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Neuronal replacement in the injured olfactory bulb

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Abstract

The adult forebrain subventricular zone contains neural stem cells that produce neurons destined for the olfactory bulb, where interneuron populations turnover throughout life. Forebrain injuries can stimulate production of these cells, and re-direct migrating precursors from the olfactory system to areas of damage, where their region-appropriate differentiation and long-term functional integration remain a matter for debate. Paradoxically, little is known about the ability of these progenitors to replace olfactory neurons lost to injury. Their innate capacity to generate bulb neurons may give them an advantage in this regard, and using injections of N-methyl-D-aspartate to kill mature olfactory bulb neurons, combined with bromodeoxyuridine labeling to monitor the fate adult-born cells, we investigated the potential for injury-induced neurogenesis in this system. Widespread degeneration of bulb neurons did not affect the rate of cell proliferation in the subventricular zone, or cause neuroblasts to divert from their normal migratory route. However migration was slowed by the injury, leading to the accumulation and differentiation of neuroblasts as NeuN+ cells in the rostral migratory stream within 2 weeks of their birth. Despite this, a subset of new neurons successfully invaded the damaged bulb tissue, where they expressed neuronal markers including NeuN, calretinin, GABA, and tyrosine hydroxylase, with some surviving here for as long as 6 months. To test for functional integration of cells born post-injury, we also performed smaller NMDA lesions in restricted portions of the bulb granule cell layer and observed adult-born NeuN+ cells in these areas within 5 weeks, and BrdU+ cells that expressed the immediate-early gene c-fos following odor stimulation. These data suggest that the normal neurogenic capacity of the adult subventricular zone can be adapted to replace subsets of olfactory neurons lost to injury.

Keywords

olfactory system; neurogenesis; subventricular zone; rostral migratory stream; odor; c-fos

Introduction

The mammalian forebrain contains two neurogenic regions that continuously produce new neurons in adulthood; the subgranular zone (SGZ) of the hippocampal dentate gyrus, and the subventricular zone (SVZ) along the walls of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002; Ming and Song, 2005). Both these areas retain astrocyte-like neural stem cells that express glial fibrillary acidic protein (GFAP) and appear to derive from the

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embryonic radial glial lineage (Ihrle and Alvarez-Buylla, 2008). Neuroblasts generated in the SGZ differentiate into dentate granule cells. Those born in the adult SVZ undergo chain migration within longitudinal channels formed by specialized astrocytes in the rostral migratory stream (RMS) to reach the olfactory bulb (Alvarez-Buylla and Garcia-Verdugo, 2002). Here they differentiate into periglomerular (PG) or granule cells, both of which function as local interneurons (Lledo et al., 2008; Whitman and Greer, 2009). Bulb granule cells, which lack axons and modulate the activity of bulb mitral and tufted cells through dendrodendritic connections, are gradually eliminated from the adult bulb through programmed cell death (PCD) and are continually replaced by new neurons (Imayoshi et al., 2008). The functional significance of this life-long neuronal turnover has been illustrated by behavioral studies correlating experience-dependent improvements in the performance of olfactory tasks with changes in the numbers of new neurons that integrate into bulbar circuits (Rocheffort et al., 2002; Alonso et al., 2006; Breton-Provencher et al., 2009). Peripheral olfactory sensory neurons (OSNs) are also continuously generated in adults. The short lifespan of these cells (~30–90 days) leads to their replacement by new OSNs generated from local progenitors in the epithelium lining the nasal cavities, a process that is influenced by sensory experience (Mackay-Sim, 1991; Jones et al., 2008). This lifelong addition of new neurons in the olfactory system, both centrally and peripherally, provides a level of anatomical plasticity that, along with refinements in existing connections, may allow adult circuits to adaptively adjust to changing olfactory environments.

Although neurogenesis in the normal adult brain is currently considered to be limited to the production of bulb neurons and dentate granule cells (Ming and Song, 2005; Lledo et al., 2008), following a variety of brain insults, endogenous SVZ and SGZ progenitors have been induced to produce neuroblasts that migrate to injured sites and appear to adopt region-appropriate, alternative neuronal phenotypes (Magavi et al., 2000; Parent et al., 2002; Nakatomi et al., 2002; Chen et al., 2004). This ability has generated considerable interest in the therapeutic potential of these cells for neuron replacement in the damaged CNS (Burns et al., 2009; Kernie and Parent, 2010). However results from experimental models of excitotoxic or ischemic injuries are somewhat conflicting. A number of studies have reported that adult SVZ-derived neuroblasts can alter their normal fate and mature as medium spiny projection neurons within the damaged striatum (Parent et al., 2002; Collin et al., 2005), while others have concluded that new cells differentiate only as interneurons or glia (Deierborg et al., 2009; Liu et al., 2009). Hence, cell-autonomous properties of neuroblasts derived from defined progenitor subpopulations in the adult SVZ may limit their repair potential (Lledo et al., 2008).

Interest in endogenous replacement of neurons in damaged, non-olfactory regions has largely overlooked the repair potential of SVZ progenitors in terms of the olfactory system itself. In previous studies, we found that excitotoxic lesions produced by injection of N-methyl-D-aspartic acid (NMDA) into the adult bulb appeared to trigger increases in the size of the RMS (Ardiles et al., 2007). This raised the question of whether injury-induced death of bulb neurons could stimulate the normal, ongoing process of neuronal replacement in this system. Our objective in the present study was to examine the adult SVZ/RMS response to bulb injury by quantifying the proliferation and migration of olfactory progenitors *in vivo*. We further explored the possibility that cells generated after injury can differentiate into functional neurons within the damaged bulb using immunoreactivity for neuronal markers, bromodeoxyuridine (BrdU) labeling, and expression of the immediate-early gene *c-fos* in response to odor stimulation.

Methods

Animal treatments

Adult female C57BL/6 mice (20–28g) were obtained from Charles River Laboratories. All treatments were carried out according to National Institutes of Health guidelines and were approved by the Florida Atlantic University Institutional Animal Care and Use Committee. Mice were anesthetized with ketamine hydrochloride/xylazine cocktail (0.08mg/g and 0.12mg/g; i.p.), positioned in a stereotaxic device, and 1.5 µl of sterile saline or NMDA (Tocris; 12mg/ml, pH 7.0) was injected into the right olfactory bulb using a Hamilton syringe (26 gauge needle) at the following coordinates: 1.3 mm anterior to the frontal pole of neocortex, 0.6 mm right of the midsagittal suture, and 1.5 mm ventral to the tissue surface (The Mouse Brain Atlas, Franklin and Paxinos). NMDA-treated mice were injected 5 days or 3 wks later with 5-bromo-2'-deoxyuridine (BrdU; 100mg/kg i.p.; Roche Applied Science) administered over a 2 hr period. Those treated at 5 days were euthanized 3 hrs after the last injection (n=3). Those treated at 3 weeks were euthanized 3 hrs (n=4), 4 days (n=4), 2 wks (n=4), 5 wks (n=6) or 6 months (n=1) later. Saline-treated mice were given identical BrdU treatments (n=2–3 per survival interval). Additional mice (3 NMDA and 2 saline) were treated as described and tissue was collected for TUNEL labeling at 5 wks post-lesion.

We also produced smaller lesions within the dorsal granule cell layer (GCL, n=10). These lesions eliminated clusters of granule cells, but preserved the bulb subependymal layer (SEL) and limited cavitation. Focal lesions were performed using a Nanofil syringe (World Precision Instruments, Sarasota, FL) with a 35 gauge needle to deliver 90–100 nl of NMDA solution (4 mg/ml) into the dorsal GCL of the right bulb at the following coordinates: 1.1 mm anterior to the frontal pole of neocortex, 0.5–0.6 mm right of the midsagittal suture, and 0.6–0.7 mm ventral to the tissue surface. Three mice were killed 24 hrs later to verify extent of lesion using Fluoro-Jade and Nissl staining. Remaining mice were injected with BrdU 8 days later as described above, following the acute phase of glial proliferation. To test for functional incorporation of new neurons, mice were given odor stimulation 5 weeks later using a 1:10 dilution of saturated isoamyl acetate vapor (Sigma, St. Louis, MO) in zero grade air (Airgas, Miami, FL). Animals were placed individually in glass chambers with odor input and output ports inside a laboratory fume hood (exhaust flow rate = 99 cu.ft/min). Following 30 min of air-only exposure, odor was delivered continuously at a flow rate of 1 liter/min for 15 min. Mice remained in the odor test chamber for an additional 75 min to allow Fos protein to accumulate, and were then euthanized (Guthrie et al. 1993). Lesions were verified microscopically from bulb sections stained with neutral red and Fluoro-Jade, or DAPI and GFAP antibody.

Tissue collection and preparation

BrdU-treated mice were overdosed with sodium pentobarbital (150 mg/kg, i.p.), and perfused with buffered saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.3). Brains were cryoprotected in 25–30% sucrose and coronal sections (30µm) were processed free-floating. The forebrains from mice given focal GCL lesions were embedded in 10% gelatin, and the gelatin fixed with 4% PFA and cryoprotected with 25% sucrose. Coronal sections through the bulbs (30 µm) were collected with left and right bulbs paired in each gelatin-embedded section. For localization of fragmented DNA, NMDA- and saline-treated mice were perfused and brain tissue was cryoprotected as described. Serial sagittal sections (25µm) were slide-mounted prior to TUNEL labeling.

Immunohistochemistry

BrdU immunolocalization was performed as described using free-floating sections to optimize antibody penetration (Sultan-Styne et al., 2009). Pretreatment included incubation

in 0.6% H₂O₂, 50% formamide/2X salt sodium citrate buffer (65°C), 2N HCl (37°C), and neutralization with 0.1 M sodium borate (pH 8.5). Tissue blocked in 5% normal rabbit serum in Tris-buffered saline (TBS, pH 7.5) containing 0.3% Triton-X-100, prior to incubation in primary antibody for 48 hours at 4°C. Sections then incubated in biotinylated rabbit anti-rat IgG (1:200; Vector Laboratories, Burlingame, CA) followed by avidin-biotin-HRP complex (1:100; Vector Laboratories ABC Elite kit) according to the manufacturer's instructions. The reaction product was visualized with Vector Laboratories IMPACT-DAB kit and sections were counterstained with neutral red.

Co-localization of BrdU with cell-specific markers was performed by combined incubation with anti-BrdU and each of the antibodies shown in Table 1. Antigens were localized by incubation for 2 hrs in species-specific AlexaFluor568- or 594-labeled secondary antibodies combined with AlexaFluor488-labeled donkey anti-rat IgG (1:1000; Invitrogen, Carlsbad, CA). Non-specific staining was evaluated in sections treated identically, except for omission of primary antibodies. Sections were examined and digitized images collected using an Olympus AX-70 fluorescence microscope equipped with a Macrofire digital camera, and a Zeiss LSM700 laser scanning confocal microscope. With the exception of orthogonal projections of image Z stacks, collected confocal images were 0.7–2.9 µm in optical depth. Figures were assembled using Adobe Photoshop CS3, with adjustments made for size, brightness and contrast.

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

For detection of apoptosis, sections containing the RMS were immersed for 5 minutes each in 100%, 95%, 70%, 50% ethanol and phosphate buffered saline (PBS, pH 7.3). Tissue was permeabilized with proteinase K (0.04mg/ml; 20 min) and treated with 3% hydrogen peroxide for 6 min. Sections incubated in buffer (30 mM Tris-HCl, 140 mM sodium cacodylate, 1 mM cobalt chloride, pH 7.2) containing 200U/ml terminal transferase (New England Biolabs, Ipswich, MA) and 14 µM biotin-14-dATP (Invitrogen) for 2.5 hrs at 37°C. Following incubation in avidin-biotin-HRP solution overnight (1:100; Vector Labs Elite Kit), reaction product was developed using the IMPACT-DAB kit. Sections were counterstained with methyl green. Staining controls included pretreatment of sections with DNase (1000U/ml, 30 min, 37°C) and omission of terminal transferase from the labeling reaction.

Quantification of cell proliferation and apoptosis in the SVZ and RMS

Mice were given BrdU at 5 days or 3 wks after NMDA lesion, and were euthanized 3 hrs later. BrdU-labeled cells were counted at 60X objective magnification in 6 serial coronal sections (30µm) through the SVZ and 9 sections through the RMS (60µm spacing; ~2.0–2.5mm anterior to bregma). SVZ cell counts were made along the lateral wall of the lateral ventricle adjacent to the striatum, beginning where the ventricle opens beneath the corpus callosum and extending caudally (+1.20 to +0.5 mm relative to bregma). BrdU+ cell density was calculated from the sample distance in mm along the lining of the ventricle. RMS cross sectional area was measured from calibrated microscope images, with boundaries determined by both BrdU and methyl green staining. BrdU+ cell density was calculated based on section thickness. All distinctly labeled cells were counted, including those that contained nuclei densely labeled throughout, and those that contained punctate labeling characteristic of label dilution after multiple cell divisions. The density of apoptotic cells in the RMS was similarly calculated from TUNEL+ cell counts made in all sagittal sections containing the RMS. Only intact TUNEL+ nuclei were quantified; labeled nuclear fragments were not counted. Group mean values, +/- standard error (SEM), were calculated from individual means. As saline animals showed no significant differences between the right and left hemispheres for either BrdU or TUNEL labeling at any time point (p=0.14 to 0.87,

paired t-tests), data was pooled for comparisons to measurements from lesioned mice. Statistical comparisons between lesioned and non-lesioned hemispheres in NMDA-treated animals were made by Student's paired t-test, and unpaired t-tests were used for comparisons to saline controls, with significance defined as $p < 0.05$.

Analysis of BrdU+ cell distribution in the RMS

Mice given BrdU 3 wks after lesion survived for 4 days, 2 or 5 wks. The RMS was distinguished from surrounding tissue in counterstained sections due to its high cell density. Area measurements were obtained from calibrated digital photomicrographs of all sagittal sections that contained the RMS (7–8 sections per hemisphere) using NIH Image (Version 1.62), and the data used to calculate volume based on section thickness. The density of BrdU+ cells within the RMS was measured at 3 different rostrocaudal locations from the same sections using a sample box of $2.25 \times 10^{-2} \text{ mm}^2$. The three sample regions were designated as the “anterior SVZ”, located between the anterior corpus callosum and dorsal striatum (+1.75 mm anterior to bregma), the RMS “elbow” (~+2.0 mm anterior to bregma), and the “anterior RMS” (+2.45 mm anterior to bregma), just caudal to the olfactory bulb. Cell counts within these areas were carried out at 100x objective magnification.

Analysis of BrdU+ cells following focal lesions in the GCL

To determine if new neurons populated areas of the GCL depleted of neurons, BrdU was administered 8 days after NMDA infusion to minimize glial incorporation. The distribution of BrdU+/NeuN+ granule cells was mapped and their density quantified in the damaged area 5 wks later. Serial coronal sections (1 in 4) stained with antibodies to GFAP and BrdU, and counterstained with DAPI, were used to identify the lesioned area. Two mice showing cavitation in the GCL were excluded from analysis. In the remaining mice, focal lesions were localized by the presence of reactive astrocytes in restricted portions of the dorsal GCL. The damaged area did not extend beyond 20 contiguous sections (range=480–570 μm) in any case. Five to six serial sections (1 in 4), adjacent to the sections stained with anti-GFAP, were used to quantify BrdU+/NeuN+ granule cells in the damaged right bulb, with control counts made from the corresponding left bulb sections, in 4 lesioned mice. Remaining serial sections through the lesioned area were immunostained to localize BrdU with Fos, Iba1, or DCX.

Digital images of bulb sections containing BrdU+/NeuN+ cells were collected at 10X objective magnification, with multiple images aligned in Photoshop to create a full image of each coronal right and left section, with tissue distance calibrated in microns. Images were rotated such that the medial mitral cell layer was oriented vertically. A pie-chart grid with a center vertical line parallel to the medial mitral cell layer was overlaid on the section image such that this line passed through the subependymal zone. The height of the GCL was measured through this line, and the grid adjusted to place the center horizontal line midway through this distance. The resulting overlay divided the GCL into 8 compartments, and within each of these zones, single-labeled BrdU+ and double-labeled BrdU+/NeuN+ cells were counted microscopically. Cells were examined at 60–100X objective magnification and plotted as single or double-labeled on the corresponding image grid. The area of each compartment was measured by tracing the perimeters of the GCL within the grid, and the density of new neurons within each compartment was calculated based on the combined sample volume of all sections analyzed. Statistical comparisons were made using cell counts from equivalent compartments in the right and left bulbs of the same animal.

Results

Elimination of bulb neurons

As described previously (Ardiles et al., 2007), Fluoro-Jade and Nissl staining revealed degenerating neurons throughout all bulb laminae by 24 hrs following a 1.5 μ l injection of NMDA into the granule cell layer (GCL). The olfactory nerve layer remained intact, and shrunken, glomerular-like structures were present at all post-lesion time points. Rapid gliosis accompanied the initial wave of neurodegeneration, and by 2 wks, treated bulbs were shrunken and displayed cavitation (Figs. 1A–B).

Degeneration of bulb neurons does not stimulate SVZ/RMS cell proliferation

Damage to the olfactory bulb could be expected to alter proliferation in the SVZ/RMS, as well as neuroblast migration patterns. Previous work has shown that migration continues even when the bulb is eliminated (Kirschenbaum et al., 1999). Unlike bulb removal, target tissue remains accessible after NMDA lesion, and the loss of granule cells could increase neurogenesis to replenish this population. However we found that bulb damage had no significant effect on proliferation in the SVZ at either 5 days or 3 wks post-injury, and instead suppressed proliferation in the RMS (Fig. 1). At 5 days, the density of BrdU+ cells in the affected RMS was only one-third of that measured in saline controls (45.6 ± 13.5 cells/100 μ m³ and 134.6 ± 3.5 cells/100 μ m³ respectively, mean $\pm$ SEM, $p < 0.001$, t-test). This change was not proportional to changes in RMS volume at this time, which increased by ~8% ipsilateral to the injury. A contralateral reduction was also apparent (100.0 ± 12.1 cells/100 μ m³, $p < 0.05$). Such effects are frequently seen after unilateral olfactory manipulations, and may be due to systemic events caused by brain lesion, or alterations in neural activity via commissural connections.

In spite of reduced proliferation, by 3 wks post-lesion the RMS in the injured hemisphere was significantly enlarged, particularly the anterior limb (Figs. 1–2). Our measurements indicated an overall ~2-fold increase in volume, similar to the effects of bulbectomy (Kirschenbaum et al., 1999), attributable to an increase in the population of migrating DCX+ neuroblasts (Fig. 2). PSA-NCAM antibody produced identical staining patterns (not shown). At this time, the density of BrdU+ cells within the enlarged stream measured one-third that of the normal RMS (43.5 ± 7.8 cells/100 μ m³ vs 133.7 ± 4.9 cells/100 μ m³, respectively (Fig. 1K). This exceeds the expected ~50% reduction in cell density caused by the volumetric changes in the RMS, indicating a partial suppression of proliferation at 3 wks. Proliferation density in the contralateral RMS recovered to normal levels. In the lesioned hemisphere, BrdU+ cells were consistently fewer in number in the anterior stream, near the injured bulb.

New cells accumulate in the RMS after injury

We followed the fate of cells born in the SVZ/RMS 3 wks after injury from 4 days to 5 wks to monitor their survival and distribution (Fig. 3). Large numbers of BrdU+ cells were distributed throughout the enlarged RMS at 4 days. To quantify differences in distribution over time, BrdU+ cell density was measured at 3 different locations within the anterior-posterior extent of the RMS at each survival interval as depicted in figures 3G–J. At 2 wks, when most surviving cells had migrated into the normal bulb, large numbers of new cells remained distributed in the RMS of lesioned mice. Consequently, the density of labeled RMS cells was significantly higher at this time, particularly within the two anterior sampling locations (Fig. 3J). By 5 wks (8 wks after lesion), BrdU+ cell density declined to the low levels measured in controls, suggesting that labeled cells had eventually migrated into the damaged bulb or died in the RMS when failing to do so. Apoptosis eliminates a proportion of migrating neuroblasts even under normal conditions (Kirschenbaum et al., 1999). To

address this possibility, we examined apoptosis in the migratory stream at 5 wks post-NMDA using TUNEL labeling (Figs. 4A–B). TUNEL+ cells were more frequently observed in the enlarged RMS, with this change roughly proportional to the increase in RMS volume, such that the density of apoptotic profiles was not significantly different between the normal and treated hemispheres (2768 $\pm$ 800 cells/mm³ vs 3177 $\pm$ 615 cells/mm³, respectively, mean $\pm$ SEM, t-test, $p=0.38$). However the total numbers of labeled nuclei were more than 2-fold higher ipsilateral to bulb injury (128 $\pm$ 12 cells/right RMS vs 49 $\pm$ 9 cells/left control RMS, $p<0.05$). Counts from saline-treated mice (52 $\pm$ 7 cells/left and right RMS, $p=0.81$) did not differ from measures in the left control RMS. TUNEL+ cells occurred throughout the lesioned bulb but these were not quantified.

Accumulation of BrdU+ cells in the enlarged RMS, without increased proliferation, suggested that migration to the damaged bulb was impaired. Many factors have been shown to regulate migration of RMS neuroblasts, including the organization of the GFAP+ glial channels they travel, and expression of neurotransmitters, guidance factors and extracellular matrix cues along the migratory route (reviewed in Whitman and Greer, 2009 and Pathania et al., 2010). We examined expression of some of these cues, as well as cell-specific markers for neuronal and glial populations, within the altered RMS. As shown in figure 4, combined immunolabeling for DCX and GFAP demonstrated that chains of neuroblasts remained enclosed within astrocytic channels, the latter network expanding to accommodate the increased number of cells. In the normal RMS, small clusters of neuroblasts were bounded by GFAP+ processes (Fig. 4C). In the damaged hemisphere, these enclosed clusters appeared to contain more DCX+ cells, and although these were bounded by astrocytic processes, the contours of the channels were irregular and showed occasional gaps in GFAP-IR (Fig. 4D). A small number of individual DCX+ cells traveled outside the glial boundaries of the RMS, and a ventrally-directed population showing chain migration diverted from the main stream at the elbow region. Anti-PSA-NCAM staining patterns were identical to those seen with DCX antibody (not shown).

Although closely associated with GFAP- or Iba1-expressing cells, the majority of BrdU+ cells in the RMS did not co-label for either glial marker at 4 days (Figs. 4E–F), and instead were labeled by DCX antibody (Fig. 4E–H). Glia within the RMS did not display morphological characteristics of activation, however reactive astrocytes and microglia were found in areas near the injury, including the anterior olfactory nucleus, and surrounding the anterior RMS. Microglia in the RMS possessed processes, often extended parallel to the direction of the migratory stream, rather than appearing enlarged and phagocytic, as they did within the damaged olfactory bulb. Calretinin+ cells with bipolar morphology, some of which co-labeled for BrdU, were also distributed in the enlarged cell stream (Fig. 4I). The transmitter GABA, normally expressed by neuroblasts (Platel et al., 2010), was immunolocalized to DCX+ cells scattered throughout the enlarged cell stream, with most exhibiting diffuse labeling in comparison to the strong labeling seen in mature GABAergic neurons in surrounding brain tissue (Figs. 4J–K). However small numbers of individual RMS cells showed intense GABA labeling, and these lacked DCX-IR. Processes within the stream stained for GABA as well; these also lacked DCX and may have been glial. The enlarged RMS displayed normal expression of the ECM molecule tenascin-C in the tissue matrix, showing prominent localization along the outer borders of the cell stream (Figs. 4L–M, de Chevigny et al., 2006).

Differentiation within the RMS

As noted above, increases in RMS volume occur with bulbectomy, and also occur in Bax-deficient mice, in which PCD in the OB and RMS is dramatically reduced. In these mice, migration is impaired, and a subpopulation of cells that accumulate in the RMS express NeuN, a marker of neuron differentiation normally expressed by new cells only after they

reach the OB (Kim et al., 2007). Although neuroblasts that fail to migrate in our NMDA-treated mice can be eliminated by PCD (as seen with TUNEL labeling) the persistence of BrdU+ cells at 2 wks suggested their arrest might be similarly accompanied by ectopic differentiation. To evaluate this we performed immunostaining for NeuN at 3–8 wks post-lesion and observed a substantial number of NeuN+ nuclei distributed throughout its anterior-posterior extent (Fig. 4N). This staining pattern was absent in the normal RMS, and at 4 days post-BrdU, these cells were not double-labeled (Figs. 4O–P). However at 2 wks both BrdU+/DCX+ neuroblasts and BrdU+/NeuN+ cells were present in the migratory stream (Figs. 4Q–V). The temporal patterns of BrdU, DCX, and NeuN expression suggest that a subpopulation of neuroblasts retained in the RMS undergo ectopic differentiation within 2 wks of their birth. The loss of these BrdU+ cells by 5 wks indicates they do not survive long-term and may be eliminated by PCD, although we cannot rule out the possibility that some undergo delayed migration to the damaged bulb.

Neuroblasts differentiate in the injured bulb

In spite of the tissue damage, DCX+ neuroblasts continued to migrate into the bulb remnant from 3 to 8 wks. As shown in figure 5, widespread loss of neurons was accompanied by reactive gliosis and cavitation, and auto-fluorescent debris indicative of degeneration was distributed in the bulb. The lesion cavity was densely bounded by GFAP+ cells and processes, and reactive astrocytes radiated outward from this. At levels posterior to cavitation, the bulb subependymal layer (SEL) consisted of a mass of DCX+ cells continuous with the enlarged RMS. Neuroblasts accumulated here and in areas where cell debris did not form a barrier, networks of neuroblasts extended into more superficial locations (Fig. 5B). The orderly, radial pattern of migration from the SEL was severely disrupted (Figs. 5C–D). Upon encountering the lesion cavity, the cell stream diverted laterally to travel along its margins. Both DCX+ and calretinin+ cells were contained in the stream (Fig. 5E). Neuroblasts approached areas containing olfactory sensory axons, which were also immunoreactive for DCX (Figs. 5E–F). As with DCX+ cells in the RMS, the mass of neuroblasts in the remnant core contained GABA-IR, with GABA+/DCX– cells scattered in the surrounding tissue (Fig. 5G). GFAP+ glia, continuous with the network in the RMS, enclosed the mass of cells at the core of the bulb, and intense IR for tenascin-R, normally expressed by granule cells (see Whitman and Greer, 2009), localized to processes throughout the remnant (not shown). Some, but not all, were co-labeled by GFAP antibody.

To monitor the fate of neuroblasts reaching the injured bulb, BrdU was given 3 wks after NMDA to minimize its incorporation by proliferating bulb glia. At 4 days, most BrdU+ nuclei were enclosed in the glial channels within the SEL (Fig. 5H). Reactive astrocytes and microglia were present throughout, and though BrdU+ cells were distributed among these, few glia were double-labeled (Fig. 5I–J). We identified occasional double-labeled Iba1+ cells, but more often we observed microglia in the process of engulfing BrdU+ nuclei, and many of these contained aggregates of autofluorescent material. At this time DCX+/BrdU+ cells were rarely seen in the bulb parenchyma, however DCX+ cells lacking BrdU were present in all lesioned bulbs (Fig. 5K). These may have been neurons that survived NMDA, as DCX is expressed normally by subpopulations of olfactory bulb neurons (Nacher et al., 2001).

Results of NeuN immunostaining showed that lesioned bulbs contained new neurons, double-labeled for BrdU at 2 wks (Figs. 5L–N). These were distributed in the tissue superficial to the SEL, though many BrdU+ cells at this time were retained here, along with DCX+ and NeuN+ cells. In examining damaged bulbs for expression of additional neuronal markers we identified BrdU+ cells at 2 wks that were also TH+ (Fig. 5O–P). Though positioned deeply in terms of their proximity to the SEL, they were often located near areas containing sensory axons and typically displayed elaborate processes. New, differentiated

neurons in the damaged bulb were similarly evident at 5 and 8 wks, and we identified BrdU+ cells that were immunoreactive for NeuN, GABA, calretinin, or TH in all NMDA-treated mice (Figs. 5Q–T).

We allowed one lesioned animal to survive to 6 months to judge the long-term survival of new bulb neurons. In this mouse, the outer bulb contained olfactory sensory axons that terminated in an array of irregular glomerular structures, with many penetrating deeply to form glomeruli embedded within fields of NeuN+ cells (Fig. 5U). These latter areas also contained deeply situated TH+ neurons with processes extending to ectopic glomeruli (Fig. 5V). BrdU+ cells were scattered in the partially reconstituted GCL, as well as in more superficial positions (Fig. 5W), and within the population of surviving BrdU+ cells were neurons that expressed GABA, NeuN or TH (Figs. 5X–Y).

New neurons populate neuron-depleted fields in the GCL

Large NMDA lesions removed mitral and tufted cells, the neurons with which adult-born granule cells form synapses, leaving new cells without synaptic targets. About half of all new cells reaching the normal bulb undergo apoptosis within 2–4 weeks of their birth, and in absence of target mitral cells, their survival is even more limited (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002; Valero et al., 2007). The smaller lesions we placed in the dorsal GCL spared most output neurons. As shown in figure 6, within 24 hrs of NMDA injection, restricted portions of the GCL contained Fluoro-Jade-stained, degenerating granule cells (Figs. 6A–B). Small numbers of degenerating neurons were located in more superficial laminae along the needle track as well. Lesioned mice were treated with BrdU 8 days after NMDA and when examined 5 wks later, two cases showing cavitation were excluded from analysis. Lesions in the four remaining mice were identified as areas containing GFAP+ reactive astrocytes in the GCL extending outward from a dense core comprised of auto-fluorescent cell debris and reactive glia, but lacking a cavity (Figs. 6C–D). Astrocytes in the surrounding GCL directed processes toward the lesion core, and reactive microglia were located throughout the area (Figs. 6D–E). BrdU+ nuclei were distributed among the reactive glia, but few cells were double-labeled. Although reactive astrocytes did not incorporate BrdU, labeled nuclei were often closely associated with their processes, suggesting that new cells might migrate on them. Combined labeling for DCX and GFAP showed that migrating neuroblasts instead associated with each other, forming chains similar to those in the RMS. Processes from astrocytes made contact with neuroblasts, but did not appear to serve as migratory scaffolds (Fig. 6F).

To determine if the injury environment prevented, or even facilitated, the addition of new neurons in the GCL, we measured the density of BrdU+/NeuN+ cells in the damaged area and compared this to the density of cells measured in the same area of the contralateral bulb. Cell counts were made microscopically in sections adjacent to those containing GFAP+ reactive astrocytes; these served to define the lesion location. NeuN+ cells were present in the area surrounding the lesion core (Fig. 6G), though the cellular organization of the GCL in this area was disrupted. Within these regions BrdU+/NeuN+ cells were also observed (Figs. 6G–I). Because lesions varied in terms of size and exact placement, it was difficult to precisely define the boundaries of equivalent areas in the left bulbs. Moreover, as reported by Kopel et al. (2009), BrdU-stained nuclei were not distributed uniformly in the GCL of either lesioned or normal bulbs; all sections contained areas with relatively high densities of new cells, and other areas that were sparsely labeled. We therefore compared bulbs by dividing sections into 8 sample “zones” as described in the Materials and Methods and calculated the mean cell density within each of these (Figs. 6J–K). All lesions were confined to the dorsolateral region shown as zone 1 in figures 6J–K (also see Fig. 6C). Numbers of bulb sections counted (1 in 4) ranged from 5 to 6, depending on lesion size, and labeled cells in all 8 sample regions were counted from matched left and right sections. Numbers of BrdU

+ cells were higher overall in the damaged GCL of the lesioned bulb (zone 1; 220 ± 44 cells, mean $\pm$ SEM) compared to the same area contralaterally (146 ± 59 cells, $p=0.18$). However numbers of BrdU+/NeuN+ cells did not differ significantly (171 ± 34 cells right zone 1 vs 136 ± 62 cells left zone 1, $p=0.23$). As shown in table 1, the density of BrdU+/NeuN+ cells did not differ from that measured in same zone in the left bulb, or in areas of the right bulb not damaged by NMDA. The proportion of BrdU+ cells lacking NeuN was higher in the damaged GCL, comprising 28% of the population of new cells, compared to only 6.8% in the same area of the control bulb. These were probable microglia, as we were able to identify double-labeled BrdU+/Iba-1+ cells.

Odor-induced Fos expression in the damaged granule cell layer

Soon after adult-born granule cells reach the bulb, they elaborate dendrites, form synapses with output neurons, develop physiological responses to odor, and demonstrate activity-dependent expression of c-fos (Carlen et al., 2002; Carleton et al., 2003; Whitman and Greer, 2007). Mitral cells deprived of input from granule cells killed by NMDA, might present available dendritic targets for newly arriving cells, permitting them to integrate. We assessed functional responses of cells in the injured GCL by stimulating 5 mice with isoamyl acetate odor to induce bulbar Fos expression. This odor was used due its extensive spatial activation of the bulb, which includes broad dorsolateral and ventromedial regions (Guthrie et al., 1993). In the intact bulb, numerous Fos+ cells were contained in the glomerular and granule cell layers in lateral and ventral bulb, and the GCL population included small numbers of BrdU+ cells. In the lesioned bulb, the damaged GCL was visible as a zone with diminished Fos labeling, in comparison to most other portions of the GCL (Figs. 6L–M). In alternate sections these areas aligned with regions containing reactive astrocytes. The low levels of Fos were likely due to the scarcity of functional granule cells; most were ablated by NMDA, and the injury may limit the incorporation of new cells. However for individual mice, the lesioned area may not have been topographically activated by the odor and lacked Fos expression for this reason. Unexpectedly, given the single dose of BrdU and the short survival interval, BrdU+/Fos+ cells were identified in the damaged GCL in 3 lesioned mice (Figs. 6N–O). In the 4–5 sections obtained through the lesion for each case, only 3–4 double-labeled cells were identified in the lesioned area, but their presence suggests that new granule cells can be functionally incorporated into injured portions of the olfactory bulb.

Discussion

We demonstrate that pathological degeneration of olfactory bulb neurons does not stimulate compensatory neurogenesis in the SVZ. Migration of new cells toward the bulb is slowed, and many neurons differentiate along the migratory route. Nevertheless, subpopulations of neuroblasts invade the injured tissue, where they mature and survive for up to 6 months. The more limited damage produced by small, focal lesions in the GCL does not prevent new cells from repopulating neuron-depleted areas, suggesting that normal mechanisms of neuronal replacement in the adult olfactory system can be adapted for repair after injury.

Suppression of cell proliferation

Forebrain injuries induce changes in SVZ cell proliferation that differ depending on the animal model, age and the type of injury (Kernie and Parent, 2010). The death of large numbers of bulb granule cells could be expected to upregulate cell production to restore the population, as has been reported after olfactory nerve axotomy (Mandairon et al., 2003). However our data demonstrate that bulb lesion has little impact on proliferation in the SVZ, and reduces the number of dividing progenitors in the RMS. These results are consistent with prior studies showing reduced proliferation following other traumatic injuries to the

olfactory system, including bulbectomy and transection of the RMS (Jankovski et al., 1998; Kirshenbaum et al., 1999). Less invasive means of increasing granule cell death also fail to stimulate progenitor proliferation, including naris occlusion (Bastien-Dionne et al., 2010), genetic loss of mitral cells (Valero et al., 2007), and transgenic expression of mutant huntingtin protein (Kohl et al., 2010). It therefore appears that levels of cell death in the olfactory bulb do not tightly regulate SVZ/RMS cell proliferation in a compensatory fashion.

The number of injury-induced signals implicated in modulating SVZ cell proliferation is large and includes a variety of cytokines and growth factors, many of which derive from activated glia at the injury site (Das and Basu, 2008; Kernie and Parent, 2010). Reactive bulb glia may similarly influence SVZ neurogenesis after bulb injury. An additional regulatory mechanism utilizes neurotransmitter signaling between neuroblasts and the astrocyte-like progenitors in the RMS. Migrating neuroblasts synthesize and release GABA non-synaptically. This activates GABA_A receptors on nearby GFAP⁺ progenitors and neighboring neuroblasts to reduce their proliferation rate (Nguyen et al., 2003; Platel et al., 2010). We observed GABA expression by RMS neuroblasts after bulb lesion, and as migration slows in the cell stream, its release by accumulating neuroblasts may increase local feedback on responsive progenitors to limit their division.

Migration impairment

A number of molecular cues that orchestrate migration of RMS neuroblasts have been identified and include factors intrinsic to the migratory pathway itself, explaining in part why it is that bulb-derived signals appear to be largely dispensable, consistent with our observations here. Even bulb removal does not eliminate rostral migration, though a number of attractive factors secreted by bulb cells have been shown to facilitate the process, including insulin-like growth factor-1 (IGF-1) and glial-derived neurotrophic factor (Kirschenbaum et al., 1999; Hurtado-Chong et al., 2009; Paratcha et al., 2006). Injury to other forebrain areas, or the RMS itself, shifts migratory patterns, diverting part of the population out of the stream and toward the damaged tissue (Alonso et al., 1999; Parent et al., 2002; Cayre et al., 2009). Their attraction to sites of inflammation is potentially mediated by glial- and macrophage-derived factors with demonstrated chemoattractive activity for neuroblasts, including fibroblast growth factor-2 and IGF-1 (Das and Basu, 2008; Gordon et al., 2009; Hurtado-Chong et al., 2009; Smirkin et al., 2010). We have previously documented increases in the expression of both these factors in the NMDA-lesioned olfactory bulb, which may facilitate the continued movement of neuroblasts toward the damaged tissue (Sultan-Styne et al., 2009).

Though directional migration was maintained, it was slowed, and the physical constraints caused by cavitation provide the most straightforward explanation for this. The lesion cavity clearly presented a barrier to rapid cell advancement, and the slowing of cells at this point established an additional obstacle for later-arriving neuroblasts to negotiate within the glial tubes. Additionally, migratory signals that rely on balanced interactions between neuroblasts and astrocytes or the ECM may not function efficiently when new cells overcrowd the pathway (Pathania et al., 2010). For example, GABA, in addition to its proliferation effects, has been shown to act on neuroblasts in an autocrine/paracrine fashion to slow migration, and as more cells accumulate, this signaling may further impair their advancement (Platel et al., 2010).

Differentiation in the RMS

We demonstrate that impaired migration is accompanied by differentiation of NeuN⁺ neurons in the RMS, even at posterior locations. This has been reported for other

manipulations that cause cells to arrest along the migratory route. For example, in Bax knockout mice, NeuN+ cells collect in areas along the lateral margins of the RMS (Kim et al., 2007). The NeuN+ cells we observed were instead distributed throughout the stream, interspersed among DCX+ cells. TH+ cells, which appear in the RMS as a result of RMS transection (Alonso et al., 1999), enzymatic removal of PSA from NCAM (Chazal et al., 2000; Petridis et al., 2004), or neonatal olfactory bulbectomy (Guthrie and Leon, 1989) were not present in the RMS.

Factors that trigger differentiation en route could involve changes in the extracellular milieu, transcriptional programs linked to time of cell birth or migration arrest, or a combination of these. Multiple lines of evidence show that as neuroblasts migrate to the OB during the first week after their birth, they gradually acquire phenotypic features of more mature cells, finally differentiating as morphologically and functionally distinct neurons after reaching the bulb (Petreanu and Alvarez-Buylla, 2002; Carleton et al., 2003; Saino-Saito et al., 2004; Platel et al., 2010; Pignatelli et al., 2009). Cells that arrest in the RMS may undergo some of the same maturational changes in place, such as occurs with developmentally delayed migration of cerebellar granule cells (Qu and Smith, 2005), a possibility supported by the time course of NeuN expression. Neuroblasts arriving at the normal OB differentiate as NeuN+ neurons between 1 and 2 weeks of their birth in the SVZ, and we observed new cells in the RMS that expressed NeuN at 2 weeks post-BrdU treatment (Petreanu and Alvarez-Buylla, 2002). As judged by the eventual loss of most BrdU+/NeuN+ cells, those differentiating in the RMS do not survive long-term, and PCD likely eliminates these young neurons from an environment in which they cannot functionally integrate.

Differentiation in the injured bulb

The present work demonstrates that adult-born cells can differentiate, albeit in small numbers, as NeuN+, GABA+, or TH+ neurons in the injured bulb within 2 wks of their generation, a pattern that is consistent with the schedule of normal differentiation reported in adult mouse studies (Petreanu and Alvarez-Buylla, 2002; Beech et al., 2004; Bagley et al., 2007; Bastien-Dionne et al., 2010). Moreover, in spite of ongoing apoptosis, we observed neurons that survived for at least 6 months in the reorganized tissue. Large lesions eliminated mitral and tufted cells, leaving new granule cells with few options in terms of the synaptic integration needed for long-term survival. What survival signals are then available to new neurons that populate the damaged tissue? The aforementioned glia-derived factors may play a contributing role, as well as small numbers of surviving neurons that may have escaped NMDA. A unique survival influence may be exerted by olfactory sensory axons and evidence in support of this can be seen in purkinje cell degeneration (pcd) mutant mice. While adult-born granule cells die in large numbers as mitral cells degenerate in pcd mutants, numbers of new PG cells that survive in the glomerular layer are equivalent to those in normal mice, suggesting that synaptic contacts from sensory axons can promote their survival here (Valero et al., 2007). Normal levels of PG cell survival also require that they be functionally activated by this input; sensory deprivation by naris occlusion, which suppresses the activity of the sensory axons, reduces their survival rate, and additionally prevents their phenotypic differentiation as TH-IR dopaminergic neurons (Saino-Saito et al., 2004; Bastien-Dionne et al., 2010). Dopaminergic PG cells are classified as type 1 PG cells that receive synapses from olfactory sensory axons (Whitman and Greer, 2007), and we observed BrdU+/TH+ cells in the injured bulb that were often located in proximity to sensory axons, or had processes extending toward them. It is tempting to speculate that sensory axons established functional synapses with these new neurons, and indeed in a prior electron microscopic study, we observed neurons in the NMDA-lesioned olfactory bulb that received synapses from olfactory sensory axons (Sultan-Styne et al., 2009).

Functional replacement of neurons lost to injury requires that adult-born cells at least partially reconstitute normal circuitry, and we attempted to reveal this capacity in the bulb by ablating a small number of granule cells while preserving output neurons. We show that cells generated after this injury were not excluded from the damaged GCL. Neuroblasts entered the lesion area in small migratory chains and many differentiated into NeuN+ cells, as they do in other injured areas of forebrain (Cayre et al., 2009; Kernie and Parent, 2010). Adult-born granule cells normally become synaptically integrated over the first 2–4 wks after their birth (Petreanu and Alvarez-Buylla, 2002; Carleton et al., 2003; Yamaguchi and Mori, 2005), and we observed a small number of new cells in the damaged GCL at 5 wks that expressed Fos after odor stimulation, suggesting their functional incorporation. The presence of new, Fos+ cells after injury was unexpected as we had anticipated that the reactive tissue environment would inhibit their integration, even with available dendritic targets. Magavi et al. (2005) have demonstrated that adult-born granule cells are most responsive to novel odor stimulation shortly after they integrate, gradually becoming less so as they continue to mature, and our detection of 5-wk-old, Fos+ cells in lesioned bulbs may have been facilitated by their greater responsiveness at this early age. Our findings suggest that with time, continued addition of new interneurons in the adult bulb could help restore functional circuitry after injury.

Conclusion

Interest in the therapeutic use of SVZ stem cells for repair of forebrain injuries has generated a large body of experimental work aimed at characterizing the replacement potential of these cells in animal models. The question we chose to ask here is, how effective are these adult stem cells at accomplishing this in the brain area they normally target? We demonstrate that adult-born neuroblasts that migrate to the damaged olfactory bulb can differentiate to express neuronal markers characteristic of bulb interneurons, can survive up to 6 months, and can be odor-activated. Some new cells express TH, suggesting their differentiation as dopaminergic neurons under the influence of olfactory sensory axons. Such bulb-specific cell interactions, combined with the autonomous capabilities of SVZ-derived neurons, may permit limited replacement of olfactory neurons in animal models of brain injury and neurodegenerative diseases (Kohl et al., 2010). Outside their normal target environment, the replacement potential of these progenitors may be more limited.

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Figure 1.

Effects of bulb injury on proliferation in the subventricular zone (svz) and rostral migratory stream (rms) at 3 hrs post-BrdU injection. (A–B). Coronal olfactory bulb sections showing the normal bulb (A) and the effects of neuronal degeneration 3 wks after NMDA (B). (C–D). Coronal sections through the svz contralateral (C) and ipsilateral (D) to the treated bulb at this time showing labeled nuclei (arrow). The asterisk indicates a blood vessel. (E–F). Sagittal views of contralateral (E) and ipsilateral (F) sections through the anterior svz and caudal rms. (G–J) Labeling in coronal sections shows that injury decreases ipsilateral rms cell proliferation at 5 days post-NMDA (H) with a partial recovery by 3 wks (J), compared to the intact hemisphere (G and I). Note the enlargement of the rms at 3 wks (dotted line in

J). (K). Bar graphs showing that the density of proliferating rms cells is reduced ipsilateral and contralateral to lesion at 5 days, before changes in RMS volume occur. The reduction at 3 wks on the damaged side remains below that expected on the basis of volumetric changes in the RMS, but recovers on the intact side. * $p < 0.05$, ** $p < 0.001$, in comparison to saline, Student's t-test. cc, corpus callosum; lv, lateral ventricle; st, striatum. Bar = 500 μ m in A–B, 150 μ m in C–D, 80 μ m in E–F, 100 μ m in G–J.

Figure 2.

Effects of bulb neuron degeneration on rostral migratory stream (rms) size. (A). Sagittal section stained with neutral red showing the rms of a saline-treated mouse. The dashed line indicates the area measured volume calculations. (B) Bar graphs showing the increase in rms volume measured over the post-lesion survival interval. Bulb lesion resulted in a ~2-fold increase in size (* $p < 0.05$ in comparison to saline, Student's t-test). (C–D). Sagittal sections through forebrain immunostained for doublecortin (DCX) 3 wks after lesion. Note the increase in the population of DCX+ neuroblasts ipsilateral to lesion. fc, frontal cortex; mob, main olfactory bulb. Bar = 400µm in A–B, 200µm in C–D.

Figure 3.

Cell migration to the injured bulb is impaired. (A–F). Sagittal sections showing the distribution of BrdU+ cells at 4 days (A–B), 2 wks (C–D) and 5 wks after BrdU treatment (E–F). Most labeled cells in the intact hemisphere have migrated into the bulb by 2 wks, whereas many BrdU+ cells persist in the rostral migratory stream (rms) ipsilateral to lesion (D). (G) Schematic diagram of the sample regions used to quantify BrdU+ cell density in the rms. (H–J). At 2 wks, the anterior sample regions (2–3) in the injured hemisphere (J) contain higher densities of BrdU+ cells compared to both contralateral (I) and saline-control measures (H). * $p < 0.01$. cc, corpus callosum; fc, frontal cortex; gcl, granule cell layer; ob, olfactory bulb; St, striatum. Bar = 400 μ m.

Figure 4.

Changes in the rostral migratory stream (rms). (A–B). TUNEL-labeled nuclei (arrows) in the rms of the contralateral (A) and ipsilateral (B) hemisphere 5 wks after unilateral bulb lesion. (C–D). Immunoreactivity (IR) for doublecortin (DCX) and glial fibrillary acidic protein (GFAP) in the contralateral (C) and ipsilateral (D) rms at 3 wks shows that the network of glial channels has expanded around the enlarged neuroblast population. (E–G). At 4 days after BrdU, most new cells in the enlarged rms do not express GFAP (E) or Iba-1 (F), and are DCX+ (arrows in G). (H). Orthogonal projection showing a DCX+/BrdU+ cell. (I). Calretinin (Cal)+ cells are distributed among DCX+ neuroblasts. (J–K). GABA-IR is expressed by neuroblasts throughout the normal (J) and enlarged RMS (K). The inset in K is a confocal image of double-labeled neuroblasts in the enlarged stream (1.1µm optical thickness). (L–M). Tenascin-C (TenC)-IR is found in the ECM of the RMS contralateral (L) and ipsilateral (M) to lesion. (N). Four-day-old BrdU+ cells are distributed in the rms ipsilateral to lesion and among them are NeuN+ cells (solid arrows). The open arrow indicates a NeuN+ cortical neuron. (O–P). Confocal images showing that NeuN and BrdU are not co-localized at 4 days post-BrdU, and that NeuN is absent in the normal rms (P). (Q). Two wks after BrdU, the stream still contains BrdU+ cells, some of which express DCX (arrows and inset). (R–S). NeuN+ cells (arrows in R) also are evident, and as shown in (T), some are BrdU+. (U–V). Confocal images of cells in the normal (U) and enlarged (V) rms at 5 wks stained for NeuN-IR and DAPI. Bar in D=50µm for A–B, 35µm for C–D. Bar in I=10µm for E–G, 6µm in H, 15µm in I. Bar in M=45µm for J–M, 11µm for inset in K. Bar in R=80µm in N, 20µm in O–P, 60µm in Q, 32µm in R. Bar in V=15µm in S, 5µm in T, 10µm in U–V.

Figure 5.

Neuroblasts in the injured olfactory bulb (OB) differentiate. (A) Coronal section 3 wks after NMDA showing the lesion cavity, GFAP+ cells, and OMP+ sensory axons, some of which penetrate deeply (arrow). (B). Sagittal section showing neuroblasts in the rostral migratory stream (rms) and subependymal layer (sel) at 3 wks. A network of neuroblasts extends outward from the sel (arrow). Sensory axons express DCX in the outer bulb. (C–D). DCX+ neuroblasts entering the damaged (C) and normal OB (D). BrdU given 4 days earlier labels some of these cells (insets in C–D). (E). DCX+ cells migrate around the cavity and along its margin (arrow). Calretinin+ (Cal) cells are contained in the cell stream (inset). Glomeruli (gl) containing DCX+ axons and Cal+ processes, are located along the cavity's edge. (F). Some neuroblasts migrate from the sel and approach glomeruli (gl, arrow). (G). GABA is expressed by DCX+ cells in the sel, and by bulb cells that are DCX- (arrow). (H–K). New cells in the lesioned bulb 4 days post-BrdU are contained within GFAP+ channels in the sel (H), and distributed among reactive astrocytes (I) and microglia (J) in the tissue. Most DCX

+ bulb cells (arrows in K) are not labeled for BrdU at 4 days; these may be bulb neurons that survived the lesion, or new cells that migrated in prior to BrdU treatment. The open arrow indicates a cell labeled only for BrdU. (L–P) Two wks after BrdU, new cells reaching the bulb remnant express NeuN (L–N, arrows in M) or tyrosine hydroxylase (TH, O–P, arrow in O). The open arrow in O indicates a glomerulus. (Q–T). New cells expressing NeuN (arrow in R), GABA, or TH (arrows in T) are seen in the deep bulb at 5 wks post-BrdU. (U–V). Bulb tissue 6 months after lesion. (U). Sensory axons have formed glomeruli in areas of NeuN+ cells. These areas also contain TH+ neurons (V) and surviving BrdU+ cells (W), some of which express TH (X) or NeuN (Y). Bar in D=310µm in A, 340µm in B, 65µm in C–D. Bar in K= 100µm in E–F, 120µm in G, 20µm in H–J, 45µm in K. Bar in P= 80µm in L and O, 45µm in M, 10µm in N and P. Bar in T=20µm in Q and T, 6µm in R, 8µm in S. Bar in Y= 100µm in U, 75µm in V, 200µm in W, 12 µm in X–Y.

Figure 6.

New cells in the damaged granule cell layer (gcl) after focal lesions. (A–B). Degenerating gcl cells labeled with Fluoro-Jade 24 hrs after NMDA. The open arrows in A indicate 2 degenerating mitral cells along the lesion track and the open arrow in B indicates the injection site. (C–D) Reactive astrocytes in the lesioned area (dotted outline in C), 6 wks after lesion and 5 wks after BrdU. BrdU+ cells in the vicinity of the injury (zone 1) are not GFAP+. Arrows in D indicate astrocytes with processes extended toward the lesion (asterisk). (E). Most BrdU+ cells (arrow) lack Iba1. The asterisk indicates the lesion core and the inset shows cells at higher magnification. (F). DCX+ neuroblasts migrate in chains (arrow) between reactive astrocytes (open arrow) in the tissue. (G–I). The damaged gcl contains BrdU+/NeuN+ cells at 5 wks (arrowheads in G; yellow material is cell debris). (H–I) Confocal images of BrdU+/NeuN+ cells in the lesion area. (J). Graphic illustrating the division of BrdU-labeled bulb sections into 8 sample zones. (K). Black and white inverted image of a section stained with BrdU antibody showing a lesion in zone 1. The lesion core and cell debris are non-specifically stained. (L–O). Fos-IR with odor stimulation. Images in L–M are taken from the dorsolateral left (L) and right (M) bulbs of a mouse lesioned in right zone 1. Fos+ cells are sparse in the damaged gcl (M). (N–O). BrdU+/Fos+ cells (arrow in N) in the damaged gcl. The open arrow in N indicates accumulated, autofluorescent cell debris at the lesion core. Bar in D=300µm in A, 100µm in B–C, 75µm in D. Bar in H=45µm in E and G, 20µm in F, 7µm in I. Bar in N=400µm in J, 140µm in K, 33 µm in L–M, 20µm in N, 12µm in O.

Table 1
Summary of primary antibodies used

Dilutions, suppliers, and catalog numbers of the antibodies used in the present

Directed against	Species	Catalogue#	Source	Dilution
BrdU	rat	OBT0030	Accurate Chemicals, Westbury, NY	1:400
Calretinin	rabbit	AB149	Millipore, Temecula, CA	1:2000
Doublecortin (DCX)	goat	sc-8066	Santa Cruz Biotech., CA	1:500
Fos	rabbit	sc-52	Santa Cruz Biotech., CA	1:300
GABA	rabbit	A2052	Sigma, St. Louis, MO	1:2000
Glial fibrillary acidic protein (GFAP)	rabbit	Z0334	DAKO, Carpinteria, CA	1:750
Iba1	rabbit	019-19741	Wako Chemicals, Richmond, VA	1:500
NeuN	mouse	MAB377	Millipore, Temecula, CA	1:500
Olfactory marker protein (OMP)	goat	544-10001	Wako Chemicals, Richmond, VA	1:7000
PSA-NCAM	mouse	MAB5324	Millipore, Temecula, CA	1:500
tenascin-C	rabbit	AB19011	Millipore, Temecula, CA	1: 100
tenascin-R	mouse	MAB1624	R and D Systems, Minneapolis, MN	1:1000
tyrosine hydroxylase (TH)	rabbit	AB152	Millipore, Temecula, CA	1:1000

Table 2
Quantification of BrdU+/NeuN+ cells in granule cell layer

Density of new neurons in subdivisions of the granule cell layer (gcl) 5 wks after BrdU. Four mice received NMDA lesions in the dorsal right olfactory bulb (zone 1) 8 days prior to BrdU treatment. Sections through the injury site were divided into sample zones as described in the Methods (refer to Fig. 7J–K), and cell density was calculated from microscopic counts of BrdU+/NeuN+ cells.

Right lesion zone 1	Left bulb zone 1	Zones 2–8 right bulb	Zones 2–8 left bulb
5922 $\pm$ 721*	5107 $\pm$ 798	4763 $\pm$ 625	5108 $\pm$ 1046

* Not significant, $p > 0.10$ for comparisons to all other group means. Values are presented as means $\pm$ SEM.