: To determine whether transplantation of olfactory mucosal cells (OMCs) is able to rescue the loss of optic nerve axons after the intraocular pressure (IOP) is elevated in rats.

Methods: The IOP was raised by injection of magnetic microspheres into the anterior chamber of the eye. OMCs cultured from the adult olfactory mucosa were transplanted into the region of the optic disc.

Results: We demonstrated that although the raised IOP returned to its normal level at six weeks, there was an irreversible 58% loss of optic nerve axons in the control group. However, the loss of the axons was reduced to 23% in the group with the transplanted OMCs. The Pattern Electroretinograms (pERG) showed that the decrement of the voltage amplitudes in association with the raised IOP was significantly alleviated in the group with transplantation of OMC.

Conclusions: Transplantation of OMCs is able to rescue loss of optic nerve axons induced by raised IOP in the rats. The pERG recording suggested that the functional activities of the axons are also protected.

Translational Relevance: The results demonstrated the ability of the transplanted OMCs to protect the loss of the optic nerve axons and the loss of function caused by raised IOPs. The findings provide a basis for future human clinical trials by autografting OMCs from autologous nasal epithelial biopsies to treat or delay glaucoma diseases.

1. Introduction

Raised intraocular pressure (IOP) damages the axons of the retinal ganglion cells (RGC) at the region of the optic nerve head (ONH) where they pass through the sclera before emerging from the eye as the optic nerve (Howell et al., 2007; Morrison et al., 2008; Quigley, 2011; Tamm et al., 2017). So far there is no treatment that can completely prevent the progression of damage in all patients (Kass et al., 2002; Kotecha et al., 2009; Leske, 2009; Musch et al., 2009). Using animals as experimental glaucoma models induced by raising IOPs to investigate pathological progress of glaucoma diseases was reviewed in details by Morrison et al. (Morrison et al., 2005) Samsel and colleagues have more recently described a method for producing an acute rise of IOP by injection of magnetic microspheres into the anterior chamber of rat eyes (Samsel et al., 2011). By using their methods, we have shown that there is a progressive loss of astrocytic cells in the ONH and a marked decline in numbers of RGC axons over the period of 4 weeks after injection of magnetic microspheres into the anterior eye chamber in rats (Dai et al., 2012a).

It is known that the olfactory system is the only part of the mammalian central nervous system where axon regeneration continues throughout adult life (Graziadei et al., 1979; Schwob et al., 1999). Olfactory ensheathing cells (OECs) and olfactory nerve fibroblasts (ONFs) are considered to play the major part in this process. Transplantation of cultured OECs and ONFs to the lesioned CNS region have been shown to induce axonal regeneration and restore lost functions (Au et al., 2007; Franklin and Barnett, 2000; Li et al., 1997; Li et al., 2003; Lu and Ashwell, 2002; Radtke et al., 2008; Ramón-Cueto et al., 2000; Steward et al., 2006; Toft et al., 2007; Ziegler et al., 2011). OECs are present in the olfactory bulb and also

in the olfactory mucosa (Barraud et al., 2007; Lu et al., 2007; Tome et al., 2007). OECs cultured from both regions have shown effectiveness in repair of CNS injuries in experimental animal models and human spinal cord injuries (Yamamoto et al., 2009). Although olfactory bulb culture is able to yield higher number of OECs, it would be less invasive in the clinical situation to biopsy tissue from the olfactory mucosa rather than obtain the olfactory bulb by craniotomy (Féron et al., 2005; Lima et al., 2010; Mackay-Sim, 2005). In our previous studies we have demonstrated that transplantation of cultured olfactory mucosal cells (OMCs) consisting mainly of OECs and ONFs before raising the IOP in a rat model could reduce the damage to ONH astrocytes and moderate the loss of optic nerve axons (Dai et al., 2012b).

In the present study we focus on a paradigm that is more relevant to the clinical situation in glaucoma by first raising the IOP, and then transplanting OMCs to explore whether the cells could also reduce damage of the optic nerve axons in this situation.

2. Results

2.1. Survival and Migration of olfactory mucosal cells (OMCs, n=8)

The GFP infection rate in OMC culture is around 40% and the labelled cells could survive for 8 weeks in vitro. The survival and the distribution of the transplanted OMCs were identified by green fluorescence of lentivirally induced GFP in cryostat sections cut in the longitudinal plane. In all cases the transplanted cells were observed at 4 weeks after transplantation at the time when the IOPs have been raised for 6 weeks. The ONH region was identified by FN immunostaining. In 3 cases a number of the cells entered various retinal layers and formed a

mass located at the optic disc; in the other 5 the cells entered various retinal layers and also migrated into the ONH (white arrow in Fig 2A). Some of the transplanted cells had bipolar morphology resembling olfactory ensheathing cells (OECs) with their long processes ensheathing single OpN axons. Double fluorescence imaging showed that at higher magnification the transplanted mucosal OECs appear as double green fluorescent tracks ensheathing OpN axons (white arrowheads in Fig 2B, neurofilament staining, red fluorescence). The ensheathment was also observed with electron microscope (Fig 2C). The OEC profiles usually ensheathed one or a small number of highly tubule-rich axons in this region of the ONH. There was no evidence that the cells migrate beyond the ONH into the myelinated OpN as we observed in the previous studies.

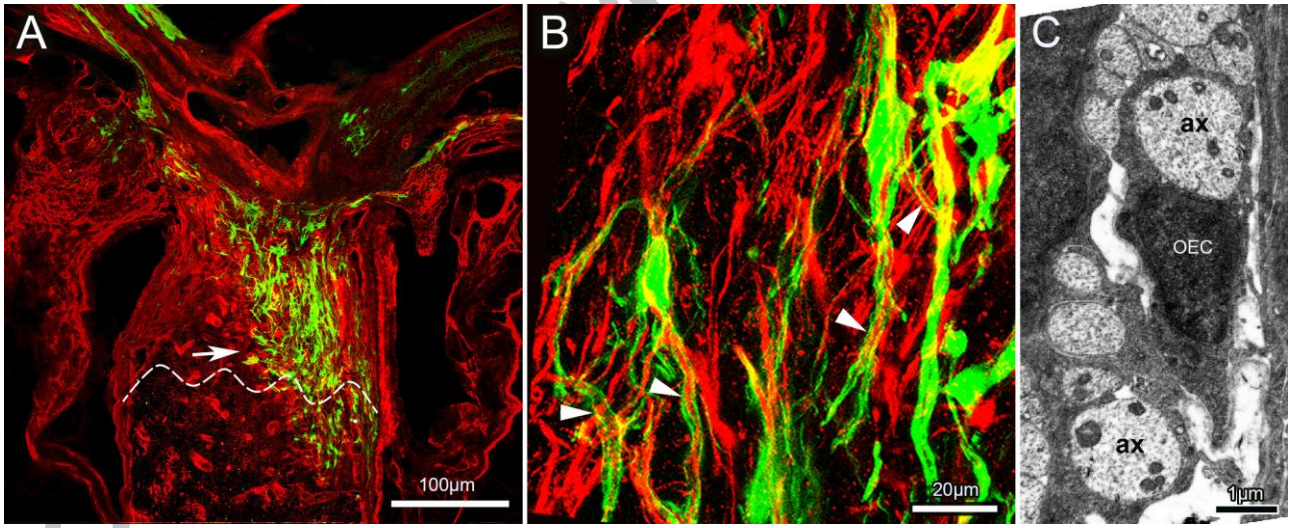

Figure 2. Confocal images of cryostat section showing pattern of migration of transplanted OMCs and their ensheathment of OpN axons, and also EM micrograph showing the ensheathment.

A, GFP labelled cells (green) migrating into the optic nerve head (white arrow, FN immunostaining, red) and form a mass occupying part of the width of the optic nerve head.

B, High magnification image to show the alignment of the transplanted cells (green) to the OpN axons (neurofilament immunostaining, red) and ensheathment of the axons in the ONH

region (white arrow in A). Arrowheads show the double tracks of green fluorescent ensheathment. C, EM micrograph to show the ensheathment of OECs (oec) to OpN axons (ax).

Survival time: 6 weeks (transplantation of OMC after raised IOP for 2 weeks; further 4 weeks' of survival after the transplantation). Cryostat longitudinal section A and B; Resin section C. Scale bars: A: 100μm; B: 20μm; C: 1μm.

2.2. Rise and fall of the IOP

The IOP was measured on the day before injection of microspheres and then measured at 1 day and then weekly for 6 weeks after injection. The measurements were compared at each time point between the rats with raised IOP alone, raised IOP+PBS injection and raised IOP+OMC transplant. The means of the 7 postoperative IOP measurements over the 6 weeks from all rats were used to represent the change of the IOP (Jia, Cepurna, Johnson, & Morrison, 2000). We did not find statistically significant differences of the IOPs between the groups.

In all groups of experimental rats the IOP had an initial sharp rise after injection of microspheres and then fell continuously towards normal (Fig 3). The IOP in the normal rats was 24.19±0.43mm Hg (mean±SEM, n=31, similar to the study from Samsel PA at al., 2011). It was raised by injection of magnetic microspheres to 47.32±2.32 (mean±SEM, n=31, $p<0.001$) at 1 day, fell to 38.55±1.21 (mean±SEM, n=31, $p<0.001$) at 1 week, then continuously fell over 2 and 3 weeks to 36.84±1.55 (mean±SEM, n=31, $p<0.000$) and 31.61±1.00 (mean±SEM, n=31, $p<0.001$) respectively, by 4 weeks it was approaching normal level at 27.74±1.28 (mean±SEM, n=31, p=NS) and, returned to the normal level at 5 weeks

25.48±0.65 (mean±SEM, n=31, p=NS) and at 6 weeks 23.42± 0.56 (mean±SEM, n=31, p=NS). From 4 to 6 weeks the IOPs were no significantly different to the level of the normal.

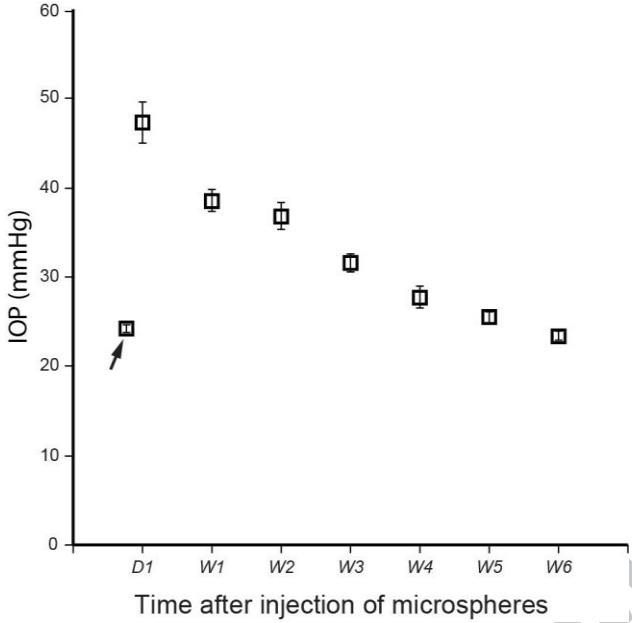

Figure 3. The time course of the rise and fall of mean IOPs of all groups. The mm Hg means of all groups at each time points (mean±SEM): Normal (arrow), 24.19±0.43; Day1, 47.32±2.32; Week 1, 38.55±1.21; Week 2, 36.84±1.55; Week 3, 31.61±1.00; Week 4, 27.74±1.28; Week 5, 25.48±0.65 and Week 6, 23.42± 0.56. From 4 to 6 weeks the IOPs returned to the normal level. *D, day. W, week*

2.3. Pattern Electroretinograms

pERG were recorded at 4 and 6 weeks after IOP was raised in all groups of rats. The representative waveforms and amplitude means of pERG are shown in Figure 4. The pERG wavefroms were similar to the previous pERG studies in rodents (Chou and Porciatti, 2012; Husain et al., 2012; Patel et al., 2017).

In the normal rats, the mean amplitude of pERG recorded was 15.61±0.80μV (mean±SD; n=4). The pERG amplitudes measured at 4 and 6 weeks after IOP was raised (n=5) were 3.14±0.36 and 1.44±0.30μV. They were 78% and 91% decrease of the normal ($p<0001$) respectively.

At 2 and 4 weeks after PBS injection (i.e. 4 and 6 weeks after IOP was raised) the pERG amplitudes in these rats were 2.89±0.21 and 1.51±0.35μV showing similar degree of decline to the rats with raised IOP alone. In the group of rats with the raised IOP+OMC transplant, the pERG amplitudes were 9.28±0.51 and 5.47±0.46μV at 2 and 4 weeks after OMCs transplantation (i.e. 4 and 6 weeks after IOP was raised) also showing amplitude decline but the reductions were 40% and 65% from the normal ($p<0.001$). The reduction in pERG amplitudes were significantly less in the rats with transplanted OMCs than the rats with raised IOP alone or PBS injection.

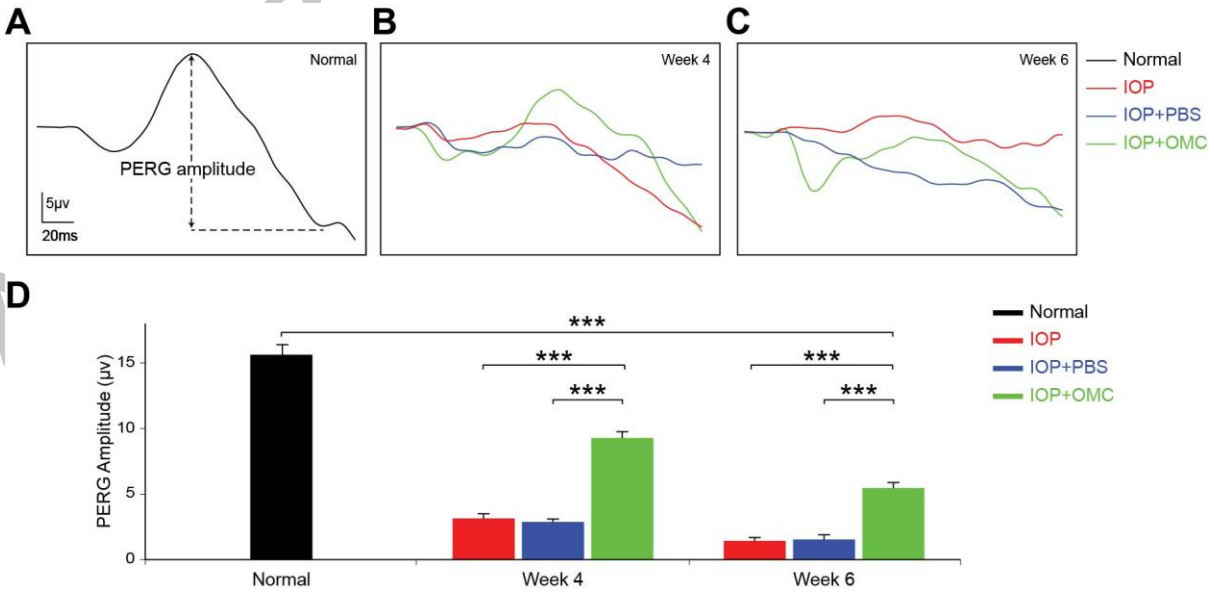

Figure 4. A,B,C, Representative pERG recordings. The recording electrode made of a gold wire loop was placed on the centre of the cornea, the reference electrode was inserted under the scleral conjunctiva around the equator of the eyes, and a ground electrode was inserted subcutaneously into the tail. Waveform of a normal rat (A); waveforms of single animals at 4 and 6 weeks after IOPs were raised (B,C): raised IOP alone (red), raised IOP+PBS injection (blue) and raised IOP+OMC transplant (green). D, Comparison of mean pERG amplitudes between normal rats (n=4, black), raised IOP alone (n=5, red), raised IOP+PBS injection (n=3, blue) and raised IOP+OMC transplant (n=3, green) at weeks 4 and 6 showing reduction in pERG amplitudes between the rats with raised IOP+OMC transplant and those without. (***, $p<0.001$).

2.4. Counts of Axons in the OpN

The RGC axons of the OpNs were counted in the cross semithin sections prepared from the normal rats and those with raised IOP for 6 weeks. Two independent observers who did not know the origin of the samples carried out the counting. For each rat the number of axons was counted in 24 *x* 1600μm^2 sampled squares which represented approximately 15% total cross sectional area in the optic nerve 2mm behind the junction with the optic disc. The summary of the mean axon counts in 24 squares (1600μm^2) from all rats in each group are presented in Figure 5. The estimate of the total number of axons was 74,330 in the normal rats (n=6), fell to 31,499 in the rats with raised IOP alone (n=6), 32,798 with raised IOP+PBS injection (n=5) and 48,050 in the rats with IOP+OMC transplant.

The lost number of axons at 6 weeks in the rats with raised IOP+OMC transplant was 35% compared to 58% in the rats with raised IOP alone and, 56% with raised IOP+PBS injection.

Transplantation of OMCs after pressure induction resulted in a significant reduction in axonal loss by 23% compared to the groups of rats without the transplant or with PBS injection.

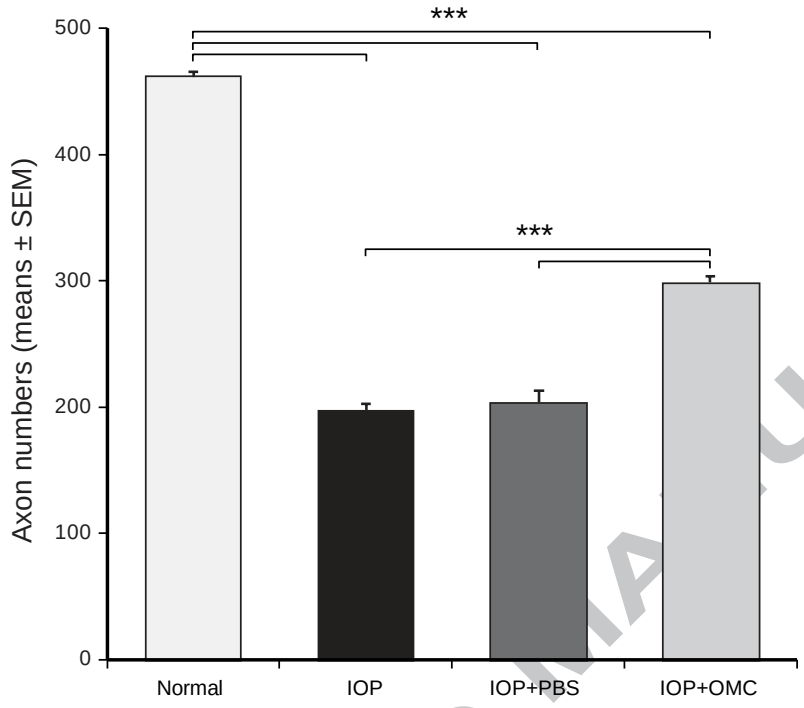

Figure 5. Summary of the mean axon numbers of 24 squares (1600μm^2) from all rats in the normal group; the rats with raised IOP alone; the rats with IOP+PBS and the rats with raised IOP+OMCs. The axon counts were 462±3 in the normal rat (n=6); 197±6 with raised IOP alone (n=6); 204±10 with raised IOP+PBS injection (n=5); 298±5 with raised IOP+OMC transplant (n=6). Axons were counted on the whole cross sectional area at the OpN level 2mm from the optic disk from the normal and operated rats 6 weeks after surgery.

3. Discussion

Our present study shows that transplantation of cultured olfactory mucosal cells (OMCs) into the region of the rat ONH reduces the loss of RGC axons in a clinically relevant glaucoma model. The less reduction of pERG amplitudes in the rats with OMC transplant compared to

those without also indicates that the transplanted OMCs are able to preserve the function of RGCs. The finding provides promising evidence that transplantation of the olfactory mucosal cells could be a possible therapeutic strategy for optic nerve repair and glaucoma treatment in the future.

3.1. Progression of RGC axon loss to irreversibility after short term rise of IOP

Our previous study (Dai et al., 2012a) and the present study show that the short term rise of IOP causes loss of RGC axons and the loss is irreversible even when the IOP returns to the normal level. This long-term damage by transient episodes of pressure-related stress has also been thoroughly discussed in a recent study from Sun et al., 2017 (Sun et al., 2017). As discussed in our previous study the induced rise of IOP stretches the elasticity of the astrocytic processes to exceed the point where the dorsal processes are torn away from their attachment to the outer sheath of ONH. With time, even as the IOP returns towards normal level the astrocytic processes can no longer grow back to re-attach to the sheath and the damage is hence irreversible (Dai et al., 2012a).

3.2. Reduction of loss of OpN axons by transplantation of OMCs

We previously showed that the loss of the OpN axons could be moderated by transplanting OMCs prior to raising IOP in the rat (Dai et al., 2012b). The transplanted cells reduced the extensive retraction of the astrocytic processes caused by raised intraocular pressure. This in turn preserved the astrocytic-axonal contact which we consider is critical for the metabolic support of the non-myelinated axons in the optic canal given the presence of giant mitochondria in astrocytes of the ONH (Li et al., 2015). The transplanted olfactory mucosal

cells also ensheathed the nonmyelinated RGC axons in both the retina and the ONH to protect them from damage (Dai et al., 2012b).

The present data demonstrate that a reduction in the loss of axons can also be achieved in a clinically relevant model - transplantation of OMCs into the ONH region after IOP has been raised. The reduction was not observed in the rats receiving PBS injection only despite having the same surgical procedure as those in the group with the transplant. Although the histology showed that in about 30% cases the transplanted OMCs were not clearly visualized in the region of the ONH, reduction of the loss of axons was still achieved in all samples. This could be due to that in addition to the reduction of damage to the astrocytes of ONH and ensheathment of the axons by the OECs, the neurotrophic factors secreted by OECs e.g. (Novikova et al., 2011; Roet et al., 2011; Sasaki et al., 2005) may also play an important role of protection to the axons (Yang et al., 2015). This observation suggests that transplanted cells might be able to reduce the damage to the axons without entering the ONH region or directly contacting the axons, and also suggests a possible role for these transplanted cells in providing neurotrophic or neurometabolic support of the axon survival in clinical glaucoma diseases. Mechanical support might also be a mechanism as OECs support and guide regeneration of olfactory axons *in situ*, although further research will be needed to confirm.

3.3. Olfactory Bulb cells vs mucosal cells in the rat glaucoma model

OECs from the olfactory bulb and the olfactory mucosa have both been shown experimentally and clinically to promote axonal regeneration, reduce glial scar formation and interact with host glia allowing re-entrance of the regenerating axons to the undamaged pathway leading to restoration of functions (Au et al., 2007; Franklin and Barnett, 2000;

Gorrie et al., 2010; Li et al., 1997; Li et al., 2003; Lu and Ashwell, 2002; Mayeur et al., 2013; Radtke et al., 2008; Ramón-Cueto et al., 2000; Steward et al., 2006; Tabakow et al., 2014; Toft et al., 2007; Ziegler et al., 2011). Studies have also shown differences between mucosal OECs and bulbar OECs at genomic level (Guerout et al., 2010), in their migration properties and ability to induce regeneration of CNS axons (Mayeur et al., 2013; Paviot et al., 2011; Richter et al., 2005). We have also found differences in their effects on axonal regeneration and functional restoration when cultured bulbar OECs and mucosal OECs were transplanted into the lesioned corticospinal tract (Yamamoto et al., 2009) and in the lesioned dorsal roots in rat (Ibrahim et al., 2014). However, in our previous study on transplanting OECs into the optic disc, we did not observe clear differences in the survival, the migration pattern and the protective effects on number of RGC axons between bulbar cells and mucosal cells.

From a clinical point of view, obtaining the OECs from the olfactory mucosa which avoids intracranial surgery is much less invasive and risky than from the olfactory bulb. Transplantation of autologous mucosa cells in patients with glaucomatous damage therefore presents a future approach both for reducing the progression of the damage to the astrocytic structure of the ONH, and for stabilising the loss of RGC axons in glaucoma.

4. Materials and methods

Adult female Sprague Dawley rats (weight 220-250g, age 10-12 weeks) were used for the study. All animals were handled according to the UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act 1986 and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

4.1. Experiment design

Total of 31 rats were used in this study. IOP measurements were carried out on all 31 rats.

(1) Axon counting (n=23): Twenty three rats were used for axon counting. They were divided into 4 groups: Normal (n=6), raised IOP alone (n=6); Raised IOP+phosphate buffered saline (PBS) injection (n=5) and raised IOP+transplanted OMCs (n =6). The axons were counted on semithin resin cross sections of the optic nerves (see below).

(2) Morphological study of the OMC transplants (n=8): Eight rats were used for Morphological study. At 2 weeks after raising the IOP, the rats received the OMC transplants. After a further 4 weeks' survival, they were sacrificed and perfused with fixative (see Cryostat section and immunostaining staining). Longitudinal cryostat sections were cut at a thickness of 16μm on a cryostat to examine the cell morphology and migration.

(3) Electron microscopy (n=3): ONH Segments of 3 rats from the group of the IOP+transplanted OMCs processed for axon counting were used.

(4) Pattern Electroretinograms (pERG) (n=11): pERG recording was performed on 11 rats. The rats were divided into 3 groups: raised IOP alone (n=5); IOP+PBS injection (n=3) and IOP+transplanted OMCs (n=3).

Experiment timeline: The total time taken for all the experiments were 6 weeks. In the group 'raised IOP alone' the animals received magnetic microspheres injections at day 1 and

were terminated at week 6; In the groups 'IOP+PBS' and 'IOP+transplanted OMCs' the animals received injection of PBS or transplantation of OMCs two weeks after receiving magnetic microspheres injections and were terminated at week 6. pERG were recorded at week 4 and week 6.

4.2. Induction and measurement of raised intraocular pressure (IOP)

The IOP was raised by injection of magnetic microspheres as described in our previous study (Dai et al., 2012b). Briefly, rats were anaesthetised with isoflurane (Abbott Laboratories Ltd., Maidenhead, Berkshire, UK). 10µl of a 30mg/ml suspension of 5µm diameter magnetic microspheres (Aldehyde-terminated Magnetic, MagicBeads™, Chi Scientific) were injected into the right anterior chamber (Fig 1) with a 33 gauge needle (Samsel at al., 2011). After injection, a small magnetic rod was used to direct the microspheres into the drainage area of the trabecular meshwork in the peripheral angle of the anterior chamber (Samsel et al., 2011). The IOP was recorded using a tonometer (Tono-Pen XL, Reichert Inc, Germany) (at) 1 day before injection as a baseline IOP value, at 1 day after, and then once a week, under local anaesthesia induced by conjunctival instillation of one drop of 0.5% proparacaine hydrochloride (Alcaine, Alcon Laboratories, Couvreur, Belgium). The mean of 8 consecutive readings was taken as the IOP value for each time point.

4.3. Mucosa cell culture

The olfactory mucosal cells (OMCs) were prepared as previously described (Dai et al., 2012b; Li et al., 2008a). Briefly, adult SD rats were decapitated under terminal anaesthesia and the olfactory mucosa dissected out and transferred to ice-cold Hanks' balanced salt solution

without calcium and magnesium supplemented with 100U/100µg/ml penicillin-streptomycin, incubated in 1ml of dispase II at 37ºC for 45min. The lamina propria was cut into small pieces, collected into 2ml of 0.25% collagenase Type I in a Dulbecco's modified Eagle medium (DMEM)/Ham's F-12 with GlutaMAX™ 1.0mg/ml insulin, 0.67mg/ml transferrin and 0.55mg/ml selenium, 100U/100µg/ml penicillin-streptomycin and 10% deactivated foetal calf serum (FCS), and incubated at 37ºC for 5min. The pieces of the lamina propria were triturated in a single cell suspension and centrifuged. The cell suspension was seeded at a density of 2.0-2.5 x 10^4 per sq cm on 35mm culture dishes coated with 0.1mg/ml poly-L-lysine (Sigma-Aldrich) and maintained in a 5 % CO_2 incubator for around 14 days at 37ºC, with the culture medium replaced every 3 days. Two days before transplantation the cells were infected with a GFP gene harbouring lentiviral construct (Cavalieri et al., 2003; Dai et al., 2012b; Ibrahim et al., 2009; Naldini et al., 1996; Ruitenberg et al., 2002). Before transplantation the cells were detached by trypsinization and a cell suspension was made up to a concentration of 2.0x10^7/ml in DMEM-F12 without FCS. During surgery, the cells were kept on ice.

4.4. Transplantation of olfactory mucosal cells (OMCs)

After 2 weeks of raising the IOP, the rats were again deeply anaesthetized with isoflurane and topical conjunctival 0.5% proparacaine hydrochloride. 3µl of OMC suspension (about 50,000 cells) was injected into the optic disc region (Fig 1) as described in a previous study (Li et al., 2008b). Briefly, the orbit was opened through the superior conjunctival fornix and the extraorbital muscles and glands separated to expose the rat optic nerve head. A fine bevelled glass pipette with an internal diameter of around 80µm guided by hand at an oblique angle for a depth of 2mm (marked on the outside of the pipette) was inserted into the eye in a

direction towards the optic nerve head and the cell suspension was injected by pressure via a 50ml syringe attached to the pipette. All animals were examined to exclude intraocular inflammation. One rat developed a cataract and was excluded from the study.

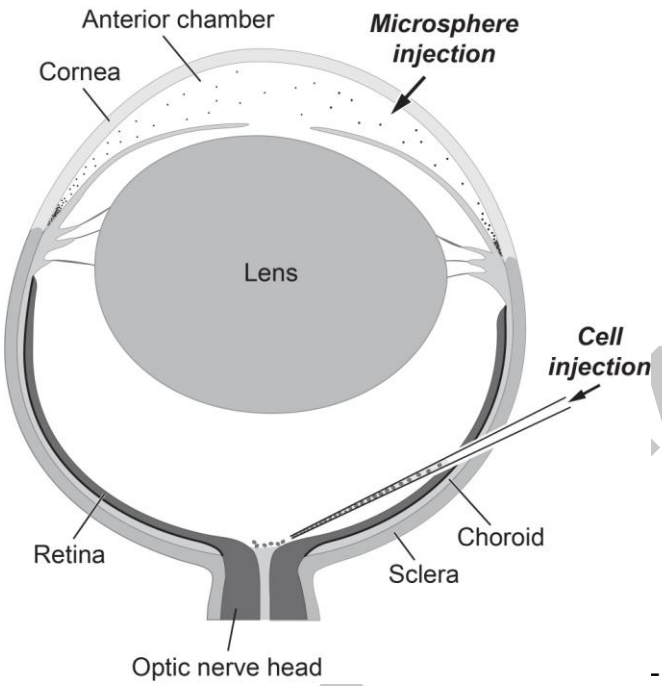

Figure 1. Diagrammatic drawing to show location of interventions of the experiment. IOP was raised by injection of magnetic microspheres into the anterior chamber (***Microsphere injection***). Cultured OMCs were injected into the optic disc region by a glass micro-pipette (***Cell injection***).

4.5. Pattern electroretinograms

Pattern electroretinogram (pERG) was recorded as described by Alsarraf et al. (Alsarraf et al., 2014). The pERG components were measured according to the previous studies (Chou and Porciatti, 2012; Husain et al., 2012; Patel et al., 2017). In brief, the recording electrode made

of a gold wire loop was placed on the centre of the cornea, the reference electrode was inserted under the scleral conjunctiva around the equator of the eyes, and a ground electrode was inserted subcutaneously into the tail. A drop of saline was frequently applied to the eye to prevent corneal dehydration. Stimulus presentation, amplification, filtering (0.1–500 Hz band-pass, without notch filtering) and data acquisition were performed with a RETIscan system (Roland Consult, Brandenburg, Germany). Stimuli were generated by white and black alternating contrast reversing bars (spatial frequency, 0.033 cycle per degree; mean luminance, 50 cd/m^2; contrast, 100%; and temporal frequency, 1 Hz) and aligned with the projection of the pupil and visual axis at a distance of 11 cm from the centre of the screen. Each pERG reading was recorded as an average of superimposed 250 sweeps. For the PERG amplitudes, measurements were made between a peak and adjacent trough of the waveform.

4.6. Resin section

Rats were transcardially perfused with 50ml of 0.01M PBS followed by 500ml of a mixture of 2% paraformaldehyde and 2% glutaraldehyde (TAAB Laboratories Equipment Ltd, England) in 0.1 M phosphate buffer (PB) under terminal anaesthesia. The rat heads were post fixed in the same fixative for a further 2 days at 4'C. The optic nerves including optic nerve heads were dissected out and postfixed in 2% osmium tetroxide for further 1.5 hours. Dehydrated tissues were embedded in epoxy resin (TAAB Laboratories Equipment Ltd, England) and hardened at 60^0 C oven for 3 days. Serial 1.5µm semithin cross sections were cut at the level 2mm from the retina and stained with 1% methylene blue and Azur II. Ultrathin sections of the ONH region with the transplant were cut and stained with 25% uranyl acetate in methanol for 2 minutes and Reynold's lead citrate for 15 minutes.

4.7. Cryostat section and immunostaining

Under terminal anaesthesia rats were transcardially perfused with 50ml of 0.01M PBS followed by 500ml of 4% paraformaldehyde (TAAB Laboratories Equipment Ltd, England) in 0.1M PB and post fixed in the same fixative overnight. The eyes (balls) and attached optic nerve were dissected out and immersed in 10%, then 20% sucrose solution until the tissue sank. 16μm cryostat sections were cut in a longitudinal plane on a cryostat (Leica CM3050). The sections were either immunostained for neurofilament or chemically stained with Sytiox® Orange Nucleic Acid Stain (S-11368; Invitrogen UK). For immunohistochemical staining the sections were incubated in a solution of rabbit anti -neurofilament heavy chains and light chains (1:500; Abcam, Cambridge, UK) at 4°C overnight, followed by washing 3 times in PBS and further incubating in a solution of secondary antibody conjugated with Alexa-546 (1:400, Invitrogen, Paisley, UK) at room temperature for 2 hours. Sytox® staining was carried out by soaking sections in the dye solution at 5μM concentration for 10 minutes. Fluorescent images were visualized and captured using a TCS SP1 Leica confocal microscope.

4.8. Axon counts in the optic nerve (OpN)

Axons were counted as described in our previous study (Dai et al., 2012a; Dai et al., 2012b). Briefly, under a x40 objective, photographs were taken across the whole cross sectional area at the OpN level 2mm from the optic disk. A photomontage of the entire nerve was constructed using the Photomerge function in Photoshop CS6. On the enlarged image on the computer screen axons were identified by the profiles of methylene blue staining of intact myelin rings encircling pale (normal) axons. The counting was carried out in 24 evenly

spaced 40μm sided grid squares by two independent observers who did not know the origin of the samples (Fig 6). A total of 23 sampled nerves were counted. They were 6 from the normal rats, 6 with raised IOP alone, 5 with raised IOP+PBS injection and 6 with raised IOP+transplantation of OMCs. The 24 1,600μm^2 squares represent 15% of the total cross sectional area of the OpN taken at the positon by average. We derived the estimate total of the axon numbers in the section the formula ∑x/15% (x=count in one square).

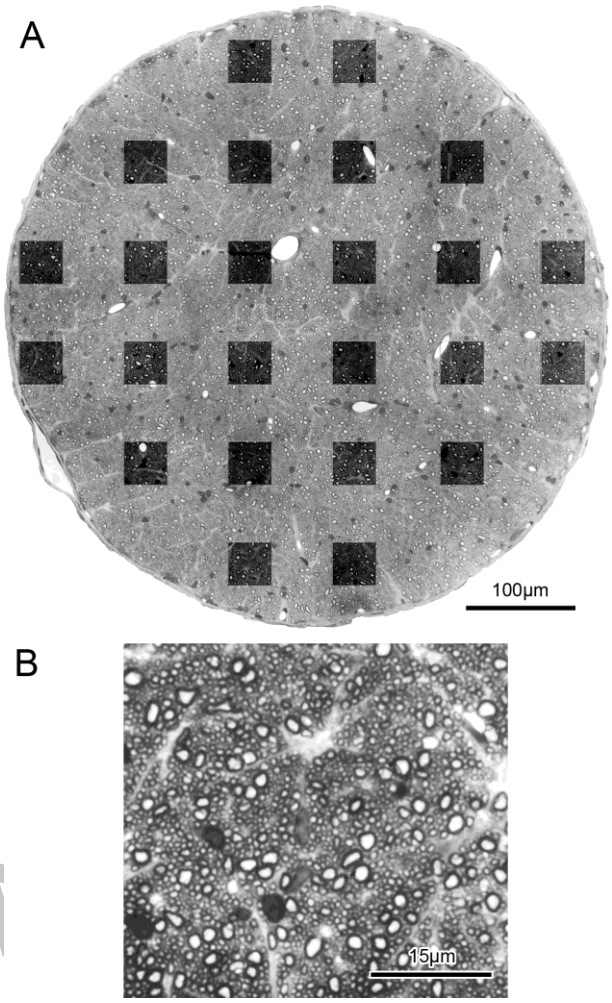

Figure 6. A: Low power montage of a 1.5μm resin embedded semithin cross section of normal OpN at 2mm below the optic disc showing sampling grids as used to count axons. Two independent observers carried out the counting blindly. B: Enlarged representative view

from one of the squares. The semithin section was stained by methylene blue and Azur II. Scale bars: 100μm for A and 15μm for B.

4.9. Statistical analyses

Statistical testing was performed with SPSS (SPSS Inc, Chicago, USA; version 17.0). One-way ANOVA used to assess if there was any statistically significant difference between the groups in terms of average IOP and the number of intact axons in the different groups.

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Figure legends:

Figure 1:

Diagrammatic drawing to show location of interventions of the experiment. IOP was raised by injection of magnetic microspheres into the anterior chamber (***Microsphere injection***). Cultured OMCs were injected into the optic disc region by a glass micro-pipette (***Cell injection***).

Figure 2:

Confocal images of cryostat section showing pattern of migration of transplanted OMCs and their ensheathment of OpN axons, and also EM micrograph showing the ensheathment.

A, GFP labelled cells (green) migrating into the optic nerve head (white arrow, FN immunostaining, red) and form a mass occupying part of the width of the optic nerve head.

B, High magnification image to show the alignment of the transplanted cells (green) to the OpN axons (neurofilament immunostaining, red) and ensheathment of the axons in the ONH region (white arrow in A). Arrowheads show the double tracks of green fluorescent ensheathment. C, EM micrograph to show the ensheathment of OECs (oec) to OpN axons (ax).

Survival time: 6 weeks (transplantation of OMC after raised IOP for 2 weeks; further 4 weeks' of survival after the transplantation). Cryostat longitudinal section A and B; Resin section C. Scale bars: A: 100µm; B: 20µm; C: 1µm

Figure 3:

Fig 3. The time course of the rise and fall of mean IOPs of all groups. The means of all groups at each time points (mean±SEM): Normal (arrow), 24.19±0.43; Day1, 47.32±2.32;

Week 1, 38.55±1.21; Week 2, 36.84±1.55; Week 3, 31.61±1.00; Week 4, 27.74±1.28; Week 5, 25.48±0.65 and Week 6, 23.42± 0.56. From 4 to 6 weeks the IOPs returned to the normal level. D, day. W, week

Figure 4:

A,B,C, Representative pERG recordings. The recording electrode made of a gold wire loop was placed on the centre of the cornea, the reference electrode was inserted under the scleral conjunctiva around the equator of the eyes, and a ground electrode was inserted subcutaneously into the tail. Waveform of a normal rat (A); waveforms of single animals at 4 and 6 weeks after IOPs were raised (B,C): raised IOP alone (red), raised IOP+PBS injection (blue) and raised IOP+OMC transplant (green). D, Comparison of mean pERG amplitudes between normal rats (n=4, black), raised IOP alone (n=5, red), raised IOP+PBS injection (n=3, blue) and raised IOP+OMC transplant (n=3, green) at weeks 4 and 6 showing reduction in pERG amplitudes between the rats with raised IOP+OMC transplant and those without. (***, $p<0.001$).

Figure 5:

Summary of the mean axon numbers of 24 squares (1600μm^2) from all rats in the normal group; the rats with raised IOP alone; the rats with IOP+PBS and the rats with raised IOP+OMCs. The axon counts were 462±3 in the normal rat (n=6); 197±6 with raised IOP alone (n=6); 204±10 with raised IOP+PBS injection (n=5); 298±5 with raised IOP+OMC transplant (n=6). Axons were counted on the whole cross sectional area at the OpN level 2mm from the optic disk from the normal and operated rats 6 weeks after surgery.

Figure 6.

A: Low power montage of a 1.5µm resin embedded semithin cross section of normal OpN at 2mm below the optic disc showing sampling grids as used to count axons. B: Enlarged representative view from one of the squares. Two independent observers carried out the counting blindly. The semithin section was stained by methylene blue and Azur II. Scale bars: 100µm for A and 15µm for B.

- Transplantation of OMCs is able to rescue loss of optic nerve axons induced by raised IOP in the rats.
- The pERG recording suggested that the functional activities of the axons are also protected.
- The results demonstrated the ability of the transplanted OMCs to protect the loss of the optic nerve axons and the loss of function caused by raised IOPs.
- The findings provide a basis for future human clinical trials by autografting OMCs from autologous nasal epithelial biopsies to treat or delay glaucoma diseases.