Chemosensory cues affect amygdaloid neurogenesis and alter behaviors in the socially monogamous prairie vole

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Abstract
The current study examined the effects of pheromonal exposure on adult neurogenesis and revealed the role of the olfactory pathways on adult neurogenesis and behavior in the socially monogamous prairie vole (Microtus ochrogaster). Subjects were injected with a cell proliferation marker [5-bromo-2′-deoxyuridine (BrdU)] and then exposed to their own soiled bedding or bedding soiled by a same- or opposite-sex conspecific. Exposure to opposite-sex bedding increased BrdU labeling in the amygdala (AMY), but not the dentate gyrus (DG), of female, but not male, voles, indicating a sex-, stimulus-, and brain region-specific effect. The removal of the main olfactory bulbs or lesioning of the vomeronasal organ (VNOX) in females reduced BrdU labeling in the AMY and DG, and inhibited the male bedding-induced BrdU labeling in the AMY, revealing the importance of an intact olfactory pathway for amygdaloid neurogenesis. VNOX increased anxiety-like behavior and altered social preference, but it did not affect social recognition memory in female voles. VNOX also reduced the percentage of BrdU-labeled cells that co-expressed the neuronal marker TuJ1 in the AMY, but not the DG. Together, our data indicate the importance of the olfactory pathway in mediating brain plasticity in the limbic system as well as its role in behavior.

Introduction
The detection of olfactory stimuli is essential for resource acquisition (e.g., food and shelter) and avoidance of predation (Wyatt, 2003; Jacobs, 2012). Olfaction is also involved in mate choice, partner bonding, and parent-offspring attachment (Wyatt, 2003; Jacobs, 2012). Although there are various definitions, pheromones are commonly defined as chemosensory stimuli conveying information among conspecífics (Dulac & Torello, 2003; Brennan & Keverne, 2004). The main olfactory epithelium and vomeronasal organ (VNO) detect such stimuli and project via the main olfactory (MOB) and accessory olfactory (AOB) bulbs, respectively, to limbic brain structures, which mediate various social behaviors (Kollack-Walker & Newman, 1995; Lonstein et al., 1998; Baum & Kelliher, 2009). Pheromonal exposure affects physiology and behavior (Taylor et al., 1992; Kelliher & Wersinger, 2009). It can also alter adult neurogenesis, i.e., the generation and functional integration of new neurons in adulthood.

The subventricular zone (SVZ) and dentate gyrus (DG) give rise to adult-generated cells that differentiate into neurons and integrate into the MOB and DG, respectively (Ming & Song, 2005). Male pheromone exposure increases SVZ cell proliferation in female mice and prairie voles (Microtus ochrogaster) (Smith et al., 2001; Mak et al., 2007). Male pheromones also increase cell survival in the MOB and AOB of female mice (Mak et al., 2007; Oboti et al., 2009). Such pheromone exposure also increases hippocampal adult neurogenesis, a phenomenon that is dependent on an intact MOB pathway (Mak et al., 2007). Although adult-generated cells also occur in non-traditional neurogenic brain regions along the olfactory pathway, namely the amygdala (AMY), medial preoptic area, and hypothalamus (Huang et al., 1998; Fowler et al., 2002; Baum & Kelliher, 2009), little is known about the effects of pheromonal exposure on adult neurogenesis within these limbic brain regions.

The current study assessed the effects of pheromones on adult neurogenesis in the AOB, MOB, and limbic brain regions that belong to the olfactory pathways in the prairie vole. The prairie vole is particularly sensitive to changes in the social environment and thus has been used to study the effects of the social environment on adult neurogenesis. For example, social isolation in females and fatherhood reduce AMY and DG cell survival (Lieberwirth et al., 2012, 2013). Furthermore, male pheromone exposure leads to an increase in SVZ cell proliferation in female prairie voles (Smith et al., 2001). In the current study, voles were exposed to their own bedding or to bedding soiled by a conspecific to assess the pheromonal effect on the number of adult-generated cells in the MOB, AOB, and limbic brain regions of the olfactory pathways. Furthermore, MOB and VNO lesions were used to assess whether an intact...
olfactory pathway is necessary for pheromone-induced changes in the number of adult-generated cells. Lastly, we assessed whether the disruption of the VNO pathway affects various behaviors (including anxiety-like and social behaviors). Investigating the effects of a disrupted olfactory pathway on adult neurogenesis and behavior will guide further research to establish a potential functional link between adult neurogenesis and behavior.

Materials and methods

Subjects

Subjects (90–120 days of age) were sexually naïve male and female prairie voles (M. ochrogaster) that were the F3 generation of a laboratory breeding colony started from field-captured animals. Subjects were pair-housed with a same-sex conspecific in plastic cages [18 × 29 × 13 (H) cm] that contained cedar chip bedding. Water and food were provided ad libitum. Females and males were housed in separate colony rooms on a 14 : 10h light : dark cycle with lights on at 07:30 h. The room temperature was kept at 21 ± 1 °C. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Florida State University and were conducted in accordance with the guidelines set forth by the National Institute of Health.

5-Bromo-2′-deoxyuridine injections, bedding exposure, and brain perfusion

All subject pairs were transferred to clean cages prior to the start of the experiment and housed individually. Twenty-four hours later, subjects were injected with a cell proliferation marker, 5-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich, St Louis, MO, USA), once a day, at 24-h intervals, for three consecutive days. BrdU injections were given intraperitoneally (150 mg/kg body weight). Throughout the 3-day BrdU injection schedule, subjects were exposed daily in their home cages to bedding soiled by the same stimulus animal. The soiled bedding (approximately 100 g) was added to the subject’s cage each morning and remained there until the end of the experiment.

At 24 h after the last BrdU injection, subjects were deeply anaesthetised and then perfused through the ascending aorta with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed and post-fixed in 4% paraformaldehyde for 2 h at 4 °C. Thereafter, brains were transferred into 30% sucrose in PB at 4 °C. Using a freezing microtome, the brains were cut coronally (40-µm section thickness) and sections were stored in 0.1 M PB with 1% sodium azide until processed for immunohistochemistry.

Experimental design

Experiment 1

This experiment was designed to examine the effects of bedding exposure on BrdU labeling in the brains of male and female prairie voles. Subjects were randomly assigned to one of three treatment groups that were exposed to: (i) their own bedding (male, n = 6; female, n = 5), (ii) bedding from a same-sex individual (male, n = 6; female, n = 7), or (iii) bedding from an opposite-sex individual (male, n = 7; female, n = 8). For the latter two groups, the bedding used was obtained from a same- or opposite-sex individual that was not a sibling of the subject. After 3 days of BrdU injections and bedding exposure, subjects were perfused, and their brains were processed for BrdU immunohistochemistry.

Experiment 2

The second experiment examined the role of the main and accessory olfactory systems on bedding-induced BrdU labeling in female prairie voles. Female subjects were randomly assigned to one of three treatment groups: (i) intact (Control), (ii) olfactory bulbectomy (OBX), and (iii) VNO lesion (VNOX). Following surgery, subjects were allowed to recover for 3 weeks before being further divided into two subgroups that were exposed to either their own bedding (Control, n = 8; OBX, n = 7; VNOX, n = 6) or to male bedding (Male; Control, n = 6; OBX, n = 10; VNOX, n = 8), respectively, for 3 days. As described in Experiment 1, all subjects received daily BrdU injections throughout the bedding exposure. Thereafter, subjects were perfused and the brains were processed for BrdU immunohistochemistry.

Experiment 3

As the AMY, especially the medial (MeA) and cortical (CoA) sub-regions, has been implicated in olfactory discrimination/memory and social behaviors (Kirkpatrick et al., 1994a; Fergusson et al., 2001; Maras & Petrulis, 2006), we examined the effects of VNOX on social and anxiety-like behaviors. In addition, we evaluated its effects on BrdU labeling and neuronal differentiation. We did not include an OBX group in this experiment, as OBX has been recognised as a reliable model for depression and in turn would lead to distinct behavioral changes (Morales-Medina et al., 2013). Female subjects were randomly assigned to one of two groups that received sham surgery (Sham; n = 13) or VNOX (n = 9). After a 3-week recovery period, subjects were injected with BrdU for three consecutive days. Thereafter, they underwent behavioral testing for social recognition (day 1), social preference (day 2), as well as locomotion and anxiety-like behaviors (day 3). Locomotion and anxiety-like behaviors were examined in the open field (OF) and elevated plus maze (EPM) tests on day 3 with a 6-h interval. Subjects were perfused at 1 h following behavioral testing. One set of brain sections was processed for BrdU single labeling and another set was processed for BrdU/TuJ1/NG2 double fluorescent labeling (see below).

Surgical procedures

The OBX was performed using a previously established procedure (Kirkpatrick et al., 1994b). Briefly, subjects were anesthetised with sodium pentobarbital (100 mg/kg body weight), and a small window was made in the skull above the MOB. While looking through a dissection microscope, the MOB was bilaterally vacuum-aspirated. This surgical procedure also removed the AOBs that reside bilaterally on the dorsal-posterior region of the MOBs. For the sham surgery, the olfactory bulbs were exposed but were left undisturbed. After the surgery, the skin incision above the window in the skull was sutured, and the subjects were allowed to recover for 3 weeks, before being assigned to experiments. Successful OBX was verified when the brains were extracted after perfusion.

The VNOX was performed using an established procedure (Curtis et al., 2001). Subjects were anesthetised and their VNO was approached via the roof of the mouth. After exposure of the incisive bone, blunt dissection was used to expose the VNO capsule. A small burr was used to enlarge the incisive foramen, through which the entire VNO capsule was removed. The incision at the
The BrdU immunohistochemistry was performed on floating sections at 120-µm intervals from the DG and AMY regions of the subjects in Experiment 3 using an established method (Fowler et al., 2005). The mouse anti-TuJ1 monoclonal IgG recognises a neuron-specific class III β-tubulin, which is an early marker of neuronal differentiation (Lee et al., 1990; Alexander et al., 1991; Pencea et al., 2001). The rabbit anti-NG2 polyclonal IgG recognises NG2, an integral membrane proteoglycan expressed in glial progenitor cells (Nishiya et al., 1995). Briefly, sections were blocked in 10% normal donkey serum in 0.1% TPB and then incubated in a cocktail of rat anti-BrdU (1 : 800), mouse anti-TuJ1 (1 : 500, Covance), and rabbit anti-NG2 (1 : 400, Millipore, Temecula, CA, USA) in 0.1% TPB with 2% normal donkey serum at 4 °C for 48 h. After rinsing in 0.1% TPB, sections were incubated in a secondary antibody cocktail with 1 : 200 dilution of Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA), Alexa Fluor 648-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch), and Alexa Fluor 488-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) in 0.1% TPB with 2% normal donkey serum for 2 h at room temperature (approximately 20 °C). Thereafter, sections were rinsed, mounted on slides in slowfade (Life Technology, Grand Island, NY, USA), and coverslipped.

**Data quantification and analysis**

The BrdU-immunoreactive (ir) cells were examined in the granular cell layer of the MOB and AOB; the DG, including the granular, polymorphic, and molecular cell layers; and the AMY, including the CoA, MeA, and central (CeA) subnuclei. Cell counts were conducted on multiple brain sections in each brain area (MOB and AOB, six sections; DG, four sections; AMY, four sections). Cell numbers and region volumes were quantified bilaterally by using an Axioskop II microscope (Zeiss) with the optical fractionator workflow (for stereological sampling parameters see Table 1) of the Stereo Investigator software (MBF Bioscience, Chicago, IL, USA), as described previously (Lieberwirth et al., 2012). Differences in the density of BrdU-ir cells across treatment groups in each brain region were analysed using a two-way ANOVA (sex × bedding for Experiment 1 and treatment × bedding for Experiment 2) followed by Student-Neuman-Keuls’s (SNK) post hoc tests. In Experiment 2, differences between the OBX sham and VNOX sham animals were first compared by an independent sample t-test. As no group differences were found, these animals were combined into a single control group for the two-way ANOVA test. In Experiment 3, individual cells stained for BrdU, BrdU/TuJ1, or BrdU/NG2 were quantified in the DG, MeA, and CoA. Cells were visualised under 40× magnification with an LSM510 confocal microscope (Zeiss). Percentages of BrdU-ir cells co-labeled for TuJ1 or NG2 were determined in Experiment 3. In Experiment 3, individual cells stained for BrdU, BrdU/TuJ1, or BrdU/NG2 were quantified in the DG, MeA, and CoA. Cells were visualised under 40× magnification with an LSM510 confocal microscope (Zeiss). Percentages of BrdU-ir cells co-labeled for TuJ1 or NG2 were determined in Experiment 3.
Results

Exposure to conspecific's bedding facilitates 5-bromo-2’-deoxyuridine labeling in a stimulus-, sex-, and brain region-specific manner

Exposure to bedding soiled by a conspecific increased the density of BrdU-ir cells in a brain region-specific manner, as treatment effects were found in the MOB, AOB, and AMY, but not DG. In the MOB, bedding exposure showed a graded effect on BrdU-ir labeling (F(2,33) = 11.23, P < 0.01). Compared with the control group exposed to its own bedding, exposure to same-sex bedding increased the density of BrdU-ir cells in the MOB, and this bedding-induced increase was even larger in response to opposite-sex bedding (Fig. 1A). Exposure to opposite-sex bedding increased the density of BrdU-ir cells in the AOB compared with the treatment groups that were exposed to their own or same-sex bedding (F(2,32) = 4.49, P < 0.05), whereas the latter two groups did not differ from each other (Fig. 1B). No sex differences and treatment by sex interactions were found in the density of BrdU-ir cells in either the MOB or AOB. In the AMY, a significant treatment effect (F(2,32) = 3.93, P < 0.05) and treatment by sex interaction (F(2,32) = 5.12, P < 0.01) were found for BrdU labeling. In female voles, exposure to male bedding, but not female bedding, significantly increased the density of BrdU-ir cells compared with the control animals exposed to their own bedding (Figs 1C and 2A-C). Such bedding effects were not found in male voles (Fig. 2E-G). In the subnuclei of the AMY, exposure to male bedding increased the density of BrdU-ir cells in the MeA and CoA, but not CeA, of females, compared with the control females exposed to their own bedding (Table 2). Finally, there were no effects of treatment, sex, and treatment by sex interaction on the density of BrdU-ir cells in the DG (Figs 1D and 2H-J).

The olfactory systems mediate male bedding-facilitated 5-bromo-2’-deoxyuridine labeling in the amygda

Following OBX or VNOX, female voles showed a significant decrease in the density of BrdU-ir cells in the AMY compared with the intact group (F(2,33) = 22.50, P < 0.01; Fig. 3A). In addition, exposure to male bedding increased the density of BrdU-ir cells in intact animals, but not in animals that received OBX or VNOX (F(2,33) = 7.32, P < 0.01, Fig. 3A). The BrdU-ir data in the MeA, CoA, and CeA subnuclei of the AMY are summarised in Table 3. In the DG, similarly to the AMY, both OBX and VNOX groups had a lower density of BrdU-labeled cells than the intact group (F(2,39) = 7.52, P < 0.01, Fig. 3B). Exposure to male bedding was not effective in altering BrdU labeling in the DG.

Vomeronasal organ lesion induces anxiety-like behavior and alters social affiliation without affecting sexual recognition

Anxiety-like behavior was significantly increased by VNOX in female prairie voles. In the OF test, VNOX females showed a lower frequency (t(18) = 2.36, P < 0.05; Fig. 4A), but a longer duration (t(18) = 2.25, P < 0.05), of corner squares entries compared with the sham-lesioned controls (Fig. 4B). In addition, the VNOX females spent less time in the center of the OF arena (t(18) = 2.55, P < 0.05) than the control females. VNOX females also showed a decrease in locomotor activity indicated by a lower number of total line crossings compared with controls (t(18) = 2.46, P < 0.05; Fig. 4C). In the EPM test, VNOX females spent a lower percentage of time in the open arms (t(17) = 2.24, P < 0.05, Fig. 4F) compared with the controls. Although VNOX females tended to show a longer latency to enter the open arm (t(18) = 1.81, P = 0.09, Fig. 4D) and to have fewer open arm entries (t(18) = 1.86, P = 0.08, Fig. 4G) compared with control females, these differences did not reach statistical significance.

In the social preference test, a significant interaction between treatment and stimulus animals was found in the cage duration (F(2,51) = 3.12, P < 0.01). The SNK post hoc test indicated that sham-lesioned subjects spent more time in the empty cage than in the male cage. In addition, sham-lesioned subjects spent more time in the empty cage than did VNOX subjects (Fig. 5A). No group differences were found in the VNOX subjects (Fig. 5A). A significant interaction between treatment and stimulus animals was also found in side-by-side contact time (F(2,40) = 3.83, P < 0.05). The SNK post hoc test indicated that sham-lesioned subjects spent more time in side-by-side contact with the female than male conspecific, whereas such a preference was not found in VNOX subjects (Fig. 5B).

In the social recognition test, there were main effects of exposure (T1, T2, T3, and new) on both the frequency (F(3,12) = 7.28, P < 0.01) and duration (F(3,12) = 5.04, P < 0.05) of olfactory investigation (Fig. 5C and D). The frequency and duration scores of T3 were significantly lower than those observed during the other exposure points. No treatment (sham vs. VNOX) effect and treatment by test interactions were found for the frequency and duration of olfactory investigation.

Table 1. Stereological quantification parameters

<table>
<thead>
<tr>
<th>Brain area</th>
<th>No. of sections</th>
<th>Counting frame (μm)</th>
<th>Grid (μm)</th>
<th>Paxinos &amp; Watson plate no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFactory bulb</td>
<td>6</td>
<td>30 × 30</td>
<td>43 × 43</td>
<td>1</td>
</tr>
<tr>
<td>AMY</td>
<td>4</td>
<td>50 × 50</td>
<td>71 × 71</td>
<td>27–33</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>4</td>
<td>30 × 30</td>
<td>43 × 43</td>
<td>29–35</td>
</tr>
</tbody>
</table>

The average mounted section thickness was about 22 μm and the optical disector height was set to 15 μm. The average Gunderson coefficient of error was less than 0.2 across all brain regions (Paxinos & Watson, 1998).
Vomeronasal organ lesion impairs cell proliferation and neuronal differentiation in the amygdala

The BrdU single labeling in Experiment 3 replicated the results in Experiment 2; VNOX significantly reduced the density of BrdU-ir cells in the DG ($t_{16} = 2.41, P < 0.05$), MeA ($t_{15} = 2.36, P < 0.05$), and CoA ($t_{15} = 2.20, P < 0.05$) compared with sham-lesioned controls (Fig. 6J). For the brain sections processed for BrdU/TuJ1/NG2 triple fluorescent labeling, the numbers of BrdU-ir cells were quantified in the DG (VNOX, 237; Sham-lesioned, 193), MeA (VNOX, 427.3; Sham-lesioned, 395.2), and CoA (VNOX, 158.1; Sham-lesioned, 115.9).

Table 2. Density of BrdU-ir cells (no./mm³) in the subnuclei of the AMY in female and male prairie voles

<table>
<thead>
<tr>
<th>Subnuclei</th>
<th>Female</th>
<th>Male</th>
<th>ANOVA (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeA</td>
<td>Own bedding</td>
<td>Same-sex bedding</td>
<td>Opposite-sex bedding</td>
</tr>
<tr>
<td></td>
<td>340.1 ± 59.7a</td>
<td>582.2 ± 151.0ab</td>
<td>910.6 ± 158.1b</td>
</tr>
<tr>
<td>CoA</td>
<td>366.6 ± 43.8a</td>
<td>1016.2 ± 219.2ab</td>
<td>1418.3 ± 195.3b</td>
</tr>
<tr>
<td>CeA</td>
<td>189.6 ± 55.2</td>
<td>355.2 ± 71.7</td>
<td>393.4 ± 52.0</td>
</tr>
</tbody>
</table>

Alphabetic letters indicate group differences following an SNK post hoc test. Mean ± SEM. S x G refers to sex x group interaction.

**Vomeronasal organ lesion impairs cell proliferation and neuronal differentiation in the amygdala**

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TABLE 3. Density of BrdU-ir cells (no./mm²) in the subnuclei of the AMY in female prairie voles

<table>
<thead>
<tr>
<th>Group</th>
<th>AMY</th>
<th>ANOVA (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeA</td>
<td>CoA</td>
</tr>
<tr>
<td>Own bedding</td>
<td>364.9 ± 42.3</td>
<td>765.0 ± 114.4</td>
</tr>
<tr>
<td>Male bedding</td>
<td>708.4 ± 129.9</td>
<td>1330.7 ± 52.4</td>
</tr>
<tr>
<td>Own bedding</td>
<td>319.5 ± 27.1</td>
<td>471.3 ± 68.2</td>
</tr>
</tbody>
</table>

Alphabetic letters indicate group differences following an SNK post hoc test. Mean ± SEM. S x G refers to sex x group interaction.

Fig. 4. VNOX induced a subtle increase in anxiety-like behaviors. In the OF test, VNOX females had fewer corner entries (A), spent less time in the center but more time in the corners (B), and had reduced line crosses in the arena (C) compared with Sham animals. In the EPM, VNOX females showed a decrease in open arm duration (F) from Sham females. The two groups did not differ in other behavioral measurements (D, E, and G). *P < 0.05. Error bars represent SEM.

Discussion

The exposure to chemosensory stimuli affects adult neurogenesis. For example, exposure to male pheromones increases the cell proliferation in the SVZ of female mice and prairie voles (Smith et al., 2001; Mak et al., 2007). Exposure to male pheromones also increases the cell survival in the MOB and AOB as well as the neuronal differentiation in the MOB of female mice (Mak et al., 2007; Oboit et al., 2009). Consistent with and in extension of these findings, data from the present study showed that the exposure to pheromones affects the number of adult-generated cells in the brain of the socially monogamous prairie vole in a stimulus- and brain region-specific manner. Specifically, the MOB of female and male voles responded to pheromones from same- and opposite-sex conspecs, the AOB only responded to an opposite-sex stimulus, whereas the DG showed no response. Interestingly, only chemosensory stimuli from an opposite-sex conspecific enhanced the BrdU labeling in the AMY, particularly in the MeA and CoA, of female, but not male, prairie voles, indicating a sex-specific effect.

Both the MOB and AOB have been shown to respond to pheromones (Veyrac et al., 2011); however, the anatomical differences in the main and accessory olfactory pathways may mediate functional differences, which in turn, may underlie the observed stimulus-specific response (Meredith, 1998; Brennan & Kendrick, 2006). The AOB receives inputs from the VNO and projects to the AMY, in particular to the MeA and CoA (Brennan & Kendrick, 2006). The AOB may be especially sensitive to reproductive stimuli (i.e. pheromones of opposite-sex, rather than same-sex, conspecifics), as the AOB probably modulates reproductive behavior through its projections to the medial preoptic area and hypothalamus via the MeA (Meredith, 1998; Choi et al., 2005; Brennan & Kendrick, 2006; Samuelsen & Meredith, 2009). Additional evidence of the involvement of the AOB in mediating reproductive behavior comes from studies showing that mating experience increased the number of adult-generated cells in the AOB of female and male mice (Corona et al., 2011; Portillo et al., 2012).

Although the pheromonal effects on adult neurogenesis have been studied in several rodent species (for review see Lieberwirth & Wang, 2012), very few studies have examined brain areas along the olfactory pathways downstream from the MOB and AOB. One interesting finding in the present study is the stimulus- and sex-specific response in the AMY of prairie voles to the exposure of...
conspicuous bedding. The AMY showed an increase in BrdU labeling in response to the chemosensory stimuli from an opposite-sex conspecific only in female, but not male, voles. In addition, this effect was subregion-specific; it was only observed in the MeA and CoA (brain regions that receive direct inputs from the AOB) but not the CeA. It should be noted that anatomical sex differences have been found in the AMY. For example, in rats and mice, males and females differ in the amygdalar volume (Hines et al., 1992; Ahmed et al., 2008; Morris et al., 2008) as well as the number of amygdaloid cells that express certain neurochemical phenotypes such as vasopressin and oxytocin (De Vries, 1984; Caffe et al., 1989; De Vries & Miller, 1998). It is possible that such sex differences in the AMY may determine its sensitivity and responsiveness to chemosensory stimuli between males and females. Such differences in the neuroanatomy of the AMY have been attributed to sex-specific reproductive behaviors (Segovia & Guillamon, 1993; Guillamon & Segovia, 1997; Keller et al., 2009). It should be noted that female prairie voles lack an estrous cycle and are induced into behavioral estrus in response to interactions with a male or male-associated sensory cues (Cohen-Parsons & Carter, 1987). As this male-induced behavioral estrus is associated with various physiological changes (Dluzen & Carter, 1979; Hnatczuk & Morrell, 1995), we cannot exclude the possibility that the observed increase in BrdU labeling in response to male bedding exposure is in part due to estrus induction (Smith et al., 2001). A caveat in the present study should be mentioned. The opposite-sex, but not same-sex, stimulus animals were housed in a different room from the subjects. Therefore, it is possible that novelty associated with male bedding exposure may also play a role in influencing the BrdU labeling in the AMY of female voles, although this did not seem to be the case in male voles.

The effects of chemosensory stimuli on adult neurogenesis depend on an intact olfactory pathway (Mak et al., 2007). Congruent with the finding that OBX reduces hippocampal adult neurogenesis (Jaako-Movits et al., 2006; Keilhoff et al., 2006; Morales-Medina et al., 2013), our data showed that OBX decreased BrdU labeling in the DG as well as in the AMY in female prairie voles. The olfactory bulbs have direct projections to the AMY (Brennan & Kendrick, 1999), which, in turn, projects to the DG (Pikkarainen et al., 1999; Pitkanen et al., 2000). Our data support the notion that an intact olfactory pathway plays an important role in regulating basal levels of cell proliferation in adult animals (Mak et al., 2007; Lieberwirth et al., 2014 Federation of European Neuroscience Societies and John Wiley & Sons Ltd European Journal of Neuroscience, 39, 1632–1641

Fig. 5. VNOX altered social preference in a three-chambered social preference test without affecting social recognition. In the social preference test, Sham females spent more time in the empty cage than the cage holding a male stimulus animal; such a preference was not observed in VNOX females (A). Furthermore, Sham females showed a longer duration of side-by-side contact with the female as compared with the male stimulus, whereas VNOX females spent a similar amount of time in side-by-side contact with the female and male stimulus (B). In the social recognition test, each female was exposed to the same juvenile three times (T1, T2, and T3) and then to a new juvenile (New). The interexposure interval was 30 min. VNOX and Sham exposed to the same juvenile three times (T1, T2, and T3) and then to a new juvenile (New). VNOX also significantly reduced the percentage of BrdU-labeled cells that co-labeled for TuJ1 in the MeA and CoA, but not DG (K). VNOX had no effect on cells double-labeled for BrdU and NG2 (L). *P < 0.05. Error bars represent SEM.

Fig. 6. Stacked confocal microscopy images illustrating fluorescent-labeled cells in the AMY (A–F) and DG of the hippocampus (G–I). Cells were labeled for BrdU (red), the neuronal marker TuJ1 (green), and the glial marker NG2 (purple). Arrows indicate BrdU/TuJ1 double-labeled cells (C and I). Arrowheads indicate BrdU/NG2 double-labeled cells (F). Scale bar = 10 μm. VNOX decreased the number of BrdU-labeled cells in the DG, as well as the MeA and anterior CoA (J). VNOX also significantly reduced the percentage of BrdU-labeled cells that co-labeled for TuJ1 in the MeA and CoA, but not DG (K). VNOX had no effect on cells double-labeled for BrdU and NG2 (L). *P < 0.05. [Color version of figure available online].
& Wang, 2012; current study). In addition, control, but not OBX, females showed increased BrdU labeling in the AMY in response to male bedding exposure, suggesting that an intact olfactory pathway is also essential for the chemosensory stimulus to affect cell proliferation (Mak et al., 2007; Lieberwirth & Wang, 2012; current study). Interestingly, this effect is brain region-specific as the bedding exposure altered cell proliferation in the AMY (an area downstream primarily from the olfactory bulbs), but not in the DG (an area downstream further from the AMY). The underlying mechanisms need to be further examined.

Our data also show that VNOX females had almost identical patterns of BrdU labeling in the DG and AMY as OBX females. Because OBX by vacuum aspiration also led to the damage of the AOB, an area that receives direct inputs from the VNO (Brennan & Kendrick, 2006), our data suggest that the accessory olfactory pathway probably plays an important role in mediating the pheromone-induced changes in adult-generated cells, although we cannot exclude the possibility that the main olfactory pathway may also be involved. It should also be mentioned that VNOX females showed a decrease in the percentage of BrdU-labeled cells that were co-labeled with TuJ1 in the AMY, but not DG, indicating an effect on adult neurogenesis selectively in downstream brain areas along the accessory olfactory pathway. Future studies should focus on assessing whether these immature neurons display long-term survival and become functionally integrated into the existing circuitry. Interestingly, previous research has suggested that immature DG neurons display distinct characteristics from mature neurons that may potentially allow these immature neurons to influence the existing circuitry independent of reaching maturity or long-term survival (Liu et al., 1996; Wang et al., 2000; Cameron & McKay, 2001; Zhao et al., 2008). Thus, it may be of interest to assess whether these immature AMY neurons may similarly play a functional role. It will also be interesting to reveal the phenotypes of BrdU-ir cells in the VNOX and control animals without behavioral testing, as the behavioral testing itself may have affected the findings.

It is worth mentioning that, although studies on adult neurogenesis have focused primarily on the DG and SVZ (see reviews by Fowler et al., 2008; Lieberwirth & Wang, 2012), newly proliferated cells have been reported in the adult AMY in several mammalian species including voles (Fowler et al., 2003; Lieberwirth et al., 2012), rats (Keilhoff et al., 2006), mice (Okuda et al., 2009), hamsters (Antzoulatos et al., 2008), and monkeys (Bernier et al., 2002; Marlatt et al., 2011). In prairie voles, at 30 min after a BrdU injection, BrdU-ir cells were found in the AMY, and some of these cells were co-labeled for immature neuronal and glial markers (Fowler et al., 2003). These data clearly indicate the presence of neuronal and glial progenitors that are locally dividing within the AMY, although one cannot exclude the possibility that these progenitor cells are migrating into the AMY from other brain regions (e.g. SVZ) (Marlatt et al., 2011).

In addition to changes in BrdU labeling, VNOX also affected behaviors in female prairie voles. VNOX caused a subtle increase in anxiety-like behaviors as assessed in both the OF and EPM tests. These data are consistent with previous findings that the ablation of the VNO increased anxiety-like behaviors in the OF and light–dark box tests in mice (Liu et al., 2010). In addition to the increase in anxiety-like behaviors, VNOX resulted in a reduction in the measure of locomotion in the OF, whereas no such decrease was observed in the EPM. Therefore, we cannot fully rule out that a decrease in exploration in VNOX females may have contributed at least in part to these findings. The VNO and its associated accessory olfactory pathway are critical for a variety of social behaviors. For example, a genetically compromised VNO system in male mice caused alterations in aggressive behavior and sexual behavior (Leybold et al., 2002). The ablation of the VNO resulted in a reduction of urine marking in male mice (Liu et al., 2010) and prevented mating-induced partner preference formation in female prairie voles (Curtis et al., 2001).

In the current study, VNOX females displayed alterations in social preferences. Control females preferred the empty chamber over the chamber holding the male conspecific, but following VNOX surgery such a preference disappeared. Furthermore, control females showed a preference to spend more time in side-by-side contact with the female compared with the male stimulus animal. VNOX animals did not show such a preference. Interestingly, VNOX did not affect social recognition memory, which is supported by previous studies that have shown that VNOX does not impair mate recognition or social discrimination (Petris et al., 1999; Woodley et al., 2004; Keller et al., 2006; Liu et al., 2010). Therefore, it seems unlikely that a deficit in social discrimination underlies the altered social preference following VNOX. One possible explanation for the lack of preference is that VNOX might not interfere with the detection of chemical differences between pheromones of conspecifics, while interfering with the extraction of the biological information from the pheromone (Liu et al., 2010).

Recent research has suggested that adult-generated cells may play an essential role in mediating behavioral functions (Imayoshi et al., 2009). For example, adult-generated MOB neurons are involved in olfactory discrimination and odor recognition, as well as recognition of conspecifics (Roche et al., 2002; Enwere et al., 2004; Mak & Weiss, 2010; Alonso et al., 2012). Similarly, adult-generated neurons in the DG have been implicated in hippocampal functions including spatial learning, object recognition, and associate memory formation (Shors et al., 2001; Clelland et al., 2009; Jessberger et al., 2009). It is reasonable to speculate that new neurons in the adult AMY, in particular in the MeA and CoA, may mediate its functions in social behavior (Kirkpatrick et al., 1994a; Ferguson et al., 2001; Maras & Petris, 2008). It should also be pointed out, however, that our data are only correlational; olfactory disruption due to VNOX (and OBX) may affect behavior via non-neurogenesis-dependent pathways. Therefore, future studies are needed to investigate the causal relationship, if any, between adult neurogenesis within the AMY and behavior (Mercadante et al., 2008).

Conflict of interest

The authors have no financial disclosures and have no potential conflicts of interest.

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Abbreviations

AMY, amygdala; AOB, accessory olfactory bulb; BrdU, 5-bromo-2′-deoxyuridine; CeA, central nucleus of the amygdala; CoA, cortical nucleus of the amygdala; DG, dentate gyrus; EPM, elevated plus maze; ir, immunoreactive; MeA, medial nucleus of the amygdala; MOB, main olfactory bulb; OBX, olfactory bulbectomy; OF, open field; PB, phosphate buffer; SNK, Student–Newman–Keul’s; SVZ, subventricular zone; TPB, Triton in 0.1 M phosphate buffer; VNO, vomeronasal organ; VNOX, vomeronasal organ lesion.


