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Newborn interneurons in the accessory olfactory bulb promote mate recognition in female mice

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In the olfactory bulb of adult rodents, local interneurons are constantly replaced by immature precursors derived from the subventricular zone. Whether any olfactory sensory process specifically relies on this cell renewal remains largely unclear. By using the well known model of mating-induced imprinting to avoid pregnancy block, which requires accessory olfactory bulb (AOB) function, we demonstrate that this olfactory memory formation critically depends on the presence of newborn granule neurons in this brain region. We show that, in adult female mice, exposure to the male urine compounds involved in mate recognition increases the number of new granule cells surviving in the AOB. This process is modulated by male signals sensed through the vomeronasal organ and, in turn, changes the activity of the downstream amygdaloid and hypothalamic nuclei involved in the pregnancy block response. Chemical depletion of newly generated bulbar interneurons causes strong impairment in mate recognition, thus resulting in a high pregnancy failure rate to familiar mating male odors. Taken together, our results indicate that adult neurogenesis is essential for specific brain functions such as persistent odor learning and mate recognition.

Keywords: pregnancy block, neurogenesis, male pheromones, Tpc2, AOB, medial amygdala, olfactory imprinting, Ara-C

INTRODUCTION

Interneurons at the first level of olfactory processing, the main olfactory bulb (MOB), are subjected to life-long replacement (Alvarez-Buylla and Garcia-Verdugo, 2002; Petreanu and Alvarez-Buylla, 2002; Gheusi et al., 2009). The integration and survival of these cells depends on olfactory activity (Petreanu and Alvarez-Buylla, 2002; Yamaguchi and Mori, 2005) and on their high excitability (Lin et al., 2010). In the MOB neurogenesis has been shown to be important for olfactory perceptual learning and long-term memory (Lazarin et al., 2009; Moreno et al., 2009) as well as social odor discrimination in mice (Sakamoto et al., 2011). We have previously shown that, similarly to the MOB, inhibitory granule cells in the accessory olfactory bulb (AOB) are constantly replaced by immature neuronal precursors migrating from the subventricular zone (SVZ; Oboti et al., 2009). In female mice the number of the surviving cells is increased by exposure to bedding soiled by male urine (Oboti et al., 2009). Exposure to male urine odors can also facilitate estrous in females, leading to altered puberty-onset, shortened estrous cycle length, and interruption of embryo implantation soon after mating, an effect that is known as selective pregnancy block or Bruce-effect (Bruce, 1966). These neuroendocrine responses are mediated by vomeronasal (VN) excitatory projections to the medial amygdala (MeA), the bed nucleus of the stria terminalis (BNST), the medial hypothalamus (MPA), and ultimately the dopaminergic neurons of the arcuate nucleus (arc) that control prolactin release by the anterior pituitary (Li et al., 1989). During mating, the granule cell inhibitory modulation of the contextual male chemosignals is enhanced so that they are no more capable of estrous induction (Brennan et al., 1990; Matsuoka et al., 1997). This male-specific pheromone recognition process involves a restricted pool of granule cells in the AOB, which inhibits mitral cell signal transmission to the forebrain areas involved in estrous regulation for several weeks (50–60 days; Brennan et al., 1990; Matsuoka et al., 1997). Long-term maintenance of this inhibition implies that, since matings may occur at shorter intervals, different or partially overlapping cohorts of cells may be necessary to each imprinting process. Here, we hypothesize that the sensory-driven granule cell addition in the AOB may provide the substrate for the pheromonal imprinting on stud male odors in order to avoid pregnancy block.

To sustain this idea, we show that adult neurogenesis in the AOB of female mice is regulated by vomeronasal inputs involved in mate pheromonal imprinting: it is enhanced by exposure to male pheromones comprised in the low molecular weight (LMW) fraction of male urine, the same compounds that are critical for mate recognition (Peele et al., 2003; Leinders-Zufall et al., 2004); it is triggered by MeA feedback sensory activity, and in turn attenuates the responses to male odors of the AOB–MeA.
downstream pathway which regulates estrous induction. In addition, we show that this response is individual-specific since AOB young granule cells are prominently activated by those male signals driving their integration.

Ultimately, we show that long-term ablation of these cells, and re-exposure of recently mated females to their stud male, leads to embryo implantation failure, like when unfamiliar pheromones are sensed. Together, our results indicate that the pheromonal imprinting process required to block estrous induction by mating male pheromones is preferentially localized on AOB newborn granule cells.

**MATERIALS AND METHODS**

**ANIMALS**

Experiments employed three strains of mice: CD-1 [females, ages: postnatal day (p) 20 or 45; males, ages: 4–6 weeks], c57bl6/j (females, age: p45), and *trpc2−/−* (females, age: p45). In all experiments CD-1 strain female (or male) mice have been used, the c57bl6/j strain has been used only in the trpc2-ko experiment. Animals of the same gender were maintained 4–6 days per cage in rooms with a 12:12 light/dark cycle, with standard diet and water ad libitum. All female subjects were kept in isolation for the duration of all experiments. All procedures were in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) and with institutional guidelines on animal welfare (DL 116/92) of the University of Turin and the University of Saarland. Experiments were designed to minimize the number of animals used.

**BrdU TREATMENTS**

To identify newly generated cells at different survival times, mice were intraperitoneally injected with bromodeoxyuridine (BrdU) in 0.1 M Tris (pH 7.4) twice a day (delay = 8 h, 100 mg/kg body weight), and sacrificed 28 days later for evaluation of neuronal survival.

**IMMUNOHISTOCHEMISTRY**

Following anesthesia, mice were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% (w/v) paraformaldehyde in PBS. Brain tissue was dissected, incubated overnight in 30% sucrose in PBS at 4˚C, embedded in O.C.T (Tissue-Tek), and snap-frozen in a dry ice/2-methylbutane bath. 25-μm thick floating sections were used in all immunohistochemical assays. Newborn cells in the olfactory bulb (OB) were labeled as described previously (Oboti et al., 2009) using blind quantification. Newborn cells were sampled in the MOB GrL on five 25-μm thick floating sections per animal using a fixed sampling volume (1000 × 400 × 25 = 10,000,000 μm³) that was positioned below the AOB, symmetrically with reference to the rostral migratory stream axis (Figure 1A). In all cases the sampled volume was located in the ventral–medial MOB, a region that is strongly activated by social odors and urine detected via the main olfactory epithelium (MOE; Xu et al., 2005). Double labeled c-Fos/BrdU cells were visualized and counted with a confocal microscope using a UPlanFL 100× (n.a. 1.3) Olympus objective. Quantification of c-Fos-positive nuclei in the MeA and MPA was performed using automated routines (ImageJ), applying a fixed threshold to the images and using the particle-counting plug-in.

Images of the anterior MeA and MPA were captured in anatomically matched brain sections (three per region for each animal). Particle number (n’ of labeled nuclei) revealed after threshold selection was counted in a standard circular sampling area (radius = 75 μm) and was then divided by the volume of tissue analyzed (π × 75² × 25 μm³). All c-Fos analysis data (BNST, MeA, MPA, arc) have been eventually normalized to the respective control values in each group (Figure 2). The amount of NeuN-positive nuclei after Ara-C or Saline treatment has been evaluated in the AOB granular layer on three tissue sections per animal (three animals per group) applying a grid over the
Non-parametric tests (Wilcoxon and Chi-square tests) have been used, data represented in the histograms are mean values ± SEM. Whenever parametric tests have been used, the Tukey-HSD post hoc comparison was performed in case of significance (P < 0.05). F-Tests have been used in simple 1:1 comparison (one dependent variable, one grouping variable with two levels). Whenever parametric tests have been used, data represented in the histograms are mean values ± SEM. Non-parametric tests (Wilcoxon and Chi-square tests) have been used to assess differences in either the proportion of time spent exploring odorant sources in the olfactory behavioral tests or the percentage of successful pregnancies, respectively. In the evaluation of the pregnancy rate in the different conditions analyzed, the expected values have been estimated using the pregnancy rates of first treated groups in the comparisons detailed in Table 1: saline-treated (1), Ara-C-mate exposure (2), Ara-C-short-term (4), saline-vnx (5). Whenever a data set has been used more than one time for any comparison (for example in the comparison of the percentages of pregnancy rate) the α value has been halved as many times as the quantity of comparisons performed. For a complete list of the results of the statistical analyses performed refer to Table 1.

CHEMOSENSORY STIMULATION

The animals exposed to male/female soiled bedding in the different experiments were given a daily renewed bedding mixture collected from the cage of an individually housed male or female. This experimental condition produced a behavioral interaction between males and females that was characterized by a significant increase in the proportion of time spent exploring odorant sources in the olfactory behavioral tests. This finding suggests that the presence of pheromones in the bedding mixtures affects odorant source exploration, potentially through interactions with the olfactory system.
**STEREOTAXIC SURGERIES**

Osmotic mini-pump installation has been performed (p40 CD-1 females) as described previously (Morgan et al., 1987) using the coordinates (referring to Bregma): anterior–posterior, 0.40 mm; medial–lateral, 0.7 mm; and dorsal–ventral, 3 mm. To assess the efficiency of the anti-mitotic administration, BrdU injections have been used in order to label proliferating cells during the treatment (after 14 days in the 28-days long-term protocol or 4 days in the 7-days short-term protocol, not shown). After each anti-mitotic infusion (28 or 7 days), the expression of BrdU in the OB was inspected and animal specimens showing even a scarce presence of BrdU-positive nuclei were discarded.

For the amygdala lesions, mice (p38 CD-1 females) were anesthetized with a ketamine (4 mg/kg body weight)–xylazine (1 mg/kg body weight) solution and placed on a stereotaxic apparatus (Kopf Instrument). Lesions were performed by injecting ibotenic acid (IB; Sigma, Italy) diluted at 10 mg/ml in saline solution (pH 7.4) using a picospritzer III system (Science Products GmbH, Hofheim, Germany) with a fine glass pipette (diameter = 40 μm). Lesions were performed into the medial nucleus of the amygdala (MeA) at the coordinates (referring to Bregma): anterior–posterior, −1.2 mm; medial–lateral, 2.1 mm; and dorsal–ventral, 5.3 mm. Sham-lesioned mice underwent the same surgical procedure with
Table 1 | Results of all statistical analyses performed.

| Experiment | Object | Figure | Test | df | Variables | Value | P  |
|------------|--------|--------|------|----|-----------|-------|----|
| 4 weeks BrdU | AOB | 1C | F | 51,3 | BrdU*week | 12.927 | <0.001 |
| 4 weeks BrdU | AOB | 1C | F | 51,6 | BrdU*layer*week | 5.251 | <0.001 |
| 4 weeks BrdU | MOB GrL | 1D | F | 15,3 | BrdU*week | 3.499 | 0.042 |
| FM bedding | AOB–GrL | 1E | F | 41,4 | BrdU*pheromone*group | 2.649 | <0.05 |
| FM bedding | AOB–GrL | 1E | F | 13,2 | BrdU*pheromone*group (Fp45) | 7.441 | <0.05 |
| FM bedding | AOB–GrL | 1E | Tukey-HSD | M bedding*Ctrl | 0.01 |
| FM bedding | AOB–GrL | 1E | Tukey-HSD | M bedding*F bedding | 0.019 |
| C-Fos/BrdU% | AOB–GrL | 1G | F | 22,1 | C-Fos*odor | 76.419 | <0.001 |
| C-Fos/BrdU% | AOB–GrL | 1G | F | 13,5 | Familiar: C-Fos*odor | 15.685 | <0.001 |
| C-Fos/BrdU% | AOB–GrL | 1G | F | 13,5 | Unfamiliar: C-Fos*odor | 7.403 | 0.02 |
| C-Fos/BrdU% | AOB–GrL | 1G | Tukey-HSD | Familiar: 3 w*2, 4–8 w | <0.05 |
| C-Fos/BrdU% | AOB–GrL | 1G | Tukey-HSD | Unfamiliar: 3 w*2, 6 w | <0.05 |
| C-Fos/BrdU% | MOB GrL | 1H | F | 30,1 | C-Fos*odor | 17.11 | <0.001 |
| C-Fos/BrdU% | MOB GrL | 1H | F | 24,4 | C-Fos*odor*cell_age | 1.427 | 0.25 |
| C-Fos/BrdU% | MOB GrL | 1H | F | 14,4 | Unfamiliar: C-Fos*odor*cell_age | 15.25 | <0.001 |
| C-Fos/BrdU% | AOB–GrL | 1I | F | 14,2 | C-Fos*delay*odor | 7.415 | <0.001 |
| C-Fos/BrdU% | AOB–GrL | 1I | T | 1 | C-Fos 2 week familiar*unfamiliar | 1.427 | 0.25 |
| C-Fos/BrdU% | AOB–GrL | 1I | T | 1 | C-Fos 3 week familiar*unfamiliar | 0.019 |
| C-Fos/BrdU% | AOB–GrL | 1I | Tukey-HSD | Familiar: 3 w*2, 4–8 w | <0.05 |
| C-Fos/BrdU% | AOB–GrL | 1I | Tukey-HSD | Unfamiliar: 3 w*2, 6 w | <0.05 |
| C-Fos/BrdU% | MOB GrL | 1I | F | 14,3 | BrdU*fraction | 7.433 | <0.01 |
| C-Fos/BrdU% | MOB GrL | 1I | F | 30,1 | C-Fos*odor | 17.11 | <0.001 |
| C-Fos/BrdU% | MOB GrL | 1I | F | 24,4 | C-Fos*odor*cell_age | 1.427 | 0.25 |
| C-Fos/BrdU% | MOB GrL | 1I | F | 14,4 | Unfamiliar: C-Fos*odor*cell_age | 15.25 | <0.001 |
| C-Fos/BrdU% | AOB–GrL | 1I | F | 14,2 | C-Fos*delay*odor | 7.415 | <0.001 |
| C-Fos/BrdU% | AOB–GrL | 1I | T | 1 | C-Fos 2 week familiar*unfamiliar | 1.427 | 0.25 |
| C-Fos/BrdU% | AOB–GrL | 1I | T | 1 | C-Fos 3 week familiar*unfamiliar | 0.019 |
| C-Fos/BrdU% | AOB–GrL | 1I | Tukey-HSD | Familiar: 3 w*2, 4–8 w | <0.05 |
| C-Fos/BrdU% | AOB–GrL | 1I | Tukey-HSD | Unfamiliar: 3 w*2, 6 w | <0.05 |
| Urine fractions | AOB–GrL | 3B | F | 14,3 | BrdU*fraction | 7.433 | <0.01 |
| Urine fractions | AOB–GrL | 3B | Tukey-HSD | Ctrl*M-p | 0.048 |
| Urine fractions | AOB–GrL | 3B | Tukey-HSD | Ctrl*bedding | 0.025 |
| Urine fractions | AOB–GrL | 3B | Tukey-HSD | F-M*M-p | 0.022 |
| Urine fractions | AOB–GrL | 3B | Tukey-HSD | F-M*M-b | 0.11 |
| Trpc2-ko | AOB–GrL | 4B | F | 13,3 | BrdU*group | 23.788 | <0.001 |
| Trpc2-ko | AOB–GrL | 4B | Tukey-HSD | Wt_ctrl*vrt_bedding | <0.001 |
| ZnSO4 | AOB–GrL | 4C | F | 16,3 | BrdU*group | 8.414 | <0.001 |
| Mea-X BrdU | AOB–GrL | 4F | F | 18,3 | Injection*exposure | 4.710 | 0.013 |
| Mea-X BrdU | AOB–GrL | 4F | Tukey-HSD | Sal_ctrl*Sal_bedding | 0.046 |
| C-Fos VNS | BNST | 2 | F | 11,3 | C-Fos*group | 33.64 | <0.001 |
| C-Fos VNS | MeA | 2 | F | 11,3 | C-Fos*group | 32.269 | <0.001 |
| C-Fos VNS | MPA | 2 | F | 11,3 | C-Fos*group | 17.462 | <0.001 |
| C-Fos VNS | Arc | 2 | F | 11,3 | C-Fos*group | 15.491 | <0.001 |
| NeuN GrL | AOB NeuN+ | 5C | T | Saline*Ara-c | 0.457 |
| Pregnancy rate | N’ pregnant | F | 6A | X² Fisher | 1 vs. 2 | 0.027 |
| Pregnancy rate | N’ pregnant | F | 6A | X² Fisher | 2 vs. 3 | 0.047 |
| Pregnancy rate | N’ pregnant | F | 6A | X² Fisher | 1 vs. 3 | 0.608 |
| Pregnancy rate | N’ pregnant | F | 6A | X² Fisher | 1 vs. 5 | 0.126 |
| Pregnancy rate | N’ pregnant | F | 6A | X² Fisher | 4 vs. 1 | 0.713 |
| Pregnancy rate | N’ pregnant | F | 6A | X² Fisher | 5 vs. 6 | 0.655 |

For the surgical vomeronasal nerve cut, mice (p68 CD-1 females) were anesthetized with a ketamine (4 mg/kg body weight)–xylazine (1 mg/kg body weight) solution and placed on a stereotaxic apparatus (Kopf Instrument). A midline incision was made over the scalp and a hole was made on the skull with a drill (Bregma: anterior–posterior, 5 mm; medial–lateral, 0 mm; and dorsal–ventral, 4.1 mm). A custom made steel blade having a width of 0.5 mm was introduced between the bulbs with a +40˚ slope referring to the vertical axis to cut the vomeronasal fibers. Occurrence of the lesion has been established by OMP immunohistochemistry 7–15 days after wound closure and recovery.

**OLFACTORY PREFERENCE TEST**
Preference for male derived volatiles before and after a 5-days bedding exposure was assessed as described previously (Moncho-Bogani et al., 2005), but two sources of chemosensory...
stimulations were presented during each day: one was a direct exposure to male soiled bedding, while the other consisted in male bedding contained in a drilled box, to prevent direct physical contact with this second odor source. Olfactory preference tests were performed for male volatile odors derived from both odor sources: one considered experienced (when direct bedding exploration was allowed), the other considered unexperienced, (as no direct contact with male bedding did occur). A 90 × 30 × 30 testing cage was used and the odor stimuli were placed at opposite sides (chosen randomly). Male odor stimuli were discharged when female mice showed higher/lower explorative responses toward one of the two, before the bedding exposure period. The duration of every nose poke on the odor source (box) and the proportion of total time (pT) spent exploring one odorant source (calculated as the time spent sniffing one odor/total time spent sniffing ratio) was calculated with a dedicated software [JWatcher; Copyright (c) 2000–2006 Daniel T. Blumstein, Janice C. Daniel, and Christopher S. Evans. All rights reserved].

OLFACTORY DISCRIMINATION TASK
The habituation–dishabituation to male derived urine volatiles was used to measure MOE-mediated olfactory discrimination following a previously described protocol (Breton-Provencher et al., 2009). Briefly, after 20 min habituation to the testing cage, mice were familiarized with a first odor (odor of habituation) in four successive sessions and then exposed once to a novel odor (odor of dishabituation). Each session was 1 min long and was followed by 10 min inter-session interval. Olfactory discrimination was analyzed using two different male odors delivered via patches of filter paper tainted with male urine and enclosed in a perforated plastic box. The two odors have been both tested alternatively as novel stimuli (during dishabituation) in separate sessions. Odor sources consisted of urine soaked filter papers contained in a drilled plastic box.

BRUCE-EFFECT, PREGNANCY BLOCK TEST
To evaluate the rates of pregnancy failure (Bruce-effect), a previously established protocol of matings and phenomornal stimulations was used (Peele et al., 2003; Leinders-Zufall et al., 2004). We have preliminarily assessed the pregnancy rate for our laboratory mice as close to 100% using 32 female mice, that were in the receptive pro-oestrus/oestrus stage, and mating them using the traditional schedule. Females were returned to their home cage 24 h after mating. At 12 h intervals (five times) each female was returned to her mating partner for 20 min. In this way exposures to the mate covered 3 days after the beginning of the mating and were timed to coincide with the prolactin peaks occurring 1 h before lights-on and lights-off (Peele et al., 2003; Leinders-Zufall et al., 2004). One group of females was exposed to an unfamiliar male to evaluate the percentage of pregnancy loss (60%). Eleven days after mating the females were killed and their uteri examined for implantation sites of the ova.

RESULTS
NEWBORN AOB GRANULE CELLS IN FEMALE MICE ARE POSITIVELY SELECTED BY MALE ODORS
The AOB, as the MOB, represents an integration site for doublecortin-positive (DCX) neuroblasts migrating from the adult SVZ, as revealed by BrdU birth dating experiments (Figure 1A; Oboti et al., 2009). When these cells reach the AOB cellular layers, they acquire a mature phenotype as indicated by BrdU/NeuN co-labeling, already evident 15 days after BrdU injection (Figure 1B). The survival of AOB newborn neurons in adult female mice has been shown to be enhanced by subsequent long-term exposure (28 days post injection of BrdU; d.p.i.) to male pheromones present in cage bedding, when direct vomeronasal contact is allowed (Oboti et al., 2009). However, by labeling newborn neurons with BrdU after 7 days of male bedding exposure, other authors reported that bulbar neurogenesis is increased afterward as an indirect proliferative effect of such sensory stimulation (Mak et al., 2007). Hence, it is currently unknown at which age the newborn granule cells begin to respond to sensory stimuli and when these neurons are integrated into functional adult AOB circuits. Moreover, the question whether these neurons support any specific sensory function dependent on vomeronasal sensing remained unanswered.

To address these points, we first analyzed the sensory-driven positive selection of newborn AOB granule cells labeled before exposure to male soiled bedding in adult females. This occurred in different moments of their maturation: 1st week (0–7th d.p.i.), 2nd week (8th–14th d.p.i.), 3rd week (15th–21st d.p.i.), and 4th week (22nd–28th d.p.i.; Figures 1C,D). We previously found that the migration of one BrdU-labeled pool of SVZ neuroblasts reaching the AOB is accomplished in the 1st week as its density reaches the maximum value at 7 d.p.i., while subsequently starts to decrease (Oboti et al., 2009). Here we show that, immediately after this peak (during the 2nd week), familiarization with male soiled bedding is more effective in promoting cell survival, indicating that, from 7 to 14 days after their genesis, most of AOB newborn cells undergo sensory-driven positive selection (Figure 1C). This effect was more prominent in the GrL of the AOB (Figure 1C) and much weaker in the MOB GrL (Figure 1D). Remarkably, such an increase in BrdU-positive cells in the AOB occurred only in adult females after exposure to male derived pheromones, whereas exposure to cues from both genders was ineffective in both adult males and pre-pubertal females (Figure 1E).

It is known that sensory experience influences neuronal integration in the neurogenic areas increasing the number of new neurons functionally involved in the activated sensory circuits (Magavi et al., 2005; Kee et al., 2007). Given that male pheromonal imprinting in mice requires a cellular substrate responding specifically to male individual odors in the AOB (Matsuoka et al., 1997), we asked whether AOB newborn granule cells could have such capability.

To answer this question, we combined immunostaining for c-Fos, a marker for neuronal excitation (Morgan et al., 1987; Kovacs, 2008), and BrdU labeling (Magavi et al., 2005; Kee et al., 2007; Figure 1F). We quantified the percentage of c-Fos/BrdU coexpression (the fraction of BrdU-positive cells expressing c-Fos) induced by familiar (experienced for 1 week) or unfamiliar (never experienced before) male soiled bedding, at different times after granule cell genesis in the AOB and MOB GrL of female mice (Figures 1G,H). We found that newborn AOB granule cells preferentially responded to experienced male individual cues around the age of 3 weeks after BrdU labeling (Figure 1G).
Moreover, this response was transient and vanished within 1 week following bedding exposure (Figure 1I). This age-specific granule cell involvement in AOB sensory processing was not evident while considering the total granule cell population (including pre-existing NeuN-positive neurons; Figure 1G, dotted lines) or granule cells aged over 3 weeks (at 28 days). Indeed, c-Fos expression levels in older cells (BrdU-positive) were not significantly increased by bedding exposure and were comparable both to control conditions (black dot in Figure 1G) or to those of NeuN-positive granule cells (4 w-bedding, 6.6 ± 0.4%; ctrl, 5.14 ± 0.59%, P = 0.21; 4 w-NeuN, 5.27 ± 0.47%; 4 w-ctrl, 5.14 ± 0.59%, P = 0.9; means ± SEM). Moreover, although c-Fos expression levels in MOB newborn granule neurons were higher after exposure to familiar chemostimuli in comparison to unfamiliar ones, the peak activity occurred at a much later time point (i.e., around 6 weeks, Figure 1H) indicating that the preferential involvement of MOB newborn interneurons in processing these odors persists for longer times and spans different cell ages (Magavi et al., 2005).

Together, these results indicate that, in female mice, the integration of AOB newborn granule cells is sensitive to male individual chemosignals during the 2nd/3rd week of cell age and occurs preferentially in the same circuits that respond to these chemosignals.

The transient nature of this effect confirms the preferential involvement in the elaboration of vomeronasal signals of AOB interneurons in this cell age. Therefore, given the relevant role of granule interneurons in gating bulbar output activity (Halem et al., 2001; Matsuoka et al., 2004; Breton-Provencher et al., 2009), AOB young granule cells are likely to convey important individual odor informations to brain centers related to estrous induction. Accordingly, after 1 week familiarization, we found that re-exposure to the same male odor stimuli (familiar), but not to novel ones, induced attenuated responses in the downstream nuclei of the vomeronasal pathway controlling estrous induction. To rule out a possible effect of odor familiarization on the extent of this response, we stimulated female mice with novel odors with or without previous contact with the familiar ones (unfamiliar or focal stimulation, respectively). As a consequence of familiar odor exposure at the end of the familiarization period, increased c-Fos expression levels have been observed in the BNST (Figure 2). This is consistent with a role of this brain region in mediating olfactory responses related to experienced stimuli (Hosokawa and Chiba, 2007). Conversely, familiar odors elicited attenuated responses the MPA, MeA, and arc (Figure 2). These results suggest that the enhanced response shown by newly generated AOB neurons to male individual odors could be linked to the activity induced by the same cues in amygdaloid and hypothalamic nuclei, both of which are known to be involved in estrous induction and thus in the pregnancy block response.

AOB NEUROGENESIS IS AFFECTED BY PHEROMONES IN THE LMW FRACTION OF MALE URINE

Individual odor signatures in scent marks are encoded by chemicals excreted with urine (Brennan and Zufall, 2006). We therefore tested the effect of male urine alone (M-U; Figure 3A) and found that it promoted AOB granule cell integration as well as soiled bedding exposure (urine, 7398.5 ± 742.2 nuclei/mm³, n = 8; ctrl, 3739.8 ± 609.9 nuclei/mm³, n = 5; P = 0.0029; means ± SEM). To gain further insight into the nature of the cues affecting AOB neurogenesis, we employed distinct male urine fractions and analyzed the density of BrdU-positive cells 28 days after injection (Figures 3A,B). These cues could include molecules present in
the LMW fraction (containing small organic molecules and small peptides) as well as high molecular weight MUPs (contained in the HMW fraction; Brennan and Zufall, 2006). We found that the M-p fraction of urine, in which all MUPs were degraded by protease treatment (Figure 3A), was still capable of increasing granule cell survival to levels comparable to those seen after exposure to male soiled bedding (M-b, Figure 3B). Consistent with this result, the HMW fraction of male urine treated with menadion to deprive it from all volatile ligands (Chamero et al., 2007) and loaded onto female LMW urine fraction (F-M), was uneffective (Figure 3B). These findings were specific for the AOB, as neither male urine (M-U) nor any of the urine fractions affected MOB GrL neuronal survival (urine, 15939.3 ± 1566.9 nuclei/mm³, n = 8; ctrl, 16949.7 ± 1295.7 nuclei/mm³, n = 5; P = 0.632; means ± SEM). Together, these results rule out an essential role for MUPs in increasing AOB neuronal survival and indicate that all activity is comprised in the LMW fraction of male urine which is primarily important for mate recognition (Peele et al., 2003; Leinders-Zufall et al., 2004).

**MALE ODORS INCREASE AOB GRANULE CELLS THROUGH BOTH PERIPHERAL AND CENTRAL INPUTS**

We have previously observed enhancement of neuronal survival in the AOB of adult female mice only if direct contact with male soiled bedding was allowed (Oboti et al., 2009), suggesting a necessary role of VNO sensory inputs. It has been proposed that experience-dependent circuitual remodeling in peripheral sensory areas, when triggered by feedback activity from higher order cortical relays, can be functionally related to sensory perception (Gilbert and Sigman, 2007). Similarly, neuronal activity in the AOB can be regulated by centrifugal inputs from the MeA, as a result of inputs converging from both the main and accessory olfactory systems (Pankevich et al., 2006; Martel and Baum, 2009). Thus, cellular activation of the AOB could be generated either by peripheral or central inputs or both (Pankevich et al., 2006).

To distinguish between these possibilities, we first compared the effects of genetic deletion of the Trpc2 cation channel (leading to impaired VN function; Leypold et al., 2002) with lesions of the MOE caused by intranasal irrigation of zinc-sulfate (ZnSO₄), see the functional ablation sites in Figure 4A; McBride et al., 2003). Adult female mice were exposed to male bedding on days 7th–14th after BrdU treatment when the effects of the sensory input on survival of AOB newborn neurons are maximal. Enhanced AOB granule cell survival after male bedding exposure was absent in trpc²⁻/⁻ mice (Figure 4B, newborn MOB granule cells were unaffected in both trpc²⁻⁻ and trpc²⁺/⁺ mice, data not shown). By contrast, ZnSO₄ lesions of the MOE (assessed by the lack of OMP staining in the MOB glomerular layer) did not abolish the enhanced neuronal survival in the AOB (Figures 4C,D). Thus, vomeronasal contact is necessary and sufficient to increase new AOB granule cells.

Next, to address the role of centrifugal inputs, we delivered excitotoxic lesions to the MeA by injections of ibotenic acid (IB, Figures 4A,E; Chauveau et al., 2008), a glutamatergic agonist, and stimulated both lesioned and sham-lesioned (saline injected) mice with male bedding from the 7th to the 14th d.p.i. (BrdU). We assessed the efficiency of the lesions using both immunohistochemical (i.e., absence of the neuronal marker NeuN in regions with massive cell loss; Jongen-Relo and Feldon, 2002; Figure 4E) and behavioral criteria. The latter consisted in the evaluation of the attraction to a specific male odor source after repeated VN contact (a function which relies on the integrity of this nucleus; Moncho-Bogani et al., 2005). Adult (p45), but not pre-pubertal (p20), female mice were attracted by male volatile odors after physical contact with male non-volatile compounds (p45: experienced, 0.64 ± 0.03 pT; unexperienced, 0.36 ± 0.03 pT; U = 0.001; p20: experienced, 0.47 ± 0.03 pT; unexperienced, 0.52 ± 0.03 pT; U = 0.327; n = 18 for p20, n = 25 for p45; means ± SEM). Such behavioral response was accompanied by concurrent neuronal activation of the medial amygdala (p45, experienced, 43 ± 1.5 nuclei/area, unexperienced, 60 ± 5.5 nuclei/area; P = 0.0409; means ± SEM). Importantly, MeA-lesioned, but not sham-lesioned (saline injected) mice, failed to show an attractive response to male odors (IB: experienced, 0.387 ± 0.06 pT, unexperienced, 0.612 ± 0.06 pT, U = 0.172; saline: experienced, 0.632 ± 0.03 pT, naïve, 0.367 ± 0.03 pT; U = 0.006; means ± SEM), thus confirming the effectiveness of the lesion.

We also found that sham-lesioned mice showed an increase in cell survival in the AOB–GrL after exposure to male soiled bedding whereas IB-treated mice did not (Figure 4F). By contrast, granule cell survival in the MOB was unaffected by either bedding exposure or IB lesioning (Figure 4G). Together, our results indicate that positive selection of new AOB granule cells depends on both centrifugal as well as centrifugal sensory activity. These findings are reminiscent of recent experiments in the MOB (Petreanu and Alvarez-Buylla, 2002; Yamaguchi and Mori, 2005; Whitman and Greer, 2007; Panzanelli et al., 2009; Veyrac et al., 2009).

**AOB NEUROGENESIS IS NECESSARY TO PREVENT MATE INDUCED PREGNANCY BLOCK**

To confirm the involvement of newborn AOB granule cells in mate memory formation, we blocked the renewal of adult-born interneurons by infusing the anti-mitotic drug Ara-C using osmotic mini-pumps (Breton-Provencher et al., 2009). Although alternative methods are available to block the genesis of new neurons in the brain (e.g., X-ray exposure or gene-coded selective deletion; Lazarini et al., 2009; Lazarini and Lledo, 2011), we favored Ara-C brain infusion because it is the most efficient way to eliminate the production of SVZ newborn cells even after short-term treatment (Doetsch et al., 1999; Breton-Provencher et al., 2009) and low doses of Ara-C that we and others used have no aversive side-effects (Moreno et al., 2009; Lazarini and Lledo, 2011). By contrast, X-ray irradiation only partially reduces SVZ neurogenesis (Lazarini et al., 2009) and genetic ablation of neurogenesis often requires injection of tamoxifen (Imayoshi et al., 2008; Sakamoto et al., 2011), a substance which is a potential source of bias when studying sexually dimorphic and estrogen responsive structures such as the vomeronasal system (Halbreich and Kahn, 2006; Hoffmann and Schuler, 2000; Kedia-Mokashi et al., 2011).

Infusion of Ara-C for 28 days following previously established protocols (Breton-Provencher et al., 2009) completely eliminated from the AOB the cells that we found to be preferentially activated by male odors (Figure 1G; Figure 5A). BrdU injections were
performed at the 14th day of the anti-mitotic treatment and the efficiency of neurogenesis ablation was assessed at the 28th day.

Female mice treated with Ara-C completely lacked BrdU and DCX labeling (Figure 5A), while saline-treated females showed a normal rate of neurogenesis in the OB. Notably, the amount of pre-existing neurons (NeuN-positive) remained unchanged in the AOB after Ara-C infusion (Figures 5B,C), indicating that the treatment specifically affected newly generated cells.

Next, after a 4-week-long ablation of bulbar neurogenesis, we tested Ara-C treated females for their ability to recognize the mate and thus avoid pregnancy block by male odor stimuli. Briefly, female mice were mated and subsequently re-exposed to the familiar partner odor during the following 3 days (Figure 6A; Bruce, 1966; Peele et al., 2003; Leinders-Zufall et al., 2004). Under control conditions (saline-treated female mice) re-exposure to the familiar mate odor did not induce pregnancy block, as indicated by a high pregnancy rate (or low pregnancy failure rate; Figure 6A, experiment 1). In stark contrast, females treated with Ara-C for 4-weeks underwent pregnancy block when re-exposed to the mate (Figures 6A,B, experiment 2), indicating that in absence of younger neurons they fail to imprint on the odors of the mating partner, as they are treated as unfamiliar ones.

However, the high pregnancy failure rate observed after neurogenesis ablation could be a result of Ara-C induced infertility. To rule out this possibility, we quantified the pregnancy rate after Ara-C treatment without subsequent exposure to the mating partner (Figure 6A, experiment 3). In this case, the pregnancy rate remained high and comparable to controls, indicating that the high pregnancy failure observed in experiment 2 was specifically induced by odor exposure after mating.

As a further control, we analyzed the effect of short-term Ara-C treatment on mate recognition efficiency (the same dose of the whole long-term treatment was given; Figure 6A, experiment 4) by starting the brain infusion 1 week before mating only. Under this condition, re-exposure to familiar odor did not induce pregnancy block. Hence, when compared with the findings of experiment 2, the inability of the short-term Ara-C treatment to induce pregnancy block (experiment 4) confirms that the lack of neurons aged between 2 and 4 weeks is ultimately responsible for switching the outcome of the pregnancy block test.

Finally, since Ara-C treatment eliminates newborn cells also in the MOB and the impact of this ablation on mate recognition is unknown, we tested the effects of mate re-exposure...
on Ara-C treated females subjected to surgical lesion of the vomeronasal nerves (VNX), a condition that alone is reported to eliminate exteroceptive pregnancy block (Figure 6A, experiments 5, 6; Figure 7A; Bellringer et al., 1980; Matsuoka et al., 2005). Consistent with a lack of MOB involvement in this recognition process, after blockade of neurogenesis (Ara-C treatment) in VNX mice, stud male odors where not effective in halting pregnancy onset after mating. Thus, in the absence of VN inputs, a lack of MOB newborn granule cells does not lead to pregnancy block by familiar pheromonal signals indicating that newborn cells in the MOB are not required for this imprinting process. As a further control that Ara-C treatment does not disrupt the ability to discriminate male volatile odors, we performed a habituation–dishabituation assay – which allows for measurement of MOE-dependent novel odor investigation, short-term odor learning, and odor discrimination – but found no significant difference (hab4 vs. dishab, t-test, \( P < 0.05 \) for Ara-C, Saline, and Saline-VNX mice) between sham-lesioned and Ara-C treated VNX female mice (Figure 7B). Together, these results indicate that the lack of bulbar newborn neurons compromises primarily AOB-mediated discrimination, as shown by the mate recognition impairment.

**DISCUSSION**

Our results highlight a direct link between VN sensory inputs and AOB neurogenesis, and a crucial role for AOB newborn neurons as preferential cellular substrate for mate pheromonal imprinting in adult female mice. Neurogenesis in the OB received considerable
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FIGURE 7 | Infusion of anti-mitotic drug Ara-C eliminates olfactory bulb neurogenesis but does not impair male odor recognition. (A) Immunostaining for OMP and BrdU in the OB after Saline/Ara-C treatment and surgical lesion of the vomeronasal nerve (experiments 1, 5, and 6 in Figure 6). The injections of BrdU have been performed 14 days after the beginning of brain infusion. In the third row, the lack of OMP/BrdU double immunoreactivity shows the efficiency of VN surgery and neurogenesis ablation in Ara-C/VNX mice compared to saline-treated mice (controls and VN-lesioned mice). Bars in (A), 500 μm for low magnification images, 40 μm for image details. (B) Habituation/dishabituation task for male volatile odor discrimination in Saline/Ara-C treated mice. Odor sources consisted of filter paper stripes soaked with male urine derived from different individuals and contained in a plastic box to prevent direct nasal contact but not odor perception. Mice were habituated to an odor with four consecutive presentations. On the fifth presentation a different odor was presented. Both saline-treated and neurogenesis-ablated mice (normal or VNX) similarly increased the investigation time in response to the new odor. Means ± SEM, *P < 0.05.

attention in the last decade, due to the suitability of the SVZ–OB model to study the functional implication of neurons generated during adulthood in sensory processes. Surprisingly, few authors considered explicitly in their analyses this unceasing cell renewal in the AOB (Bayer, 1983; Bonfanti et al., 1997; Peretto et al., 1999; Martinez-Marcos et al., 2001; Sakamoto et al., 2011; Veyrac and Bakker, 2011). Furthermore, the functional role of newborn neurons in the perception of social odors and pheromones has been evaluated only in the MOB (Shingo et al., 2003; Mak et al., 2007; Larsen et al., 2008; Feierstein et al., 2010; Mak and Weiss, 2010) with the exception of a few reports considering both the AOB and the MOB (Oboti et al., 2009; Nunez-Parra et al., 2011; Sakamoto et al., 2011). Therefore, to our knowledge, an integrated study focused both at a cellular and behavioral level on the VNS circuitry, is still lacking. Moreover, a possible involvement of AOB neuronal turn-over in the well known granule-to-mitral AOB synaptic plasticity occurring during mating, which is the neural correlate of mate recognition (Brennan and Keverne, 1997; Matsuoka et al., 2004), remained untested.

Here we have hypothesized that this memory formation process, established in a short time window during mating, could be promoted by the presence of renewed interneurons in the AOB of adult female mice.

Accordingly, we report firstly that in adult female mice the functional integration of these cells is triggered by the exposure to male odors: such sensory-driven positive selection acted preferentially on 2–3 weeks old granule cells (Figure 1A). In addition, shortly after their integration, these neurons became particularly responsive toward the experienced sensory stimuli (as shown by BrdU and c-Fos imaging, Figure 1G). Thus, the timing of this process is coherent with a rapid involvement of these interneurons in AOB circuits underlying individual recognition.

Moreover, the same sensory activity increased the survival of 3 weeks old MOB granule cell but their preferential involvement in OB sensory processing occurred only around the 6th week of age. Therefore, it is likely that only young AOB granule cells are selectively and rapidly involved in the elaboration of male individual chemosensory signals.

More generally, such neuronal selectivity and perceptual resolution of peripheral sensory areas is modified through sensory experience via feedback connections between higher and lower cortical levels (Gilbert and Sigman, 2007). Thus, we argued that if VN sensory inputs and AOB neurogenesis would have been similarly linked, the hypothesis of the functional involvement of AOB newborn cells in male odor recognition would have gained likelihood.

Concerning the sensory structures involved in this olfactory task, it is known that the main and accessory olfactory pathways act synergistically in pheromonal odor processing through convergent projections to the medial amygdaloid region (MeA; Moncho-Bogani et al., 2005). From here, a feedback loop is formed by excitatory centrifugal afferents contacting the AOB granular layer which, in turn, directly regulates the local activity in response to external stimuli (Pankevich et al., 2006; Fan and Luo, 2009; Martel and Baum, 2009). Hence, AOB neuronal activity, and neurogenesis as well, can be alternatively modulated by either direct peripheral sensory stimulation through VNO–MeA activation, or indirectly by MOE–MeA centrifugal inputs.
Thereafter, by selective functional ablation of the main sensory relays involved in social odor perception (MOE, VNO, MeA), we reported that the neurogenic process observed in the AOB is actively guided by VNO–MeA sensory activity (but not MOE–MeA) underlying its substantial correlation with vomeronasal sensory processing.

Sustained by these evidences, we have halted the production of new neurons in the brain by intracerebroventricular (ICV) Ara-C infusion and tested the ability of adult female mice to recognize their mating partners in order to avoid ectoceptive implantation failure. The drug infusion period covered the time window for AOB granule cell peak responsiveness to male individual odors.

As a result of the neurogenesis blockade, we found a high rate of pregnancy failure in recently mated females after exposure to the mating partner. This effect was specifically due to the re-exposure to the stud male since no such pregnancy failure did follow when Ara-C was given alone. Although Ara-C-treated female mice showed a subtle decrease in pregnancy rates in comparison to saline-treated females (Figure 6A, experiments 3, 4 vs. 1; 60 vs. 77% = 17%), the strong decrease in pregnancy rate evident after re-exposure to the mating male goes far beyond these differences (Figure 6A; 2 vs. 3, 4; 21 vs. 60% = 39%). Nevertheless, this pregnancy failure rate does not reach the levels observed in our control conditions and in other reports (60–70%; Bruce, 1966; Brennan and Zufall, 2006), possibly because older cells (aged over 28 days) spared by the anti-mitotic treatment could constitute a residual substrate for the imprinting process.

Notably, the memory established on the stud male urine odors during mating is localized specifically at the granule-to-mitral synaptic interface in the AOB (Kaba et al., 1989). Thus, our results strongly support a preferential role of AOB newly generated neurons in this olfactory recognition process (Figure 6B), despite a capability of the main olfactory system to detect mate’s pheromones (Brennan and Zufall, 2006) and widespread anti-neurogenic CNS effects following Ara-C ICV infusion. In addition, a lack of involvement of MOB interneurons was further supported by the fact that the 4-week long anti-mitotic treatment left undamaged those new granule cells (6 weeks old) highly responding to male familiar cues in this region (Figure 1H). Although this observation alone does not rule out the involvement of these neurons in mate recognition, a role of the MOB in the avoidance of pregnancy block was shown only 6.5 days after mating (Serguera et al., 2008) and therefore is not compatible with our mate exposure protocol. Eventually, female mice treated with Ara-C were still able to discriminate male individual odors, thus implying the lack of cells aged up to 4 weeks does not disturb normal olfactory recognition of individual males. Nevertheless, to confirm this idea and test the effect of neurogenesis ablation only in the MOB, we have blocked vomeronasal inputs by lesioning the vomeronasal nerves in Ara-C treated female mice. This procedure was sufficient to prevent the high rate of pregnancy block by stud male exposure, ruling out the involvement of MOB newborn interneurons (aged 1–4 weeks) in mate recognition and confirming the key role of AOB interneurons in this process (Kaba et al., 1989; Hudson, 1993).

In conclusion our experiments demonstrate the importance of newly generated AOB inhibitory interneurons in the mating pheromonal imprinting process. Therefore, we provide evidence that neurogenesis in the adult brain is indeed of extraordinary functional significance, playing a critical role in the context of AOB-mediated sensory processing, persistent odor learning, and mate recognition. Our results also imply that newborn AOB interneurons are necessary to convey nerve signals that, ultimately, lead to determined neuroendocrine responses in higher circuits of the vomeronasal pathway. The survival of new neurons in the AOB seems to be regulated by both the detection of species-specific, sensory vomeronasal stimuli, included in the LMW urine fraction, that requires an intact Trpc2 cation channel, and central inputs from the medial amygdala. The identification of the function of newly generated AOB interneurons in pheromonal mate recognition provides an attractive cellular substrate to begin to dissect the molecular logic of this feat.

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