Remodeling of the piriform cortex after lesion in adult rodents
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Denervation of the piriform cortex by bulbotomy causes a series of important cellular changes in the inhibitory interneurons of layer I and transsynaptic apoptosis of a large number of pyramidal neurons in outer layer II within 24 h. In this study, we report that following the marked loss of neurons in outer layer II, the piriform cortex is reconstituted by the addition of newly formed neurons that restore the number to a preinjury level within 30 days. We provide evidence that the number of newly divided neuronal progenitors increases after injury and further show that a population of doublecortin-positive cells that resides in the piriform cortex decreases after injury. Taken together, these findings suggest that the piriform cortex has significant neurogenic potential that is activated following sensory denervation and may contribute toward the replacement of interneurons (Koliatsos, unpublished observations, V.E. Koliatsos) suggests that these interneurons may also play a role in cortical remodeling after denervation lesions are formed. Indeed, the piriform cortex is associated closely with the two canonical neurogenic niches in the adult central nervous system – that is, the rostral migratory stream (RMS) and the subventricular zone (SVZ) – and there is evidence that certain cells in the piriform cortex remain mitotically active into adulthood [8] and doublecortin (DCX) (+) progenitor cells that reside in the adult piriform cortex [9–11] appear to respond to injury [12].

In this study, we extend the time period in which we examine cellular changes in the rodent olfactory system following bulbotomy. We confirm a significant loss in the number of layer II pyramidal neurons 1 day after lesion and further show that transsynaptic cell death progresses up to 7 days postlesion. More importantly, we report that layer II pyramidal neurons are reconstituted with an efficiency that leads to full replenishment of apoptotic neurons within 30 days postlesion. We further explore whether restitution of layer II pyramidal neurons proceeds through neurogenic mechanisms and provide evidence that the adult piriform cortex may function as a neurogenic niche that recruits newly generated progenitors from canonical neurogenic zones and is itself comprised of DCX (+) progenitor cells that decrease in number after bulbotomy. The loss of DCX (+) progenitor cells in conjunction with the gain of neurons in layer II suggests that these progenitors mature in response to injury.
Methods
Animal subjects and surgical procedures
All animal studies were carried out in accordance with NIH guidelines for the care and use of laboratory animals. Sprague–Dawley rats (male; 2–3 months of age; Charles River Laboratories, Frederick, Maryland, USA) were anesthetized with a gas mixture of isoflurane (4%), nitrous oxide (66%), and oxygen (33%). Proper anesthesia was determined by checking corneal and hindlimb reflexes. Rats were then prepared under aseptic conditions for surgery on a Kopf stereotaxic device (David Kopf Instruments, Tujunga, California, USA), where they received isoflurane (2%) and oxygen (30%) through nose cone and were subjected to a unilateral bulbotomy or sham procedure as described previously [5]. Bulbotomies entail a coronal transection of the olfactory peduncle 1 mm anterior to the frontal pole with the aid of a small surgical blade under direct visualization.

Time course of changes in the number of neurons in layer II of the piriform cortex after bulbotomy
Bulbotomized or sham rats were prepared as described [5] and allowed to survive for 1, 7, 14, 30, or 90 days after injury (n=3 per bulbotomy or sham procedure at each time point). Animals were euthanized by brief transcardial perfusion with 4% paraformaldehyde, followed by immersion fixation in Carnoy’s fluid (ethanol: chloroform: glacial acetic acid at a ratio of 60:30:10) as described [13] to maximize histological quality for Nissl staining. Blocks through the forebrain were embedded in paraffin and sectioned as described [13]. Layer II of the piriform cortex was outlined on 10 systematically selected serial sections from the rostral beginning of the piriform cortex to the level of the commissural decussation as described [2]. The optical fractionator probe [14] was used for stereological estimates of layer II neurons using our published protocols [2,5,13]. The number of layer II neurons per animal was aggregated in a lesion type versus sham arms of the experiment using analysis of variance (ANOVA), followed by the Neuman–Keuls or Bonferroni post-hoc test as described [13].

BrdU and immunocytochemistry for phenotypic markers of neuronal precursors and maturing neurons
BrdU was administered (100 mg/kg intraperitoneally; Roche, Indianapolis, Indiana, USA) to bulbotomized and sham animals (n=5 per group at each time point) for 3 days preceding or following bulbotomy. Animals injected with BrdU before injury were euthanized 24 h after bullotomy to assess the levels of basal neurogenesis. Animals injected with BrdU following injury were allowed to survive for 4 or 14 days after injury. Tissues were prepared with brief transaortic perfusion-fixation with PBS, followed by 4% paraformaldehyde. Coronal sections were cut at 30 μm using a freezing microtome. Qualitative assessments of BrdU-labeled nuclei in the piriform cortex were based on immunocytochemistry using a monoclonal anti-BrdU (1:1000; Sigma, St Louis, Missouri, USA) or polyclonal sheep anti-BrdU (1:100; Abcam, Cambridge, Massachusetts, USA) antibodies. The time course of precursor differentiation into mature neurons was assessed by double staining with BrdU and phenotypic markers of developing neurons: DCX (1:1000; migrating neuroblasts/immature neurons; Cell Signaling, Danvers, Massachusetts, USA), PSA-NCAM (1:500; developing/migrating neurons; Millipore, Billerica, Massachusetts, USA); neuronal nuclear antigen (NeuN, 1:200; mature neurons; Millipore); glial-fibrillary acidic protein (1:2000; astrocytes; Dako, Carpinteria, California, USA); and Iba-1 (1:600; microglia, Biocare Medical, Concord, California, USA) according to well-established protocols in our lab [15].

Counting DCX-expressing cells in the piriform cortex
Bulbotomized and sham mice (male; 2–3 months of age) that express Discoma spp. reef coral red fluorescent protein (DsRed) under the control of the DCX promoter [C57BL/6J-Tg(DCX-DsRed)14 Qlu/J; Jackson Labs, Bar Harbor, Maine, USA] were euthanized 3 and 7 days after injury by transaortic perfusion-fixation as above (n=3 animals per group at each time point). Brain tissues were prepared for counts of DCX-DsRed (+) cells by a blinded observer using stereological methods. The rostral–caudal length of the piriform cortex was outlined on every 10th section and the numbers of DsRed (+) cell profiles were quantified using Stereo Investigator software (MBF Bioscience, Williston, Vermont, USA) from the lateral edge of the forebrain to the olfactory tubercle. Differences between bulbotomized and sham mice were studied using a Student’s t-test.

Results
Piriform cortex neurons are reconstituted after bulbotomy-induced apoptosis
To address the possibility that the piriform cortex is regenerated after bulbotomy, we assessed cell death in the piriform cortex 1, 7, 14, 30, and 90 days after unilateral deafferentation of the olfactory bulb or sham procedures (n=3 per group at each time point). The total number of neurons in the compact layer II of the piriform cortex at the lesion site was analyzed using the optical fractionator stereological probe as described [14]. Apoptotic profiles visible in animals euthanized 1 day following the procedure were not counted.

Variance in cell numbers at time points following bulbotomy or sham procedures were analyzed using ANOVA. The overall variance among groups was significant (Fig. 1, P=0.0072). Bonferroni post-hoc testing showed significant differences between bulbotomy and sham groups at 1 and 7 days postlesion (Fig. 1, P=0.0003). Cell death was greatest at 7 days, with 71 380...
neurons dying by transsynaptic apoptosis \((P = 0.045)\). By 14 days, however, the number of neurons in bulbotomized animals increased, resulting in no significant differences when compared with sham-treated animals at 14, 30, and 90 days after bulbotomy.

Nissl staining of the piriform cortex shows the apoptotic effect of bulbotomy in the outer layer II 1 day after lesion (Fig. 1b, top) and a large deficit in cell number at 7 days (Fig. 1b, middle), but not 4 weeks (Fig. 1b, bottom) postlesion. In accordance with findings from our initial study [2], 58 700 neurons degenerated 1 day after bulbotomy. By 7 days, the total number of apoptotic neurons increased to 71 380. This increase, however, is not significantly different from 1 day postlesion. Reconstitution of pyramidal neurons in layer II of the piriform cortex was achieved by 30 days postlesion as evidenced by the addition of 55 300 neurons when compared with day 7.

### Injury-induced neurogenesis in rats

To directly address the possibility that the piriform cortex undergoes neurogenesis, we administered BrdU to rats for 3 days before or after bulbotomy. In animals injected before bulbotomy, there were no significant differences in the numbers of BrdU (+) cells between bulbotomy and sham procedures. In animals injected following bulbotomy, there was a marked difference in the density of BrdU (+) cells in the piriform cortex and olfactory tract lesion sites as early as 1 day and as late as 10 days following the final BrdU administration, which corresponds to 4 and 14 days after bulbotomy (Fig. 2).

Cells dually labeled for BrdU and the migrating neuroblast marker DCX were found interspersed among fibers in the olfactory tract and in superficial layer I immediately next to the olfactory tract 4 days after bulbotomy. At 14 days after bulbotomy, BrdU and DCX (+) cells appeared in the superficial outer layer II of the piriform cortex (Fig. 2c). In addition, immunoreactivity for PSA-NCAM, a marker of migrating neuroblasts and immature neurons, was markedly increased in the olfactory tract and layer I 4 days after bulbotomy and continued to be present at 14 days postlesion. At 14 days, dually labeled cells for PSA-NCAM and BrdU were identified in layer Ila (Fig. 2d). Colocalization of BrdU and NeuN, a marker for mature neurons, was not present in layer II neurons at day 4, but was present by day 14 following bulbotomy (Fig. 2c). BrdU (+) cells colocalized with glial-fibrillary acidic protein in layer I were rare, but were observed at 4 days after lesion formation. Iba-1 (+) macrophages were also BrdU (+), but infrequently and these cells did not cross the pial level into the parenchyma of the piriform cortex.

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The piriform cortex is fully reconstituted by 1 month after bulbotomy. (a) Total cell numbers in layer II were obtained using an optical fractionator-based stereological method. Differences between bulbotomy and sham groups across various survival times were analyzed with ANOVA \((P = 0.0072)\), followed by post-hoc Bonferroni testing. At 1 (242 054 sham vs. 183 347 bulbotomy) and 7 (241 741 sham vs. 170 362 bulbotomy) days postlesion, the total number of neurons in layer II was significantly less than that observed in sham-operated animals \((P = 0.0003\) and 0.045, respectively). Data are represented as mean ± SD \((n = 3)\). (b) The thickness of layer II of piriform cortex, as outlined with black lines, is markedly reduced 7 days after bulbotomy (middle). Reduced thickness was mainly caused by the death of pyramidal neurons in sublayer IIX (top). The piriform cortex in lesioned animals (bottom) recovered to the same size as control sham animals at 30 days after bulbotomy. ANOVA, analysis of variance.
DCX expression in the piriform cortex of mice

We further investigated conditional neurogenesis in response to bulbotomy using DCX-DsRed transgenic mice. Bulbotomized and sham animals were allowed to survive for 3 or 7 days postlesion and brain tissue was processed for fluorescence microscopy after perfusion fixation. DsRed expression and colocalization with DCX was confirmed in uninjured adult mice in the canonical neurogenic regions of the dentate gyrus (DG) (Fig. 3b) and SVZ (Fig. 3c). Interestingly, DCX expression was also observed extending from the external capsule/lateral ventricle through the dorsal endopiriform cortex to the superficial layers of the piriform cortex (Fig. 3d).

At 3 days after injury, the number of DCX (+) cells was comparable in bulbotomized (1533 ± 815) and sham (1450 ± 166) animals (Fig. 3a). However, the distribution of cells was different between bulbotomized and sham groups. DCX (+) cells in sham animals clustered and localized to layer II of the piriform cortex (Fig. 3e), whereas DCX (+) cells in bulbotomized animals appeared to be diffusely labeled in superficial cortical layers and clustered in layer II (Fig. 3f). By 7 days after injury, the number of DCX (+) cells in sham animals was not significantly different from that observed at day 3 (1454 ± 175). In contrast, there was a marked 76% decrease in the number of DCX (+) cells from 3 to 7 days in bulbotomized animals (Fig. 3g; 490 ± 547) following bulbotomy. This represents a significant difference between bulbotomized and sham animals on day 7 (P = 0.04).

Discussion

The findings presented here show that sensory denervation of the olfactory bulb by bulbotomy induces apoptosis in layer II of the piriform cortex, resulting in a significant decrease in the number of pyramidal neurons from 1 to 7 days after injury. By 30 days after injury, layer II pyramidal neurons are reconstituted and the number of neurons returns to preinjury levels. BrdU labeling shows that after injury, animals had BrdU (+) cells dually labeled with the neural progenitor markers PSA-NCAM and DCX in the piriform cortex, whereas sham animals did not. Moreover, in transgenic mice expressing DCX-DsRed, we observed a prominent pool of DCX (+) progenitors in the piriform cortex that decreased in number after bulbotomy, consistent with the idea that these progenitors mature in response to injury.

Our previous work has established olfactory denervation as a model of cell death in which pyramidal cells in the piriform cortex undergo classical apoptosis within 24 h after bullectomy. The upregulation of nNOS in GABAergic interneurons in the outer plexiform layer of the piriform cortex has been associated with transsynaptic signaling of apoptosis in layer II pyramidal neurons after olfactory bullectomy or bulbotomy. Here, we further characterized our injury paradigm and extended our outcome measures to 30 days after injury. At these later time points, we found that the significant lesion-induced loss of layer II pyramidal neurons is reversed. The replacement of layer II pyramidal neurons after olfactory bulb injury represents an ideal paradigm to examine the mechanisms of neural repair in the injured brain, especially the limbic cortex.

Neurogenesis in the adult mammalian nervous system is primarily established in two consensus regions, that is the subgranular zone of the hippocampus that supplies neurons to the DG and the SVZ – RMS that repopulates the olfactory bulb [16,17]. The functional significance of neurogenesis in the forebrain remains an open question. For example, the proliferation and migration of SVZ precursors persists in adult animals even after removing the olfactory bulb [18]. It is, therefore, possible that the SVZ may supply other brain regions and play a broader role in forebrain plasticity and repair. Consistent with this idea, there may be alternative migratory streams originating from the SVZ that guide newly generated cells to cortical regions. In primates, Bernier et al. [19] have identified a ‘temporal stream’ that extends from the temporal horn of the lateral ventricle to the amygdala. Luzzati et al. [20] further reported chains of neuroblasts that migrate through the subcortical parenchyma to telencephalic regions in the rabbit. Two alternative streams, the ventrocaudal (from the ‘elbow’ of the RMS to the rostral piriform cortex) and caudoventral (from the caudal lateral ventricle to the caudal piriform cortex), have been shown to populate the piriform cortex in rats [11,21,22]. Interestingly, the piriform cortex and amygdala are precise anatomical intermediaries between the two canonical neurogenic zones in the adult central nervous system.

The primary cell types generated during neurogenesis in adult rats [23] and primates [19], including humans [24], are interneurons, predominantly granular and periglomerular interneurons in the olfactory bulb and granular as well as nongranular GABAergic interneurons in the hippocampus [25]. After sensory denervation of the piriform cortex, however, we observe large, basophilic neuronal profiles that are not indicative of interneurons. These findings are in line with other accounts showing that after bullectomy, the number of DCX/PSA-NCAM-positive cells decrease and the number of NeuN-positive cells increase, whereas no significant change is observed in the number of glutamate decarboxylase-positive interneurons [12]. The decrease in the number of neuronal progenitors and the restoration of principal neurons to layer II appears to be a phenomenon unique to the neuroplasticity of the piriform cortex.

To explore the nature of repair in the piriform cortex after bulb lesions, we asked whether the regenerative response observed was because of classical neurogenesis, that is de-novo generation of neuronal progenitors. To this effect, we carried out BrdU labeling studies in...
In accordance with established regimens in the field. At 4 days after injury, BrdU was colocalized with the immature neuroblast markers DCX and PSA-NCAM and double-labeled cells were observed along the olfactory tract and in superficial layers of the piriform cortex. By 2 weeks, BrdU was colocalized with the postmitotic neuronal marker NeuN within layer II of the piriform cortex. This shifting pattern may be representative of neurogenic responses in the SVZ and subsequent migration along alternative pathways stemming from the RMS [11,19–22]. The time frame for neuronal maturation is in accord with previous reports where BrdU/NeuN (+) cells are present in the piriform cortex of rats 10 days after BrdU injection [11]. Alternatively, de-novo neurogenic responses within the piriform cortex itself could account for the presence of BrdU (+) cells; however, the lack of BrdU intercalation in sham animals suggests that a non-pathogenic, constitutive stem cell ‘pool’ does not exist in the piriform cortex.

We further investigated lesion-induced changes in neurogenesis using mice that express DsRed under the...
control of the DCX promoter. DCX expression was observed in numerous brain regions including the canonical SVZ and DG, as well as the endopiriform and piriform areas. The number of DCX (+) cells was not significantly different between sham and bulbotomized animals 3 days after injury. However, by 7 days, the number of DCX (+) cells was significantly less when compared with both sham and day 3 bulbotomized animals. These data suggest that a pool of DCX (+) progenitors residing in the piriform cortex may be induced to differentiate in response to injury in accord with previous findings [12]. It is possible that several mechanisms are responsible for the reconstitution of layer II neurons: the mobilization of a quiescent population of DCX (+) progenitors residing in the piriform cortex, lesion-induced de-novo neurogenesis in the SVZ followed by migration to the piriform cortex, and, possibly, the maturation of oligodendrocytes progenitor cells into mature neurons [26]. On the basis of the data presented here and reports in the literature, we believe that activated DCX (+) progenitors mature into NeuN (+) neurons that reconstitute layer II. We provide evidence for lesion-induced neurogenesis and targeted migration as well as activation and maturation of quiescent neural progenitor pools in the piriform cortex and/or adjacent neurogenic zones.

Bulbotomy results in a decreased number of DCX (+) neuroblasts. (a) Whisker plot of the total number of DCX (+) cells in the piriform cortex in sham versus lesioned animals 3 and 7 days after bulbotomy. At 3 days, the number of DCX (+) cells was comparable in sham (1450 ± 165) and lesioned animals (1533 ± 815). By 7 days, the number of DCX (+) cells in lesioned animals decreased markedly (490 ± 546), resulting in a significant difference in the number of DCX (+) cells between groups by 7 days (P = 0.04). (b–g) Expression of DCX-DsRed in the canonical neurogenic regions, dentate gyrus (b), and subventricular zone (c; red: DsRed, green: DCX immunostain), as well as basal expression pattern in normal piriform cortex (d). (e) Expression of DCX in sham animals is largely confined to layer II (compact layer) of the piriform cortex. (f) Three days after bulbotomy, DCX expression is more diffuse and DCX (+) cells are observed in superficial cortical layers in addition to layer II. (g) By 7 days after bulbotomy, DCX expression is largely reduced. DCX, doublecortin.
The exciting combination of both cell death and regeneration-neurogenesis in the piriform cortex after lesions suggests a new paradigm of thinking about brain insults as complex processes that unleash both destructive and regenerative mechanisms. The tandem occurrence of apoptotic cell death and neurogenesis introduces an in-vivo model of cortical plasticity with widespread implications across traumatic and degenerative diseases of the nervous system.

Acknowledgements
This work was supported by the NIH-funded grants: RO1 AG16263 and R21 MH086020.

Conflicts of interest
There are no conflicts of interest.

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