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Radial glia phagocytose axonal debris from degenerating over-extending axons in the developing olfactory bulb

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Abstract

Axon targeting during the development of the olfactory system is not always accurate and numerous axons over-extend past the target layer into the deeper layers of the olfactory bulb. To date, the fate of the mis-targeted axons has not been determined. We hypothesised that following over-extension, the axons degenerate, and that cells within the deeper layers of the olfactory bulb phagocytose the axonal debris. We utilised a line of transgenic mice that expresses ZsGreen fluorescent protein in primary olfactory axons. We found that over-extending axons closely followed the filaments of radial glia present in the olfactory bulb during embryonic development. Following over-extension into deeper layers of the olfactory bulb, axons degenerated and radial glia responded by phagocytosing the resulting debris. We used in vitro analysis to confirm that the radial glia had phagocytosed debris from olfactory axons. We also investigated if the fate of over-extending axons was altered when the development of the olfactory bulb was perturbed. In mice that lacked Sox10, a transcription factor essential for normal olfactory bulb development, we observed a disruption to the morphology and positioning of radial glia and an accumulation of olfactory axon debris within the bulb. Our results demonstrate that during early development of the olfactory system, radial glia play an important role in removing over-extended axons from the deeper layers of the olfactory bulb.

Introduction

The primary olfactory system comprises a complex neural network in which primary olfactory neurons express one of ~1000 odorant receptors (Buck and Axel, 1991). The neurons are mosaically distributed in restricted regions throughout the olfactory epithelium. Olfactory axons extend inwards through the lamina propria, where the form fascicles and project towards the nerve fibre layer of the olfactory bulb. Upon entering the olfactory bulb, axons defasciculate and sort out depending on their receptor type (Mombaerts et al., 1996). Here they establish synapses with secondary neurons of the olfactory bulb (Au et al., 2002; Malun and Brunjes, 1996). During development of this complex topographical map, axon targeting is not always accurate and numerous axons project to inappropriate sites within the target layer (Tenne-Brown and Key, 1999) or over-extend a considerable distance past their target into the deeper layers of the olfactory bulb (Chan et al., 2011; Gong and Shipley, 1995; Graziadei et al., 1980; Santacana et al., 1992; St John and Key, 2005). While the mis-targeted axons are likely to undergo pruning, their fate has not been determined and it is not known which cells are responsible for removing the cellular debris from the deeper layers of the olfactory bulb.

A critical point of the development of the olfactory bulb is when the developing telencephalon fuses with the olfactory nerve. This is generally observed at embryonic day 11 (E11) of mouse development (Treloar et al., 1996; Valverde et al., 1992). The olfactory nerve consists mainly of primary olfactory axons and the accompanying glia of the olfactory system, olfactory ensheathing cells (OECs) (Doucette, 1989; Valverde et al., 1992). The OECs penetrate only into the nerve fibre layer, which lines the exterior surface of the developing olfactory bulb, and within this layer the OECs are thought to contribute to the

sorting and targeting of axons (Chehrehasa et al., 2010; Chuah and Au, 1991; Doucette, 1984; Ekberg et al., 2012). The transcription factor Sox10, containing the SRY box as HMG-type DNA-binding domain is an important factor for embryonic development (Britsch et al., 2001; Herbarth et al., 1998; Kuhlbrodt et al., 1998). It has also been found to be an important regulator of the development and migration of OECs and loss of Sox10 leads to a reduced OEC population and disturbed axon targeting (Barraud et al., 2013). In adult rats, OECs phagocytose the cellular debris from degraded olfactory axons that occurs during normal turnover of primary olfactory neurons (Su et al., 2013). However, as OECs are restricted to the exterior region of the olfactory bulb during development, they cannot be responsible for clearing the pruned axons that over-extended into the deeper layers of the olfactory bulb; thus, other cells must be involved.

Within the developing olfactory bulb, radial glia project from the centrally located ventricle towards the exterior surface of the olfactory bulb. In the cerebral cortex of the lizard brain, radial glia contribute to the removal of cellular debris arising from neuronal degeneration (Nacher et al., 1999). Hence, we hypothesised that radial glia interact with over-extending primary olfactory axons in the developing olfactory bulb, and that the radial glia remove the cellular debris that arises when the over-extended axons are degraded.

We have tested this hypothesis in a line of transgenic reporter mice that we previously generated, olfactory marker protein (OMP)-ZsGreen mice, in which the OMP promoter drives the expression of the reporter fluorescent protein ZsGreen and providing outstanding visualisation of olfactory neurons (Ekberg et al., 2011). We now show that in these mice the ZsGreen protein remains intact and strongly fluorescent during axon degradation. Our results reveal that the over-extending axons in the developing olfactory bulb closely follow the

filaments of the radial glia into the deeper layers of the olfactory bulb and that the radial glia remove the cellular debris that arises when the axons are degraded. We also re-examined the Sox10 knockout mice (Barraud et al., 2013; Britsch et al., 2001), and found that the radial glia had perturbed morphology and that axon debris accumulated within the olfactory bulb. This perturbation of the radial glia is likely to contribute to the increased accumulation of primary olfactory axons in deeper layers of the olfactory bulb in these mice.

Materials and Methods

Animal strains

We used several lines of transgenic mice of both sexes: (1) OMP-ZsGreen mice in which olfactory neurons selectively fluoresce green (Ekberg et al., 2011; Windus et al., 2011; Windus et al., 2010); (2) Sox10lacZ/lacZ mice ($Sox10^{tm1Weg}$ knockout) (Britsch et al., 2001), along with their respective wild-type littermates. Sox10lacZ/lacZ mice were obtained through many generations of backcrossing; (3) S100β-DsRed mice in which glia selectively fluoresce red (Windus et al., 2007). 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

To harvest embryos, pregnant mice were euthanized by cervical dislocation, embryos were removed and decapitated. The tissues were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 1 h at room temp, then cryoprotected in 30% sucrose in PBS with 0.1% azide at 4 ºC. Heads were mounted in embedding 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 isolation of radial glia

Radial glia were isolated from crosses of S100β-DsRed and OMP-ZsGreen mice of both sexes at P1. The pups were euthanized by cervical dislocation and the inner olfactory bulb

was removed through suction via a sterile syringe. We used DsRed fluorescence (expressed by OECs) to guide the dissection and to ensure the nerve fibre layer was left intact. Tissue was minced and plated onto glass coverslip-bottom chamber wells (X-well Tissue Culture Chambers, Sarstedt) pre-coated with Matrigel basement membrane (1:10) and incubated in media containing Dulbecco's modified eagle medium (DMEM, Gibco), 10% fetal bovine serum, G5 supplement (Invitrogen), gentamicin and L-glutamine at 37°C in 5% CO_2. Cell preparations from four different animals were separately isolated and upon attachment to the basement membrane the cells were immediately fixed in 4% PFA in PBS for 5 min and then immunostained.

Antibody characterization

Immunochemistry was performed on tissue sections and cell cultures as previously described (Chehrehasa et al., 2012). The following primary antibodies were used. Rabbit anti-brain lipid binding protein (BLBP) antibody (RRID: AB 880078) which has been previously used as a marker for radial glia in the developing brain (Anthony et al., 2004) and is also expressed by olfactory ensheathing cells (Chehrehasa et al., 2010; Murdoch and Roskams, 2007). Anti-beta tubulin III antibody (RRID: AB 727049) is highly reactive with neuron beta tubulin III and positively stains olfactory neurons. Anti-olfactory marker protein (OMP) antibody (RRID: AB 664696) has been used extensively as a marker for olfactory neurons. The specificity of both anti-beta tubulin III and anti-OMP antibodies were also verified by examining their co-expression with the OMP-ZsGreen transgenic mice in which olfactory neurons are fluorescent green.

The secondary antibodies used were anti-rabbit Alexa Fluor 647 (10 μg/ml, Invitrogen RRID:AB_10561706), anti-goat Alexa Fluor 488 (5 μg/ml, Invitrogen RRID:AB_10564074),

anti-goat Alexa Fluor 594 (5 μg/ml, Invitrogen RRID:AB_2313737), anti-chicken biotin (10 μg/ml, Abcam RRID:AB_954958) and streptavidin 647 (10 μg/ml, Invitrogen RRID: AB_2336066) as a tertiary antibody. Cell nuclei were stained with 4'6-diamidino-2-pheylindole (DAPI).

Image capture

Confocal images were taken using an Olympus FV1000 microscope and processed on FV10-ASW 3.1 Viewer software. Epifluorescent images were taken on a Ziess AxioImager Z1 with images merged to form mosaic view of the larger field of view. Brightness and contrast were adjusted using the FV10-ASW 3.1 Viewer software, AxioVision software and ImageJ 1.40g software (RRID: nif-0000-30467). Image panels were created by using Adobe Illustrator CS5 15.0.2, and schematics on Adobe Photoshop CS3 10.0.1.

Statistical analyses

Statistical analyses using a two sample Student's two-tailed t-test was performed to compare the morphology of the olfactory bulb between Sox10 KO and their wild-type litter mates.

Results

Olfactory axons over-extend into the developing olfactory bulb

To visualise the trajectory of the primary olfactory axons we used the OMP-ZsGreen transgenic line of mice that we previously generated (Ekberg et al., 2011). In these mice, ZsGreen fluorescent protein is expressed under the control of the promoter of olfactory marker protein (OMP) and thus primary olfactory neurons and their axons express ZsGreen (Fig. 1A) (Ekberg et al., 2011), however, a few neurons in the olfactory epithelium express OMP but not ZsGreen (Fig. 1B-C). OMP protein has been demonstrated to be expressed during early olfactory development and hence has been reliable as a detection tool (Graziadei et al., 1980). In OMP-ZsGreen mice, the ZsGreen expression enables easy visualisation of primary olfactory axons as early as E10.75 (Fig. 1M and N), long before they reach the telencephalon (Miller et al., 2010). Thus in the OMP-ZsGreen mice, immature as well as mature olfactory sensory axons are visualised. After axons reached the telencephalon, the strong expression of ZsGreen in primary olfactory axons enabled clear visualisation of the axons that over-extended past the nerve fibre layer and inappropriately entered the deeper layers of the developing olfactory bulb (Fig. 1D). Over-extending axons could also be detected with OMP immunolabelling, however, the ZsGreen expression provided superior detail of the axons (Fig. 1E, arrow). Immunolabelling for ß-tubulin III, which is a cytoskeleton protein and a neuron-specific marker, showed that almost all neurons within the epithelium expressed both ZsGreen and ß-tubulin III (Fig. 1F-G). It should be acknowledged that OMP-ZsGreen was not expressed in some immature neurons that expressed ß-tubulin III (Fig. 1G). However, the ZsGreen expression provided superior detail of the axons (Fig. 1F, arrow) compared to the ß-tubulin III immunolabelling.

We first confirmed that, during the early development of the olfactory bulb, numerous axons over-extended past their target layer, the nerve fibre layer, and projected into the deeper layers of the olfactory bulb (Fig. 1J-L, arrows). The axons over-extending the furthest distance into the deeper bulbar regions were degenerating and the degradation products accumulated around the surface of the ventricle in the centre of the olfactory bulb (Fig. 1J-L, double-headed arrows).

We used immunohistochemistry in an attempt to confirm the origin of the ZsGreen fluorescent axonal debris with immunolabelling for OMP and ß-tubulin III, however the very fine debris was not labelled by the antibodies (Fig. 1D-E, H-I, double-headed arrows). We concluded that this was likely due to the OMP and ß-tubulin III proteins having been degraded more rapidly than the ZsGreen fluorescent protein. However, as the primary olfactory neurons and their axons are the only cells that express ZsGreen fluorescent protein, the debris in the olfactory bulb must have arisen from the axons.

Axonal debris is phagocytosed by radial glia during olfactory bulb development

The distribution of the ZsGreen fluorescent axonal debris near the ventricle suggested that the debris was contained within cells at both E13.5 (Fig. 1K) and E15.5 (Fig. 1L) indicating that cells in the region had phagocytosed the debris. During the early development of the olfactory bulb, radial glia are the principal cells within that region (Puche and Shipley, 2001). To detect radial glia, we used immunolabelling for brain lipid-binding protein (BLBP) which is a cytoplasmic protein and a marker for radial glia (Feng et al., 1994) (Fig. 2A-C). The radial glia were organised in a distinct pattern with their cell bodies close to the ventricle and their processes extended towards the outer surface of the olfactory bulb (Fig. 2D-F). The

ZsGreen fluorescent axonal fragments clearly co-localised with the radial glia (Fig. 2G-L). Over-extended axons and radial glia were viewed from early development. At these ages the presence of the developing olfactory bulb layers, including the nerve fibre layer (NFL), the external plexiform layer (EPL) and the glomerular layer (GL) can be detected (Fig. 2F) From E11.5, the distribution of the intact olfactory axons and the degradation products followed the organisation of the radial glia. Axons were intact in the outer layers of the olfactory bulb where the radial glial processes are localised (Fig. 1J-L, arrows) whereas accumulated degraded axon fragments were found deeper within the bulb, close to the ventricle, where the glial cell bodies reside (Fig. 1J-L, double-headed arrows). We therefore examined the relationship between the over-extending axons and the radial glia in more detail. As the axons over-extended into the olfactory bulb, they initially interacted with the radial glial processes in the developing EPL (Fig. 3A-C, arrows); as they extended further into the region that was densely populated with radial glial cell bodies, the axons became fragmented (Fig. 3D-H) and co-localised with the glial perikarya (Fig. 3I-K double-headed arrows; three-dimensional reconstruction shown in Supplementary movie 1). In the region close to the ventricle where the cell bodies of the radial glia are located, the axon debris was almost always associated with BLBP immunostaining (Fig. 3K). We next used antibodies against lysosomal-associated membrane protein 1 (Lamp1) which verified that the ZsGreen debris was located within lysosomes of the radial glia (Fig. 3L-N, arrows). These results indicate that the degraded axonal debris was internalised at sites distant from the radial glial cell bodies and transported to the perikarya for processing within lysosomes and other intracellular compartments.

Radial glia isolated from the olfactory bulb contain fragments from olfactory axons

To verify that the radial glia were phagocytosing the axonal debris, we dissected and isolated radial glia from crossed S100β-DsRed and OMP-ZsGreen transgenic mice pups. In these

crosses, OECs are easily identified by expression of DsRed (Windus et al., 2007) whereas radial glia do not express DsRed. The radial glia were removed from the central region of the olfactory bulb while leaving the nerve fibre layer intact. The cells were plated out, fixed immediately upon adhesion and then immunolabelled for BLBP expression. Different cell types were present in the dissociated cell mix, but the radial glia were easily identified by the expression of BLBP and the lack of DsRed expression, with 46.4% +/- 4.1 of the cells in the preparation being identified as radial glia (Fig. 4). In tissue sections of the olfactory bulb, OECs strongly express DsRed and BLBP (Fig. 4G-H, arrow), whereas radial glia only express BLBP (Fig. 4H, arrow with tail). All BLBP-positive radial glia contained ZsGreen debris within the cytosol (Fig. 4D, F). Higher magnification imaging clearly showed the axonal debris within the cytosol of the BLBP-labelled radial glia (Fig. 4C-F). Cells that did not express BLBP did not contain ZsGreen fluorescent axonal debris (arrow in Fig. 4B) indicating that the radial glia were the only cells within the central region of the olfactory bulb that were contributing to the phagocytosis of axonal debris.

Sox10 knockout results in disrupted development of the olfactory bulb and altered morphology of radial glia.

Mutations in the transcription factor Sox10 leads to Kallmann's syndrome in humans, a neurodevelopmental disorder characterised by perturbed migration of GnRH-releasing hypothalamic neurons, anosmia and disrupted development of the olfactory bulb (Barraud et al., 2010; Cadman et al., 2007; Cariboni et al., 2007; Hardelin and Dode, 2008). Importantly, in Sox10 knockout mice, olfactory axon targeting is perturbed (Barraud et al., 2013; Pingault et al., 2013), suggesting that regulatory mechanisms determining correct axon targeting may be disrupted. Before investigating how the absence of Sox10 affected the fate of over-extending olfactory axons, we first compared the overall morphology of the developing

olfactory bulb in Sox10 knockout mice and wild-type littermates. In wild-type mice, the nerve fibre layer was thick and a distinct external plexiform layer was present (Fig. 5A, E). Nuclear staining showed that there were fewer cell bodies within the external plexiform layer compared to the adjacent regions (Fig. 5C). In contrast, in the absence of Sox10, the nerve fibre layer was thin and the external plexiform layer was not obvious (Fig. 5B, F). The region corresponding to the position where the external plexiform layer should be located was densely packed with cell nuclei (Fig. 5D, dotted lines). However, we also noted that in the Sox10 knockout embryos there were some areas where the nerve fibre layer was slightly thicker (Fig. 5B, arrowhead). Immediately adjacent to these thicker areas, a more distinct external plexiform layer could be observed. After statistical analyses we found that there was a significant 48.2% decrease in the thickness of the NFL in Sox10 KO embryos compared to the wild-type littermates (t-test $p<0.01$ $n=3$) and that there was a significant 58.3% increase in the number of cells in the EPL region in the Sox10 KO embryos compared to the wild-type littermates (t-test $p<0.001$ $n=3$).

We focussed particularly on comparing the morphology and anatomical distribution of glia in the olfactory bulb in the absence and presence of Sox10. Here, we studied two types of glia; radial glia and olfactory ensheathing cells (OECs). It has been previously shown that differentiation and distribution of OECs is perturbed in the absence of Sox10 (Barraud et al., 2013). OECs are present in the nerve fibre layer of the olfactory bulb, but not in the deeper regions where radial glia reside. BLBP is expressed in both OECs and radial glia (Chehrehasa et al., 2010; Feng et al., 1994), however, as the two glial cell types are not present in the same anatomical location the two populations can easily be distinguished in vivo. We confirmed that loss of Sox10 results in perturbed OEC distribution. We showed that in wild-type E16.5 embryos, the thick nerve fibre layer was highly populated by OECs (Fig. 5A, E). In contrast,

in Sox10 knockout littermate embryos, the OEC population of the nerve fibre layer was dramatically reduced in many regions. Only a few limited regions of the nerve fibre layer were thickly populated by OECs (Fig. 5B, arrowhead). We then examined the distribution of radial glia in the olfactory bulbs of Sox10 knockout mice and wild-type littermates. As discussed earlier, in wild-type embryos, processes of radial glia were restricted to the central region of the olfactory bulb (Fig. 5A, arrow). In contrast, in the absence of Sox10, the radial glial processes (Fig. 5B, double-headed arrow) extended all the way to the nerve fibre layer (Fig. 5B, arrow). Only adjacent to the very thickest areas of the nerve fibre layer, where we showed that OECs were present, did we observe a more normal morphology of radial glia; here, processes of radial glia were shorter and did not reach the nerve fibre layer (Fig. 5B, F). This suggests that there may be a repulsive cue produced by the OECs within the nerve fibre layer that repels the processes of the radial glia and contributes to the formation of the external plexiform layer. In the wild-type embryos the radial glia possessed a more uniform morphology with the filaments and cell bodies radially aligned as they projected away from the ventricle (Fig. 5I). In comparison, in the olfactory bulbs of Sox10 knockout embryos the radial glia were less aligned, appeared disorganised and the cell bodies were often located further from the ventricle than in the wild-type embryos (Fig. 5J, arrow). Thus, loss of Sox10 from OECs results in fewer OECs populating the nerve fibre layer and there is a perturbation in the development of the radial glia within the deeper layers of the olfactory bulb.

Axonal debris accumulates in the Sox10 knockout embryos

We next examined the fate of the axons that over-extended past the nerve fibre layer into the deeper regions of the olfactory bulb in Sox10 knockout mice. As the OMP-ZsGreen mice were of a different background strain to the Sox10 mice, back-crossing the two strains was

considered to be too time consuming, so for this series of experiments, we instead used OMP immunostaining to visualise the initial degradation of the axonal debris.

Similar to the results shown in Fig. 1, E16.5 wild-type embryos exhibited axon over-extension where olfactory axons entered the developing external plexiform layer of the olfactory bulb (Fig. 6A-C, G). Intact axons (Fig. 6A, G, arrows) as well as some fragmented and degraded axonal debris (Fig. 6A, G, double headed arrows) were present in the external plexiform layer. Over-extending intact axons were also present in the Sox10 knockout littermate embryos (Fig. 6D, arrow), but there was a striking difference in the amount of fragmented axonal debris between wild-type and knockout mice. In the Sox10 knockout embryos, immunolabelling for OMP showed considerably more axonal debris present within the deeper layers of the olfactory bulb (Fig. 6D-F, H, double headed arrow) in comparison to wild-type littermates. Since we previously showed that there are fewer OMP-positive neurons in the Sox10 knockout mice at E16.5 than in wild-type mice (Barraud et al., 2013), the increased accumulation of the axonal debris is not due to an increased total number of olfactory axons but more likely due to a perturbation in the phagocytic clearance of the axonal debris by the radial glia (summarised in Fig. 7A, B).

Discussion

In this study, we investigated the fate of olfactory axons that during development miss their target and over-extend into deeper layers of the olfactory bulb, and the behaviour of glial cells in contact with these axons. We showed that during olfactory bulb development, the mis-targeted axons are degraded into axonal debris which is phagocytosed by radial glia that reside in the central region of the bulb. We also demonstrated that in Sox10 knockout mice, the development of radial glia was perturbed and axonal debris accumulated within the deeper layers of the olfactory bulb indicating that the radial glia did not clear the axonal debris from mis-targeted axons with the same efficiency as during normal development.

Previous studies showed that during development, primary olfactory axons over-extend past their target layer and enter the deeper layers of the olfactory bulb (Chan et al., 2011; Gong and Shipley, 1995; Graziadei et al., 1980; Santacana et al., 1992; Tenne-Brown and Key, 1999). These studies, however, did not examine any relationship between the over-extending axons and other cells within the olfactory bulb, such as radial glia and olfactory ensheathing cells. We now show that olfactory axons that extend past their target closely follow the filaments of the radial glia that reside in the deeper layers of the olfactory bulb. In the cerebral cortex, radial glia actively guide the migration of neurons to their final destination and also phagocytose cellular debris (Nacher et al., 1999). Previous studies also suggested that radial glia may play a role together with olfactory ensheathing cells in establishing the glomerular layer of the olfactory bulb by ramifying their glial processes to interact with the axons of primary olfactory neurons (Bailey et al., 1999). Thus, it is possible that the radial glia play a crucial role in guiding the extension of olfactory axons to their correct targets in the olfactory bulb during development.

To determine the fate of the over-extending axons, we used the previously generated line of OMP-ZsGreen mice, in which olfactory neurons and their axons selectively express a bright green fluorescent coral protein. We analysed cryostat sections of the developing olfactory tract and found that ZsGreen fluorescence provided exceptional clarity and detail of olfactory axons, significantly superior to immunolabelling for β-tubulin III, a marker that has previously been used for identifying axon debris within cells (Su et al., 2013). One reason for this was that ß-tubulin III over-saturated the images with axons that were not of interest. This was especially true in the olfactory bulb which is populated with numerous different neurons (Fig. 1H). We also postulate that this was due to more rapid degradation of the β-tubulin III protein in comparison to the ZsGreen coral protein. We found that mis-targeted olfactory axons that extended past their bulbar target were degraded and that the resulting axonal debris was internalised by radial glia. After internalisation, the debris co-localised with the lysosomal marker Lamp1 within the radial glia, suggesting that the debris was degraded by the glial cells. It has been previously shown that adult rat OECs also phagocytose axonal debris in vivo, and that the debris co-localises with Lamp1 within the cells (Su et al., 2013). During embryonic development, however, olfactory ensheathing cells are not in contact with over-extending axons and therefore cannot be responsible for phagocytosis of debris arising from degeneration of these axons. At this stage, it is not clear whether the axons are pruned or entirely degenerated, however, the entire part of the axons localised within the olfactory bulb appears to degenerate following mis-targeting.

We have previously shown that the transcription factor Sox10 is critical for normal development of the olfactory bulb (Barraud et al., 2013). Sox10, a crucial regulator of normal embryonic development and cell fate, is expressed in neural crest-derived OECs (Barraud et

al., 2010), and in Sox10 knockout mouse embryos, the OEC population displays disturbed development, resulting in a significantly thinner than normal nerve fibre layer in the olfactory bulb (Barraud et al., 2013). However, the effect on axon over-extension in the absence of a properly formed nerve fibre layer was not investigated. Because the primary olfactory axons are in close contact with radial glia in the developing bulb, we also examined the behaviour of the radial glia at the time in development that mis-targeting of axons occurs in Sox10 knockout animals. We found that the development of normal radial glial morphology was perturbed. In Sox10 knockout mice at E16.5, the filaments of the radial glia projected almost all the way to the nerve fibre layer and hence, no distinct external plexiform layer was present, contrary to what was witnessed in the wild-type littermates. Since the radial glia themselves do not express Sox10 (Barraud et al., 2013), the effect of the loss of Sox10 must be indirect, effecting radial glia morphology and maturation in a manner that renders them unable to perform normal function. Our analyses revealed that there is an accumulation of axon debris within the EPL of the Sox10 knockout mice. We consider that there are two possibilities that may account for the accumulation of axon debris. First, the perturbed development of the radial glia reduces their ability to phagocytose axon debris and hence it accumulates in the EPL. Second, the loss of OECs from the nerve fibre layer of the Sox10 knockout mice could perturb the ability of the in-growing primary sensory axons to find their target in the developing glomerular layer. OECs and radial glia are suggested to interact together with axons to contribute to the formation of glomeruli (Bailey et al., 1999) and thus the loss of OECs may lead to poor formation of the target zone. Without an appropriate stop signal, more axons would over-project into the deeper layers of the olfactory bulb. While previous analysis revealed that there were fewer olfactory sensory neurons in the Sox10 knockout mice (Barraud et al., 2013), the resultant effect of a loss of a stop signal could be an overall increased number of over-projecting axons. However, while the loss of a stop signal

may be a contributing factor we detected very few intact over-projecting axons; instead there was considerable debris from axons in the EPL of the Sox10 knockout mice. We therefore favour the first model in which reduced phagocytosis by radial glia leads to accumulation of the axon debris but with the caveat that it is a complex system with numerous different cell types each of which may play a contributing role.

In summary, we have investigated the fate of primary olfactory axons that extend past their targets in the olfactory bulb during embryonic development. We found that the distal region of many axons that over-extend closely follow the filaments of central radial glia and that these axons eventually degenerated and the fragments were phagocytosed by the radial glia. We demonstrated that knockout of Sox10 led to perturbed development of the external plexiform layer and of normal radial glial morphology and resulted in an accumulation of axonal debris from degenerating olfactory axons deep within the bulb. Together these results show that radial glia are active participants in the regulation of primary olfactory axon fate within the olfactory bulb.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest

Role of Authors

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: D.A, J.SJ, J.E, F.C; Acquisition of data: D.A; Analysis and interpretation of data: D.A; Drafting of the article: D.A, J.SJ, J.E, C.S, M.W; Obtained funding: J.E, D.A.

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Figure Legends

Figure 1. Z-stack laser scanning confocal images (LSCM) of over-extending primary olfactory axons degrading in deeper layers of the olfactory bulb and epifluorescent mosaic images of whole embryo and embryo head. Panels show sagittal sections of embryonic mouse heads, dorsal is to the top and rostral is to the left. (A) At E15.5, OMP-ZsGreen fluorescence labels primary olfactory neurons lining the olfactory mucosa (OM) and their axons that project into the olfactory bulb (OB). Cell nuclei are labelled with DAPI (blue); the image is a mosaic of several images. Regions similar to those outlined by the dotted squares are shown in B-C and D-E. (B-C) In the olfactory epithelium (OE) and ventral nerve fibre layer (NFL), ZsGreen fluorescence shows fine detail of the primary olfactory axons (arrow) and co-localises with anti-OMP immunostaining. (D-E) In the rostral olfactory bulb, primary olfactory axons that over-extended past the NFL into the deeper layers of the olfactory bulb expressed ZsGreen and were positive for anti-OMP immunostaining; (E) anti-OMP immunostaining labelled primary olfactory axons but ZsGreen fluorescence was stronger and enabled visualisation of some axons (arrow) and axon fragments (double-headed arrow) that were not labelled by OMP antibodies. (F-G) ß-tubulin III immunostaining co-localised with OMP-ZsGreen fluorescence on primary olfactory neurons and their axons (arrow) in the mucosa, (H-I) but did not co-localise with the ZsGreen axonal debris (double-headed arrow) situated adjacent to the ventricle (V). (J-L) During development, primary olfactory axons over-extended past the NFL into the deeper layers of the developing olfactory bulb (arrow) and green fluorescent axonal debris (double-headed arrow) accumulated in the central region of the olfactory bulb in cells lining the ventricle (V). OMP-ZsGreen fluorescence was observed as early as E10.75 as shown in the mosaic image in M and the high magnification image of the developing OE in N. Image properties: A: epifluorescent mosaic, B-C: 26 slice

Z-stack (1.26 μm/slice), D-E: 71 slice Z-stack (0.55 μm/slice), F-G: 47 slice Z-stack (0.55 μm/slice), H: 20 slice Z-stack (1.26 μm/slice), I: 58 slice Z-stack (0.51 μm/slice), J: 28 slice Z-stack (1.26 μm/slice), K: 24 slice Z-stack (1.26 μm/slice), L: 19 slice Z-stack (1.26 μm/slice), M: epifluorescent mosaic, N: 59 slice Z-stack (0.19 μm/slice). Scale bar is 300 μm for A, 50 μm for B-C, 30 μm for D-E, 20 μm for F-G, 70 μm for H, 10 μm for I, 60 μm for J-L, 600 μm for M and 20 μm for N.

Figure 2. Z-stack LSCM images of radial glia and axonal debris accumulating in radial glia in the developing olfactory bulb. Sagittal views of the olfactory bulb at E12.5 (A), E13.5 (B) and E15.5 (C); rostral is to the left, dorsal is to the top in all panels. (A-C) BLBP immunostaining highlights the presence of radial glia and olfactory ensheathing cells in the developing olfactory bulb. (D-E) High magnification images show the nerve fibre layer and the radial glia filaments which extend towards the NFL. At this point the external plexiform layer (EPL) and glomeruli layer (GL) are not distinguishable. (F) E15.5, the EPL and GL are distinguishable (dotted lines). (G-L) High magnification images showing ZsGreen axon debris (double-headed arrow, G-I) associated with radial glia (J-L) near the ventricular surface (V). Image properties: A: 8 slice Z-stack (4.77 μm/slice), B: 4 slice Z-stack (30.41 μm/slice), C: 2 slice Z-stack (30.41 μm/slice), D: 61 slice Z-stack (0.51 μm/slice), E: 25 slice Z-stack (0.55 μm/slice), F: 22 slice Z-stack (0.55 μm/slice), G and J: 15 slice Z-Stack (0.55 μm/slice), H and K: 44 slice Z-stack (0.56 μm/slice), I and L: 65 slice Z-stack (0.51 μm/slice). Scale bar is 133 μm for A, 200 μm for B, 193 μm for C, 60 μm for D, 40 μm for E and F, 9 for G, H and J, K, and 20 μm, μm for I and L.

Figure 3. Z-stack LSCM images of over-extending primary olfactory axons interacting with radial glia filaments and axon debris in radial glia. (A-C) In the EPL, axons over-extended

past the NFL (to left of image) and projected along a radial glia filament. (D) Over-extending axons (arrow) are degraded into axonal debris fragments (double-headed arrow). (E-H) Axon debris (circles) was not continuous with intact axons (arrows). (I-K) Double headed arrow highlights axon debris which is internalised within the radial glia (BLBP positive); three-dimensional reconstruction is shown in Supplemental movie 1. (L-N) The ZsGreen debris co-localised with anti Lamp1 immunostaining (arrows). Image properties: A-C: 39 slice Z-stack (0.51 μm/slice), D-H: 30 slice Z-stack (0.51 μm/slice), I-K: 10 slice Z-stack (0.51 μm/slice), L-N: 53 slice Z-stack (0.56 μm/slice). Scale bar is 9 μm for A-C, 30 μm for D, 23 μm for E-H, 9 μm for I-K and 10 μm for L-N.

Figure 4. Z-stack LSCM images of primary olfactory axon debris, internalised within radial glia. (A-B) Low magnification view of a heterogeneous cell population isolated from the ventricular surface. (A) Nuclei stained with DAPI; ZsGreen fluorescence shows axonal debris; (B) radial glia immunostained with anti-BLBP (white). Cells not labelled with anti-BLBP did not contain ZsGreen debris (arrow). (C-D) Higher magnification view of ZsGreen axonal debris contained within radial glia immunostained with anti-BLBP. (E-F) A single radial glia immunostained with anti-BLBP and containing ZsGreen axon debris. (G-H) A comparison between DsRed-positive OECs and radial glia. Radial glia (arrow with tail) do not express DsRed (blue) but strongly express BLBP (white), whereas OECs express both DsRed and BLBP (arrow). Image properties: A-B: 14 slice Z-stack (4.77 μm/slice), C-D: 42 slice Z-stack (0.25 μm/slice), E-F: 51 slice Z-stack (0.28 μm/slice), G-H: single plane image. Scale bar is 120 μm for A-B, 40 μm for C-D, 10 μm for E-F and 20 μm for G-H.

Figure 5. Z-stack LSCM images showing that loss of Sox10, disrupts development of radial glia. (A) Coronal sections of an olfactory bulb of wild-type E16.5 embryo immunostained

with anti-BLBP to show olfactory ensheathing cells within the normal nerve fibre layer (NFL) around the periphery and the radial glia (arrow) in the central region of the olfactory bulb. (B) Olfactory bulb of Sox10 knockout embryo. The NFL is thinner (arrow) and the radial glia extended their filaments to the NFL (double-headed arrow). The arrowhead is highlighting a slightly thicker NFL area. (C, E, G) Higher magnification view of the developing external plexiform layer (EPL) in a wild-type embryo. The dotted lines highlight the EPL; radial glia filaments are largely absent from the EPL at this age and the NFL is thickly populated by OECs. (D, F, H) In contrast in Sox10 knockout embryos the region of the EPL was less defined and radial glia filaments (arrows) extended to the NFL which was thinner and poorly populated with few OECs. (I, J) Sox 10 wild type and knockout bulbs showing the distinct differences in radial glia morphology. (I) In wild type embryos, the radial glia and their filaments project radially from the ventricle to the NFL. (J) In Sox10 knockout embryos, the cells bodies of many radial glia are located further from the ventricle (V) and the cells appeared disorganised in their alignment (arrow points to a cell that is perpendicular to the radial alignment seen in I). Image properties: A: 8 slice Z-stack (4.77 μm/slice), C, E, G: 42 slice Z-stack (0.55 μm/slice), D, F, H: 49 slice Z-stack (0.55 μm/slice), I: 24 slice Z-stack (1.26 μm/slice), J: 22 slice Z-stack (1.26 μm/slice). Scale bar is 180 μm A-B, 40 μm for C-H and 60 μm for I-J.

Figure 6. Z-stack LSCM images showing that loss of Sox10 results in increased axon extension and axon debris in the deeper layers of the olfactory bulb. (A-C) Immunostaining of wild-type E16.5 olfactory bulb with anti-OMP antibodies revealed axon over-extension (arrow) past the NFL in wild-type embryos and the presence of degraded axons (double-headed arrow); (B) is immunostained with anti-BLBP and merged images are shown in C. Orientation and region of interest are similar to those shown in Fig. 5C-H. (D-F) In Sox10

knockout littermate embryos, anti-OMP immunostaining revealed that there were fewer axons within the NFL (asterisk) and more degraded primary olfactory axons in the deeper layers (double-headed arrow) although intact axons were also present (arrow). (G-H) Higher magnification view of boxed areas in C and F, showing the difference in axon degradation within the olfactory bulb. (G) In wild-type embryos, a few axons (arrow) and axon debris (double-headed arrow) were present in the EPL. (H) In Sox10 knockouts there was an increased amount and spread of ZsGreen debris (double-headed arrow) and little evidence of intact axons. (E,H) In Sox10 knockouts, radial glia bodies were present much closer to the nerve fibre layer but were not detected in similar regions in wild-type littermates (B,G). Image properties: A-C, G: 52 slice Z-stack (0.55 μm/slice), D-F, H: 57 slice Z-stack (0.55 μm/slice). Scale bar for A-F is 40 μm and scale bar for G-H is 15 μm.

Figure 7. Schematics summarising the changes observed between the wild-type and Sox10 KO embryos. (A) In the wild-type embryos there is a mass accumulation of axonal debris as it is taken up by the radial glia. The radial glia have short processes and are uniformly aligned. (B) In the Sox10 knockout embryos, there are fewer olfactory ensheathing cells in the NFL, the radial glia and external plexiform layer do not develop properly and there is more axonal debris. The radial glia also display a disrupted orientation.