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Response of olfactory axons to loss of synaptic targets in the adult mouse

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Abstract

Glomerular convergence has been proposed to rely on interactions between like olfactory axons, however topographic targeting is influenced by guidance molecules encountered in the olfactory bulb. Disruption of these cues during development misdirects sensory axons, however little is known about the role of bulb-derived signals in later life, as new axons arise during turnover of the olfactory sensory neuron (OSN) population. To evaluate the contribution of bulb neurons in maintaining topographic projections in adults, we ablated them with N-methyl-D-aspartate (NMDA) in P2-IRES-tauLacZ mice and examined how sensory axons responded to loss of their postsynaptic partners. NMDA lesion eliminated bulb neurons without damage to sensory axons or olfactory ensheathing glia. P2 axons contained within glomeruli at the time of lesion maintained convergence at these locations; there was no evidence of compensatory growth into the remnant tissue. Delayed apoptosis of OSNs in the target-deprived epithelium led to declines in P2 neuron number as well as the gradual atrophy, and in some cases complete loss, of P2 glomeruli in lesioned bulbs by three weeks. Increased cell proliferation in the epithelium partially restored the OSN population, and by eight weeks, new P2 axons distributed within diverse locations in the bulb remnant and within the anterior olfactory nucleus. Prior studies have suggested that initial development of olfactory topography does not rely on synapse formation with target neurons, however the present data demonstrate that continued maintenance of the sensory map requires the presence of sufficient numbers and/or types of available bulbar synaptic targets.

Keywords

axon guidance; glomeruli; topography; convergence; targeting; olfactory bulb

Introduction

Functional organization of the peripheral olfactory pathway relies on selective expression of odorant receptors by olfactory sensory neurons (OSNs) in the nasal cavity. Odorant receptors (ORs) are G-protein coupled receptors, and their expression determines not only the odor response properties of individual OSNs, but their central projection patterns as well (Buck, 1996; Mombaerts et al., 1996; Wang et al., 1998). Evidence to date suggests that in mice, each OSN expresses only one of ~1000 different OR genes (Mombaerts, 2004). Though scattered within fairly broad domains in the olfactory epithelium, OSNs expressing the same receptor project their axons to topographically conserved regions of the olfactory bulb, where they coalesce into glomeruli. Here they establish synaptic contacts with the dendrites of mitral,

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tufted and periglomerular neurons. Spatial patterns of glomerular activation that reflect stimulation of select sets of OSNs are thought to form the basis of odor coding in the olfactory bulb (Buck, 1996; Wachowiak et al., 2003).

Odorant receptor proteins, which localize to OSN axons as well as dendrites (Barnea et al., 2004), are critical to the organization of olfactory afferents. Genetic deletion of OR coding sequences disrupts axon convergence, producing afferents that fail to coalesce into glomeruli, while in most cases, substitution of one coding sequence for another shifts glomerular terminations to novel bulb locations (Mombaerts et al., 1996; Wang et al., 1998). However, guidance molecules that govern sensory mapping in other systems have been shown to also control the patterning of olfactory axons. Bulb neurons and olfactory ensheathing glia (OEG) express Eph receptors and semaphorins, while OSNs express ephrins and neuropilins (Cutforth et al., 2000; Schwarting et al., 2000; St. Johns et al., 2002). The crossing of mouse strains deficient in guidance molecules with strains expressing a labeled OR allele that permits visualization of their axons (Mombaerts et al., 1996), has demonstrated shifts in normal glomerular targeting (Walz et al., 2002; Cutforth et al., 2003; Schwarting et al., 2004). A mechanism linking OR expression with sensitivity to guidance cues has been proposed based on recent studies of G protein signaling pathways in OSNs (Imai et al., 2006; Chesler et al., 2007). OR signaling through the G protein G_s increases OSN expression of specific guidance molecules, including neuropilin-1, through a transcriptional mechanism mediated by cAMP. Genetic disruption of these intracellular pathways eliminates or shifts the targeting of sensory neurons expressing a common OR, presumably by altering their responsiveness to guidance cues (Imai et al., 2006; Chesler et al., 2007).

As with most sensory systems, topographic olfactory connections are established before birth but undergo postnatal refinements, some of which are activity-dependent, to achieve normal adult organization (Royal and Key, 1999; Zhou et al., 2004). However, new olfactory connections are continually made throughout life. OSNs have a limited lifespan and are replaced by neurons generated from proliferating basal cells in the epithelium (Farbman, 1990; Mackay-Sim and Kittel, 1991). In spite of continual afferent turnover, the fidelity of the glomerular map remains stable, suggesting that mechanisms governing map formation persist throughout life. The regenerative capacity of the system has allowed examination of bulbar re-innervation patterns following injuries that trigger widespread death and synchronous replacement of OSNs in adults. These include olfactory nerve transection and chemical destruction of the epithelium, manipulations leave the bulb intact, but nevertheless cause perturbations in glomerular targeting following re-innervation (Costanzo, 2000; Christensen et al., 2001; St. John and Key, 2003a). They also deafferent the bulb, and trigger inflammatory responses in the epithelium (Getchell et al., 2002). In contrast, temporary genetic ablation of only those sensory neurons expressing the P2 OR has shown that re-innervating P2 axons target the postnatal bulb correctly (Gogos et al., 2000). This outcome suggests that guidance cues are normally present in older animals, but may be altered by deafferentation, or function imperfectly following widespread peripheral injury, a situation that improves with time (St. John and Key, 2003a).

To directly assess the role of postsynaptic neurons in the maintenance of sensory axon distribution, the present study examines the convergence and targeting of intact P2 axons in adult mice using N-methyl-D-aspartate (NMDA) to eliminate bulb neurons without damaging OSNs and their axons. Our findings demonstrate that P2 axons that have already coalesced into glomeruli do not stray upon loss of postsynaptic contacts, and additionally support the notion that neuronal targets provide extrinsic signals that influence OSN survival and turnover (Schwob et al., 1992; Wieler and Farbman, 1999; Carson et al., 2005). In contrast to the effects of olfactory nerve transection (Costanzo, 2000), P2 axons that reach the neuron-depleted bulb

after lesion show disrupted distributions, indicating that map topography is only maintained when a sufficient complement of appropriate neuronal targets remain available.

MATERIALS AND METHODS

Animal Treatments

Homozygous mice of the P2-IRES-tauLacZ strain (P2 mice; generously provided by Dr. Peter Mombaerts at the Rockefeller University, New York) were bred in our facility. Unilateral olfactory bulb lesions were carried out using NMDA according to the methods of Sofroniew et al., (1990). This study used ketamine, a non-competitive NMDA open channel blocker, as the surgical anesthetic. Due to ketamine's ability to interact with NMDA receptors, we first carried out preliminary experiments to determine if this method of anesthesia interfered with NMDA ablation of olfactory bulb neurons. Adult male and female mice (3–10 months old) were anesthetized with ketamine hydrochloride/xylazine (0.08 mg/g and 0.12 mg/g; i.p.; n=6) or with sodium pentobarbital (65 mg/kg; n=6) and placed in a Stoelting stereotaxic device equipped with a mouse adapter and warming pad (37°C). An opening was drilled in the frontal bone overlying the right olfactory bulb at 1.3 mm anterior to the frontal pole of neocortex, and 0.6 mm lateral (right) of the midsagittal suture. A Hamilton syringe (26 gauge) was lowered 1.5 mm ventral to the bulb surface and 1.5 µl of N-methyl-D-aspartic acid (12 mg/ml in phosphate buffered saline (PBS), pH adjusted to 7.0; Tocris, Ellisville, MO) was injected over a 7-minute period, and the needle remained in place for 5 minutes before being withdrawn. Four days or one week later (n=3 per treatment per time point), animals were anesthetized with sodium pentobarbital (150 mg/kg), and intracardially perfused with PBS followed by 2% paraformaldehyde in 0.1 M PIPES buffer (pH 6.9). Brains with attached bulbs were dissected and cryoprotected in 20% sucrose in 0.1 M sodium phosphate buffer (PB). The rostral forebrain was embedded in OCT compound (Sakura Finetek, Torrance, CA) and frozen in isopentane (−55°C). Serial coronal cryosections through the olfactory bulbs (20 µm) were collected on charged glass slides. Alternate series of sections were stained using neutral red and the dye FluoroJade (below) to microscopically evaluate neuronal degeneration. Results were equivalent for both types of anesthesia, with massive loss of neurons by four days, and loss of tissue volume accompanied by cavitation by one week. Brief ketamine anesthesia did not limit the effectiveness of NMDA using the concentration, volume and delivery method described, and surgical recovery was faster with this method. All subsequent lesions were carried out with ketamine/xylazine anesthesia, and mice with significant sparing of bulb tissue were excluded from the study. Inhalant anesthetics were not used to avoid the possibility of direct damage to the sensory epithelium.

Adult male and female mice received a single injection of NMDA (1.5 µl) into the right olfactory bulb and were killed 1 day (n=5), 4 days (n=4) or 1, 2, 3 or 8 weeks later (n=4–5 per time point), as described above. Eighteen control mice (3 per time point) received 1.5 µl of sterile saline. Brains and olfactory bulbs were cryoprotected and frozen as described. Nasal cavities were decalcified in 0.25 M ethylenediamine tetraacetic acid (EDTA, pH 7.5) prior to cryoprotection and freezing. In some cases, forebrains were dissected with nasal cavities attached. All procedures were carried out in accordance with National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the Florida Atlantic University Institutional Animal Care and Use Committee.

X-gal staining

Reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Sections through the nasal cavities and olfactory bulbs (20 µm) rinsed for 15 min in X-Gal buffer (0.1 M PBS, 2 mM MgCl₂, 5 mM EGTA, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) followed by incubation at 37°C in X-Gal buffer containing 5 mM potassium

ferricyanide, 5 mM ferrocyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). Tissue was counterstained with neutral red. Counts of P2 neurons in the epithelium were made at 40x objective magnification from 20 sections per animal, spaced at 60 μ m intervals. Sections processed for X-gal staining, as well as other histological procedures, were examined and photographed using an Olympus AX70 fluorescent and bright field microscope equipped with a Magnafire digital camera. Images were imported into Adobe Photoshop 7.0 for labeling and assembly of composite figures, with adjustments made for brightness and contrast.

FluoroJade labeling

Sections through the olfactory bulbs were stained with the anionic fluorochrome Fluoro-Jade, as described by Schmued et al., 1997. Air-dried sections were rehydrated in decreasing concentrations of ethanol, rinsed in deionized water, and treated with 0.06% potassium permanganate for 15 min, followed by incubation in 0.001% FluoroJade (Histo-Chem Inc, Jefferson, AR) for 30 min at room temperature (RT).

Terminal transferase mediated-UTP-nick end labeling of fragmented DNA (TUNEL)

Sections were treated with 0.3% Triton-X-100 for 10 min, followed by equilibration in terminal transferase buffer (TTB) for 15 min (30 mM Tris-HCl, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.2). Sections were then incubated for 2 hrs at 37°C in TTB containing 200 U/ml terminal transferase (New England Biolabs, Ipswich, MA), 0.2 mg/ml BSA, and 0.5 μ l/ml digoxigenin (DIG)-11-dUTP (Roche Applied Science, Indianapolis, IN). Tissue incubated with mouse antibody to DIG (1:100; Roche Applied Science) at 4°C overnight, and labeling was localized using donkey anti-mouse IgG conjugated to AlexaFluor 555 (1:500; Molecular Probes, Eugene, OR). Controls included treatment of some sections with DNase (1000 U/ml) for 30 min at 37°C prior to incubation in terminal transferase, or omission of terminal transferase from the labeling reaction. TUNEL-labeled cells were counted on each side of the epithelium for each animal in five representative sections per animal, sampled at equivalent rostrocaudal levels of the nasal cavity (based on the presence of turbinates), spaced at ~300 μ m intervals. Counts were made throughout the full extent of the epithelium at 100x objective magnification using an Olympus AX70 fluorescent microscope. Only densely labeled, round nuclei were counted as apoptotic cells. Group means ($\pm$ S.E.M.) were generated from animal totals to characterize patterns of apoptosis as a function of survival time after lesion.

Immunohistochemistry

Sections were quenched in 1% H₂O₂ in PB, blocked in 3% normal serum, then incubated with rabbit anti-glial fibrillary acidic protein (anti-GFAP, 1:500; Dako USA, Carpinteria, CA), or mouse anti-NeuN (1:300; Chemicon, Temecula, CA). Primary incubations were carried out overnight at 4°C in PBS containing 3% BSA or normal serum, and 0.3% Triton-X-100, followed by treatment with species-appropriate secondary IgGs conjugated to biotin (1:1000; Vector Laboratories, Burlingame, CA). Tissue then incubated in avidin-biotin-HRP complex (Vectastain Elite kit), followed by chromagen development in PB containing 0.01% 3,3'-diaminobenzidine, 0.005% H₂O₂, 0.03% cobalt chloride and 0.015% nickel ammonium sulfate. For double labeling, additional mice were lesioned as described. One saline-treated mouse and three NMDA-treated mice were perfused with 4% paraformaldehyde in PB (pH 7.3) at 1, 2, 3 or 8 weeks after lesion. Nasal cavities were decalcified, cryoprotected, embedded and frozen. Tissue sections (30 μ m) were processed free-floating using goat anti-OMP (1:7000), in combination with rabbit anti-growth associated protein-43 (anti-GAP-43, 1:500; Chemicon, Temecula, CA). Labeling was visualized using secondary antibodies conjugated to AlexaFluor 488 or 568 (1:1000; Molecular Probes, Eugene, OR).

For combined TUNEL labeling and immunostaining, free-floating sections were first processed for TUNEL as described above, with antibody to OMP or GAP43 added to the incubation solution containing DIG antibody. TUNEL labeling was detected with AlexaFluor 568-conjugated donkey anti-mouse IgG. OMP or GAP-43 immunostaining was localized with AlexaFluor 488-conjugated donkey anti-goat- or anti-rabbit-IgG, respectively. Sections were examined and images collected using a BioRad Radiance 2100 laser scanning confocal microscope with LaserSharp software.

For Ki-67 immunostaining, nasal tissues from four saline- and four NMDA-treated mice were collected at three weeks post-lesion and processed for Ki-67 immunostaining using a rabbit antibody to Ki-67 (Dr. S.J. Watson; Isgor and Watson, 2005). Frozen coronal sections (14µm) through the epithelium were collected onto charged slides and postfixed. Slides were first rinsed with 0.05% Triton X-100/TBS solution (Tris buffered saline, 0.1 M Tris-HCl, 0.9% NaCl, pH 7.4). For epitope retrieval, slides then incubated at 91°C in a water bath for 1 h in 10% Na-Citrate buffer, pH 6.0. Slides cooled to room temperature (RT), and were treated with 0.05% Triton X-100 in TBS containing 0.6% H₂O₂ for 30 min. Sections were blocked in 2% normal goat serum for 1 hr, followed by incubation in primary antibody to Ki-67 (1:20,000) overnight at 4°C. After rinsing, sections incubated in goat anti-rabbit IgG (Santa Cruz Biotech, Santa Cruz, CA) for 1 hr at RT, followed by incubation with ABC complex and chromagen development according to the manufacturer's instructions (Santa Cruz Biotech ABC kit). Control staining was evaluated by omission of primary antibody. Counts of labeled nuclei in equivalent portions of the left and right epithelia were made at 100X magnification along 1 mm of the nasal septum, from the dorsal recess extending ventrally. Counts were restricted to the deepest half of the epithelium. Six sections per mouse, spaced at 168–210µm intervals, were quantified to generate mean numbers of labeled cells per mm, per animal, with group means calculated from these values.

In situ hybridization/Quantitative densitometry

Sections through the olfactory epithelium were hybridized with ³⁵S-labeled OMP or GAP-43 cRNA. Olfactory bulb sections were hybridized with ³⁵S-labeled neuropeptide Y (NPY) cRNA. The 218 base OMP riboprobe was complementary to positions 692–900 of the GenBank sequence **NM-012616**. The NPY cRNA comprised 720 bases complementary to GenBank sequence **M20373**, and the GAP-43 cRNA was complementary to positions 334–897 of GenBank sequence **M16228**. Hybridization was carried out as described (Guthrie and Gall, 2003), and sections were processed for film (Kodak Biomax MR) followed by emulsion (Kodak NTB) autoradiography, with exposure times of 3–7 days and 4–10 weeks, respectively. Autoradiograms were digitized and labeling densities calibrated relative to radiolabeled standards (American Radiolabeled Corp., St. Louis, MO), using NIH Image analysis software (Wayne Rasband, NIH). Density measurements were made throughout the full extent of both the left and right olfactory epithelia from a minimum of six sections per animal (~300 µm spacing). Changes in mean hybridization density in the epithelium ipsilateral to lesion were expressed as a percentage of measures made in the left, control epithelium of each animal. Group means ± S.E.M. were calculated from these values, and significance of the lesion effect was evaluated using one-way analysis of variance (ANOVA), followed by Student's Neuman-Keuls test for post hoc comparisons. ANOVA analysis revealed no significant difference in labeling ratios obtained from saline-treated mice at any time point for OMP (p=0.13) or GAP-43 (p=0.35) cRNA hybridization, and these values were combined for comparisons to experimental groups. Labeling specificity was verified by hybridization of sense RNA sequences.

Olfactory epithelium thickness

Sections processed for X-gal staining and counterstained with neutral red were examined at 60X objective magnification using bright field optics. A calibrated reticule was used to measure the depth of the olfactory epithelium from the basal lamina to the luminal surface, at 10 equivalent locations along the left and right sides of the nasal septum, beginning at the dorsal recess and extending ventrally for 1.5–1.7 mm. Measurements were made from a minimum of five sections per animal, spaced at ~300 μ m intervals. Mean left and right values for each animal were used to generate group means at post-lesion intervals, with Student's paired t-test used for comparisons.

RESULTS

Elimination of bulb neurons by NMDA

NMDA receptors are expressed by neuronal populations throughout the adult olfactory bulb (Sassoe-Pognetto et al., 2003; Murphy et al., 2005; Christie and Westbrook, 2006), and as described for other brain regions (Sofroniew et al., 1990), bulb NMDA injection caused rapid neuronal death. Neutral red and Fluoro-Jade (FJ)-staining demonstrated that most mitral cells had disappeared within 24 hours (Fig. 1). At this time, all laminae contained large numbers of degenerating cells intensely labeled with FJ, with the exception of the olfactory nerve layer (ONL), which lacked staining at all post-lesion time points. Diffuse FJ staining also occurred in the external plexiform layer (EPL) and within glomeruli, indicating dendritic degeneration, and within the lateral olfactory tract (LOT), indicating degeneration of mitral/tufted cell axons. Labeled cells remained evident in the glomerular and granule cell layers four days after lesion (Fig. 1H). By one week, the bulb was smaller at dissection, laminar organization had deteriorated (Fig. 1D), and cell death was largely complete, with only a few cells labeled by FJ seen in the granule cell layer (Fig. 1I). From 2–3 weeks, small glomerular-like structures persisted, usually in dorsal and ventrolateral locations, though these were few in number (Figs. 1E–F). Treated bulbs showed cavitation at the injection site, as well as evidence of infiltration by erythrocytes, probable macrophages and other peripheral cells. The lesion cavity expanded in size out to three weeks, with the remaining bulb present as a rim of tissue around it. Cell staining in sections caudal to the lesion showed that the subependymal layer (SEL) had expanded in size (not shown), with cells apparently piling up behind the lesion cavity.

At eight weeks, FJ non-specifically stained networks of glial processes around the lesion cavity. Neutral red staining showed glomerular-like structures scattered throughout lesioned bulbs, particularly in superficial locations. The ONL remained recognizable at all time points, however it varied in depth over the post-lesion interval and along different aspects of lesioned bulbs. The accessory olfactory bulb (AOB) was also partially or completely lesioned in all NMDA-treated mice, as were rostral portions of the anterior olfactory nucleus (AON) and ventral neocortex. However, 20% of experimental mice showed some sparing of neurons in caudal ventral bulb, indicating that NMDA did not always reach this area.

The absence of cell degeneration in the ONL suggested that olfactory ensheathing glia (OEG) were not damaged by NMDA. This was verified by examining bulbs for expression of neuropeptide Y (NPY) (Gall et al., 1986; Ubink et al., 1994). Bulb neurons expressing NPY mRNA were rapidly killed by lesion while ensheathing glia remained at all time points (Fig. 2A–C). Shrinkage of the bulb compacted the OEG population into a dense band of cells around the remaining tissue. Elimination of bulb neurons was verified by immunostaining for the nuclear protein NeuN, a neuronal marker expressed by all classes of bulb neurons with the exception of mitral cells, which were readily identifiable in neutral red-stained material. By one week, most NeuN-immunoreactivity (IR) had disappeared from lesioned bulbs, with small numbers of stained cells still seen in the deteriorating glomerular and granule cell layers (Fig.

2E). Though the vast majority of bulb neurons were eliminated by NMDA, some NeuN-labeled nuclei were still present in the main bulb at three weeks (Fig. 2F), and in portions of the AOB that were not heavily damaged. These are likely to be neurons that survived NMDA, however we cannot rule out the possibility that neurons migrated into the remnant from the rostral migratory stream after lesion, despite the cavitation that developed (Lois and Alvarez-Buylla, 1994). At eight weeks, NeuN+ cells were observed deep in the remnant, caudal and ventral to the lesion cavity. Their distribution in and around the SEL suggested that they had indeed migrated into the remaining tissue. Rare NeuN-labeled profiles were seen in more superficial areas that contained glomeruli, but these were surprisingly scarce in light of the number of foci that appeared to be present. As NeuN is a pan-neuronal marker that labels cell nuclei, we were not able to further characterize these cells in terms of phenotype.

Widespread gliosis was detected as increased GFAP-IR within one day of lesion, and by four days, reactive astrocytes were found in all bulb laminae except the ONL (Fig. 2H). These persisted in degenerating portions of glomerular and granule cell layers at one week, and by three weeks, a network of GFAP+ processes had formed around the lesion cavity (Fig. 2I). GFAP-IR in the ONL, indicative of OEG and their processes, was seen at all time points. All bulbs contralateral to NMDA lesion appeared normal. Saline-injected bulbs were normal except for a limited amount of gliosis around the needle track.

Distribution of P2 axons after loss of postsynaptic partners

Inhibition of synaptic transmission in P2 neurons by conditional expression of tetanus toxin causes axons that have previously converged into glomeruli to separate and wander (Yu et al., 2004). To determine if loss of synaptic contacts would trigger similar alterations, we examined the distribution of P2 axons in lesioned adult bulbs using histochemical localization of β -galactosidase. Examination of stained serial sections indicated that most mice (50%) contained two medial and two lateral P2 glomeruli in the non-injected bulb. Some of the mice contained one medial and one lateral P2 glomerulus, while others contained three at each location. This variability in numbers of OR-specific glomeruli across mice has been noted previously (Royal and Key, 1999; Schaefer et al. 2001), and for this reason, changes in the distribution of P2 axons in lesioned bulbs are described here relative to the contralateral bulb of the same animal. Figures 3 and 4 illustrate some of these changes. Though bulb neurons were rapidly eliminated by NMDA, P2 axons that remained present in the glomerular layer maintained convergence into foci 2–3 weeks later (Figs. 3D, 3F, 4A). Reductions in the size of X-gal stained foci were evident by one week and by two weeks, stained foci in treated bulbs averaged only ~50 μ m in diameter compared to ~120 μ m in control bulbs (Figs. 3C-D). At this time numbers of foci remained equivalent in both bulbs. At three weeks, stained foci were reduced in size (Figs. 3F and 4A), and in some animals, in number as well. They ranged from 20–50 μ m in diameter, while X-gal stained glomeruli in contralateral bulbs averaged 100–150 μ m. In three of five mice that survived for three weeks, numbers of P2 foci in both bulbs were equivalent. However in one lesioned mouse, the treated bulb contained only two small medial clusters of stained axons; lateral P2 fibers were absent. In the fifth mouse, X-gal stained fibers were entirely lacking in the bulb remnant, though a few stained axon bundles were present in the ipsilateral epithelium. A correlation between the amount of target tissue remaining and the persistence of P2 innervation is likely. This animal sustained the most extensive lesion of all mice in this group, which involved the entire main bulb, the AOB, anterior AON, and rostroventral neocortex.

Although all NMDA-lesioned bulbs were shrunken and lacked normal lamination, as shown in figures 3F and 4A, P2 axons that persisted out to three weeks appeared to maintain coalescence at their original positions, when compared to locations of P2 glomeruli in contralateral bulbs. Despite of the loss of postsynaptic contacts, sensory axons did not appear

to stray from P2 glomerular structures. Individual axons were observed wandering around the perimeter of P2 foci, but these fibers appeared to be in transit to the second, nearby glomerulus in regions containing two P2 glomeruli. Sprouting axons, in the form of distinct stained fibers emerging from the parent structure, as described by Yu et al. (2004), were not observed invading adjacent locations or penetrating into deeper portions of the tissue (Figs. 4F–G). Our analysis was limited to X-gal staining, and does not eliminate the possibility of more subtle rearrangements that may only be demonstrable with more sensitive techniques, such as electron microscopy.

P2 foci were present in the bulb remnant eight weeks after lesion, but by this time, the original distribution was disrupted and glomerular structures were observed in diverse locations. Examples are shown in figure 4. Foci were typically small in diameter (~20µm; Figs. 4B–C), but some were as large as 90µm. Numbers of foci per bulb varied across mice, ranging from six to approximately twelve, with no apparent symmetry in terms of their medial-lateral distribution. In four cases, P2 foci were located in the medial or lateral halves of the remnant, but their dorsoventral and rostrocaudal locations varied widely, as did their depth within the tissue. X-gal stained fibers coursed through the nerve layer of lesioned bulbs, but also diverted to penetrate deep into the tissue parenchyma, coming close to the SEL. Comparisons to normal topography are complicated by disrupted bulb anatomy, and some glomerular structures observed eight weeks may have persisted at established locations as new axons tracked along prior connections. Individual P2 foci appeared to be densely occupied by stained fibers, rather than sparsely innervated, but without double-labeling for multiple OR axon populations we cannot address the issue of heterogeneous innervation, nor do we know if observed foci are transient as opposed to maintained structures.

In the fifth mouse that survived to eight weeks, the bulb lesion was incomplete. Though not used for quantitative data, this case proved interesting in terms of axon targeting. As shown in figure 4D, a subpopulation of P2 fibers entering the medial bulb diverted from the main group to innervate a ventrally located glomerulus within a spared region. The normal medial target area was damaged but not completely ablated, and some P2 axons succeeded in converging at this location as well. P2 glomeruli are not normally located near the ventral midline (Schwartz et al., 2004), and their convergence here indicates these fibers established a new glomerulus, or displaced ventrally projecting axons from this location. This partial lesion may have created competitive interactions among new axons for available target space, or distorted the patterning of neuronal guidance signals. Though evidence suggests that the zonal location of OSNs in the periphery determines dorsoventral positioning of glomeruli (Miyamichi et al., 2005), we did not observe significant restriction of foci along this axis; P2 foci were found at both more dorsal and more ventral locations than normal (Figs. 4B–C), in so far as axes appeared to be maintained.

Olfactory projections in the absence of target neurons

To better assess the global response of the epithelium to target neuron loss, we examined the distribution of mature and immature sensory axons in lesioned bulbs using immunostaining for OMP and GAP-43 respectively (Schwob, 2002). As shown in figure 5, OMP-IR revealed irregularities in glomerular size and shape within one week of lesion. By three weeks, glomerular structures were found in small clusters at dorsal and ventral locations but, either as a consequence of axon withdrawal or degeneration, were essentially lacking in medial and lateral bulb (Figs. 5C and G). OMP+ axons remained present, but were restricted to the ONL. In some animals, loss of tissue volume and expansion of the lesion cavity left only a thin rim of tissue along the medial aspect of the remnant, along which a band of OMP+ fibers traveled (Fig. 5C). By eight weeks, OMP+ foci had reappeared throughout the bulb remnant, with considerable variation, both within and across animals, in their size, shape and location (Figs

5D and H). Sizes ranged from ~20 μm up to large, irregularly shaped clusters of fibers of measuring ~400 μm along a single dimension. As has been observed following neonatal bullectomy (Graziadei et al., 1978), OMP+ axons invaded more caudal forebrain regions as well; stained foci were found in the AON of two mice at this time (Fig. 5Q).

GAP-43-IR remained restricted to the ONL of lesioned bulbs for the first week. At two weeks, small numbers of stained fibers extended more deeply into the remaining tissue (Fig. 5J). A week later, the ONL was densely occupied by GAP-43+ axons, and numerous stained fibers emerged from this layer to penetrate the tissue parenchyma, with some traveling along the outer margins of the lesion cavity. Small bundles of coalescing GAP-43+ terminals were also observed in the tissue parenchyma (Fig. 5O). Immunostaining for GAP-43 and OMP overlapped in the ONL at this time. GAP-43+ fibers occurred throughout the ONL at three weeks, as well as in numerous foci within the remnant. Double labeling demonstrated that most, but not all, of the foci present at eight weeks were composed of both GAP-43+ and OMP+ fibers, a situation seen during early glomerular development (Kim and Greer, 2000; Fig. 5P), suggesting that elaboration of new projections after lesion recapitulates developmental patterns of bulb innervation.

Cell survival and proliferation in the epithelium lacking neuronal targets

The most straightforward explanation for the loss of P2 axons over time was a concomitant loss of the sensory neurons themselves. Thus we evaluated numbers of P2-expressing OSNs in the target-deprived epithelium. Figure 6 illustrates the declines in P2 sensory neuron number seen after bulb neuron ablation. Numbers of X-gal stained neurons were significantly reduced by one week, when numbers reached 39.5% ($\pm 2.3\%$) of normal. At three weeks, only 21.5% ($\pm 9.0\%$) of the population remained (Figs. 6A and D). Over the next five weeks, numbers of P2 neurons recovered slightly, reaching 29% ($\pm 5\%$) of numbers counted in the contralateral epithelium (Fig. 6E). When present, P2 neurons appeared restricted to their normal expression zone within the olfactory epithelium (zone 2).

Losses in P2 neurons were paralleled by atrophy of the deprived epithelium, as evidenced by a reduction, and later a partial recovery, in epithelial thickness and in OMP-IR (Figs. 6F–H). By two weeks, the deprived epithelium exhibited a 37% decrease in thickness (53.3 $\pm$ 1.8 μm left side vs. 33.4 $\pm$ 3.4 μm right side; $p < 0.001$; paired t-test). By eight weeks, this decrease averaged only 22% (52.5 $\pm$ 6.6 μm left side vs. 41.9 $\pm$ 5.2 μm right side; $p < 0.01$; paired t-test). GAP-43-IR demonstrated an expression pattern inverse to that of OMP; as the numbers of mature sensory neurons expressing OMP declined, numbers of immature neurons expressing GAP-43 increased (Figs. 6I–K). Qualitative differences seen with immunostaining were verified by quantitative measurements of OMP and GAP-43 cRNA hybridization in the epithelium (Fig. 7). Density of OMP cRNA labeling declined to 44% of normal at two weeks after lesion, while increases in GAP-43 cRNA hybridization peaked at this time (213% of contralateral levels). Thereafter, a slight recovery toward normal expression levels occurred for both transcripts.

As shown in figure 8, numbers of TUNEL-labeled nuclei in the epithelium increased after NMDA lesion, in accordance with results reported by Carson et al. (2005). Increases were detectable by four days, with a significant increase seen at one week (217 $\pm$ 65 cells left side vs. 3.5 $\pm$ 1 cells right side; $p = 0.043$, Student's paired t-test). At 2–3 weeks, numbers of stained profiles in the deprived epithelium averaged 22 per animal, and by eight weeks this had declined to 14 cells. In contralateral epithelia and samples from saline controls, cell count totals ranged from zero to eight. All regions showed the effects of target lesion, however changes in both TUNEL and OMP-IR suggested that medial areas of endoturbinates II, II' and III, and portions of ectoturbinate 3, were particularly impacted. Combined labeling for TUNEL and OMP- or GAP-43-IR revealed that the majority of apoptotic cells did not co-label with either of these

markers, although TUNEL-labeled profiles were observed at epithelial depths occupied by adjacent OMP+ or GAP-43+ neurons (Figs. 8C, 8E). However there were small numbers of double-labeled neurons seen at all time points examined (Figs. 8D, 8F).

Increased apoptosis was followed by an upregulation in cell proliferation (Fig. 8G), as evidenced by a 2.2-fold increase in the numbers of Ki-67 expressing cells within the basal epithelium by three weeks (123.5 ± 12.3 (SEM) cells/mm right vs 58.5 ± 9.7 cells/mm left, $p=0.0017$, Student's t-test). The density of labeled cells in contralateral epithelia did not differ significantly from densities measured in right or left samples from saline-treated mice ($p=0.165$, t-test) at this time.

DISCUSSION

The widespread use of guidance molecules in the developing nervous system makes this an attractive mechanism for the patterning olfactory axons throughout life, and recent evidence of mechanisms that link OR expression with OSN sensitivity to guidance molecules points to a role for target neurons, ensheathing glia, and other cell types that serve as sources of these cues, in the development and life-long stability of the olfactory glomerular map (Imai et al., 2006; Serizawa et al., 2006; Chesler et al., 2007). Maintenance of precise topography is likely to require contributions from all of these cellular elements.

Synaptic disconnection and axon rearrangements

Although axons in the glomerular layer gradually retract or degenerate shortly after lesion, our results show that elimination of synaptic contacts does not cause axons within previously established glomeruli to wander beyond these foci. Prior evidence that bulb neurons are not essential for convergence comes from studies showing that like olfactory axons can coalesce in the absence of some developing bulb neurons (Bulfone et al., 1998), and even in novel tissue environments. Regenerating P2 axons form ectopic foci in forebrain following neonatal bullectomy, and within fibrous tissue masses found in the nasal cavities of mutant *extratoes* mice (St. John et al., 2003b). However, during development, some axons stray from their neighbors to travel separate paths to the same target, or overshoot the glomerular layer, elaborating branches in the EPL (Royal and Key, 1999). Axons will also rapidly wander from established glomeruli as synaptic contacts are disassembled following selective inactivation of transmitter release (Yu et al., 2004). These observations raised the possibility that intact olfactory axons might stray when postsynaptic contacts were lost, and that newly-arriving axons would rapidly extend beyond established foci to locate potential targets. Our global ablation of neurons did not leave many alternative targets available however, and perhaps a more selective lesion of only those neurons innervated by P2 axons might have revealed diverging fibers. As shown by Yu et al. (2004), inhibition of functional transmission does not disrupt convergence when all OSNs are functionally blocked. Convergence fails only in a competitive environment in which P2 connections are selectively inactivated while other sensory neurons maintain functional contacts.

Glial elements in the bulb were spared by NMDA, and these cells may have provided signals that maintained or restricted sensory axons in the degenerating glomerular layer after lesion. Reactive astrocytes express extracellular matrix molecules that inhibit axon growth after CNS injury (Fitch and Silver, 1997), and bulb radial glia have been proposed to participate in glomerular formation by providing axon stop signals during development (Gonzalez and Silver, 1994). The transformation of glomerular astrocytes into reactive astrocytes may have presented a growth barrier that inhibited axon extension within and beyond this layer. Such rearrangements may not be advantageous in a system designed to regularly replace connections. Recovery after damage might be better served by maintaining portions of topographic pathways for later-generated axons to travel, than by allowing surviving afferents

to redistribute inappropriately. In this way, contacts with correct topography might restore functional organization within a reasonable time frame, particularly as damage stimulates sensory neuron replacement (Carr and Farbman, 1991; Schwob et al., 1992). The nature of olfactory coding also may make rearrangements detrimental from a neural coding standpoint. Normal olfactory function relies on spatial organization of sensory input, as well as the organization of second-order neuronal circuits that receive this input (Belluscio et al., 2002). Unlike sensory systems that contain continuously mapped information, sprouting of intact axons from one glomerulus to another could alter the bulb's combinatorial code. Luo and colleagues have elegantly demonstrated sensory input stability in the *Drosophila* antennal lobe. When an identified glomerulus is depleted of input, intact axons from adjacent glomeruli do not expand into the denervated territory (Berdnik et al., 2006).

Distribution of new P2 axons in the neuron-depleted bulb

Our results indicate that a full complement of bulb target neurons is necessary to maintain normal afferent distribution, if for no other reason than it provides new axons with an array of potential synapse formation sites that allow them to terminate at preferred locations. Axons generated after peripheral lesions exhibit less drastic disturbances in patterning. Mis-targeting occurs, but as long as bulb neurons remain available, topography is partially restored over time, albeit imperfectly (Costanzo, 2000; St. John and Key, 2003b). The availability of established dendritic targets in adult mice has similarities to *Drosophila* antennal lobe development. In this system, projection neurons are patterned and their dendrites topographically specified, prior to being contacted by select sets of sensory axons (Jefferis et al., 2004).

Though small numbers of NeuN+ cells survived NMDA lesion, over time P2 glomeruli atrophied and in some cases disappeared, indicating that numbers of surviving neurons, and any positional cues, trophic factors or synaptic sites they provided, were not sufficient to maintain normal P2 innervation patterns. Axons elaborated shortly after NMDA lesion have access to peripheral pathways that include pre-existing P2 projections. Ensheathing glia also persist, and their expression of guidance molecules like semaphorin 3A (Schwartz et al., 2004) may continue to direct axons appropriately as they reach the bulb remnant. Nevertheless, by eight weeks, new P2 axons coalesce at "inappropriate" locations, rather than remaining within their normal ventrolateral and ventromedial domains, a task they accomplish during development in transgenic mice lacking bulb neuronal populations (Bulfone et al., 1998). Some axons arriving at the neuron-depleted bulb may initially target these areas, as they do in the aforementioned mice, with those that survive long enough continuing onward, arresting and persisting at locations that contain spared (or new) bulb neurons, or alternative synaptic targets, such as AON neurons.

Neurons in the olfactory system express a number of known guidance molecules that act to pattern sensory afferents, maintain separate fasciculation of vomeronasal and olfactory nerve projections, and restrict bulb innervation to the glomerular layer (Walz et al., 2002; Cutforth et al., 2003; Cloutier et al., 2004). Mice deficient in semaphorin or ephrin signaling display defects in axon targeting (Walz et al., 2002; Cutforth et al., 2003; Schwartz et al., 2004). Results of these studies and others (Costanzo, 2000; Gogos et al., 2000) are consistent with a role for bulb neurons and glia in axon mapping. Ablation of bulb neurons eliminates neuron-derived signals, including those detected on contact, but may also indirectly affect targeting by reducing the numbers of surviving OSNs, by limiting the availability of factors that promote axon growth, or by altering OSN activity levels. A decline in P2 neuron number has implications for fasciculation and convergence mechanisms that are proposed to rely on interactions requiring a minimal number of like axons (Ebrahimi and Chess, 2000; Feinstein and Mombaerts, 2004). Potential trophic limitations on axon growth do not appear to hinder re-innervation of the remnant and in fact, olfactory axons are notorious for their ability to grow

in the absence of normal targets, providing they do not encounter scar tissue (Graziadei et al., 1978; Pasterkamp et al., 1998). P2 fibers in NMDA-lesioned adult mice behave in much the same way as those in neonatally bulbectomized mice; in the absence of bulb neurons, they continue onward to invade areas of forebrain (St. John and Key, 2003b). Activity clearly plays a role in establishing and refining the olfactory map, and can regulate OSN expression of some guidance molecules (Yu et. al., 2004; Zhou et al., 2004; Serizawa et al., 2006), however whether target ablation alters OSN spontaneous transmitter release or activity is not known.

Epithelial cell survival and proliferation in the absence of neuronal targets

OSNs have a limited lifespan, die by apoptosis, and are replaced by neurons generated from proliferating globose basal cells (Farbman, 1990; Mackay-Sim and Kittel, 1991a; Schwob, 2002). Maintenance of the population throughout life is known to depend on the presence of synaptic targets and olfactory bulbectomy, which simultaneously severs OSN axons, results in rapid apoptotic OSN death (Holcomb et al., 1995; Carson et al., 2005). Scar tissue prevents new axons from contacting the forebrain after adult bulbectomy, and their inability to form synaptic contacts shortens OSN lifespan and increases neuronal turnover rate, prompting the hypothesis that target connections provide trophic signals that allow sensory neurons to achieve a normal life span (Verhaagen et al., 1990; Schwob et al., 1992; Hayward et al., 2004). Bulb NMDA lesion produces similar changes in the epithelium in terms apoptosis, OMP and GAP-43 expression, and basal cell proliferation, though these changes are more gradual when compared to bulbectomy. Partial recovery of these parameters at eight weeks is correlated with the invasion of remaining tissue by new sensory axons, and the small but persistent increase in TUNEL labeling seen at this time indicates that the lifespan of some neurons is shortened in the absence of bulbar targets. Similar results have been reported following LOT transections that eliminate subpopulations of mitral cells (Weiler and Farbman, 1999).

Increased apoptosis can account for the gradual loss of P2 neurons from the deprived epithelium, although OR gene switching in response to limited function cannot be ruled out (Shykind et al., 2004). Partial recovery of their numbers at later intervals suggests that contacts made in the remaining tissue provide trophic support for their continued survival. Some axons may form functional connections at these ectopic locations; OR-specific neurons that fail to maintain functional contacts with the bulb are eliminated over time (Yu et al., 2004; Zhou et al., 2004). As is the case with LOT transection, ensheathing glia are spared by NMDA lesion, and any trophic support these, and other non-neuronal cells may provide, clearly does not compensate for lost bulb neurons in terms of maintaining the full OSN population. Limitations in trophic support may also indirectly affect the way in which olfactory axons respond to guidance molecules, as well as how they interact with each other; normal coalescence has been proposed to require a minimal number of like axons (Ebrahimi and Chess, 2000).

In summary, our results directly demonstrate that intact olfactory axons expressing the same OR do not stray beyond established foci when their postsynaptic targets are eliminated in adults. Over time, the absence of sufficient numbers or types of target neurons increases cell death in the target-deprived epithelium, leading to the gradual loss of glomeruli. New axons can form foci in the bulb remnant, but even with only partial loss of target populations, they do so at atypical locations. Map stability therefore relies on a normal distribution of available synaptic targets in the olfactory bulb.

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Figure 1.

Bulb sections stained with neutral red (top panels) or Fluoro-Jade (FJ, lower panels), showing neuronal degeneration after NMDA injection. Normal sections are shown in A and G. Treated bulbs are shown at 24 hrs (B), 4 days (C and H), 1 week (D and I), 2 weeks (E), and 3 weeks (F). Most mitral cells are lost within 24 hrs. By 4 days, glomerular layer (gl) organization breaks down, with some dying neurons still evident here (arrow) and in the granule cell layer (gcl). Degeneration is largely complete by 1 week, with the exception of some granule cells (arrow in I). Laminar organization is disrupted, though irregular, glomerular-like structures (asterisks) can still be observed at 1–3 weeks. Increasing cell density in the bulb remnant reflects reactive gliosis. onl, olfactory nerve layer; epl, external plexiform layer; mcl, mitral cell layer. Bar= 100 μ m.

Figure 2.

A–C. Bulb sections showing hybridization of ³⁵S-labeled NPY cRNA at 1 day (A), 1 week (B), and 2 weeks (C) after NMDA treatment. Normal bulbs are on the right and lesioned bulbs to the left. Most neurons expressing NPY are eliminated by NMDA within 24 hrs, but olfactory ensheathing glia in the nerve layer persist. The asterisk in B indicates the lesion cavity. **D–F.** NeuN-immunoreactivity (IR) in the normal bulb (D) and in lesioned bulbs at 1 week (E) and 3 weeks (F). Most neurons are eliminated by NMDA within the first week. Boxed areas in E–F indicate regions where stained nuclei remain; insets show these at higher magnification (arrows). **G–I.** GFAP-IR in the normal bulb (G), and in lesioned bulbs at 4 days (H) and 3 weeks (I). The arrowhead in G indicates a normal astrocyte in the external plexiform layer (epl). Reactive astrocytes are seen throughout the bulb 4 days after NMDA injection (H, open arrow). At 3 weeks, shrinkage of the lesioned bulb has reduced the distance between the olfactory nerve layer (onl) and the lesion cavity (asterisk in J), which is surrounded by GFAP

+ processes. gcl, granule cell layer; gl, glomerular layer. Bar =1.0 mm in A–C and 100 μ m in D–I.

Figure 3.

X-gal staining showing the positions of medial P2 glomeruli in normal (left side) and NMDA-injected bulbs (right). Staining is shown at 1 week (A–B), 2 weeks (C–D), and 3 weeks (E–F). Note that though stained glomeruli become smaller, their relative positions within lesioned bulbs remain constant out to 3 weeks (arrows in F). Open arrows indicate glomerular structures, not stained by X-gal. The asterisk in F indicates the lesion cavity. aob, accessory olfactory bulb; gl, glomerular layer; gcl, granule cell layer; m, mitral cell layer; onl, olfactory nerve layer. Bar= 500 μ m.

Figure 4.

X-gal staining showing P2 foci (arrows) in NMDA-lesioned bulbs. **A.** A horizontal section showing the position of a medial glomerulus in the lesioned bulb (right, arrow) relative to the same glomerulus in the normal bulb (left) at 3 weeks. **B–D.** Sections from mice that survived 8 weeks. P2 foci are seen in some atypical locations in the bulb remnant, including one located dorsomedially in B. Four small foci are shown in a single section from another mouse in C. The section in D was taken from a mouse in which part of ventral bulb was spared. P2 axons converge in an abnormal, ventral location. **E–H.** P2 axon convergence in the normal bulb (E), and in lesioned bulbs (F–H). Convergence is maintained 2 weeks after glomerular layer (gl) neuronal targets are lost (F). Numbers of stained axons decline and foci become smaller by 3

weeks (G). The open arrow indicates a second area of convergence in a mouse that contained two medial P2 glomeruli in the normal bulb. X-gal stained axons do not appear to stray from the core group after lesion. **H.** Small P2 foci are seen in neuron-depleted bulbs at 8 weeks (arrow). Other, unstained foci can be seen nearby (asterisks). epl, external plexiform layer; gcl, granule cell layer; onl, olfactory nerve layer. Bar = 400 μ m in A–D, and 100 μ m in E–J.

Figure 5.

Horizontal (A–B, I–J) and coronal (C–D, K–L) bulb sections showing changes in sensory innervation with target neuron loss. Lateral is to the left. **A–D.** OMP-immunoreactivity (IR; red) in sections through normal bulb (A), and at 1 (B), 3 (C) and 8 weeks after NMDA treatment. Note the irregular appearance of glomeruli at 1 week. By 3 weeks, OMP+ glomeruli have disappeared from most of the lesioned bulb (C) but at 8 weeks, the remnant contains numerous stained foci (D). Areas indicated by arrows are shown at higher magnification below in panels E–H. **I–L.** GAP-43-IR (green) in sections through normal (I) and lesioned bulbs at 2 (J), 3 (K) and 8 weeks (L) after NMDA. GAP-43+ fibers have penetrated into the remnant by 3 weeks (open arrow in K), and stained foci are seen at 8 weeks (arrow in L). **M–P.** Double labeling for OMP (red) and GAP-43 (green) in normal (M), and lesioned bulbs at 1 (N), 3 (O) and 8 weeks (P). At 3 weeks (O), GAP43+ fibers extend into the bulb parenchyma (open arrow), with some coalescing into small foci with OMP+ fibers (arrowhead). At 8 weeks, many foci contain both OMP+ and GAP-43+ fibers (yellow-orange; arrowhead in P). **Q.** Ectopic distribution of OMP+ foci (open arrow) within the anterior olfactory nucleus (aon) is seen in some mice at 8 weeks. Caudal medial bulb located to the right of this area (not shown). (Q). Asterisks indicate the lesion cavity. gl, glomerular layer; gcl, granule cell layer, onl, olfactory nerve layer. Bar = 500 μ m in A–D and I–L, and 104 μ m in E–H and M–, and 220 μ m in Q.

Figure 6.

A–E. X-gal staining in the epithelium of P2 mice. **A.** Coronal section showing P2 neurons in the epithelium ipsilateral (left) and contralateral (right) to bulb NMDA lesion at 3 weeks. A portion of the nasal septum and the medial tips of endoturbinates III are shown. Stained neurons in the septum of a saline-treated mouse (**B**), and mice that survived 4 days (**C**), 3 weeks (**D**) and 8 weeks (**E**) after lesion. **F–H.** OMP-IR in the septum of a saline mouse (**F**) and in the deprived epithelium at 3 weeks (**G**) and 8 weeks after NMDA (**H**). **I–K.** Double labeling showing the increase in GAP43+ neurons (green) that accompanies the decline in numbers of OMP+ neurons (red). A normal section is shown in **I**. Staining ipsilateral to lesion is shown at 3 weeks (**J**), and 8 weeks (**K**). Bar = 82 μ m in **A**, 49 μ m in **B–E**, 25 μ m in **F–H**, 27 μ m in **I–K**.

Figure. 7.

Autoradiograms showing changes in OMP- (A–B) or GAP-43- (C–D) mRNA expression in the epithelium after NMDA lesion. The target-deprived epithelium is on the left in all panels. A and C show patterns of labeling at 3 weeks; B and D show labeling at 8 weeks. **E.** Line graph illustrating changes in the density of hybridization of OMP and GAP-43 cRNAs in epithelium after lesion. Measures made ipsilateral to NMDA injection are expressed as a percentage of measures from the contralateral side. Values from saline-injected mice are shown at the zero time point. Group means $\pm$ S.E.M. are plotted. ** $p < 0.01$, * $p < 0.001$ vs. measures from saline mice; Student's Neuman-Keuls test. Bar = 1.0 mm.

Figure 8.

Apoptosis and proliferation in the target-deprived epithelium. **A–B.** TUNEL-labeling in the normal (A) and deprived epithelium one week after NMDA bulb lesion (B). Open arrows indicate labeled nuclei. **C–F.** Merged confocal microscope images showing TUNEL labeling and OMP-IR (C–D) or GAP-43-IR (E–F). Optical section thickness = 2.1 μm . After NMDA lesion, most TUNEL-labeled cells (open arrows) do not label for either OMP- (C) or GAP-43-IR (E), even though they are located near adjacent sensory neurons. Small numbers of double-labeled cells are seen at one week post-lesion (D, F, arrows). **G.** Ki-67-IR indicates increased cell proliferation within the ipsilateral septal epithelium (left) by 3 weeks. Arrows indicate

labeled nuclei. Individual stained cells are shown at higher magnification in the inset. Bar = 25 μm in A–B; 20 μm in C–F, 84 μm in G, and 7 μm in inset.