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Olfactory glia enhance neonatal axon regeneration

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Abstract

Olfactory ensheathing cells (OECs) migrate with olfactory axons that extend from the nasal epithelium into the olfactory bulb. Unlike other glia, OECs are thought to migrate ahead of growing axons instead of following defined axonal paths. However it remains unknown how the presence of axons and OECs influences the growth and migration of each other during regeneration. We have developed a regeneration model in neonatal mice to examine whether (i) the presence of OECs ahead of olfactory axons affects axonal growth and (ii) the presence of olfactory axons alters the distribution of OECs. We performed unilateral bulbectomy to ablate olfactory axons followed by methimazole administration to further delay neuronal growth. In this model OECs filled the cavity left by the bulbectomy before new axons extended into the cavity. We found that delaying axon growth increased the rate at which OECs filled the cavity. The axons subsequently grew over a significantly larger region and formed more distinct fascicles and glomeruli in comparison with growth in animals that had undergone only bulbectomy. In vitro, we confirmed (i) that olfactory axon growth was more rapid when OECs were more widely distributed than the axons and (ii) that OECs migrated faster in the absence of axons. These results demonstrate that the distribution of OECs can be increased by repressing by growth of olfactory axons and that olfactory axon growth is significantly enhanced if a permissive OEC environment is present prior to axon growth.

Key words: glia, olfactory bulb, methimazole, bulbectomy, neuron, fascicle

1. Introduction

In the mammalian olfactory system, olfactory ensheathing cells (OECs) are unique glia which are thought to contribute to successful growth of olfactory sensory axons throughout life (Boyd et al., 2005; Graziadei and Graziadei, 1979; Mackay-Sim and Kittel, 1991). In the peripheral nervous system, OECs surround fascicles of olfactory sensory axons whereas in the central nervous system, OECs assist in the sorting of olfactory axons within nerve fibre layer of the olfactory bulb (Chuah and Au, 1991; Doucette, 1984; Farbman and Squinto, 1985).

During development of the olfactory system, OECs have been reported to migrate slightly ahead of the primary olfactory axons en route to the olfactory bulb (Tennent and Chuah, 1996). This is in contrast to Schwann cells which migrate along already defined axonal pathways during development of the peripheral nervous system (Jessen and Mirsky, 2005). The OECs are thought to promote axon growth by providing a cellular substrate containing molecules that facilitate axonal adhesion and extension and by expressing growth-promoting agents such brain derived neurotrophic factor, glia-derived nexin and nerve growth factor (Boruch et al., 2001; Chuah et al., 2004; Chung et al., 2004; Doucette, 1990; Kafitz and Greer, 1999; Tisay and Key, 1999; Woodhall et al., 2001).

The axon growth promoting ability of OECs combined with their unique feature of being able to migrate from the peripheral nervous system into the central nervous system has led to the investigation of OECs for neural regeneration therapies. Several studies have now shown that OECs transplanted into the injured spinal cord have promoted axon regeneration, although to a limited extent (Bartolomei and Greer,

2000; Gudino-Cabrera et al., 2000; Ramer et al., 2004). Significantly, while some reports have shown that OECs migrate substantial distances following transplant into injured spinal cord (Boruch et al., 2001; Ramon-Cueto and Nieto-Sampedro, 1994; Resnick et al., 2003) others have shown limited migration of OECs (Lakatos et al., 2003; Ruitenberg et al., 2002). Thus further understanding is required of the factors that control migration of OECs and subsequent axon growth. Schwann cells, which have also been used in transplantation studies but with less success than OECs in some models (Lankford et al., 2008), tend to follow axons rather than migrate ahead of axons (Jessen and Mirsky, 2005). It is possible that the remarkable regeneration observed in some of the OEC transplantation studies is due to the unique migratory properties of OECs, in particular their ability to migrate ahead of growing axons, a property that could potentially be further enhanced to optimize axonal regeneration.

We hypothesized that the presence of OECs ahead of olfactory axons extending towards the bulb would create a permissive environment enhancing axonal growth. We tested this hypothesis by studying the growth of new axons within three different animal models. In the first model, we used unilateral olfactory bulbectomy to examine axon growth when OECs were present slightly ahead of axons. In the second model, we combined unilateral bulbectomy with later treatment of methimazole which resulted in delayed regeneration of neurons. In this model, the OECs populated the cavity left by bulbectomy prior to the growth of olfactory axons. In the third model, we transplanted OECs into the cavity following bulbectomy. We used these models to determine how the establishment of a permissive glia environment affected axon growth. Our results show that the early arrival of OECs into the cavity and the

formation of a continuous mass of OECs significantly promoted subsequent axon growth.

2. Material and methods

2.1 Animals

Transgenic mice expressing ZsGreen in primary olfactory sensory neurons were previously generated. In these mice, the full length (5.5kb) olfactory marker protein (OMP) promoter (Danciger et al., 1989) drove the expression of ZsGreen fluorescent protein, from pZsGreen-Express Vector (Clontech, Palo Alto, CA). The transgene was liberated from the vector using EcoR1 restriction sites and injected into fertilised mouse oocytes at the Transgenic Animal Service of Queensland (University of Queensland, Brisbane). Successful integration of the transgene was confirmed by expression of ZsGreen fluorescence of the olfactory septum in living neonatal animals. S100ß-DsRed transgenic mice were previously generated (Windus et al., 2007; Windus et al., 2010); some S100ß-DsRed mice were crossed against the OMP-ZsGreen transgenic mice.

2.2 Surgical ablation of olfactory bulb and methimazole administration

The olfactory bulb was removed unilaterally in P4.5 mice using a protocol developed in our laboratory as described previously (Chehrehasa et al., 2007, 2008; Chehrehasa et al., 2005; Chehrehasa et al., 2006). This procedure is known as olfactory bulbectomy. Postnatal animals were chosen in preference to adults as the regeneration of olfactory neurons is more rapid and uniform in postnatal animals (Hendricks et al., 1994) and thus the effects of the various models would more readily be detected. The animals were divided in two groups: (1) olfactory bulbectomy and (2) olfactory bulbectomy followed by methimazole (bulbectomy-methimazole) (Fig. 1). In the bulbectomy-methimazole group, the animals were allowed to recover for 4 days after

bulbectomy and then injected with a single dose of methimazole (i.p, 50 mg/kg, 10 mg/ml in PBS) (Fig. 1A).

2.3 OEC and Schwann cell transplantation

To prepare cultures of OECs, axon fascicles (with small amounts of surrounding tissue attached) were isolated from the lamina propria underlying the olfactory epithelium of postnatal day 7 S100ß-DsRed mice (males and females) using fine forceps and purified using an established protocol (Puche and Key, 1996; Tisay and Key, 1999; Windus et al., 2007; Windus et al., 2010). In addition to this protocol we have used OMP-ZsGreen transgenic mice crossed with S100ß-DsRed mice; the ZsGreen fluorescence was used to visualise the primary olfactory axon fascicles to facilitate the dissection and then to confirm that the isolated tissue contained olfactory axon fascicles, rather than other peripheral nerves which would not express ZsGreen. The cultures of the OECs have been previously characterised and routinely have a purity of at least 95% (Windus et al., 2007; Windus et al., 2010). Schwann cells were isolated from dorsal root ganglion. The cells were maintained in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum, G5 supplement (Gibco), gentamicin (Gibco, 50 mg/ml) and L-glutamine (200 μM) at 37°C with 5% CO_2 for 2-3 weeks in wells that had been coated with Matrigel basement membrane matrix (BD Biosciences Australia, North Ryde, New South Wales). One week before transplantation, fetal bovine serum was gradually eliminated and the cells grown in serum-free medium for a few days. Immediately following bulbectomy $1x10^5$ cells (50,000 cells/μl) were injected into the cavity and the access to the cavity was sealed by a small piece of gelfoam (Cat # MS002, Johnson & Johnson Medical limited, UK). Twelve days after transplantation, the animals were sacrificed and processed for

analysis. All animal procedures were carried out with the approval of, and in accordance with, the Griffith University Animal Ethics Committee, and in accordance with the Australian Commonwealth Office of the Gene Technology Regulator and the guidelines of the National Health and medical Research Council of Australia.

2.4 Animal preparation

The mice were killed by cervical dislocation at different time points (Fig. 1A). The heads were fixed by immersion in 4% paraformaldehyde in PBS at room temp for 2-4 hr. Following fixation, they were decalcified in 20% disodium ethylene diaminetetraacetic acid (EDTA) in PBS. The heads were placed in an embedding matrix (O.C.T. compound, Miles Scientific, Naperville, IL) and snap frozen by immersion in iso-pentane that had been cooled with liquid nitrogen. Cryostat sections (30 μm) of the nasal cavity and brain were cut, mounted on to gelatinized slides and stored at –20 °C before processing for immunochemistry.

2.5 Immunohistochemistry

Immunohistochemistry was performed as previously described (Chehrehasa et al., 2007, 2008; Chehrehasa et al., 2009; Chehrehasa et al., 2005; Chehrehasa et al., 2006). Cryostat sections were incubated with following antibodies, polyclonal rabbit anti-p75NTR (1:500; Chemicon, Temecula, CA), polyclonal rabbit anti-S100 (1:400; Dako, Denmark), polyclonal rabbit anti-brain lipid binding protein (BLBP) (1:1000; Millipore Corporation, Billerica, MA), polyclonal anti-GFAP (1:500; Dako, Denmark), and polyclonal anti-Iba1 (1:500; Wako, Japan) and incubated with the secondary antibody Alexa Fluor 647 (1:200; Invitrogen, USA).

2.6 Quantification of regeneration

Within the cavity left by the bulbectomy, OECs and axons were present with OECs being present alone or with axons, depending on the model and stage of regeneration. The first and the last coronal sections of the operated cavity in which OECs or regenerating olfactory axons were detectable were considered the starting and ending points respectively. The length of the region was determined by the number of serial coronal sections between two points, thus for example, the length of axons that entered the cavity was determined by counting the number of sections from the rostral to caudal region of the cavity in which axons were detected. The cross-sectional area of the region of interest was quantified by drawing an area of interest around the ZsGreen-expressing axons that were present within the cavity and using Axiovision software 4.7.2 (Zeiss, Germany) to quantify the region. The regeneration of olfactory sensory neurons was quantified in two regions of olfactory epithelium in several coronal sections from each animal by counting the number of OMP-ZsGreen positive neurons in the field of view using a x20 objective. The number of OECs within the region of the operated cavity was quantified by counting the number of DAPI-stained nuclei co-labelled with S100ß antibody in several regions throughout the cavity.

2.7 OEC migration assay

Peripheral OECs were isolated from the lamina propria underlying the olfactory epithelium of the posterior half of the nasal septum. Fine forceps were used to tease away olfactory nerve fascicles from the lamina propria. The lamina propria tissue was incubated in glass-bottomed 24-well plates coated with Matrigel basement membrane matrix and maintained in DMEM containing 10% fetal bovine serum (P2 tissue), G5 supplement (Gibco), gentamicin (Gibco, 50 mg/ml) and L-glutamine (200 μM) at 37

°C with 5% CO_2 for 3-5 days. Explants of olfactory mucosa including the olfactory epithelium and LP were dissected from embryonic day 14 mice using with either OMP-ZsGreen x S100ß-DsRed mice or OMP-ZsGreen mice. The explants from OMP-ZsGreen x S100ß-DsRed mice were directly plated into glass-bottomed 24-well plates coated with Matrigel; the explants from OMP-ZsGreen mice were plated onto monolayers of DsRed-OECs purified from S100ß-DsRed mice and maintained in the same medium as described above. Axon outgrowth occurred within 24 hr of plating and images were taken after 48 hr. During imaging, culture plates were maintained at 37 °C in an incubator chamber with 5% CO_2. Time-lapse image sequences of primary OEC and olfactory neuron cultures were collected and analysed with Axiovision Rel 4.6.3 (Zeiss, Germany). The migration rate of any given cell was calculated by using the distance measurement tool to trace the total distance travelled by the cell nucleus over the total recording period. Similarly, axon length was calculated using the distance measurement tool.

2.8 In vitro axon extension assay

Peripheral OECs were isolated from the lamina propria underlying the olfactory epithelium and Schwann cells were isolated from the trigeminal nerve of S100ß-DsRed mice; thus both the OECs and trigeminal Schwann cells expressed DsRed. The tissues were separately dissociated and underwent fluorescence activated cell sorting to give rise to cultures with at least 99% purity. The OECs and Schwann cells were seeded at the same cell density in 24-well plates that had been coated with Matrigel. Explants of olfactory epithelium were dissected from postnatal day 1 OMP-ZsGreen mice and were plated on to either the monolayers of the DsRed-OECs or DsRed-Schwann cells or on to wells coated with Matrigel alone as control. The explants were

cultured in medium containing Neurobasal medium (Gibco Invitrogen Corporation) supplemented with 0.4% methyl cellulose (BDH Chemical Ltd), 1x B27 serum-free supplement (Gibco Invitrogen Corporation), 0.8 mM L-glutamine (Gibco Invitrogen Corporation), 10 mM HEPES and 5 μg/ml gentamycin (Gibco Invitrogen Corporation) at 37 ^{o}C and 5% CO_2. Axon outgrowth occurred within 24 hr of plating; axons were imaged at that time point using a Spot 2 digital camera (Spot Diagnostic Instruments, Inc., Sterling Heights, MI) mounted on an Olympus IX70 inverted microscope and the length of axons was measured using the Spot software.

2.9 Statisticial analysis

ANOVA followed by Tukey (HSD) or in some cases a two tailed t-test were used to compare all-Pairwise samples. All data are expressed as mean ± S.E.M.

2.10 Image capture and image preparation

Images of live explant tissue were collected on a Zeiss AxioImager Z1 with an Axiocam MRm digital camera using Axiovision software (Zeiss, Germany) and Zeiss EC Plan-NeoFLUAR 20/0.75 x 20 objectives. Images of immunostained sections were captured using an Axio Imager Z1 epi-fluorescence microscope with Apotome and an Axiocam MRm camera (Carl Zeiss, Germany) or by confocal laser scanning microscopy (Olympus FV10-MCPSU, Germany). Figures were compiled in Adobe Photoshop 7.0 and Adobe Illustrator 10.0 (Adobe Systems Incorporated).

3. Results

3.1 The combination of bulbectomy and methimazole delays olfactory axon growth

In order to determine the effect of OECs on axon growth, we set out to develop a model in which axon growth would be delayed while the OECs from the peripheral region of the olfactory nerve would be able to populate the cavity left after bulbectomy. During normal development of early postnatal animals, primary olfactory axons extend into the olfactory bulb together with migrating OECs, with some of the axons having reached their targets in the glomerular layer, while others are still growing along the olfactory nerve (Fig. 1A). In comparison, when unilateral bulbectomy is performed in neonatal mice, the neurons whose axons have already reached the olfactory bulb die off and are rapidly replaced by new neurons that arise from stem cells lining the basal layer of the olfactory epithelium. The numerous axons that were still in the process of extending to the bulb at the time of bulbectomy, however, continue their growth and the OECs that are present along the olfactory nerve are stimulated to enter the cavity left by the bulbectomy. Thus in the bulbectomy-only model, the axons extend together with the OECs that enter the cavity (Fig. 1B). In order to establish an olfactory neuron regeneration model in which OECs could enter the cavity before the extension of olfactory axons, we combined unilateral bulbectomy followed 4 days later by administration of methimazole (Fig. 1C). The administration of methimazole leads to the degeneration of the apical layer of the olfactory epithelium including the primary olfactory neurons and supporting cells (Brittebo, 1995). However, the OECs which are present along the peripheral olfactory nerve are unaffected and similar to the bulbectomy-only model

they enter the cavity left by the bulbectomy. Several days later the apical layer, including the neurons, regenerates from stem cells lining the basal layer. Thus in this model with dual periods of neuronal death, the bulbectomy provides the cavity for OECs to populate while the second wave of neuronal death caused by the methimazole provides time for the OECs to enter the cavity without the axons (Fig. 1D). To allow for easy visualisation of primary olfactory sensory axons, we used neonatal OMP-ZsGreen transgenic mice, in which the OMP promoter drives expression of the bright fluorescent protein ZsGreen in primary olfactory neurons (Fig. 1E). In the bulbectomy-only model, the regeneration of the neurons was rapid and by 8 days after bulbectomy the olfactory epithelium contained numerous neurons and axons observed entering the lamina propria (Fig. 1F). In contrast, the combined approach of bulbectomy with methimazole delayed the regeneration of the epithelium as expected (Fig. 1G-J). Five days after bulbectomy and one day after injection of methimazole, ZsGreen expression on the bulbectomized side of the olfactory epithelium revealed a dramatic loss of receptor neurons compared to control, with the majority of cells of the olfactory epithelium having been detached from the olfactory mucosa (Fig. 1G). By day 3-5 after methimazole injection, the olfactory epithelium became thinner and more disrupted, with only olfactory nerve bundles remaining in the lamina propria (arrow, Fig. 1H,I). It should be noted that the coral protein ZsGreen is particularly stable and is retained for several days in macrophages and other cells that have phagocytosed the axon debris. Thus ZsGreen fluorescence is detectable even though the primary olfactory neurons are completely absent from the olfactory epithelium. Eight days after methimazole treatment, olfactory sensory neurons had regenerated and were present in the olfactory epithelium, however the number of new neurons in these animals was significantly less than the animals which

had experienced only bulbectomy-only (32% fewer neurons, $p<0.01$; Fig. 1J, Fig. 4N). The results confirmed that the bulbectomy-methimazole model delayed the regeneration phase of olfactory sensory neurons by around 4-5 days.

We next examined the cavity left by the olfactory bulbectomy to determine whether the delayed axon growth affected the rate at which OECs filled the cavity and the subsequent axon extension. We have previously shown that following bulbectomy in neonatal animals, olfactory axons and OECs take 2-3 days to enter the cavity (Chehrehasa et al., 2006) and therefore we examined mice at 12 days post bulbectomy since there should be considerable regeneration by this time. Primary olfactory axons were easily visualised using ZsGreen fluorescence and OECs were visualised by immunolabelling with brain lipid binding protein (BLBP) (Murdoch and Roskams, 2007, 2008). Twelve days after bulbectomy in bulbectomy-only animals, the OECs together with the axons had entered the cavity left by bulbectomy and occupied the peripheral regions of the rostral half of the cavity (Fig. 2A,C,D). Neither the OECs nor the axons were present in the central region of the cavity (asterisk, Fig. 2A). The axons were present in loose fascicles within the cavity and formed indistinct structures resembling glomeruli (Fig. 2D). In the bulbectomy-methimazole animals on the other hand, the OECs filled the entire cavity left by the bulbectomy (Fig. 2B,E). While there were some differences in the distribution of the OECs within the cavity, the OECs were clearly present in all regions (Fig. 2E). In contrast to the bulbectomy-only model, axons in the bulbectomy-methimazole animals were present in the central region as well as in the peripheral region of the cavity (Fig. 2B,F). Although the OEC distribution was more extensive than that of the axons, it was clear that axons were present in many more regions than in the bulbectomy-only model (Fig. 2C-F). Of

particular interest was the arrangement of the axons in the bulbectomy-methimazole animals in comparison to the bulbectomy-only model. In bulbectomy-methimazole mice, the axons formed distinct fascicles that terminated in tight glomerular-like structures within the central region of the cavity (Fig. 2F); this was clearly different from the less organised arrangement of axons within the bulbectomy-only animals (Fig. 2D).

3.2 OECs are present ahead of axons

As it was apparent that the distribution of OECs in the bulbectomy-methimazole animals was more extensive than the axons at 12 days post bulbectomy, we examined earlier stages to track the distribution of the OECs in both the bulbectomy-only and bulbectomy-methimazole animals. At 7 days post bulbectomy in the bulbectomy-only animals, the OECs and axons were present together within the ventral region of the cavity (Fig. 2G). The distribution of the OECs in relation to the axons confirmed that OECs were always slightly more widespread than that of the axons such that axons were always surrounded by OECs (arrows, Fig. 2G). This indicates that OECs in the bulbectomy-only model were in the process of migrating ahead of the axons at the time of the analysis. In stark contrast, at earlier time points in the bulbectomy-methimazole animals, the OECs were present in the throughout the cavity while the axons were largely absent (Fig. 2H-J). At 5 days post bulbectomy and 1 day post methimazole, very few axons were present in the cavity (Fig. 2H-I). By 7 days post bulbectomy and 3 days post methimazole, the OECs formed an extensive mass of cells into which the axons were beginning to penetrate (Fig. 2J). With increasing time after methimazole treatment the newly extending axons clearly penetrated the mass of OECs (Fig. 3C,F), but at all time points and in all regions of the cavity, OECs were

always detected deeper to the axons indicating that as expected, due to the delayed axonal growth, OECs were present ahead of axons. Thus these results demonstrate that in the bulbectomy-only model, OECs are present together with axons with the OECs forming a slightly more widespread distribution than the axons. In contrast, the combined bulbectomy-methimazole treatment resulted in the OECs filling the cavity well ahead of the arrival of the axons due to the delayed regeneration of the neurons.

We next verified that the vast majority of cells within the cavity were OECs. Immunolabeling of the bulbar cavity by p75NTR (Fig. 3A-C) and S100β (Fig. 3D-F) antibodies to detect mature OECs confirmed that OECs were the dominant cell type in the cavity. To exclude the presence of other glial cells within the cavity, we immunolabelled adjacent sections of the bulbar cavity with anti-Iba1 and anti-GFAP antibodies, which label microglia/macrophages and astrocytes, respectively. Although GFAP is a marker of OECs in rat (Li et al., 2005; Liu et al., 2005), in mouse, GFAP is not expressed by OECs in the outer nerve fibre of olfactory bulb in which S100 and p75NTR positive OECs were detectable (Au et al., 2002; Hisaoka et al., 2004). This was similar in our transgenic mice. Anti-Iba1 and anti-GFAP antibodies revealed that although a small proportion of cells within the cavity were microglia, macrophages and astrocytes (Fig. 3H,I), the majority of cells were OECs that expressed p75NTR (Fig. 3G). These results were also confirmed at different time points after methimazole treatment (data not shown).

3.3 The distribution of OECs is more extensive when axon growth is delayed

We considered that the more extensive region over which the OECs were present in the bulbectomy-methimazole animals could have been due to either a higher density

of OECs being present or a faster migration rate of the OECs, allowing them to spread over a larger area than in the bulbectomy-only model. We therefore quantified the overall density of OECs within the cavity by counting the number of DAPI-stained nuclei of S100ß-positive OECs per unit area. We found that the density of OECs in bulbectomy-methimazole animals was significantly less than the density of OECs in the cavity of bulbectomy-only animals (Fig. 3J-L). However as the region over which OECs and axons were present was greater in the bulbectomy-methimazole animals than the bulbectomy-only animals (Fig. 4M), the overall number of OECs in both animals was similar and not significantly different. This indicates that the larger volume occupied by OECs in the bulbectomy-methimazole model in comparison to the bulbectomy-only model was due to increased spread of the OECs rather than an overall increased number of OECs.

The well structured arrangement of the axons in the rostral region of the cavity in the bulbectomy-methimazole animals (Fig. 2B) led us to further examine the distribution of axons throughout the cavity in animals 12 days post bulbectomy. In bulbectomy-only animals, olfactory axons did not project into the very caudal region of the cavity such that at the plane equivalent to the accessory olfactory bulb on the control side axons were never seen on the bulbectomy side (Fig. 4A). However, in the bulbectomy-methimazole animals, axons extended to the plane equivalent to the accessory olfactory bulb (Fig. 4B). Thus these results suggest that not only did the bulbectomy-methimazole treatment result in a more structured arrangement of axons, but also that the distribution of axons was more extensive in comparison to the axon growth in bulbectomy-only animals. As the axons in the bulbectomy-only model were limited to areas where OECs were present, it appeared that the olfactory axons

required contact with OECs for their growth and that secreted molecules alone were insufficient to stimulate their growth. In addition, we speculated that the superior axon growth in the bulbectomy-methimazole model was due to the arrangement and distribution of the glial cells rather than simply the presence of higher numbers of glia cells relative to axons at the time that axons penetrate the cavity. This was tested by transplanting purified DsRed-fluorescent OECs into the bulbectomized cavity of OMP-ZsGreen mice immediately after bulbectomy. After 12 days, the transplanted OECs (identifiable because they expressed DsRed) were present in regions throughout the cavity and intermingled with the new growing axons. In particular, the transplanted OECs were present in the rostral region of the cavity close to the cribriform plate (Fig. 4C) as well as caudally where they were in close association with the axons (Fig. 4D,E). The transplanted OECs mingled with the endogenous OECs with up to 50% of the OECs being transplanted cells in some areas (Fig. 4F-G). However, the distribution of the transplanted OECs was inconsistent, with the OECs being concentrated in small patches that were not in contact with each other. Thus in the bulbectomy-methimazole animals the endogenous OECs were distributed throughout the cavity with considerable cell-cell contact, whereas in the OEC transplant animals the transplanted OECs were unevenly distributed. The axon growth in the OEC transplanted animals was indistinct with the fascicles being loosely defined and the glomeruli being difficult to discern (Fig. 4C,D). Instead, the distribution of the axons was similar to that seen in bulbectomy-only animals (Fig. 2A,D) and was clearly different from the distinct arrangements of axons seen in bulbectomy-methimazole animals (Fig. 2B,F).

Considering that in OEC cultures contaminating Schwann cells can sometimes be present or that endogenous Schwann cells could enter the bulbectomy cavity following surgery (Kawaja et al., 2009), we determined whether the axon growth was different if Schwann cells were transplanted instead of OECs. We therefore transplanted DsRed fluorescent Schwann cells (SC) purified from S100β-DsRed mice (Fig. 4H-K). In contrast to OECs, 12 days after transplant the SCs were not present in the rostral region of the cavity near the cribriform plate where the axons entered the cavity (Fig. 4H). Instead the transplanted SCs were restricted to the external regions of regenerating axons in the caudal part of the cavity (Fig. 4I-K). However, it was still apparent that the transplanted SCs were permissive for axon growth and that the extent of axon growth was enhanced in comparison to the bulbectomy-only animals.

3.4 Axon extension is increased by the presence of a larger OEC environment

We quantified the distribution of regenerating axons within the cavity of the different models by measuring the rostral-caudal length and the cross-sectional area in the coronal plane of the region in which the axons were present. The axons in bulbectomy-methimazole, Schwann cell and OEC-transplanted animals all projected significantly deeper into the cavity left by the bulbectomy than in the bulbectomy-only model ($p<0.01$ OBX-M; $p<0.01$ OEC-tr; $p<0.05$ SC-tr: Fig. 4L), but were not significantly different from each other. However, the cross-sectional area of the region occupied by the axons was significantly greater in the bulbectomy-methimazole animals compared to all other treatments ($p<0.05$; Fig. 4M). Considering that the bulbectomy-methimazole animals had significantly fewer neurons within the olfactory epithelium compared to bulbectomy-only animals (Fig. 4N), these results

demonstrate that despite this, these axons extended over a greater volume of the cavity in comparison to axons in bulbectomy-only animals.

As the bulbectomy-methimazole model resulted in OECs initially migrating in the absence of axons, we hypothesised that the migration of OECs is greater in the absence of axons. We tested this using an in vitro OEC migration assay. Explants of embryonic olfactory epithelium were obtained from OMP-ZsGreen x S100β-DsRed mice. OECs in the S100β-DsRed mice express the bright red fluorescent protein DsRed (Windus et al., 2010), whereas the axons express green fluorescent protein ZsGreen. In these explants, olfactory axons migrated out from restricted regions of the external edges of the explant whereas OECs migrated out uniformly around the explant and often in the absence of axons (Fig. 5A,B). Therefore we measured the rate of migration for OECs that were migrating with axons (unfilled arrowhead, Fig. 5A) or without axons (filled arrowhead, Fig. 5A). We found that in the absence of axons, OECs migrated significantly faster than OECs in close association with axons (Fig. 5C) and therefore the OECs that were not in contact with axons were more dispersed than OECs that were in contact with axons (compare arrow vs arrowhead, Fig. 5B). Hence, these results confirm that OEC migration is more rapid in the absence of axons.

In the bulbectomy-methimazole animal model, the regenerating axons grew more extensively despite their being fewer axons. Therefore this raised the possibility that axons grow better when there is an established OEC environment. We tested this hypothesis in vitro by comparing axon outgrowth when olfactory epithelium explants were plated with, or without, an existing monolayer of OECs. In the absence of the

existing OEC monolayer, the axons extend out with the limited number of migrating OECs (Fig. 5F) whereas when plated on a monolayer of OECs, the out-growing axons immediately contact an extensive OEC environment (Fig. 5G). In this assay, the outgrowth of olfactory axons was significantly more extensive when grown over the pre-existing monolayer of OECs ($p<0.05$, Fig. 5D). These results are therefore consistent with bulbectomy-methimazole results which indicate that enhanced axon growth occurs when a permissive environment of OECs is present.

While our preparations of DsRed OEC cultures have been previously thoroughly characterised (Windus et al., 2007; Windus et al., 2010), it remains possible that contaminating Schwann cells could sometimes be present as has been reported in other studies (Kawaja et al., 2009). We therefore determined whether the growth of olfactory axons on a monolayer of Schwann cells was similar to the growth on a monolayer of OECs. As the trigeminal nerve is a possible source of contaminating Schwann cells for OEC cultures prepared from the peripheral olfactory nerve, we therefore prepared FACS purified cultures of Schwann cells dissected from the region of trigeminal nerve which was clearly anatomically separate from the olfactory epithelium and therefore could not contain OECs. We cultured explants of olfactory epithelium from OMP-ZsGreen mice with a monolayer of either trigeminal Schwann cells or OECs purified from S100β-DsRed mice (Fig. 5E), or on Matrigel-coated wells without a monolayer of glial cells. We measured the length of outgrowing axons 24 hr after plating (Fig. 5A) and found that the outgrowth of olfactory axons was significantly more extensive when grown over the monolayer of OECs ($p<0.01$, Fig. 5E) when compared to axons grown on Schwann cells or Matrigel-coated wells. In

particular, the presence of the monolayer of trigeminal Schwann cells did not alter the extension of olfactory axons in comparison to axons grown on Matrigel-coated wells.

4. Discussion

In the present study we have shown that olfactory axon growth after bulbectomy was dramatically enhanced when a permissive glial environment was present prior to axon growth. Delaying neuron regeneration resulted in OECs filling the cavity and forming an extensive glial environment in which subsequent axon growth was more widespread.

Our results have shown that the presence of the permissive glial environment not only dramatically enhanced the growth of olfactory axons, but also resulted in axons projecting in well-formed fascicles and terminating in distinct glomerular-like structures (Fig. 6C). In comparison, in bulbectomized animals where axonal growth was not delayed, there was limited growth of axons (Fig. 6A) and while the axons did form some fascicles and glomerular-like structures they were less distinct than in the animals with bulbectomy-methimazole. Thus, delay of axonal growth after bulbectomy resulted in a phenotype that more closely resembled an uninjured animal than bulbectomy alone. It should be noted that the bulbectomy removes the entire olfactory bulb including the mitral/tufted cells and thus while the axons form "glomerular-like structures", the second order neurons are not present. This demonstrates that the signals for axons to condense and form balls of neuropil are at least partially independent of mitral/tufted cells. As the formation of the glomerular-like structures was more distinct in the bulbectomy-methimazole animals, it suggests that the presence of the OECs contributes to the successful formation of glomeruli.

During development of the olfactory system, olfactory sensory neurons and OECs arise from the olfactory placode (Doucette, 1990; Farbman and Squinto, 1985) and maintain a close physical relationship as they project toward the telencephalon (Doucette, 1990). It has been shown that the OECs migrate ahead of the axons (Tennent and Chuah, 1996) and thus it would appear that the growth of olfactory axons is reliant on the OECs. Since that study was published, there have been no other reports on whether OECs always maintain a lead over the axonal growth cones, or whether the migration pattern of OECs in relation to olfactory axons varies depending on the milieu in the primary olfactory nervous system. In the both the bulbectomy-only and bulbectomy-methimazole models, the distribution of OECs was always more widespread than that of the axons. While OECs were observed without the presence of axons, the reverse was never observed and axons were always observed with surrounding OECs. These results demonstrate that the OECs were more widespread than the axons during regeneration in these models.

We determined that in the delayed axon growth model that the distribution of OECs and the extent of axonal growth were both enhanced when OECs were present ahead of the axons, strongly suggesting that for optimal axonal growth, this scenario is favourable. However, it is not merely the presence of OECs ahead of axons that is important for improved axon growth. While transplantation of OECs into the bulbar cavity increased the extent of axon growth into the cavity (Fig. 6B) it was not as great as in bulbectomy-methimazole animals and the formation of fascicles and glomeruli were not as distinct. The transplanted OECs aggregated in patches that were in close association with the olfactory axons, but the transplanted OECs did not establish continuous cell-cell interactions with each other over a large area. In contrast, in the

bulbectomy-methimazole treated mice the endogenous OECs filled the bulbar cavity where they formed an extensive mass of cells. As OECs have previously been shown to undergo contact-mediated migration (Cao et al., 2007; Windus et al., 2007), it is likely that the in the bulbectomy-methimazole treated mice the OECs underwent contact-mediated migration or proliferation to create the extensive OEC environment. In contrast, the transplanted cells were likely to have been unable to establish sufficient proximity to each other as a single group within the bulbar cavity and instead migrated in separated groups. These results indicate that it is not only the presence of OECs over a larger area that is beneficial to axon growth, but that it is important for the OECs to form an environment with continuous cell-cell contact. In vitro, we have observed that primary olfactory axons always follow OECs and that increasing the migration rate of OECs leads to faster axon extension as the axons follow the OECs (St John lab, unpublished data). It is therefore possible that the same OEC-axon relationship exists in vivo which leads to the superior axon growth when a extensive OEC environment is available.

It is possible that the permissive glial environment formed after bulbectomy could have contained Schwann cells since they proliferate and migrate in response to CNS injury (Lisak et al., 2006). In addition, Schwann cells can potentially contaminate OEC cultures (Kawaja et al., 2009) and since they are phenotypically very similar to OECs it is at present not possible to definitively verify that Schwann cells are not present within OEC cultures. We did however address this by transplanting Schwann cells into the cavity and while axon growth was improved, it was clear that superior growth and closer interaction with axons were achieved when OECs were present. We further verified that the cultures of OECs were unlikely to contain large amounts of

Schwann cells by demonstrating that the growth of primary olfactory axons on monolayers of OECs is significantly greater compared to axons that are grown on monolayers of Schwann cells from the trigeminal nerve. In particular the results showed that trigeminal Schwann cells offered no advantage to axon growth compared to when axons were grown over the matrix provided by coating the wells with Matrigel. These in vitro axon assay results are therefore consistent with the axon growth that was observed in the Schwann cell and the OEC-transplanted animals in which axon growth was superior when transplanted OECs were present within the cavity left from bulbectomy. While our results do not preclude that Schwann cells were not present, is unlikely that they were present in large proportions within the cavity. Similarly, other cells such as fibroblasts could also have been present, but as the vast majority of cells were S100 positive (see Fig. 3K), fibroblasts would have been present at a very low percentage at best. Hence while cells other than OECs may have been present, they are unlikely to have played a major role.

The ability of the OECs to rapidly respond to the bulbectomy is of interest. It has previously been reported that when olfactory nerve transection is performed, the OECs do not respond dramatically but instead remain in place and continue to provide conduits for the new growing axons (Li et al., 2005). However, following bulbectomy when the inhibitory cues or physical barriers which restricted OEC movement or proliferation were removed, the OECs rapidly responded by filling the now vacant space. Thus it is clear that OECs are not merely providing static support for axons, but are instead able to dynamically respond to a changing environment with the responses varying depending on the situation. Our results have shown that the region that the OECs occupied in the cavity was considerably larger when axon growth was delayed,

suggesting that OEC occupied the cavity more rapidly in the absence of axons. To support this, we confirmed that OEC migration in vitro was faster in the absence of olfactory axons. The ability of OECs to migrate more rapidly in the absence of axons is similar to the response of Schwann cells following peripheral nerve injury. It has been shown that the migration of Schwann cells was significantly increased when a pre-denervated nerve graft which contained Schwann cells, but not axons, was provided (Tomita et al., 2009). In this situation the Schwann cells encountered a glial environment that was free of axons and it is therefore possible that the increased migration of Schwann cells occurred, at least partially, in a similar manner to the OECs in which higher migration occurred in the absence of axons. While our in vitro data demonstrates that OEC migration is faster in the absence of axons and we have previously shown that OECs undergo contact mediated migration (Windus et al., 2007), is unclear whether the same occurs in vivo. It is possible that the OECs filled the cavity by either active migration or by passive mechanisms as the OECs proliferated. We will determine the active and passive mechanisms by which OECs become distributed in future work.

In spinal injury transplant therapies, OECs have been introduced as cell suspensions or in matrices into the injury site where they disperse and integrate with the host tissue (Boruch et al., 2001; Ramon-Cueto and Nieto-Sampedro, 1994; Sasaki et al., 2004). However, unfortunately they do not maintain a high degree of cell-cell contact with each other and therefore do not form a continuous mass. Instead the OECs tend to be interspersed amongst other cells and remain in close association with axons (Ruitenberg et al., 2002; Sasaki et al., 2004). Our results in the olfactory bulbectomy+methimazole model demonstrated that in the absence of axons the OECs

rapidly formed an extensive mass and that the presentation of OECs in this format resulted in subsequent superior axon growth. It would therefore be of interest to examine whether the delivery of OECs that would encourage their migration as a extensive mass within spinal transplant models would result in improved axon growth.

4.1. Conclusions

In summary, we have shown here that temporarily delaying olfactory axon growth enabled OECs to respond rapidly and form a large permissive glial environment which subsequently resulted in enhanced axon growth. Together these data demonstrate that olfactory axon growth is dependent on the extent of the distribution of OECs and strongly suggest that a situation where the presence of OECs ahead of olfactory axons en route to the bulb is required for axonal growth and guidance.

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7. Figure legends

Figure 1. Bulbectomy combined with methimazole treatment delays regeneration of olfactory sensory neurons. A: In the normal olfactory nerve of postnatal mice, OECs ensheathe bundles of olfactory sensory axons throughout the nerve; mature axons terminate in glomeruli within the olfactory bulb (OB) while immature axons grow along the nerve. OE, olfactory epithelium. B: Olfactory bulbectomy (OBX) leads to the death of mature olfactory sensory neurons which are then regenerated from stem cells in the olfactory epithelium. Immature neurons whose axons have not reached the olfactory bulb continue to grow towards the bulb. The axons and OECs then grow into the cavity left by bulbectomy. C: To delay axon growth, unilateral olfactory bulbectomy was performed on postnatal pups and a single injection of methimazole was given 4 days later; animals were harvested at different time points. D: In combined bulbectomy-methimazole animals (OBX-M), the methimazole treatment results in a second wave of neuronal death and leads to a delayed regeneration of olfactory neurons. Thus OECs migrate into the cavity without axons; axons subsequently regenerate. E-J: Coronal sections through the olfactory epithelium of neonatal mice, with nasal cavity to the right. All are OMP-ZsGreen mice whose olfactory sensory neurons are green; cell nuclei are stained with DAPI (blue). E: control animal; F: bulbectomy-only animal; G-J: bulbectomy-methimazole animal (e.g. 5 days after bulbectomy, 1 day after methimazole = 5dOBX, 1dM). E: In control olfactory epithelium, cell bodies of olfactory sensory neurons (green) occupy the middle compartment of olfactory epithelium; they send their dendrite to the nasal cavity (NC) and the axons enter the lamina propria (LP) where they fasciculate together (arrow). F: Eight days after bulbectomy, the olfactory sensory neurons had

regenerated and axons (arrow) were present leaving the basal layer of the epithelium. G: Five days after bulbectomy and one day after methimazole injection, olfactory sensory neurons were degenerating with numerous cells (punctuate nuclei in NC) having detached from the epithelium and shed into the nasal cavity (NC). H-I: Three to five days after methimazole treatment, no OMP-ZsGreen sensory neurons were evident, with only cellular debris (green) being present. Some ZsGreen protein was detected in olfactory nerve bundles within the lamina propria (arrow). J: Eight days after methimazole treatment, olfactory sensory neurons had regenerated although there were fewer neurons compared to the animals with bulbectomy-only treatment (F). Scale bar is 30 μm.

Figure 2. OEC distribution is more widespread in the absence of axons. Panels are coronal sections through the olfactory bulb (OB) of control side (left) and bulbar cavity (right) of operated animals 12 days after bulbectomy; OECs were immunolabelled with anti-BLBP (magenta); axons expressed ZsGreen. A: Following bulbectomy alone (OBX), OECs (magenta) and axons (green) were present around the periphery of the rostral region of the cavity left by bulbectomy. B: In the combined bulbectomy-methimazole treatment (OBX-M) animals, OECs and olfactory axons filled the rostral region of the cavity, the co-localization of olfactory axons and OECs were dominated by the very bright green axons in some areas. C-F: Higher magnification of single labels of the rostral regions of the cavity; C-D are bulbectomy-only animals; E-F are bulbectomy-methimazole animals. E: The olfactory axons in bulbectomy-only animals did not form distinct fascicles (arrow) and irregular shaped glomerular-like structures were present (arrow with tail). F: in bulbectomy-methimazole animals the axons formed distinct fascicles (arrow) and glomerular-like

structures (arrow with tail). G: 7 days following bulbectomy, OECs and axons were present together in the cavity. OECs always surrounded axons (arrows). H: 1 day after methimazole treatment (5 days OBX), OECs were distributed throughout the cavity and not confined to the area of olfactory axons. The medial-ventral region of the control olfactory bulb (OB) is partially in view at the left; the olfactory epithelium (OE) is at the bottom. I: A higher magnification of (H). J: The olfactory axons had stalled 3 days after methimazole treatment, but OEC migration had continued. Arrow indicates direction of OEC migration. Scale bar is 630 μm in A-B; 170 μm in C-F; 520 in H; 210 μm in G, I-J.

Figure 3. OECs filled the cavity left by bulbectomy. Panels are higher magnification views of coronal sections through the bulbar cavity, with dorsal to the top and lateral to the right. All are OMP-Zsgreen mice whose olfactory sensory neurons are green. A: Immunolabeling of the bulbar cavity by p75NTR (magenta, A-C) and S100β (magenta, D-F) antibodies confirmed that OECs had filled the cavity by 3 days after methimazole treatment. C,F: the olfactory axons had projected through the OECs by 6 days after methimazole treatment. G-I: Immunolabelling of adjacent sections with p75 (magenta, G), GFAP (magenta, arrow, H) and Iba1 (magenta, arrow, I) antibodies revealed that the cavity primarily contained p75-positive OECs with only a small proportion of glial cells being microglia and astrocytes. J: OECs labelled by S100β antibody in bulbectomy-only animals; K: OECs in bulbectomy-methimazole treated animals. L: There were significantly less OECs per unit area in bulbectomy-methimazole compared to bulbectomy-only animals ($p<0.01$). Nuclei are stained with DAPI. Scale bar is 40 μm in D-F, J-K; 75 μm in A-C, G-I.

Figure 4. Axon extension is greater in bulbectomy-methimazole animals. Panels are coronal sections through the bulbar cavity of operated animals. A: in bulbectomy-only (OBX) animals, axons did not extend to the caudal region of cavity and were never observed at the level of the accessory olfactory bulb (AOB) on the control unoperated side. B: in bulbectomy-methimazole (OBX-M) animals, axons (arrow) extended to the level of the AOB. C-D: in animals that received OEC transplant following bulbectomy, OECs were present throughout the cavity including the rostral cavity (C) and caudal cavity (D) however the distribution of transplanted OECs was not continuous. E: Higher magnification view of axons integrating with the transplanted OECs. F-G: The distribution of anti-S100 antibody staining (magenta) was more widespread than DsRed fluorescent protein expression (red; single label shown in G) indicating that the transplanted DsRed OECs mingled with endogenous host OECs; axons are green. H: 12 days after transplantation of Schwann cells following bulbectomy, the Schwann cells were not present in the rostral region of cavity. I: Schwann cells did not interact with axons in some regions of the caudal cavity. J-K: higher magnification views of SCs in the caudal cavity shows axons (green) in addition to the SCs (red) of the same section as shown in I. Nuclei are stained with DAPI (blue). Scale bar is 630 μm in A-B; 150 μm in C, H; 250 μm in D, I; 100 μm in E, J, K; 50 μm in F, G. L: The olfactory axons in OBX-M, the Schwann cell transplanted (SC-tr) and the OEC transplanted (OEC-tr) animals projected significantly deeper into the cavity in comparison to OBX animals (** $p<0.01$ for OEC-tr and * $p<0.05$ for SC-tr ; Tukey HSD). M: The olfactory axons in the OBX-M animals projected significantly deeper into the cavity left by the OBX (* $p<0.01$) in comparison to OBX and OEC and SC cells transplant animals. N: There were

significantly fewer neurons within the olfactory epithelium of OBX-M mice compared to OBX animals (** $p<0.01$).

Figure 5. OECs migrate faster without axons in vitro. A: Olfactory sensory neurons in explants of mouse olfactory epithelium from OMP-ZsGreen x S100ß-DsRed mice extend axons (green, arrow) over the surface of OECs (red, unfilled arrowhead). S100ß-DsRed positive OECs were also found to migrate out from the explant tissue and across the matrix without being accompanied by olfactory axons (filled arrowhead). B: single label image of OECs of the same field of view as shown in (A); OECs in contact with axons (arrow) were at higher density than OECs that were not in contact with axons (arrowhead). C: Quantification of the migration rate of OECs in the absence or presence of axons in vitro; (n= 23); * $p<0.05$, two-tailed t-test. D: Quantification of the average axon length of olfactory neurons when grown from an explant or on a pre-existing monolayer of OECs; (n= 24-27); ** $p<0.01$, two-tailed t-test. E: Quantification of the average axon length of olfactory neurons when grown from an explant on wells coated with Matrigel alone (Matri) or with a monolayer of OEC or SCs; (n= 41-46); ** $p<0.01$ for OEC; Tukey HSD). F: When grown from a single explant, olfactory axon extension (arrow) was restricted to the region occupied by the limited number of OECs (unfilled arrowhead) that migrated out of the explant. G: In comparison olfactory axon extension (arrow) was significantly increased when the explant was plated on a pre-existing monolayer of S100ß-DsRed positive OECs (unfilled arrowhead). Scale bar is 40 μm in A-B, 20 μm in F-G.

Figure 6. Improved OEC migration leads to enhanced axon growth. Bulbectomy leads to death of olfactory sensory neurons followed by regeneration of new neurons in the

olfactory epithelium. A: In bulbectomy-only mice, OECs and axons entered the cavity together. B: In bulbectomy treated mice combined with OEC transplantation into the bulbar cavity, OECs were aggregated in patches; axon growth was more wide spread than in bulbectomy-only mice. C: In bulbectomy-methimazole treated animals, there was a delay in the regeneration of olfactory sensory neurons and the OECs entered the cavity without axons. OEC distribution was more extensive and subsequent axon growth was enhanced despite their being fewer neurons.