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Axon Mis-targeting in the Olfactory Bulb During Regeneration of Olfactory Neuroepithelium

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Abstract

During development, primary olfactory axons typically grow to their topographically correct target zone without extensive remodelling. Similarly, in adults, new axons arising from the normal turnover of sensory neurons essentially project to their target without error. In the present study we have examined axon targeting in the olfactory pathway following extensive chemical ablation of the olfactory neuroepithelium in the P2-tau:LacZ line of mice. These mice express LacZ in the P2 subpopulation of primary olfactory neurons whose axons target topographically fixed glomeruli on the medial and lateral surfaces of the olfactory bulb. Intraperitoneal injections of dichlobenil selectively destroyed the sensory neuroepithelium of the nasal cavity without direct physical insult to the olfactory neuron pathway. Primary olfactory neurons regenerated and LacZ staining revealed the trajectory of the P2 axons. Rather than project solely to their topographically appropriate glomeruli, the regenerating P2 axons now terminated in numerous inappropriate glomeruli which were widely dispersed over the olfactory bulb. While these errors in targeting were refined over time, there was still considerable mis-targeting after four months of regeneration.

Key words: glomerulus, guidance, navigation, neuron

Introduction

Primary olfactory neurons in mouse express one of ~1000 odorant receptors. These neurons, which are mosaically distributed throughout at least one of four zones of the olfactory neuroepithelium (Ressler *et al.*, 1993; Vassar *et al.*, 1993; Strotmann *et al.*, 1994), project axons that target sites within the olfactory bulb specific for each odorant receptor (Ressler *et al.*, 1994; Vassar *et al.*, 1994; Nagao *et al.*, 2000). While axons appear to grow to their target area with minimal error, there is considerable local mis-routing, as axons branch and enter multiple glomeruli as well as overshoot the glomerular layer and continue growing into deeper inappropriate laminae of the olfactory bulb (Santacana *et al.*, 1992; Royal and Key, 1999; Tenne-Brown and Key, 1999). These errors in targeting continue until around postnatal day 12, after which the glomeruli exhibit the beginnings of a mature organization (Kim and Greer, 2000).

The primary olfactory neurons have a limited life span and there is continual turnover of the neurons as they are replaced by stem cells in the basal layer of the olfactory neuroepithelium (Schultz, 1941; Graziadei and Graziadei, 1979). Hence, throughout life, primary olfactory axons enter the adult olfactory bulb and project to glomerular targets that are appropriate for each odorant receptor and they do so largely without error. Similar specificity in targeting is observed even when neurons expressing the same odorant receptor are selectively ablated by genetic techniques (Gogos *et al.*, 2000). However, what happens during widespread regeneration, for example following severe upper respiratory tract infection or trauma to the nasal cavity? Are signaling mechanisms still expressed which allow axons to sort out and find their target? In humans who suffer such diseases, the restoration of olfaction is often inhibited, resulting in anosmia or dysosmia that can persist for several months to years (see review by Wolfensberger and Hummel, 2002). It would therefore seem that in some situations regeneration is not occurring sufficiently to allow functional restoration of olfaction.

Recent animal studies have yielded contradictory results regarding the targeting ability of regenerating axons. Following physical lesioning of the olfactory nerve, regenerating primary sensory olfactory axons fail to target their original topographically correct glomerular site (Costanzo, 2000; Christensen *et al.*, 2001). Instead, axons converge at inappropriate positions in the bulb. One of the problems

with this technique is that a physical lesion can produce scarring that prevents normal axon re-growth. An alternative approach is to chemically induce degeneration of the olfactory neurons. When Triton-X was used to deafferent the olfactory bulb in H-OMP-LacZ-6 mice, the primary olfactory axons appeared to correctly reinnervate glomeruli within the same region of the olfactory bulb (Cummings *et al.*, 2000). In these mice LacZ was expressed by a large subpopulation of olfactory neurons that projected to numerous glomeruli on the ventromedial and ventrolateral surfaces of the olfactory bulb (Treloar *et al.*, 1996; Cummings *et al.*, 2000). This projection pattern made it difficult to determine if regenerated axons innervated the same glomerulus both pre- and post-lesion. Alternatively, methyl bromide inhalation can induce lesion of the olfactory neuroepithelium, probably by disrupting the P450 cytochrome system, leading to almost complete loss of primary olfactory neurons (see review by Schwob, 2002). Following methyl bromide lesioning, primary olfactory neurons regenerate within appropriate epithelial zones and at least one large subpopulation of axons recognised by the RB-8 antibody re-establishes a broad topography (Christensen *et al.*, 2001). However, again it was unclear whether targeting at the level of single glomeruli was restored post-lesion. A subsequent genetic approach to delete only P2 neurons revealed that regenerating axons were able to correctly reinnervate their original target site in the olfactory bulb (Gogos *et al.*, 2000). The selective ablation of a discrete subpopulation of olfactory neurons is quite unnatural and provides little insight into regeneration normally experienced in wild-type mice. Moreover, the fact that this genetic approach spared ~5% of the P2 subpopulation of olfactory neurons leaves open the possibility that the remaining neurons were providing a source of guidance cues for regenerating axons. Considering that axon targeting appears to rely on axon–axon interactions mediated in part by odorant receptors (Key and St John, 2002) the presence of unablated P2 neurons provides a convenient substrate for axon targeting during regeneration.

We have examined axon targeting in a model in which the olfactory neuroepithelium is selectively degenerated and the P2 neurons are completely lost, while sparing the olfactory pathway between the epithelium and bulb of direct physical trauma. The olfactory neuroepithelium was chemically degenerated using dichlobenil (2,6-dichlorobenzonitrile), which acts by irreversibly binding in Bowman's glands and inducing lesions via a cytochrome P450-dependent mechanism (Brandt *et al.*, 1990; Brittebo, 1997; Mancuso *et al.*, 1997). We demonstrate that regenerating P2 axons project to numerous inappropriate glomeruli over a large rostro-caudal and ventro-dorsal area of the bulb. While improvements in targeting occur with time, even after 4 months numerous aberrant projections were still present.

Materials and methods

Chemical ablation of primary olfactory neurons

Adult (7- to 15-week-old) female homozygous P2-IRES-tau-LacZ transgenic mice were distributed amongst five groups, with six mice per group. Four mice in each group were given intraperitoneal injections of dichlobenil (2,6-dichlobenzonitrile, 25 μg/g body wt) in dimethyl sulphoxide (2 μl/g body wt) on days 1 and 5. Two mice in each group served as controls and were given intraperitoneal injections of dimethyl sulphoxide using the same injection regime. The groups of mice were killed by CO_2 asphyxiation respectively at 2, 4, 8, 12 and 18 weeks after the last injection. Heads were immersion-fixed for 4 h at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Following fixation, heads were decalcified in 20% disodium ethylene diaminetetraacetic acid in PBS (pH 7.4). Heads were placed in embedding matrix (OCT compound; Miles Scientific, Naperville, IL) and snap frozen by immersion in iso-pentane that had been cooled by liquid nitrogen. All procedures were carried out with the approval of, and in accordance with, the University of Queensland Animal Ethics Experimentation Committee. Serial coronal sections (30 μm) of animals at each age were cut on a cryostat microtome, collected on slides coated with 2% gelatine and 0.1% chromalum, air dried overnight and stored at –25°C.

Histochemistry

X-Gal staining was performed as previously described (Royal and Key, 1999). Sections were counterstained with nuclear fast red.

Immunohistochemistry was performed using the protocol described in Key and Akeson (1993). Sections were reacted overnight at 4°C with goat anti-olfactory marker protein (OMP) (1:15 000) (Keller and Margolis, 1975), followed by rabbit anti-goat immunoglobulins conjugated to biotin (Vector Laboratories, Burlingame, CA), then incubated in Vectastain Elite avidin–biotin–horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories) and reacted with 3,3-diaminobenzidine (0.5 mg/ml) in the presence of 0.02% H_2O_2.

For double-label immunofluorescence, sections were incubated in the primary antibodies rabbit anti-β-galactosidase (20 μg/ml; Cortex Biochem, Inc., San Leandro, CA) and goat anti-OMP (1:1000). Sections were incubated with secondary antibodies conjugated to TRITC or Cy5 as appropriate. Fluorescence images were collected using a Biorad MRC 1024 confocal laser scanning microscope. Z-series images for TRITC and Cy5 were collected every 1.5 μm through the depth of the section and merged.

Lectin staining with biotinylated *Dolichos bifloris* agglutinin was performed as previously described (Key and Akeson, 1993).

Image preparation

Images were collected with a Spot 2 digital camera (Spot Diagnostic Instruments, Inc., Sterling Heights, MI) fitted on an Olympus BH2 microscope with differential interference contrast optics. Images were colour balanced and montages constructed using Adobe Photoshop 7.0 software (Adobe Systems Incorporated, San Jose, CA) without further digital manipulation.

Analysis of regeneration following chemical ablation of primary olfactory neurons

For each animal, every second section was analysed for the presence of P2-LacZ-positive axons within glomeruli. A P2-positive glomerulus was defined as any glomerulus containing P2-LacZ axons. Glomeruli on the lateral and medial surfaces in both bulbs for each animal were examined. The mean rostro-caudal extent of glomeruli on the lateral and medial surfaces in which P2-LacZ axons were present was measured by determining the most rostral and most caudal sections in which P2-LacZ axons could be detected in glomeruli. The dorso-ventral extent of P2-LacZ-positive glomeruli was determined by measuring the distance between the most dorsal and the most ventral glomeruli within a single 30-μm-thick section. The measurement was carried out using Spot 2 software version 3.5.6 (Spot Diagnostic Instruments). For each section examined, the number of glomeruli in which P2-LacZ axons were present was counted. Data are presented as means for each treatment. Data for control animals were similar, and were therefore combined as one group.

Results

Axon regeneration following chemical lesion of the olfactory nerve

Following administration of dichlobenil by intraperitoneal injection into P2-IRES-tau:LacZ mice, animals were allowed to survive for between 2 and 18 weeks. During the first 2–4 weeks we observed the loss of primary olfactory neurons as both the thickness of the olfactory neuroepithelium decreased and the blue X-Gal stained P2 neurons disappeared (compare Figure 1A,B). In the olfactory bulb, OMP immunostaining revealed the widespread loss of axon terminations in the glomerular layer of treated (Figure 1F) but not control (Figure 1E) animals. Due to the absence of Bowman's glands in the vomeronasal organ, dichlobenil does not affect the population of accessory olfactory sensory neurons. The assessory olfactory bulb therefore provides a valuable control for the specificity of dichlobenil since the accessory olfactory axons, vomeronasal nerve pathway and accessory olfactory bulb (Figure 1G) were unaffected by the treatment. Between 8–18 weeks following treatment, the olfactory neuroepithelium was repopulated with primary olfactory neurons, including the P2 subpopulation (Figure 1C,D). As the primary olfactory axons re-grew into the bulb, the P2 axons converged and formed small loci at multiple inappropriate sites (Figure 1H–J) as opposed to the typical one to two large glomeruli in control animals (Figure 1O). While large P2 glomeruli were observed at 18 weeks recovery (Figure 1K), numerous small loci continued to be present as well (Figure 1L–N). Between 12 and 18 weeks, 43% of olfactory bulbs had at least one large P2 glomerulus on the lateral surface and 31% had at least one large glomerulus on the medial surface. Whereas in control animals P2 axons filled glomeruli (Figure 1P), in lesioned animals P2 axons formed small fascicles that terminated in subregions of glomeruli that were also innervated by other OMP-positive primary olfactory axons (Figure 1Q).

The P2-LacZ axons terminated in multiple loci that were located over a wide rostro-caudal distance. We examined the location of glomeruli containing P2 axons and found that between 8 and 18 weeks of regeneration P2 axons terminated in loci that were both more rostral and more caudal compared with the location of P2 glomeruli in control animals (Figure 2A). In control animals the position of the glomeruli typically exhibits ~15% variability compared to the length of the olfactory bulb whereas in treated animals this approaches 40%. The extent of the rostro-caudal mis-targeting was particularly evident when P2 axons were found on both the medial and lateral sides of the olfactory bulb within the same coronal section (Figure 2A at 8–12 weeks). These glomeruli are normally at least 500 μm apart in the rostro-caudal plane in control animals. In addition, P2 axons terminated in widely dispersed glomeruli about the dorso-ventral axis on both the medial and lateral sides of the olfactory bulb (Figure 2B). While the spread of P2 axons in both the rostro-caudal plane (Figure 2A) and the dorso-ventral plane (Figure 2B) peaked at around 8–12 weeks regeneration, the number of glomeruli containing mis-targeted P2 axons continued to increase up to 18 weeks on both the lateral (Figure 3A) and medial (Figure 3B) surfaces. Thus, while the P2 axons appeared to be more restricted in their target zone, more glomeruli were inappropriately targeted.

Discussion

In order to further understand targeting in the regenerating primary olfactory system we ablated the olfactory neuroepithelium using the herbicide dichlobenil (2,6-dichlorobenzonitrile). Dichlobenil irreversibly binds in Bowman's glands inducing glandular death via a cytochrome P450-dependent mechanism which indirectly results in degeneration of the olfactory neuroepithelium (Brandt *et al.*, 1990; Brittebo, 1997; Mancuso *et al.*, 1997). Administration of dichlobenil has previously been shown to alter the expression of P-450 within sustentacular cells (Walters *et al.*, 1993) but leaves the basal lamina, and nerves were unaffected (Mancuso *et al.*, 1997). The timing of the degeneration of the olfactory neuroepithelium is similar to other methods of chemical lesioning such as methyl bromide (Schwob *et al.*,

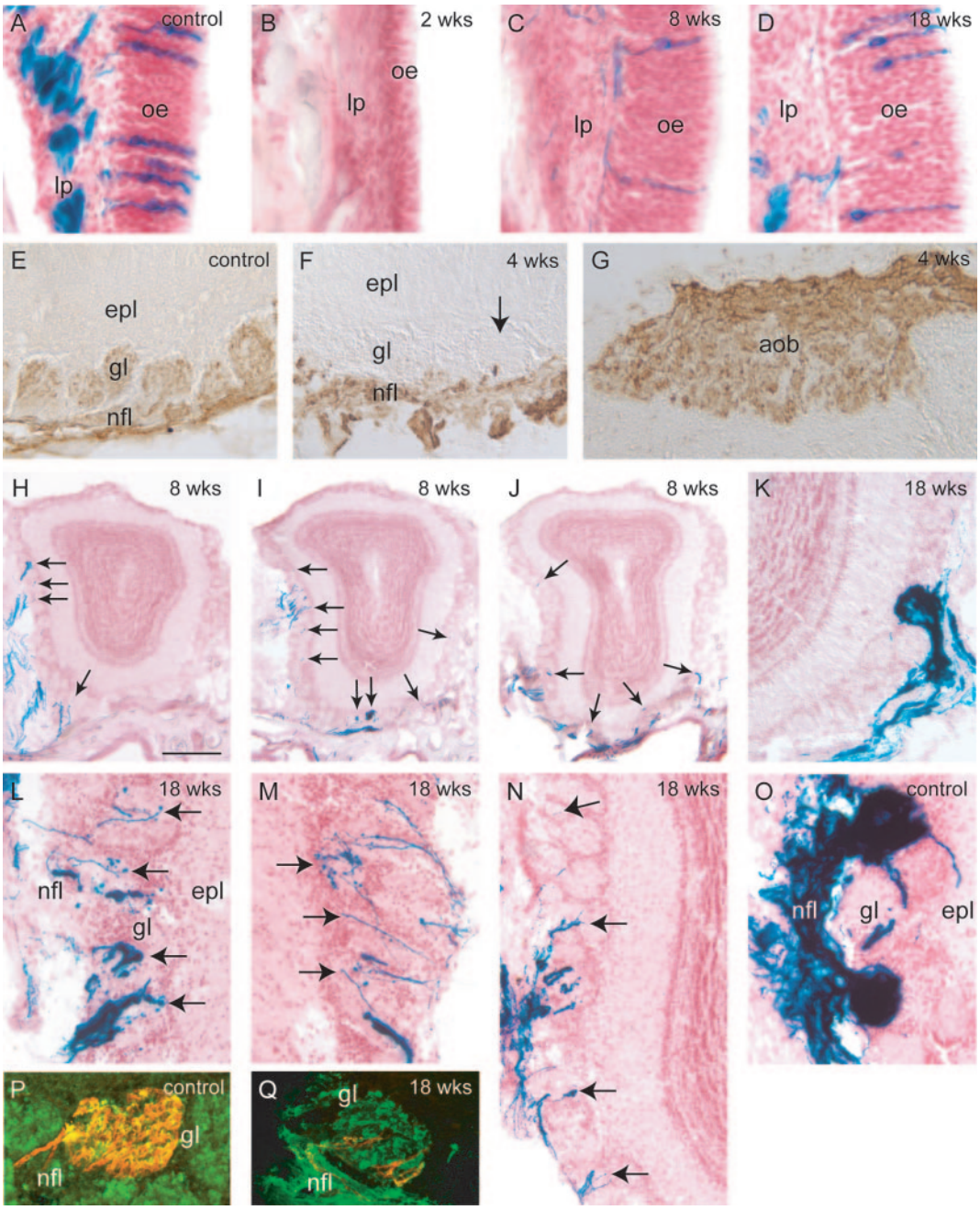

Figure 1 Axon regeneration following chemical ablation of primary olfactory neurons. Panels are coronal sections through the olfactory bulbs and nasal cavity. **(A)** Olfactory neuroepithelium (oe) in a control animal with P2-LacZ positive neurons. **(B)** Two weeks following chemical ablation treatment, the olfactory neuroepithelium was reduced in thickness and P2-LacZ neurons were not present. **(C)** Eight weeks following ablation, the olfactory neuroepithelium had regenerated and some P2-LacZ neurons were present. **(D)** The olfactory neuroepithelium at 18 weeks following ablation. **(E)** OMP immunostaining in a control animal labelled all glomeruli. **(F)** At 4 weeks following chemical ablation OMP staining was considerably reduced. OMP was present in the nerve fibre layer (nfl), but was absent from glomeruli (arrow). **(G)** The accessory olfactory system was not affected by the chemical ablation treatment. Lectin histochemistry using *Dolichos biflorus* agglutinin labelled the entire accessory olfactory bulb (aob). (H–J) At 8 weeks post-chemical ablation, P2-LacZ axons (arrows) terminated in numerous glomeruli on the lateral, ventral and medial surfaces of the olfactory bulb. Panels **H**, **I** and **J** are each separated by 240 μm, with panel H being most rostral and panel J most caudal. **(K)** Eighteen weeks following chemical ablation, P2-LacZ axons often terminated in glomeruli in the topographically correct position. However, small bundles of P2-LacZ axons (arrows) were also found in numerous glomeruli on both the lateral **(L)** and medial **(M)** surfaces of the olfactory bulbs. **(N)** P2-LacZ axons were not restricted to a small region, but terminated in numerous glomeruli over a wide extent of the dorso-ventral bulbar surface. **(O)** In a control animal, P2-LacZ axons terminated in two glomeruli on the lateral side of the olfactory bulb. **(P)** Double label immunohistochemistry in a control animal. OMP antibodies (green) label all primary olfactory axons within glomeruli while P2-LacZ axons (red) terminate in a single glomerulus. **(Q)** In lesioned animals, P2-LacZ axons (red) partially innervate glomeruli. Panels A–D, H–O are counterstained with nuclear fast red. Epl: external plexiform layer; gl: glomerular layer; lp: lamina propria. Bar is 20 μm in A–D, P, Q; 40 μm in E–F, L–O; 80 μm in G; 330 μm in H; 400 μm in I; 420 μm in J; 170 μm in K.

Figure 2

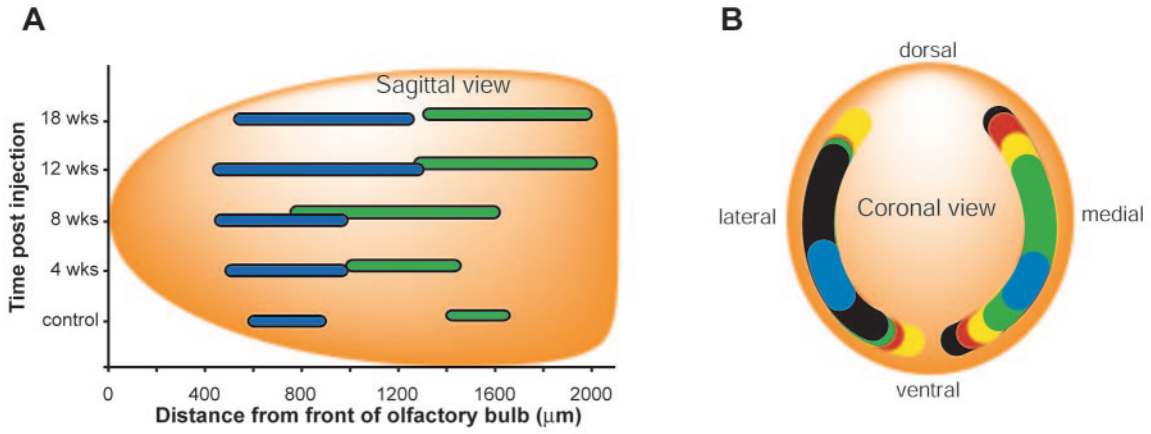

Figure 3

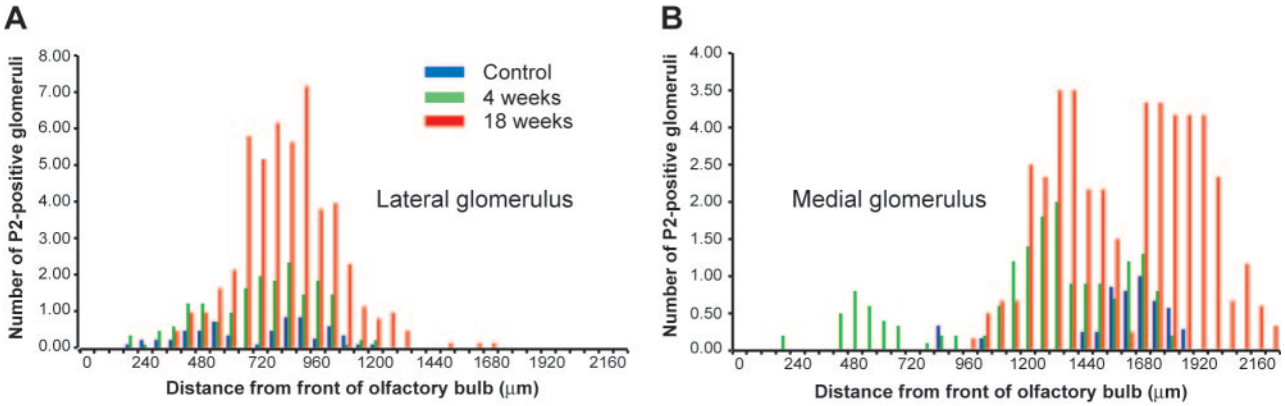

Figure 2 Distribution of P2-LacZ-positive glomeruli following chemical ablation of primary olfactory neurons. (A) The mean rostro-caudal extent of lateral (blue) and medial (green) glomeruli in which P2-LacZ axons were present as measured from the front of the olfactory bulb. (B) A schematic representation of a coronal view of the olfactory bulb showing the differences in the dorso-ventral distribution of lateral and medial glomeruli in which P2-LacZ axons were present; control (blue), 4 weeks (green), 8 weeks (yellow), 12 weeks (black), 18 weeks (red).

Figure 3 P2 axons terminate in numerous glomeruli. The mean number of glomeruli containing P2-LacZ axons per 30 μm coronal section on the lateral (A) and medial (B) surfaces of the olfactory bulb in control mice (blue) and experimental mice at 4 weeks (green) and 18 weeks (red) following chemical ablation.

1999) and Triton-X (Kawano and Margolis, 1982; Cummings *et al.*, 2000). The administration of the toxicant killed almost the entire population of primary olfactory neurons and removed all P2-LacZ neurons within 2 weeks without any obvious damage to the basal layer of the neuroepithelium or lamina propria. Thus, while this technique ablated the primary olfactory neurons it left the olfactory pathway free from direct physical damage. The specificity of the response was remarkable since the vomeronasal neuroepithelium and pathway were undamaged by the treatment.

Following surgical transection of the mouse olfactory nerve, regenerating P2-LacZ axons project to numerous inappropriate glomeruli leading to their partial innervation by these axons (Costanzo, 2000). However, transection of the olfactory nerve causes extensive physical damage to the olfactory nerve pathway and even to the olfactory bulb which can indirectly affect axon growth and guidance. Regenerating olfactory axons may not be able to target correctly due to indirect physical damage, tissue scarring, inflammatory responses, and possibly even death or disorganization of olfactory ensheathing cells. In contrast, chemical lesioning leaves the olfactory nerve pathway free of direct physical trauma. However, when lesioned with Triton-X100, the targeting of regenerating axons appeared not to be greatly disrupted (Cummings *et al.*, 2000). While

many neurons degenerated after 1 week with this technique, numerous cells remained throughout the epithelium. It is therefore possible that in this model regenerating axons may have been able to follow unlesioned axons. In comparison, lesioning with methyl bromide can cause the loss of >95% of primary olfactory neurons (Schwob *et al.*, 1995), and yet it does not greatly disrupt the topography of the regenerated RB-8 subpopulation of neurons (see Christensen *et al.*, 2001; Schwob, 2002). As noted above, the Triton-X100 and methyl bromide studies used markers for large subpopulations of neurons.

The results we obtained following chemical lesioning using dichlobenil in P2-LacZ mice are similar to those observed in the same mice following surgical transection of the olfactory nerve. Numerous P2-LacZ axons aberrantly targeted glomeruli that were distributed over a broad region of the olfactory bulb, both dorso-ventrally and rostro-caudally. Other studies suggest that, following widespread regeneration, axons tend to project to more anterior glomeruli (see review by Schwob, 2002). In our study, regenerated P2 axons did initially target more anterior glomeruli, particularly on the medial surface of the olfactory bulb. However, with increased recovery time the most anterior position glomeruli in which P2 axons were present was similar to control animals. In addition, P2 axons now projected to much more posterior glomeruli as well (Figure 2a and 3). With increased recovery time, the extent of mis-targeting was greatly reduced as was also observed following nerve transection (Costanzo, 2000). A significant part of this improved specificity of targeting may be due to activity-dependent competition leading to the elimination of mis-targeted axons that fail to make functional synaptic connections (Zhao and Reed, 2001). In addition, mis-targeted neurons may die. P2 neurons whose axons fail to reach the P2 glomerulus have previously been shown to die over a 10 week period (Wang *et al.*, 1998).

Why is it that axons are not able to sort out correctly from other axons during the initial stages of widespread regeneration? It is clear that the adult olfactory bulb still expresses the necessary targeting cues since many P2 axons are still able to innervate their topographically correct P2 glomeruli on the medial and lateral surfaces of the olfactory bulb. However, in the face of massive in-growth of regenerating axons it would appear that there are errors in the sorting out of specific subpopulations which then affects their convergence on to one or two glomeruli. This is clearly not the case during the early postnatal period since we have shown that in bulbectomised neonates P2 axons are able to sort out and converge to discrete loci (St John *et al.*, 2003). When only P2 olfactory neurons are genetically ablated in adults, the regenerating P2 axons are able to converge and target topographically appropriate glomeruli (Gogos *et al.*, 2000). This would indicate that the adult olfactory bulb possesses the necessary guidance cues and that regenerating primary olfactory axons are able to read these cues. It would seem that in any paradigm that induces extensive axon reorganization, either via lesioning of the olfactory nerve (Costanzo, 2000; Christensen *et al.*, 2001) or by chemical destruction by dichlobenil of the olfactory neuroepithelium then axon targeting is disrupted. Thus, an ordered sorting of olfactory axons en route to the olfactory bulb seems essential for producing a topographical map. It now remains to be determined what changes occur in the expression of guidance cues along the olfactory nerve pathway following extensive degeneration of the olfactory neuroepithelium.

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