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Published in:
Neuroscience Letters

DOI:
[10.1016/j.neulet.2014.01.043](https://doi.org/10.1016/j.neulet.2014.01.043)

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Recommended citation(APA):
Chehrehasa, F., Ekberg, J. A. K., & St John, J. A. (2014). A novel method using intranasal delivery of EdU demonstrates that accessory olfactory ensheathing cells respond to injury by proliferation. *Neuroscience Letters*, 563, 90-95. <https://doi.org/10.1016/j.neulet.2014.01.043>

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**A novel method using intranasal delivery of EdU demonstrates that accessory olfactory
ensheathing cells respond to injury by proliferation**

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Abstract

Olfactory ensheathing cells (OECs) play an important role in the continuous regeneration of the primary olfactory nervous system throughout life and for regeneration of olfactory neurons after injury. While it is known that several individual OEC subpopulations with distinct properties exist in different anatomical locations, it remains unclear how these different subpopulations respond to a major injury. We have examined the proliferation of OECs from one distinct location, the peripheral accessory olfactory nervous system, following large-scale injury (bulbectomy) in mice. We used crosses of two transgenic reporter mouse lines, S100 β -DsRed and OMP-ZsGreen, to visualise OECs, and main/accessory olfactory neurons, respectively. We surgically removed one olfactory bulb including the accessory olfactory bulb to induce degeneration, and found that accessory OECs in the nerve bundles that terminate in the accessory olfactory bulb responded by increased proliferation with a peak occurring 2 days after the injury. To label proliferating cells we used the thymidine analogue ethynyl deoxyuridine (EdU) using intranasal delivery instead of intraperitoneal injection. We compared and quantified the number of proliferating cells at different regions at one and four days after EdU labelling by the two different methods and found that intranasal delivery method was as effective as intraperitoneal injection. We demonstrated that accessory OECs actively respond to widespread degeneration of accessory olfactory axons by proliferating. These results have important implications for selecting the source of OECs for neural regeneration therapies and show that intranasal delivery of EdU is an efficient and reliable method for assessing proliferation of olfactory glia.

Keywords: accessory olfactory bulb; glia; vomeronasal; regeneration; axon

Introduction

Olfactory ensheathing cells (OECs) are the glial cells of the olfactory nervous system that ensheath fascicles of main and accessory olfactory axons. OECs exhibit unique growth-promoting and migratory properties amongst glia and are now regarded as essential for the turnover of olfactory neurons and for regeneration after injury [1].

OECs express a variety of neurotrophic factors [2-3] which are important for the extension and guidance of axons and have led to the investigation of OECs for neural regeneration therapies. We have previously shown that following unilateral bulbectomy, there is a massive influx of OECs from the main olfactory system into the cavity left by the removal of the olfactory bulb [4-5]. The OECs proliferate and migrate in different regions of the olfactory nerve towards the cavity in response to widespread degeneration of the entire olfactory nerve pathway.

While it is clear that OECs in the main olfactory nervous system in rodents proliferate in response to widespread degeneration of olfactory axons, little is known about how OECs in the accessory olfactory nervous system, which is responsible for detecting pheromones, respond to neuronal injury. The cell bodies of accessory olfactory neurons reside in a distinct region of the olfactory epithelium, the vomeronasal organ. Their axons extend into the accessory olfactory bulb in the brain, localised caudally to the main olfactory bulb. We have previously shown that accessory olfactory neurons degenerate in response to unilateral bulbectomy as the accessory olfactory bulb is also removed by the procedure [6]. We therefore hypothesised that OECs ensheathing accessory olfactory neurons respond to widespread neuronal degeneration and investigated the response following bulbectomy.

To label proliferating cells, we have used a novel method of delivery of 5-ethynyl-2'-deoxyuridine (EdU). Currently, the method of application is intraperitoneal injection of EdU which can be

difficult in neonatal mice due to lack of skin elasticity and hence there can often be a leakage of the injected fluid. We have now developed an intranasal delivery method and found that EdU is incorporated into dividing cells following intranasal application as effectively as after intraperitoneal injection.

Overall, our results showed that the OECs of the peripheral accessory olfactory nervous system proliferate in response to large-scale neuronal injury, similar to the OECs of the main olfactory nervous system. These findings have increased our understanding of how different subpopulations of OECs respond to injury, which is highly warranted for future developments of new nerve repair therapies.

Materials and methods

Animals

We used postnatal pups of crosses of two transgenic reporter lines of mice, OMP-ZsGreen [7-8] and S100 β -DsRed [9]. All procedures were carried out with the approval of the Griffith University Animal Ethics Committee.

EdU delivery

The animals received EdU (5-ethyl-2'-deoxyuridine, Invitrogen) at a dose of 50 mg/kg body weight in 20 mg/ml in PBS. For comparison of intranasal versus intraperitoneal injection, the same dose was applied for the two different delivery methods. For intranasal delivery, the pups received 8 μ L to each nostril. Intraperitoneal delivery was injected as previously described [10] with 16 μ L injected. For acute EdU labelling, postnatal day 4 pups received either intranasal or intraperitoneal EdU, and euthanased at P5 to harvest tissues. For long term EdU labelling, pups received intranasal or intraperitoneal EdU at P4 and then harvested at P8. Three animals were used for each treatment at each timepoint.

EdU administration to quantify OEC proliferation

Animals that had undergone bulbectomy treatment were given EdU intranasally at 2, 4, 7 or 9 days after treatment and sacrificed 4 h later. The EdU Click-iT reaction (Invitrogen) was performed as previously described [10].

Surgical ablation of olfactory bulb (unilateral bulbectomy)

The olfactory bulb was removed unilaterally at postnatal day (P) 4.5 as described previously [6]. For each time point littermates were used with three unoperated control pups and three pups that

underwent surgery. Mice were killed by cervical dislocation and the heads were fixed in paraformaldehyde, frozen and sections cut (30 μm) on a cryostat.

Image capture and image preparation

Images were captured using an Axio Imager Z1 epi-fluorescence microscope or an Olympus FV1000 microscope.

Quantification of OECs

For each time point, three animals with at least three sections per animal were examined, with the total number of cells counted being at least 30-90 for each data point. Regions of interest (200 x 150 μm^2) from the ventral nasal septum below the main olfactory epithelium where accessory olfactory axons bundle are present were selected as indicated in Fig. 3C. The total numbers of OECs and EdU-labelled OECs were counted. The percentage of EdU-labelled cells was calculated in each region using equal surface areas. Statistical significance was assessed using ANOVA and Tukey's post hoc test.

Results

Intranasal delivery of EdU labels proliferating cells in different tissues

After intranasal delivery of EdU into P4 pups, numerous EdU-labelled cells could be visualised in the nasal cavity and the labelling pattern was similar to that seen with intraperitoneal injection of EdU (Fig. 1A-B). Cells in the olfactory epithelium were labelled (Fig. 1A, C) as well as other cell types throughout the nasal cavity and olfactory bulb (Fig. 1A). EdU-labelled cells that had proliferated in the cartilage of the nasal septum (Fig. 1D) and skeletal muscle attached to the skull were detected (Fig. 1E). Throughout all tissues examined, EdU co-localised with cell nuclei (DAPI labelling). The extensive labelling of cells that had proliferated in the olfactory epithelium demonstrates effective penetration of EdU by the intranasal delivery method (Fig. 1A). In the olfactory epithelium, cells located in the basal and apical layers of epithelium were labelled with EdU, including sustentacular (arrowhead, Fig. 1C) and horizontal basal cells (arrow with tail, Fig. 1C). Other EdU-labelled cells were also distributed throughout the lamina propria underneath the olfactory epithelium (Fig. 1C). At four days after intranasal delivery of EdU, numerous cells were labelled in the olfactory bulb, rostral migratory stream (RMS), and epidermis (Fig. 1F-H). In the olfactory bulb, EdU-labelled cells were present in the nerve fibre layer, where OECs are localised (Fig. 1F). These results showed that the EdU-positive cells retained the labelling after several days similar to what we observe using the intraperitoneal injection method.

Quantification of EdU positive cells

To determine the efficiency of intranasal delivery of EdU in comparison to intraperitoneal injection, we quantified the number of EdU-labelled cells, in different tissues. The quantification showed that there were no significant differences in the number of proliferating cells in the two methods in different tissues (Fig. 2A-B, ANOVA and post hoc t test, $p > 0.05$ for individual comparisons), indicating the efficiency of intranasal delivery of EdU similar to the standard method of delivery.

Removal of the olfactory bulb induced proliferation of accessory OECs

To determine whether proliferation of OECs from the accessory olfactory system is stimulated by a major injury, we performed unilateral bulbectomy in postnatal mice. Unilateral ablation of an olfactory bulb which includes removal of the accessory olfactory bulb (bulbectomy) results in widespread death of olfactory neurons as well as obliteration of almost all central OECs; however, the contralateral olfactory bulb is relatively unaffected. Postnatal animals were chosen in preference to adults as their regeneration of olfactory neurons is more rapid and uniform [5]. In unilaterally bulbectomised mice, the resulting damage of the sensory axons led to degeneration of the neurons in the ipsilateral olfactory epithelium and vomeronasal organ which became thinner and had fewer ZsGreen-positive accessory olfactory neurons (left vomeronasal epithelium, Fig. 3B, D) compared to the contralateral control side (right vomeronasal epithelium, Fig. 3B, F). Subsequently, the accessory olfactory axons degenerated completely on the bulbectomy side (Fig. 3E) whereas the accessory olfactory axons on the contralateral control side maintained their uniform distribution throughout the accessory olfactory nerve bundles (Fig. 3G).

To determine whether accessory OECs proliferated in response to the bulbectomy, proliferating cells were labelled by intranasally delivered EdU at days 2-9 after bulbectomy and harvested 4 h later. OECs that underwent proliferation at other times would remain unlabelled. In S100 β -DsRed transgenic mice, accessory OECs are easily identified by their expression of DsRed, their morphology and their association with accessory olfactory axons (see Fig. 3E, G). Based on these characteristics, accessory OECs in the regions indicated in Fig. 3C (arrows) were clearly labelled by EdU. As expected, not all OECs were proliferating at the time of EdU exposure and thus EdU-negative OECs were also observed (Fig. 4; arrows with tail in Fig. 4A, D, G). To quantify the proportion of OECs that had proliferated, we examined only the accessory OECs in the nerve bundles that were clearly separated from the main olfactory region (see arrows in Fig. 3C). After

bulbectomy, numerous EdU-positive OECs were present in these accessory olfactory nerve bundles at all periods examined. On the contralateral control side, there were fewer OECs labelled with EdU (Fig. 4D-F), and in unoperated control animals even fewer EdU-labelled OECs were seen (Figure 4G-I).

Quantification of the number of EdU-positive cells in the different conditions showed that at days 2-9 after bulbectomy, significantly more proliferating accessory OECs were detected in the ipsilateral nasal epithelium compared to contralateral control and unoperated controls ($p < 0.01$ at 2-4 days, $p < 0.05$ at 7-9 days, Fig. 4J). The peak of proliferation occurred at day 2 after surgery with EdU-labelled OECs comprising 11% of the total population of accessory OECs. Accessory OECs also proliferated in the lamina propria contralateral to the bulbectomy with 6-7% of OECs being EdU-positive (Fig. 4J). This percentage was significantly higher than in unoperated animals (control; 3% at day 2, Fig. 4J), suggesting that the contralateral olfactory nervous system is also affected by bulbectomy. By day 9 after bulbectomy, the number of proliferating accessory OECs in the bulbectomised side was still significantly higher than the contralateral control and unoperated controls although it was reduced towards normal proliferation rate (~2% as seen in unoperated control animals).

These findings demonstrate that accessory OECs respond to large-scale injury to the central part of the olfactory nervous system by increased proliferation, with particularly high levels of proliferation occurring for 2-7 days after bulbectomy.

Discussion

EdU is an alternative thymidine analogue for labeling proliferating cells and is commonly delivered by intraperitoneal injection [11-14]. Here, we report a new method of delivery of EdU to label proliferating cells in the olfactory nervous system and associated tissue. Our results confirmed that intranasal delivery of EdU labelled the same proportion of cells that had proliferated as the standard intraperitoneal injection method and cells were labelled by EdU not only in the nasal cavity but in surrounding skin, muscle, and cartilage. These results illustrate that intranasal delivery of EdU can be used for acute and long term labelling of proliferating cells.

The surgical removal of one olfactory bulb including the accessory olfactory bulb rapidly resulted in the loss of neurons from the vomeronasal organ as well as loss of axons within the accessory olfactory nerve bundles on side from which the bulb was removed. To ensure accurate identification of the accessory olfactory nerve bundles we examined only those axon bundles that were located ventral to the main olfactory epithelium. We found that the accessory OECs rapidly responded to the ablation of the accessory olfactory bulb by undergoing proliferation with the peak of proliferation occurring two days after bulbectomy, when 11 % of accessory OECs underwent proliferation. This percentage is similar to that we have previously reported for OECs from the main olfactory nervous system (10 %) [4]. Other studies which have examined the proliferation of OECs have reported low levels or negligible levels of proliferation after destruction of the olfactory epithelium [15] or in nerve transection models in adult rats [16]. The differences are likely to reflect in part that we have used young postnatal animals whereas other studies have used adult animals, but nevertheless it is clear from our study that OECs are able to detect the sudden loss of axons and to respond by rapid proliferation.

Our results also indicate that there are differences between the accessory and main olfactory OECs in the time at which proliferation reaches its peak. We have previously reported that for main

olfactory system, OECs within nerve bundles in the lamina propria, the peak of proliferation occurred at day 5 after bulbectomy [4], whereas in the current study the peak of proliferation of the accessory OECs occurred two days after surgery. These differences are potentially of importance for the purification of OECs for neural transplant therapies. Main olfactory OECs are often purified from the lamina propria lining the septum [17-18]; however in the more caudal regions of the nasal cavity the accessory olfactory nerve bundles also project along the dorsal septum and they lie basally to the main olfactory nerve bundles. Thus, purification of OECs from the dorsal septal region may therefore include OECs from the main as well as the accessory olfactory nerve bundles. If the accessory OECs respond and proliferate earlier than the main OECs then the proportion of accessory OECs could be higher than previously considered. As we have shown that OECs from different regions have different cell-cell interaction properties [19], it is essential for cell transplant therapies that the composition and overall properties of purified cells is comprehensively determined to ensure reproducibility of experimental outcomes.

In summary, we have shown that intranasal delivery of EdU is an efficient method for labelling proliferating cells in young postnatal pups. We have used this method to determine that accessory OECs rapidly respond to unilateral bulbectomy of the accessory olfactory bulb and that they proliferate in the accessory nerve bundles that project out of the vomeronasal organ.

Acknowledgements

This work was supported by an Australian Research Council Fellowship to J.E. The project was planned and written by all authors; the experiments and analyses were conducted by F.C.

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Figure legends

Figure 1. Proliferating cells were detected throughout the olfactory and nasal tissue after intranasal delivery of EdU. Panels are coronal sections through the nasal cavity and rostral brain. (A) One day after intranasal delivery of EdU, cells that had proliferated throughout the nasal cavity were labelled. (B) One day after intraperitoneal EdU, the distribution of EdU-positive cells was similar to the intranasal delivery method. (C-E) Higher magnification of different tissues, including olfactory mucosa (C), cartilage within the nasal septum (D) and skeletal muscle (E). (C) Within the olfactory mucosa, cells lining the basal layer of the olfactory epithelium (OE) (arrow) and within the lamina propria (LP) (arrow with tail) were labelled; and sustentacular cells in the apical epithelium were also labelled (arrowhead). (F-H) Four days post intranasal delivery, EdU-positive cells (magenta, arrows) were present in the olfactory bulb (OB) (F), an extension of rostral migratory stream (RMS) (G) and the basal cells of the epidermis (H). GL, glomerular layer; OM, olfactory mucosa; NFL, nerve fibre layer; S, septum. Scale bar: 500 μm in A-B and 35 μm (C-H).

Figure 2. Acute and long-term labelling of proliferating cells by intranasal and intraperitoneal delivery of EdU. Quantification of the number of EdU-positive cells which were labelled by intranasal (black columns) or intraperitoneal (grey columns) delivery in the olfactory epithelium (OE), olfactory bulb (OB), skeletal muscle (SM), cartilage (CT) and respiratory epithelium (RE). One and four days after labelling, similar numbers of proliferating cells were labelled by the two methods (A-B). There was no significant difference between the number of EdU-positive cells at either 1 or 4 days labelling, $p > 0.05$; ANOVA; error bars represent the standard error of the mean.

Figure 3. Accessory OECs proliferate after unilateral bulboectomy. Panels are coronal sections through the main olfactory and vomeronasal (VNO) regions of postnatal OMP-ZsGreen x S100 β -DsRed mice that had undergone unilateral bulboectomy and then labelled with EdU 4 h prior to

harvest. Primary olfactory neurons and accessory olfactory neurons are green; OECs are red; nuclei are stained with DAPI (blue). (A) Seven days after bulbectomy, the regenerating olfactory axons (arrow) penetrated the ventral region of the cavity left after bulbectomy; the contralateral olfactory bulb (OB) is intact. (B) The rostral nasal cavity two days after bulbectomy showing the main olfactory epithelium (MOE) and the vomeronasal organ (VNO), boxed areas are shown in D and F. (C) The ventral septum showing the vomeronasal nerve bundles (arrows) which are distinctly separated from the MOE. Boxed areas are shown in E and G. (D) Neurons (green) within the vomeronasal epithelium on the bulbectomy side were degenerated. (E) Within the vomeronasal nerve bundles on the bulbectomy side, the accessory olfactory axons (arrow) were degenerated. (F-G) On the contralateral side, the neurons within the vomeronasal epithelium were healthy (F) and the axons within the vomeronasal nerve bundles were intact (arrow in G). S, septum. Scale bar is 800 μm in A; 600 μm in B; 220 μm in C; 40 μm in D, F; 20 μm in E, G.

Figure 4. Accessory OECs in the lamina propria proliferated after bulbectomy. (A-I) Panels are coronal sections through the nasal septum in the region ventral to the main olfactory epithelium of unilaterally bulbectomised mice, 2 days after surgery and 4 h after intranasal EdU delivery. Images show OECs (red), and EdU (yellow); merged images are in A, D G. Numerous OECs (red) within the accessory olfactory axons bundles were co-labelled with EdU (yellow, arrows) on the operated side (A-C); not all OECs were labelled by EdU (arrow with tail). Fewer OECs were co-labelled with EdU on the contralateral control side (D-F) and in unoperated control animals (G-I); not all OECs were labelled by EdU (arrow with tail). (J) Quantification of the percentage of OECs that were labelled by EdU on the ipsilateral operated side, the contralateral control side and in unoperated control animals. There was a significant difference between the operated side and both the contralateral control side and unoperated controls at all time points; ** $p < 0.01$; Tukey HSD for 2-4 days and * $p < 0.05$ for 7-10 days; significant difference was present between the contralateral

control side and unoperated control only at 2 days after bulbectomy, * $p < 0.05$; error bars are standard error of the mean. Scale bar is 50 μm .