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## SVZ-derived newly generated neurons populate several olfactory and limbic forebrain regions

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### Abstract

Neurogenesis persists in several regions of the adult mammalian brain. Although the hippocampus and olfactory bulb are most commonly studied in the context of adult neurogenesis, there is an increasing body of evidence in support of neurogenesis occurring outside of these two regions. The current study expands upon previous data by showing newborn neurons with a mature phenotype are located in several olfactory and limbic structures outside of the hippocampus and olfactory bulb, where we previously described DCX/BrdU immature neurons. Notably, newborn neurons with a mature neuronal phenotype are found in the olfactory tubercles, anterior olfactory nuclei, tenia tecta, islands of Calleja, amygdala and lateral entorhinal cortex. The appearance of newborn neurons with a mature phenotype in these regions suggests that these structures are destinations, and that newborn neurons are not simply passing through these structures. In light of the increasing body of evidence for neurogenesis in these, and other olfactory, limbic and striatal structures, we hypothesize that brain regions displaying adult neurogenesis are functionally linked.

### Keywords

adult neurogenesis; rostral migratory stream; lesions; BrdU; NeuN; doublecortin

### Introduction

There are two well-described neurogenic regions of the adult brain, the subventricular zone (SVZ) and the subgranular zone. Granule cells are generated in the subgranular zone of the dentate gyrus and migrate a short distance to the granule cell layer where they are integrated into existing hippocampal circuitry [1–4]. The SVZ is a much more robust and widespread neurogenic region in that newly generated neurons have been shown to migrate a much longer distance and in larger numbers to the olfactory bulb [5]. Functional studies of these newly generated neurons in the hippocampus and olfactory bulb have indicated that they are

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incorporated into the existing circuitry and have similar electrophysiological characteristics as the mature cells in these structures [4,6–8]

Altman [9] provided initial evidence for adult neurogenesis in the SVZ using [<sup>3</sup>H] thymidine-labeling. Subsequently, numerous reports showed that there is a robust rostral migratory stream (RMS) from the SVZ to the olfactory bulb [10–14]. These previous studies have examined newly generated neurons emanating from the SVZ and along the RMS using neuron-specific molecular markers such as, Tuj1, TUC-4, PSA-NCAM, doublecortin (DCX) and Bcl-2 combined with the mitotic marker bromodeoxyuridine (BrdU) to confirm that these newborn cells are newly generated neurons [15–17]. Recent data have shown that neurons born in specific regions of the SVZ have an inherent program to migrate to specific areas of the olfactory bulb [18]. Thus, despite the popular belief that progenitor cells in the SVZ are “stem cells”, it appears as though the SVZ progenitor cells have a specific signal for where the cells they generate are intended to reside within specific regions of the olfactory bulb. Thus, cells that are migrating along the RMS appear to have a predetermined destination [18].

The fact that the newly generated neurons migrating along the RMS have a specific destination suggests that they have a specific function. Gheusi [19] and others have shown that newly generated neurons in the olfactory bulb are important for olfactory discrimination learning [19–21]. Moreover, Shapiro et al [22] confirmed that olfactory enrichment increases the number of newly generated neurons in the olfactory bulb [20] and also showed that olfactory enrichment enhances the differentiation of newborn neurons in the piriform cortex. In a separate paper, Shapiro et al [23] elucidated the migratory route of newly generated neurons originating from the most ventrocaudal portion of the SVZ, and showed their migration to the piriform cortex. It is interesting to note that Merkle et al [18] detected few if any cells in the olfactory bulb that originated from this most ventrocaudal portion of the lateral ventricle. Taken together, these data suggest that the final destination for the newly generated neurons arising from the progenitor cells located in this caudal portion of the SVZ is different than that for the other parts of the SVZ.

There are several lines of evidence showing that newly generated neurons derived from the SVZ migrate to numerous forebrain regions as reported for monkeys, rodents and rabbits. These include: the amygdala [24,25], striatum [26], piriform cortex [22–24,27,28] and the olfactory tubercles [23,24]. In addition, Yang et al. [17] examined newly generated neurons near the lateral ventricles in the adult mouse brain and found that a population of neurons also migrates dorsally to the corpus callosum and ventrally to the nucleus accumbens, ventromedial striatum, ventrolateral septum, and bed nucleus of the stria terminalis. Thus, there is accumulating evidence for the existence of newly generated neurons derived from the adult SVZ that migrate to other brain regions besides the olfactory bulb.

The goal of this study was to further describe the distribution of newly generated cells derived from the SVZ in several olfactory and limbic structures and to determine whether they differentiate into neurons. BrdU-labeling combined with double-labeling for immature and mature neuronal markers was used to elucidate the phenotype and morphology of these newborn cells. Laser-scanning confocal microscopy was used to confirm that the BrdU-label was contained within neurons and not within satellite cells. Lesions were made to interrupt the migratory pathway to some of these destinations to provide additional evidence that the populations of newly generated cells observed in some of these structures were not born locally.

## Methods

### BrdU Injections

**Single BrdU injections**—At 4h prior to sacrifice, a single BrdU injection (i.p. 100 mg/kg) was given to adult C57/B16 mice (2 months old) mice (N=4). This short time point was examined to determine if cells might be generated locally within the examined olfactory and limbic structures.

**Daily BrdU injections**—Male C57/B16 mice (2 months old; N=20) were used for this study. The mice were allowed access to food and water ad libitum and were kept on a normal 12 hr light/dark cycle. BrdU was injected i.p. (Sigma, St. Louis, Missouri, USA; 100mg per kg), once daily, beginning on day 1 and continuing to day 4. The mice were perfused a week after the last BrdU injection by first anesthetizing with xylazine and ketamine, followed by transcardial perfusions with 50 ml of 0.9% NaCl followed by 4% paraformaldehyde. Brains were post-fixed overnight and cryoprotected with 30% sucrose in 0.1 M phosphate buffer (PB, pH 7.4) for 48 hr, embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, California, USA), and sectioned on a cryostat in the coronal plane at 40  $\mu$ m. Sections were placed on slides or collected free-floating into an antifreeze solution and stored at  $-80^{\circ}$  C.

**Lesions**—Lesions (N=4) were made in the RMS of adult CD-1 mice to determine the effect on neurogenesis in the piriform cortex and adjacent brain structures by assessing the number of DCX-labeled cells. Briefly, animals were first anesthetized using xylazine/ketamine (10 mg/kg) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The scalp was excised above the cranium, and a drill was used to cut a hole in the cranium. Then, a sterile #11 scalpel blade was inserted into the hole 2.90 mm deep to produce a lesion between the lateral ventricle and the rostral piriform cortex at  $-1.80$  mm to  $-1.00$  mm from bregma [29]. After suturing their skin, the animals were placed back into their home cages and their weight was monitored twice daily to ensure that they were eating and drinking, and that no infections or other adverse effects of the lesion occurred. The animals were allowed to survive for 21 days post-lesion, to ensure that the only DCX-labeled cells would be the ones that were born after the lesion [15]. Age-matched controls (N=4) that had the hole drilled into their skull, but with no lesion, were used for comparison. The animals were perfused as described above. Analysis of DAB reacted DCX-labeled tissue was performed using a brightfield microscope (Carl Zeiss, Inc, Thornwood, NY, USA).

### Immunohistochemistry

For DCX/BrdU- and NeuN/BrdU-double-labeling, every sixth 40  $\mu$ m section was examined. The protocol for immunostaining was the same as that previously described [22]. This double labeling procedure was used to identify whether BrdU-labeled cells had an immature, or mature neuronal phenotype.

### Laser-scanning confocal microscopy

Fluorescent-tagged secondary antibodies were used to allow simultaneous visualization of immunoreaction product using 2 separate laser channels of a confocal scanning microscope (Olympus, Tokyo, Japan). These channels were sequentially scanned to avoid cross-excitation. This method was employed to verify that the BrdU-labeled nucleus was contained within the NeuN-labeled or DCX-labeled perikaryal cytoplasm, and not within satellite cells, as previously suggested for cortical BrdU-labeled cells [30].

## Results

The results from the current study using DCX/BrdU preparations support previous studies that newly born neurons are found in several olfactory/limbic forebrain regions besides the hippocampus, piriform cortex and olfactory bulb. In addition, the results from the NeuN/BrdU preparations extend upon previous findings and suggest that the newly born neurons in these brain regions develop a mature phenotype. The results from lesions ventral to the elbow of the RMS showing a decreased number of DCX-labeled cells in the piriform cortex ipsilateral to the lesion support the idea that the newborn neurons in this region are migrating from the SVZ.

### Olfactory/limbic forebrain regions have newly generated neurons with immature neuronal phenotype

DCX/BrdU double-labeled cells were observed in other gray matter regions of the adult rodent forebrain besides the piriform cortex and olfactory bulb. These included: the striatum (Fig. 1i), the lateral entorhinal cortex (Fig. 1j), olfactory tubercle (Fig. 1k), amygdalopiriform area, amygdalohippocampal area, presubiculum, parasubiculum, subiculum, islands of Calleja, tenia tecta, anterior olfactory nucleus and the accessory olfactory bulb (some data not shown). DCX/BrdU double-labeled cells were also found in multiple (e.g., anterior, basolateral, basomedial) amygdaloid nuclei (Fig. 1h). These double-labeled cells were relatively small (6–10  $\mu\text{m}$ ), had a thin shell of perikaryal cytoplasm and were mostly bi-polar or multi-polar. Their processes were typically less than 20  $\mu\text{m}$  in length and were not usually branched. The DCX/BrdU double-labeled cells observed in the striatum had nuclear sizes of 7–10  $\mu\text{m}$  and had thin shells of perikaryal cytoplasm, consistent with previous data from Dayer et al. (26). Their processes were typically unbranched and less than 15  $\mu\text{m}$  in length (Fig. 1j). The DCX/BrdU double-labeled cells found in the islands of Calleja, olfactory tubercle and anterior olfactory nucleus were typically unipolar or bipolar in appearance, and their processes were less than 20  $\mu\text{m}$  in length. No such cells were found in any of the regions examined at 4 hours after the BrdU-injection.

### Lesion Study of the RMS and DCX-labeled cells in the piriform cortex

Data from mice with lesions of the RMS showed an almost complete absence of DCX-labeled cells in the rostral portion of the ipsilateral piriform cortex of lesioned mice, as compared to non-lesioned control mice (Fig. 2). Thus, intersecting the RMS from the rostral portion of the piriform cortex reduces the number of DCX-labeled cells in this region. In addition, the rostral piriform cortex contra-lateral to the lesioned hemisphere showed an increased number of DCX-labeled cells (Fig. 2).

### Olfactory/limbic forebrain regions have newly generated neurons with mature neuronal phenotype

NeuN/BrdU double-labeled cells were found in brain regions outside the olfactory bulb and hippocampus and included several regions where DCX/BrdU-labeled cells were observed. These regions include: anterior olfactory nucleus (Fig. 3), lateral to the olfactory ventricle, the insula Calleja magna complex (Fig. 3) located along the most medial portion of the mouse brain, the ventral tenia tecta (not shown) the deep layers (layers V & VI) of the lateral entorhinal cortex (Fig. 4) and several amygdaloid nuclei, including the basolateral nucleus and the central amygdaloid nucleus (Fig. 4). Laser-scanning confocal microscopy confirmed that the BrdU-labeling was located within the NeuN-labeled cell body. Orthogonal views further confirmed these observations (Figs. 3D,H and 4D,H). It is pertinent to note that in the olfactory tubercles, of 57 BrdU-labeled cells examined, only 1 (< 2%) was confirmed to be double-labeled for NeuN. Alternatively, in the other structures examined, ~5–10% of the BrdU-labeled cells were double-labeled for NeuN (minimum of 25 BrdU-labeled cells examined per region). Thus,

NeuN/BrdU-labeled cells are found throughout several regions of the rostral/caudal extent of the adult mouse brain at 11 days after the first of 4 daily BrdU-injections.

## Discussion

The results from this study show that newborn neurons with a mature phenotype are found in several olfactory and limbic regions that were previously reported to contain newly born neurons derived from the SVZ. This suggests that these brain regions where NeuN/BrdU-labeled cells are found represent destinations for these newly born neurons and not simply a reflection of their being in transit to another brain region. The functional significance for adult neurogenesis in these olfactory and limbic brain structures remains elusive.

### Putative role of adult neurogenesis in the olfactory system

Data from the current study and others show that adult neurogenesis has been observed in key regions of the olfactory system. In rodents, lagomorphs and non-human primates, olfaction is still one of the predominant senses. The fact that the neural epithelium in the nasal mucosa consists of a local pool of constantly regenerating neurons [31] may provide a clue as to why neurogenesis occurs in brain structures that are part of the olfactory system. The axons from the continually regenerating olfactory receptor cells project to the olfactory bulb, where a robust pool of newly generated neurons occurs that is derived from the SVZ via the RMS [5]. It should be noted that evidence is mounting for the existence of this pathway in humans [32–34]. In addition, Rochefort et al. [20] have shown in adult mice that olfactory enrichment enhances the survival and differentiation of newborn neurons in the olfactory bulb, and it has been shown that newborn neurons in the olfactory bulb are important for olfactory discrimination learning [19]. Thus, newborn neurons in the olfactory bulb are functionally integrated into the existing olfactory circuitry indicating that they contribute to the modulation of the olfactory bulb's output, the lateral olfactory tract.

The axons of the mitral cells of the olfactory bulb make up the lateral olfactory tract and their major projection is to the olfactory cortex, comprised mainly of the anterior olfactory nuclei, olfactory tubercle, tenia tecta and piriform cortex [35]. The fact that neurogenesis has been detected in these regions, albeit at relatively lower levels than that found in the olfactory bulb [22–24,36], suggests that newborn neurons in these regions might be involved in processing the signals encoded by the recently generated neurons in the olfactory bulb. It should be noted that neurons with hilar basal dendrites born after seizures in the dentate gyrus, have recently been modeled to show that just a few neurons integrated into the dentate circuitry can have profound effects on the excitability of the hippocampus (37). Thus, newly generated mature neurons observed in several olfactory structures receiving lateral olfactory tract input might play a role in the dynamic plasticity of the olfactory system.

### Putative relationship between olfactory and limbic system neurogenesis

Similar to olfaction, the limbic system is phylogenetically one of the oldest systems in mammals. Interestingly, the lateral olfactory tract also sends projections to two key components of the limbic system, the amygdala and the entorhinal cortex [38,39]. The latter structure has a direct projection to the hippocampus, a structure with newly generated neurons that mature to become functional granule cells [6]. The fact that the data from the present study show newly generated mature neurons in the lateral entorhinal cortex and amygdala demonstrates a neurogenic link between the olfactory and limbic systems.

We propose that neurogenesis in each of these limbic structures plays a role in processing olfactory data. First, the hippocampus is anatomically situated to consolidate olfactory and emotional information, and functional data support this idea [35,40–46]. Second, the amygdala

is known to be involved in fear response [47], and this structure may need to remain highly plastic in order to attach the appropriate emotional response to continuously varying stimuli, including olfactory stimuli. Indeed, fear conditioning has been shown to be dependent on newborn neurons in the hippocampus [48,49]. Considering the reliance that lower mammals, such as rodents, have on olfaction, it is obligatory that olfactory information is encoded with an emotional component. This is supported by the idea that the amygdala receives extensive projections from the olfactory cortex [50]. Third, the entorhinal cortex is another limbic structure that is anatomically linked to the olfactory system [39,51–53] in that it shares reciprocal connections with the piriform cortex [54–56]. Moreover, the entorhinal cortex sends a major projection to the dentate gyrus and amygdala [43,53,57]. Thus, the common thread amongst the structures where newly generated mature neurons are found in the adult brain is that they populate mainly limbic and olfactory structures. Therefore, we hypothesize that newborn neurons in olfactory and limbic structures are involved in synchronizing novel olfactory and emotional information.

### **Striatal adult neurogenesis and its putative function**

An obvious exception to our proposed hypotheses is the presence of adult neurogenesis in the striatum [26,58]. It is pertinent to note that the islands of Calleja are considered to be a part of the striatal system [59–61]. The islands of Calleja are in the rostral portion of the rodent forebrain, situated very close to many olfactory structures. Moreover, it has been shown that the striatum forms reciprocal connections with several olfactory and limbic structures [62–64]. Considering the basic biological drives of feeding and reproduction, mammals have an inherent need to process an adequate response to novel olfactory and emotional stimuli. Therefore, the newborn neurons in the striatum and islands of Calleja might be involved in encoding the appropriate reward component to novel olfactory and emotional information, which is then consolidated into memories by the hippocampus, thus providing a link between the neurogenic regions of the adult brain.

### **Conclusion**

The results from this study support previous data showing newborn neurons in multiple regions throughout the adult brain. This study also extends these findings by showing that mature newborn neurons are also found in these regions as indicated by double-labeling for NeuN and BrdU. Hypotheses are posed in an attempt to consolidate the existing literature on adult neurogenesis into a cogent theory for the putative role of neurogenesis in many regions of the adult brain. Future studies will need to simultaneously examine several neurogenic regions following olfactory sensory manipulations to obtain functional data for newborn neurons in the adult brain.

### **Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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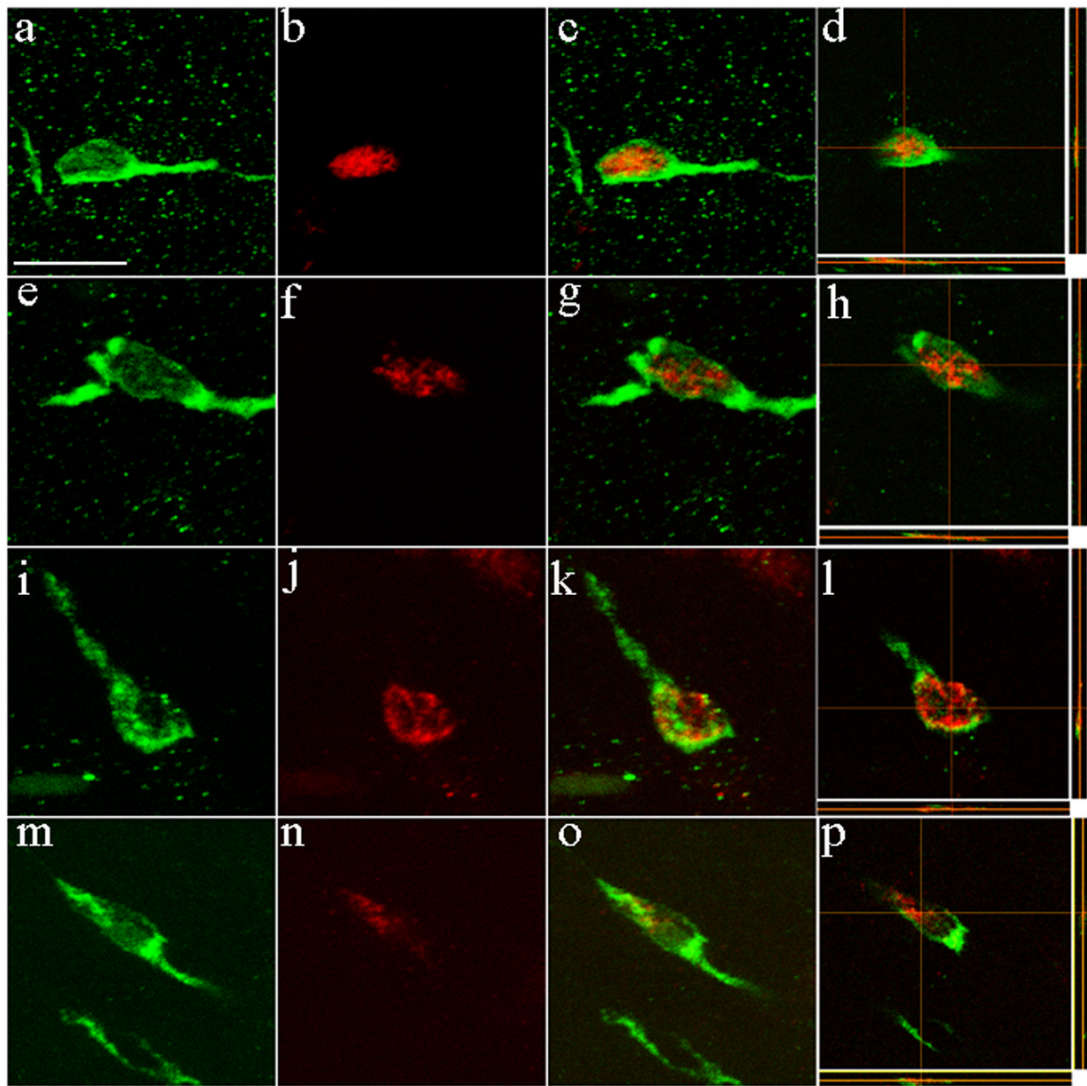
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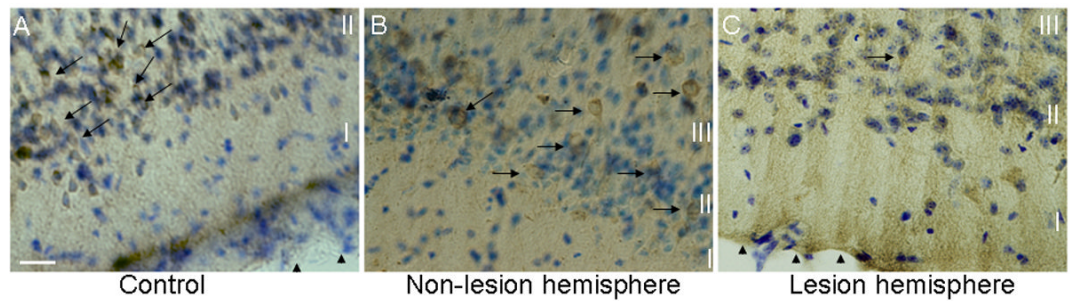
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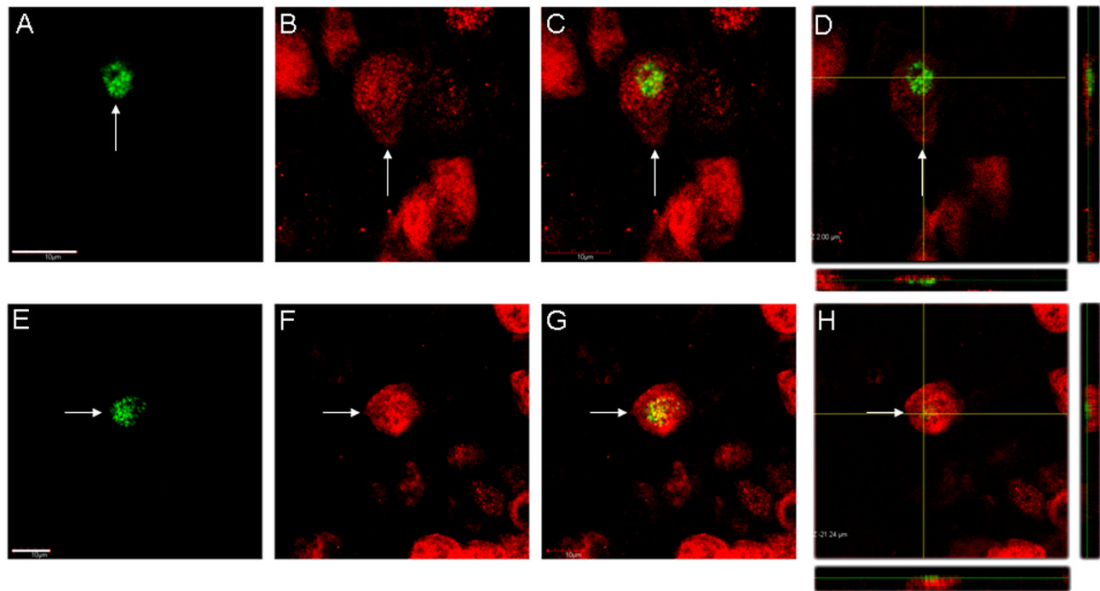
**Figure 1.**

Confocal Z-stack images of DCX/BrdU double-labeled cells in several telencephalic structures in the adult mouse brain. The images are organized such that the DCX immunolabeling (green) is shown in the first column, the BrdU immunolabeling (red) is found in the second column, the merged image is in the third column and the last column shows a modified merged image with cross hairs to indicate where the orthogonal images (thin rectangular panels to the right and at the bottom) were obtained. (a–d) basolateral amygdaloid nucleus, (e–h) lateral entorhinal cortex, (i–l) striatum, (m–p) olfactory tubercle. Note that these cells have a relatively small (less than 5  $\mu\text{m}$ ) nucleus, a thin shell of perikaryal cytoplasm, and have only 1 or 2 rudimentary processes. Scale bar in **a** represents 6  $\mu\text{m}$  for (a–d); 5  $\mu\text{m}$  for (e–h); 4  $\mu\text{m}$  for (i–l); 6  $\mu\text{m}$  for (m–p).



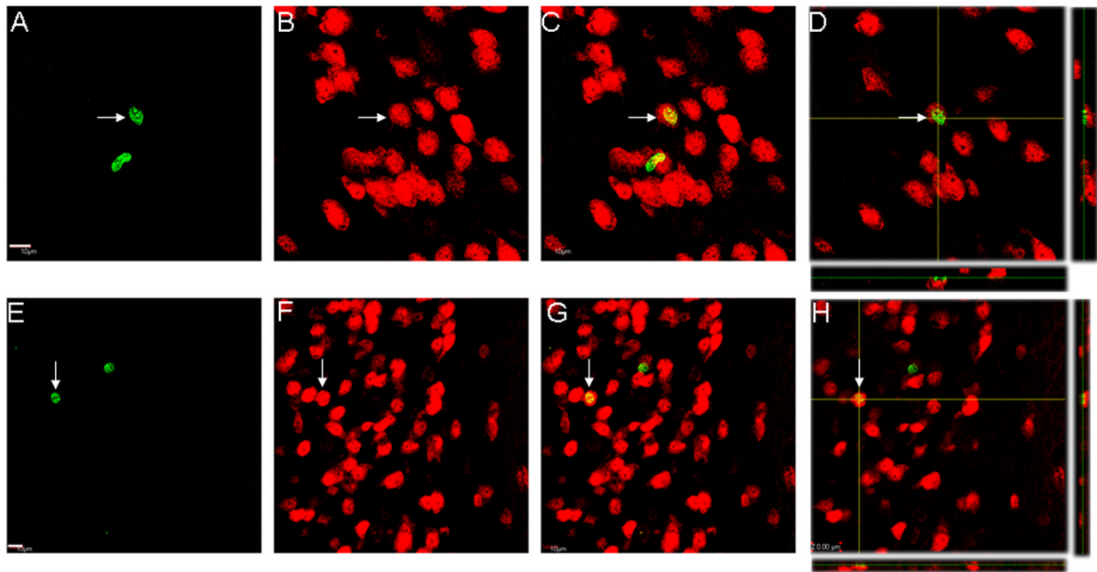
**Figure 2.**

Bright-field micrographs of DCX-immunolabeled and Nissl counterstained sections in the piriform cortex from lesioned and non-lesioned mice. In **A**, DCX-labeled cells (arrows) in the piriform cortex of a control mouse are shown. Note the pial surface (arrowheads) in the lower right portion of the micrograph. In **B**, DCX-labeled cells (arrows) are shown from the piriform cortex non-lesioned hemisphere of a mouse that received a lesion of the RMS on the contra-lateral side. Note that DCX-labeled cells are abundant. In **C**, the piriform cortex is shown in the hemisphere ipsilateral to the lesion. There are reduced numbers of DCX-labeled cells (arrow) in the piriform cortex at 21 days after a lesion bisecting the RMS. Note the pial surface (arrowheads) in the lower left corner. Scale bar in **A** = 25  $\mu$ m for all three panels.



**Figure 3.**

Confocal Z-stack and orthogonal images of NeuN/BrdU double-labeled cells in the anterior olfactory nuclei and islands of Calleja. In **A–D**, a NeuN(red)/BrdU(green) double-labeled cell (arrow) is shown from the dorsal portion of the anterior olfactory nuclei. The merged view is shown in **C** and the orthogonal view through the middle of the cell is shown in **D**. In **E–H**, a NeuN(red)/BrdU(green) double-labeled cell(arrow) is shown from the most medial portion of the islands of Calleja, also known as the insula Calleja magna complex. The merged view is shown in **G** and the orthogonal view through a portion of the cell body is shown in **H**. Scale bar in **A** = 10  $\mu\text{m}$  for **A–D**. Scale bar in **E** = 10  $\mu\text{m}$  for **E–H**.



**Figure 4.**

Confocal Z-stack and orthogonal images of NeuN/BrdU double-labeled cells in the limbic system. In **A–D**, a NeuN(red)/BrdU(green) double-labeled cell(arrow) is shown from the central amygdaloid nuclei. The merged view is shown in **C** and the orthogonal view through the center of the cell is shown in **D**. Note that there is another BrdU-labeled cell that is not doubled-labeled for NeuN. In **E–H**, a NeuN(red)/BrdU(green) double-labeled cell (arrow) is shown from the lateral entorhinal cortex. The merged view is shown in **G** and the orthogonal view through this cell body is shown in **H**. Note that there is another BrdU-labeled cell that is not doubled-labeled for NeuN. Scale bar in **A** = 10  $\mu\text{m}$  for **A–D**. Scale bar in **E** = 10  $\mu\text{m}$  for **E–H**.