Differing phagocytic capacities of accessory and main olfactory ensheathing cells and the implication for olfactory glia transplantation therapies

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Abstract

The rodent olfactory systems comprise the main olfactory system for the detection of odours and the accessory olfactory system which detects pheromones. In both systems, olfactory axon fascicles are ensheathed by olfactory glia, termed olfactory ensheathing cells (OECs), which are crucial for the growth and maintenance of the olfactory nerve. The growth-promoting and phagocytic characteristics of OECs make them potential candidates for neural repair therapies such as transplantation to repair the injured spinal cord. However, transplanting mixed populations of glia with unknown properties may lead to variations in outcomes for neural repair. As the phagocytic capacity of the accessory OECs has not yet been determined, we compared the phagocytic capacity of accessory and main OECs in vivo and in vitro. In normal healthy animals, the accessory OECs accumulated considerably less axon debris than main OECs in vivo. Analysis of freshly dissected OECs showed that accessory OECs contained 20% less fluorescent axon debris than main OECs. However, when assayed in vitro with exogenous axon debris added to the culture, the accessory OECs phagocytosed almost 20% more debris than main OECs. After surgical removal of one olfactory bulb which induced the degradation of main and accessory olfactory sensory axons, the accessory OECs responded by phagocytosing the axon debris. We conclude that while accessory OECs have the capacity to phagocytose axon debris, there are distinct differences in their phagocytic capacity compared to main OECs. These distinct differences may be of importance when preparing OECs for neural transplant repair therapies.

Key words: Olfactory nerve; neuron; apoptosis; bulbectomy; axon, phagocytosis

Introduction

The olfactory system is characterised by its ability for the sensory neurons to regenerate continuously throughout life in the normal healthy animal as well as after injury and disease. Olfactory ensheathing cells (OECs) are the glia of the olfactory nerves and are intimately associated with the axons of the sensory neurons that line the nasal cavity. The OECs do not myelinate individual axons, but instead ensheathe large bundles of numerous axons. OECs are considered crucial for the regenerative ability of the olfactory system because they support the growth and guidance of axons by their expression of numerous molecules (Boruch et al., 2001; Chuah and Zheng, 1992; Doucette, 1990; Kafitz and Greer, 1999). Due to the continual turnover of olfactory sensory neurons, there is a constant need for removal of the debris arising from degraded axons. Studies have shown that OECs, rather than macrophages, are the main phagocytic cells responsible for the removal of cellular debris throughout development (Nazareth et al., 2015), in the adult (Su et al., 2013) and after injury (Nazareth et al., 2015; Wewetzer et al., 2005).

The numerous molecular and behavioural characteristics of OECs are seen as potentially useful for neural regeneration therapies. The transplant of OECs into the spinal cord has shown promising therapeutic outcomes in humans (Tabakow et al., 2014) and in animal models (Granger et al., 2012; Li et al., 2003; Li et al., 1998; Ramon-Cueto, 2000), however, other studies have reported no functional or anatomical improvements (Chhabra et al., 2009; Collazos-Castro et al., 2005; Lu et al., 2006). One contributing reason for the variation in outcomes of OEC transplant therapies may be that the preparation of pure cultures of OECs is difficult. OECs are often purified from the olfactory epithelium lining the nasal cavity (Feron et al., 2005; Granger et al., 2012; Stamegna et al., 2011; Tharion et al., 2011) or from the

nerve fibre layer of the olfactory bulb (Huang et al., 2012; Tabakow et al., 2014; Toft et al., 2007). In particular in animal models, variations in cell preparations can arise because in both the nasal and olfactory bulb regions the nerve fascicles of the accessory olfactory system and the main olfactory system project through the same anatomical regions (Fig. 1). Similar to the main olfactory system, OECs also ensheathe the accessory olfactory nerve. However, while these accessory cells express many molecules similar to OECs of the main olfactory system they also have distinct characteristics and anatomically they remain physically separate from the fascicles of the main olfactory nerve (Chehrehasa et al., 2008; Chehrehasa et al., 2006; Cloutier et al., 2004; Knoll et al., 2001; St John et al., 2006). The purification methods typically used to obtain OECs for neural transplant therapies do not separate out the contaminating accessory OECs and therefore cultures of OECs for neural transplant are likely to contain OECs from both the main and accessory olfactory systems. The potential mix of these different OECs may contribute to variation in outcomes of neural transplant therapies considering these cell preparations will likely contain differing proportions of accessory OECs.

The phagocytic capacity of OECs is one characteristic which may be particularly useful for neural repair therapies as the OECs can potentially increase the removal of cell debris from the injury site and thereby improve conditions for axon regrowth (Lankford et al., 2008). However, the phagocytic ability of accessory OECs has not been determined. We have previously generated a transgenic reporter line of mice, OMP-ZsGreen, in which olfactory sensory neurons of the main and accessory olfactory systems express ZsGreen fluorescent protein (Ekberg et al., 2011). In these mice, cell debris arising from olfactory axons retains expression of ZsGreen which is then able to be detected in radial glia that phagocytose the debris during development (Amaya et al., 2015) and in main olfactory OECs in the embryo

(Nazareth et al., 2015). In order to determine the phagocytic capabilities of OECs of the accessory olfactory system, we have now examined the accessory olfactory nerve of OMP-ZsGreen mice and compared them to OECs of the main olfactory system.

Materials and Methods

Animal strains

Transgenic reporter mice of two separate lines were used: S100ß-DsRed mice, expressing DsRed in cells that express S100ß including OECs (Windus et al., 2007) and OMP-ZsGreen mice, expressing ZsGreen in primary olfactory sensory neurons (Ekberg et al., 2011; Windus et al., 2011). The progeny of S100ß-DsRed mice crossed with OMP-ZsGreen mice were used for most experiments. All procedures were carried out with the approval of the Griffith University Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia and the Australian Commonwealth Office of the Gene Technology Regulator.

Tissue preparation

Postnatal pups were decapitated and the tissues fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) for 1 h at room temp or overnight at 4 ºC and then cryoprotected in 30% sucrose in PBS with 0.1% azide at 4 ºC. For wholemount preparations, heads were bisected in the parasagittal plane to expose the septum and olfactory bulb. For sectioning, heads were embedded in mounting matrix (O.C.T, Tissue-Tek) and snap frozen by immersion into 2-methyl butane which had being cooled with liquid nitrogen. Serial sagittal or coronal sections (30 μm) were cut, mounted on slides and stored at -20 ºC before processing for immunohistochemistry.

In vitro fresh dissections of OECs from OMP-Green x S100ß-DsRed pups.

Accessory OECs were dissected from the accessory olfactory bulb of postnatal day 4 (P4) OMP-ZsGreen x S100ß-DsRed pups using the ZsGreen expression as a guide to accurately

localise the accessory olfactory bulb. OECs from the main olfactory bulb were dissected from the lateral region of the nerve fibre layer to ensure that there was no contamination by OECs from the accessory olfactory nerve which runs along the nasal septum. Cells were dissociated using Tryple Express (Life Technologies) into single cell suspension, plated onto glass cover-slipped bottom culture dishes (Sarstedt) in medium containing DMEM, 10% fetal bovine serum, G5 supplement (Life Technologies), gentamicin, L-glutamine and incubated in 5% CO_2 at 37 ºC for 1-2 h to allow attachment of cells to the plate surface and then fixed with 4% PFA. For quantification of the proportion of OECs that contained cell debris, tissue was dissected from each of 3 animals and plated separately into 3 different cultures. For quantification of the intensity of fluorescence within OECs, five separate cultures were prepared with the cells from two animals mixed together for each of the five cultures (5x2 animals).

In vitro phagocytosis of axon debris by OECs.

For each dissociated culture of accessory and main OECs were prepared from the accessory and main olfactory bulbs of three S100ß-DsRed P4 pups as described above and replated at density of 5,000 cells per well in 8-well glass-cover-slipped bottomed chambers (Sarstedt). Axonal debris was generated by dissecting out the nerve fibre layer of the olfactory bulb of an OMP-ZsGreen mouse and partially digesting it in TrypLE Express (Life Technologies) and collagenase (0.1 mg/ml, Life Technologies) for 30 min. The reaction was stopped by addition of fetal bovine serum (Bovogen), centrifuged for 5 min, resuspended in DMEM and triturated using a syringe with a 27 gauge needle. The same amount of Zs-Green olfactory axonal debris (50 µL) was added to the medium of the dissociated cultures of DsRed OECs. After addition of the ZsGreen axon debris, live cells were imaged using laser scanning confocal microscopy as described below. For assays to quantify ZsGreen fluorescence

intensity within cells, four separate cultures were prepared with cells plated onto 8-well glass-cover-slipped bottomed chambers (2,000 cells per chamber) and incubated with the ZsGreen debris for 24 h. The OEC cultures were washed to remove external axonal debris and fixed in 4% PFA.

Quantification of ZsGreen fluorescence intensity within OECs

Confocal microscope images were taken of the dissociated cells with the laser and imaging parameters held constant (473 laser at 6.0% power and PMT voltage of 607) and with the levels set so that saturation of the green fluorescent channel (to visualise axon debris) did not occur. Quantification of the amount of internalised axonal debris was performed using ImageJ software to measure the mean intensity of green fluorescence (8 bit images were used giving a range of 0-255) within the entire cell boundaries as defined by the DsRed fluorescence of the cells. For the freshly dissected and plated cell assay, the proportion of cells that contained any ZsGreen debris was determined by counting the total number of cells and the number of cells that contained ZsGreen debris.

Immunochemistry

Immunohistochemistry on tissue sections and cell cultures was performed as previously described (Chehrehasa et al., 2012; Windus et al., 2011; Windus et al., 2007). The antibodies used were: rabbit anti-brain lipid binding protein (1:500; BLBP; Abcam Ab32423, AB_880078; Garcia-Ovejero et al., 2013); mouse monoclonal anti-blood group A (1:10; CSL, 02611305); rabbit anti-p75ntr (1 μg/mL; Promega G3231, AB_430853, Tripathi and McTigue, 2008); rabbit-anti Lamp1 (4 μg/mL; Sigma, L1418, AB_477157, Stewart et al., 2012); rabbit anti-early endosome antigen marker 1 (1 μg/mL; EEA1, Abcam Ab2900,

AB_2262056, Mu et al., 1995); followed by the secondary antibodies anti-rabbit Alexa Fluor 647 (5 µg/mL; Life Technologies, A-21244). Cell nuclei were stained with 4'6-diamidino-2-pheylindole (DAPI).

Surgical ablation of olfactory bulb (unilateral bulbectomy)

Surgical ablation of one entire olfactory bulb (unilateral bulbectomy) was performed on postnatal day 4.5 using our established method (Chehrehasa et al., 2006). Immersion of the postnatal pups in ice for 8 min resulted in deep anaesthesia (Danneman and Mandrell, 1997) and then a midline incision was cut to expose the cranial bones through which a 0.5 mm hole was cut above one olfactory bulb using a surgical drill with a 0.5 mm burr. A pulled glass pipette attached to a low pressure vacuum pump was used to aspirate the entire olfactory bulb including the accessory olfactory bulb and then the skin covering the site was sutured and the anti-inflammatory drug Flunix was administered via intramuscular injection (active ingredient flunixin, 1 mg/kg body weight; Parnell Laboratories). Three pups were harvested three days after surgery at P7.

Image capture

For fixed tissue preparations, confocal mages were taken using an Olympus FV1000 microscope and processed on FV10-ASW 3.01.01.09 Viewer software. For live cell assays, confocal imaging was performed using an Olympus CV1000 confocal microscope. In order to enhance presentation, brightness and contrast were adjusted uniformly across the field of view using the FV10-ASW 3.1 Viewer software and ImageJ 1.47t software. For images comparing treatments, the images were captured using the same confocal settings and the same z depth. Image panels were created by using Adobe Illustrator CS5 15.0.2.

Results

We used the OMP-ZsGreen transgenic reporter line of mice previously generated in our laboratory (Ekberg et al., 2011) to easily visualise the axons that arise from the accessory olfactory neurons which line the vomeronasal organ (VNO). The VNO is positioned in the ventral region of the nasal septum (Fig. 2A) and the nerve fascicles that arise from the VNO project dorso-caudally to their terminations in the dorsal region of the olfactory bulb (Fig. 2A-B). As the VNO axons project along the septum they pass through the region of the main olfactory neurons and are in close proximity to the nerve fascicles that arise from the main olfactory neurons (arrow, Fig. 2A). The VNO axons can be identified using immunohistochemistry as they express blood group A carbohydrate while main olfactory neurons do not (St John et al., 2006). Within the accessory olfactory bulb (Fig. 2B-D), anti-BGA immunostaining labelled the axons in the densely clustered glomeruli (arrow, Fig. 2C), but did not label axons in glomeruli of the main olfactory bulb (arrowhead, Fig. 2B-D). Within the septum of the nasal cavity, anti-BGA immunostaining labelled the fascicles of VNO axons (arrow, Fig. 2E-G) but did not label fascicles of main olfactory axons (arrowhead, Fig. 2E-G). The fascicles of VNO axons could also be easily distinguished by morphology as they are much larger than the fascicles of the main olfactory axons (compare arrow with arrowhead, Fig. 2E). The VNO fascicles could also be identified by their location as they lie in the basal portion of the lamina propria adjacent to the cartilage whereas the main olfactory nerve fascicles are positioned more apically within the lamina propria (Fig. 2E).

To detect phagocytosis of axon debris by the OECs of the accessory olfactory system, we examined the distribution of the ZsGreen protein in the VNO and main olfactory fascicles in

postnatal day 7 offspring of crosses between OMP-ZsGreen and S100ß-DsRed mice. Postnatal animals were chosen as this is the period in which there is not only large scale olfactory axon targeting but also considerable mis-targeting of axons followed by their degradation and phagocytosis as shown for main OECs (Nazareth et al., 2015; Su et al., 2013; Wewetzer et al., 2005). In a parasagittal section that revealed the numerous axon fascicles within the lamina propria, the VNO nerve fascicles (arrow, Fig. 2H) projected more dorsally in comparison to the main olfactory fascicles (arrow with tail, Fig. 2H). The distribution of the ZsGreen protein was distinctly different between the VNO and main olfactory fascicles. In the VNO fascicles, the ZsGreen appeared to be uniformly distributed (arrow, Fig. 2H) while the main olfactory fascicles had numerous small foci that gave the fascicles a much brighter appearance (arrow with tail, Fig. 2H). Higher magnification imaging of the DsRed OECs revealed that small ZsGreen protein accumulations were contained within OECs of the VNO nerve (arrows, Fig. 2I, three-dimensional reconstruction is shown in Movie 1). In stark contrast, in the main olfactory nerve fascicles ZsGreen debris from degrading axons was widely accumulated within the main olfactory OECs (arrows with tails, Fig. 2I). In coronal sections through the septum, the distinct differences in the accumulation of axon debris were apparent, with VNO fascicles having a small number of ZsGreen accumulations, while main olfactory fascicles had numerous accumulations of ZsGreen (arrow with tail, Fig. 2J-L).

We next examined OECs within the accessory olfactory bulb (AOB) (Fig. 3A-B). The OMP-ZsGreen gave clear visualisation of the axons within the AOB while the S100ß-DsRed was strongly expressed by OECs in the AOB. We confirmed the identity of the OECs by immunostaining with anti-brain lipid binding protein (BLBP) antibodies which is a marker of OECs (Chehrehasa et al., 2010; Murdoch and Roskams, 2007) (Fig. 3C). Higher magnification imaging of the OECs in the AOB revealed they contained numerous

accumulations of ZsGreen axon debris (arrows, Fig. 3D; three-dimensional reconstruction is shown in Movie 2). In contrast, in OECs of the main olfactory bulb, there were large accumulations of ZsGreen axon debris (Fig. 2E). In the postnatal period, axon targeting is often inaccurate so we examined whether there was a correlation between the occurrence of over-projecting axons and the accumulation of axon debris within OECs. In the AOB, few axons over-projected into the deeper layers (Fig. 3F) whereas in the main olfactory bulb, numerous axons over-projected into the deeper layers (Fig. 3G) indicating that the occurrence of axon mis-targeting is higher in the main olfactory bulb compared to the accessory olfactory bulb.

To determine the fate of the axon debris within the accessory OECs, we performed immunohistochemistry using antibodies against lysosomal associated membrane protein 1 (lamp1) and found that some of the ZsGreen debris co-localised with lamp1 (Fig. 3H-I). To verify that the accessory OECs had internalised the ZsGreen axon debris, we next dissected out the nerve fibre layer of the AOB, dissociated the cells and fixed them after they attached to the culture plate. The dissociated OECs clearly contained accumulations of ZsGreen axon debris (Fig. 4A). As the accessory OECs appeared to contain less debris in vivo compared to main olfactory OECs, we next dissected out and dissociated cells from the nerve fibre layer of the accessory olfactory bulb and the main olfactory bulb. We counted the number of cells that contained any ZsGreen debris and found that 75-90% of OECs contained cell debris with no significant difference between accessory and main OECs ($p>0.05$) (Fig. 4B). We then quantified the intensity of ZsGreen fluorescence within the cells that contained ZsGreen debris. Accessory OECs had significantly less fluorescence compared to main OECs (Fig. 4C, $p<0.001$). We prepared cultures of DsRed OECs from S100ß-DsRed mice (in these mice there is no ZsGreen debris) and used immunostaining with antibodies against p75ntr, which is

a marker of OECs to verify the identity of the OECs (Fig. 4D). Next, we used the cultures of accessory OECs to determine if phagocytosis of axon debris occurred in vitro. Axon debris was prepared from the nerve fibre layer of the main olfactory bulb from OMP-ZsGreen mice, so that the axon debris expressed ZsGreen fluorescent protein. We used timelapse live cell confocal microscopy to track the fate of the ZsGreen debris. The accessory OECs exhibited dynamic movements with active lamellipodia that explored the local environment (Movie 3). ZsGreen debris that came into contact with the OECs was often phagocytosed by the lamellipodia (arrow with tail, Fig. 4E-H) or along the shaft of the process (arrow, Fig. 4E) emanating from the OEC. The ZsGreen debris was then transported to the cell body (Fig. 4G-H; Movie 3). We further assayed the phagocytic activity of accessory OECs by adding ZsGreen axonal debris to the accessory and main OEC cultures and quantifying the intensity of ZsGreen fluorescence internalised by the OECs after 24 h incubation (Fig. 4I). Accessory OECs contained significantly more ZsGreen fluorescence compared to main OECs (Fig. 4J, $p<0.05$, t-test). Immunostaining of these in vitro accessory OECs with antibodies against early endosome antigen 1 (EEA1) revealed some ZsGreen debris co-localised with EEA1 (Fig. 4K-M).

The in vivo and ex vivo analyses of accessory OECs indicated that they contained less debris than main OECs in healthy animals (Figs. 1-2, Fig. 4A-C). However, the in vitro assays showed that accessory OECs are active phagocytes when exposed to exogenous cell debris. We therefore examined the reaction of accessory OECs in vivo when there was widespread degeneration of axons. We performed a unilateral bulbectomy in which the accessory and main olfactory bulb on one side were ablated (Fig. 5A). The neurons on the unoperated control side were not affected (Fig. 5B, C) while the neurons on the bulbectomy side (OBX side) degenerated in both the VNO and main olfactory epithelia (Fig. 5B, D). Examination of

the accessory axon fascicles that were present in the ventral septum (Fig. 5B) showed that on the control side the axons remained intact and there were few accumulations of ZsGreen debris within OECs (arrows, Fig. 5E-G). In contrast, on the bulbectomy side, intact axons were not detected and instead there were numerous accumulations of ZsGreen debris that localised with the accessory OECs (arrows, Fig. 5H-J). Next, we examined the fate of accessory axons compared to the main olfactory axons. On the control side, the intact main olfactory axons were ensheathed by OECs that contained small accumulations of ZsGreen axon debris (arrows, Fig. 5K-L). On the bulbectomy side where the neurons in the main olfactory epithelium had degenerated, the axons were absent from the fascicles and the OECs contained large accumulations of ZsGreen debris (arrows, Fig. 5M-N). These results support the in vitro analyses as they demonstrate that the accessory OECs reacted to the injury by rapidly phagocytosing the axon debris. Thus both the main and accessory OECs are active phagocytic cells after injury albeit with differing capacities for phagocytosis.

Discussion

We have demonstrated that the OECs of the accessory olfactory system are active phagocytic cells that remove cell debris derived from degraded sensory axons. In the normal healthy animal, the level of phagocytosis occurring in accessory OECs appears to be less than that of the main OECs. This likely reflects a decreased need for clearance of axonal debris considering that we have identified a reduced incidence of axon mis-targeting in the accessory olfactory bulb compared to the main olfactory bulb. However after widespread injury, the accessory OECs respond to rapidly remove the degraded sensory axons. In vitro assays demonstrated the dynamic activity of accessory OECs as they phagocytosed axon debris and quantification of the amount of phagocytosis indicated that accessory OECs were more effective at internalising debris compared to main OECs in vitro. As there is the considerable potential for accessory OECs to be included in a preparation of purified OECs for neural transplant therapies, these results are important as they confirm that OECs from both the main and accessory olfactory systems are active phagocytic cells, albeit with different capacities, in the normal healthy animal as well as during injury.

OECs of the main olfactory system are known to be active phagocytic cells during embryonic (Nazareth et al., 2015) and postnatal development (Wewetzer et al., 2005) as well as the adult (Su et al., 2013). Indeed, OECs are the main phagocytic cells of the olfactory nerves and macrophages play a minor role in removing olfactory axon cell debris (Nazareth et al., 2015; Su et al., 2013). Given that main olfactory neurons are constantly dying and being regenerated throughout life (Holcomb et al., 1995; Mackay-Sim and Kittel, 1991), there is a continual need for removal of the axon debris which is performed by the resident phagocytic cells, the OECs, thus reducing or eliminating the requirement for macrophages to infiltrate

into the olfactory fascicles. Even after widespread injury, the OECs are the main phagocytic cells and macrophages play a very limited role and do not demonstrate any large scale mobilisation or infiltration of the main olfactory fascicles (Nazareth et al., 2015; Su et al., 2013). Similarly, we now show that OECs are also the main phagocytic cells in the accessory olfactory system and that macrophages play a minor role in the accessory olfactory nerves.

During development, olfactory axon targeting is inaccurate (Gong and Shipley, 1995; Graziadei et al., 1980; Santacana et al., 1992; St John and Key, 2005; Tenne-Brown and Key, 1999) and it has been shown that in the postnatal rat, more than one-third of main olfactory axons project to multiple targets or over-project into the deeper layers of the main olfactory bulb (Tenne-Brown and Key, 1999). Accessory olfactory axon targeting is also inaccurate (Ekberg et al., 2011), but our results indicate that the incidence of axons that over-project past the glomerular layer of the accessory olfactory bulb may be less than that which occurs in the main olfactory system. However, the incidence of mis-targeting within the confines of the accessory olfactory bulb is not known. The accuracy of accessory olfactory axon targeting was not the focus of the current study, however we considered that with fewer over-projecting accessory axons compared to the main olfactory system there would be a reduced need to remove debris from apoptotic accessory olfactory axons. This was reflected in the accumulation of ZsGreen debris within the OECs where we found that there was a distinct difference in the distribution of axon debris, with main OECs having numerous accumulations of debris while accessory OECs had considerably less. The in vitro analyses of freshly dissected OECs confirmed this finding. However, when assayed with exogenously added axon debris in vitro, the accessory OECs were clearly capable of phagocytosis and rapidly internalised the exogenously added cell debris. When the two OEC types were compared in vitro, the accessory OECs showed an almost 20% higher capacity for

phagocytosis compared to the main OECs. The strong phagocytic activity of accessory OECs was confirmed by the in vivo ablation model after which the accessory OECs rapidly phagocytosed axon debris without contribution from macrophages. Thus while accessory OECs phagocytose less debris in healthy animals in vivo, they have the capacity to respond after widespread injury.

The phagocytic activity of OECs is potentially useful in the spinal cord (Lankford et al., 2008) where the clearing of debris likely improves neurite outgrowth and neuron survival (He et al., 2014; Tello Velasquez et al., 2014). The transplantation of cells with a high capacity for phagocytic activity could reduce the endogenous inflammatory response and reduce the recruitment of macrophages to the injury site (Lankford et al., 2008). Sustained macrophage infiltration within the injured spinal cord is considered to hinder regeneration (Hulsebosch, 2002), therefore the use of OECs may be beneficial in both reducing the requirement for macrophages as phagocytic cells while also serving to aid axon growth across the injury site (Lopez-Vales et al., 2004). While there have been numerous studies examining the potential therapeutic use of OECs (Ekberg and St John, 2014; Mackay-Sim and St John, 2011), a major remaining limitation is the lack of an ability to easily purify OECs. OECs are obtained from either the olfactory mucosa lining the nasal septum or from the nerve fibre layer of the main olfactory bulb. In animal studies, accessory olfactory OECs are likely to be included in purified OEC preparations derived from both of these regions due to their anatomically close locations (Fig. 1). In addition, Schwann cells from the trigeminal nerve as well as fibroblasts are potentially included in these cell preparations (Bock et al., 2007; Tabakow et al., 2013). Apart from the difficulties in purifying these cells, the use of markers to identify the cells in culture is also troublesome. The most widely used marker is p75ntr which is expressed by main and accessory OECs, as well as Schwann cells and fibroblasts (Garcia-Escudero et al.,

2012). Other markers are less reliable and do not offer definitive identification (Garcia-Escudero et al., 2012). Given the potential therapeutic success of OEC transplantation to restore function in a human with a severed spinal cord (Tabakow et al., 2014), for future animal trials of spinal cord repair there is an urgent need to improve the purification and identification of OECs to maximise the likelihood of obtaining consistent results. While we have shown that accessory OECs have high capacity for phagocytosis of axon debris and may therefore be useful for transplant therapies, it may be better to devise strategies to reduce their inclusion and thereby reduce the potential variations in the cell preparations. For example, the accessory OECs could be easily avoided by dissecting olfactory mucosa from the lateral turbinates rather than from the nasal septum, or by dissecting the lateral regions of the nerve fibre layer of the olfactory bulb. However, both the nasal septum and medial regions of the nerve fibre layer are often favoured as they give rise to large numbers of highly proliferative cells (Choi et al., 2008; Feron et al., 2005). Therefore additional techniques that improve proliferation of OECs in vitro need to be considered if the use of more discrete populations of OECs are to be used. For example, we have recently shown that for main OECs derived from the nasal mucosa, low-dose curcumin stimulates not only the proliferation of OECs but also potently stimulates their phagocytic activity (Tello Velasquez et al., 2014).

Conclusion

We have shown that while accessory OECs phagocytose less axon debris in healthy animals in vivo in comparison to main OECs, the accessory OECs are indeed active phagocytic cells that are primarily responsible for removing cell debris particularly after widespread injury. While the inclusion of accessory OECs may therefore be potentially beneficial for cell transplantation therapies, it is also possible to prepare OEC cultures without accessory OECs

by dissecting cells from discrete regions of the olfactory system. Without such discrete dissections, the likely inclusion of accessory OECs in cell preparations for spinal cord repair needs to be considered as they are a potential source of variation that may confound interpretations of results.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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Figure Legends

Figure 1. The accessory and main olfactory systems in mice. (A) Schematic sagittal view of the mouse olfactory systems. In the accessory olfactory system (brown), neurons within the vomeronasal organ (VNO) project axon fascicles to the accessory olfactory bulb (AOB). The accessory olfactory axon fascicles pass through the region of the main olfactory system (orange). The main olfactory neurons populate the olfactory mucosa (OM) and their axons project to the external layer of the olfactory bulb (OB). (B) In a schematic coronal view of the olfactory systems, the close relationships between the fascicles of accessory (brown) and main (orange) olfactory axons are shown.

Figure 2. Accessory OECs phagocytose axon debris. Panels show parasagittal or coronal confocal z-stack views through the olfactory system of postnatal day 7 animals. Olfactory neurons are green; OECs are red; DAPI (blue) stains nuclei. (A) In a parasagittal section of an OMP-ZsGreen transgenic mice, accessory olfactory sensory neurons express ZsGreen protein in the cell bodies within the vomeronasal organ (VNO) and axons; neurons of the main olfactory system also express ZsGreen in the main olfactory epithelium (MOE) and in the main olfactory bulb (OB); rostral is to the left, dorsal to the top. Arrow points to region shown in panel H. (B-D) In coronal sections, the accessory olfactory axons (arrow) within the accessory olfactory bulb (AOB) were identified using anti-blood group A (BGA, magenta) antibodies; main olfactory axons (arrow with tail in B-D) do not express BGA. (E-G) In coronal sections through the nasal septum (S) the large bundles of the accessory olfactory axons were identified by anti-BGA staining (arrow), while the smaller axon bundles of main olfactory axons did not express BGA (arrow with tail). (H) Higher magnification view of a parasagittal section shows the large accessory axon bundles (arrow) projecting amongst the

smaller main olfactory bundles (arrow with tail). (I) In the parasagittal plane of an OMP-ZsGreen x S100ß-DsRed mouse, main olfactory bundles were characterised by the numerous accumulations of axon debris (arrow with tail) within OECs (red) which gave the main olfactory bundles a distinct spotted appearance. In comparison, there were few accumulations of axon debris within OECs of the accessory fascicles (arrows). Three-dimensional reconstruction is shown in Movie 1. (J-L) In coronal sections the distinct difference in accumulation of axon debris in main (arrow with tail) versus accessory (arrow) OECs (red) was apparent with main olfactory bundles (dotted oval) having numerous accumulations while accessory bundles had little accumulation of debris in OECs (arrow). NC, nasal cavity. Scale bar is 500 μm in A, 170 μm in B-D, 125 μm in E-G, 72 μm in H, 25 μm in I, 12 μm in J-L.

Figure 3. OECs in the accessory olfactory bulb phagocytose axon debris in vivo. Panels show parasagittal sections of OMP-ZsGreen x S100ß-DsRed postnatal day 7 pups. Olfactory neurons are green; OECs are red; DAPI (blue) stains nuclei in panels A-C. (A) Low power view of the nasal cavity and olfactory bulb. Dashed boxed areas are shown in panels F and G. (B) The expression of DsRed provided clear visualisation of OECs within the AOB while the ZsGreen provided visualisation of the olfactory axons. (C) Immunostaining with anti-BLBP antibodies confirmed the DsRed cells were OECs. (D) High magnification view of OECs within the AOB showed accumulation of ZsGreen axon debris (arrows) in OECs. Three-dimensional reconstruction of panel D is shown in Movie 2. (E) In the nerve fibre layer (NFL) of the main olfactory bulb the OECs accumulated large amounts of ZsGreen axon debris (arrows). (F) In the AOB, some accessory olfactory axons over-projected into the deeper layers (arrows). (G) In the main olfactory bulb, numerous sensory axons (arrows) over-projected past the NFL into the deeper layers. (H-I) Immunostaining with anti-lamp1

antibodies showed that some ZsGreen axon debris within OECs co-localised with lamp1 (arrows). Scale bar is 500 μm in A, 130 μm in B, 13 μm in C, 8.5 μm in D-E, 50 μm in F-G, 30 μm in H-I.

Figure 4. Accessory OECs phagocytose axon debris in vitro. (A) OECs from the nerve fibre layer of the AOB that were dissected out from OMP-ZsGreen x S100ß-DsRed P7 pups and imaged immediately upon attachment to the culture plate contained ZsGreen axon debris. (B) Percentage of OECs that contained ZsGreen debris in freshly dissected OECs from the nerve fibre layer of the AOB and main olfactory bulb; n=3 cultures; 45-60 cells analysed per culture; $p>0.05$; error bars are standard error of the mean. (C) Quantification of the intensity of ZsGreen fluorescence in freshly dissected OECs; n=5 cultures; 8-15 cells analysed per culture; ***$p<0.001$; error bars are standard error of the mean. (D) In cultures of accessory OECs prepared from S100ß-DsRed mice, immunostaining with anti-p75ntr antibodies confirmed the identity of the accessory OECs. (E-H) Timelapse sequence of DsRed accessory OECs with exogenously added ZsGreen cell debris. ZsGreen debris was phagocytosed by lamellipodia (arrow with tail) or along the shaft (arrow) of the cells and accumulated within the cell bodies of OECs. Time (h:min) is from the start of the imaging sequence; timelapse movie is shown in Movie 3. (I) Exogenous ZsGreen cell debris added to cultures of DsRed accessory OECs was phagocytosed (arrows). (J) Quantification of the intensity of green fluorescence within accessory and main OECs 24 h after addition of exogenous debris, n=4 cultures; 8-10 cells analysed per culture; *$p=0.027$; error bars are standard error of the mean. (K-M) Immunostaining of cultured accessory OECs with anti-EEA1 antibodies (blue) showed co-localisation of some green axon debris (arrows) within OECs (red). Scale bar is 40 μm in A, 90 μm in D, 60 μm in E-H, 70 in I, 14 μm in K-M.

Figure 5. Accessory olfactory OECs phagocytose axon debris after unilateral bulbectomy. Panels show coronal sections of the nasal cavity of mice that had unilateral bulbectomy at P4 and examined at P7. Olfactory neurons are green; OECs are red; DAPI (blue) stains nuclei. (A) One olfactory bulb (OB) was surgically removed (olfactory bulbectomy (OBX) side; arrow points to location of surgery); the other side remained intact (control side). (B) In the rostral nasal cavity, the main olfactory epithelium (MOE) and epithelium lining the VNO on the control side (left nasal cavity) was populated by numerous neurons (green). On the OBX side (right nasal cavity), the neurons in the MOE and VNO were degraded. Locations of following panels are indicated by the letters. (C) The epithelium lining the VNO on the control side was populated by neurons; whereas (D) on the OBX side the neurons were degraded; some immature neurons were evident (arrow). (E) In nerve fascicles of accessory olfactory axons on the control side, there were infrequent accumulations of axon debris in OECs (arrow). (F-G) Higher magnification shows the accumulation of axon debris (arrows) within OECs; double label in F, ZsGreen alone in G. (H) In nerve fascicles of accessory olfactory axons on the OBX side, few intact axons were detected and there were large accumulations of axon debris (arrows) in OECs. (I-J) Higher magnification view showing the axon debris (arrows) within OECs. (K) The MOE on the control side was populated by numerous neurons and the axons projected into the lamina propria (LP) where they formed fascicles ensheathed by OECs (arrows). (L) Higher magnification view of an axon bundle of the control side showing accumulations of small amounts of ZsGreen debris (arrows) in main olfactory OECs. (M) On the OBX side, the neurons in the MOE were degraded and debris (arrows) from the axons accumulated in the main OECs. (M) Higher magnification of a main olfactory axon fascicle on the OBX side showing increased accumulation of axon debris (arrows) in OECs. Scale bar is 580 μm in A, 540 μm in B, 25 μm in C-D, K, M, 15 μm in E, H, 6 μm in F-G, I-J, 10 μm in L, N.

Movie 1. Three-dimensional reconstruction of the confocal z-stack (9.66 μm deep) of Fig. 2I showing nerve fascicles of the accessory versus main olfactory systems with internalised green fluorescent axonal debris.

Movie 2. Three-dimensional reconstruction of the confocal z-stack (6.58 μm deep) of Fig. 3D showing OECs of the accessory olfactory bulb with internalised green fluorescent axonal debris.

Movie 3. Timelapse movie accessory OECs phagocytosing exogenously added ZsGreen cell debris. Time is in h:min after addition of exogenous debris. Select cropped frames are shown in Fig. 4E-H.