

# Exposure to young preferentially activates adult-born neurons in the main olfactory bulb of sheep mothers

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**Abstract** New neurons are continuously added to the main olfactory bulb (MOB) of the mammalian brain. While their function has been demonstrated in olfactory learning, it is less known in an ethological context such as mothering. We addressed this question by investigating whether in sheep mothers the adult-generated olfactory neurons contribute to the processing of odors involved in attraction to lambs and in memorization of its individual signature. Parturient ewes, after having 2 days of contact with their lamb and being separated from them for 3 h, were exposed for 2 h either to their own lamb, an unfamiliar lamb or a familiar adult sheep and then sacrificed. A control group was composed of mothers not exposed to any lambs for 5 h before sacrifice. Bromodeoxyuridine, a marker of cell division, was injected 3 months before parturition and revealed through immunocytochemistry in combination with markers of activation or neuronal maturation. The percentage of adult-born cells activated in the granular layer of the MOB was compared between the four groups. Results show that the whole population of olfactory neuroblasts and in particular the 3-month-old neuroblasts, are preferentially activated by lamb exposure and that the preferential activation is specific to olfactory neurogenesis since no activation was observed in newborn neurons of the

dentate gyrus. However, neither neuroblasts nor mature neurons of the MOB differentiate between familiar and unfamiliar lamb exposure. Therefore, our data shows that adult-born neurons contribute to the processing of infantile odors which are determinant for maternal behavior.

**Keywords** Brain plasticity · Hippocampus · Maternal behavior · Neurogenesis · Olfactory learning · Olfactory bulb

## Introduction

In several mammalian species, odor cues emitted by newborn are essential to establish maternal behavior at parturition and coordinate early mother-infant interactions. In most parturient females, infantile odors are highly attractive and are potent stimuli, promoting the contact with the young to ensure a normal development of maternal care (Corona and Lévy 2015; Lévy et al. 2004; Numan et al. 2006). This rapid change of preference occurring at parturition is regulated by the perception of chemosensory stimuli by the main olfactory system. For instance in sheep, primiparous mothers rendered anosmic before parturition show reduced maternal behavior (Lévy et al. 1995b) and removing amniotic fluid from the neonate's coat prevents acceptance behavior (Lévy and Poindron 1987). In some species, like sheep, odors also provide a basis for individual recognition of the offspring. Ewes develop discriminative maternal care, called maternal selectivity, favoring their own young at suckling while rejecting other young. This recognition is based on learning the olfactory characteristics of the lamb which takes place within the first hours after parturition (Lévy and Keller 2009; Lévy et al. 2004). Extensive neurochemical and electrophysiological changes occurring in the main olfactory

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system (MOB) at parturition indicate the existence of a coding of lamb odors (Kendrick et al. 1992; Lévy et al. 1993).

In addition to these changes in the MOB, another form of brain plasticity, is the capacity of the brain to manufacture new neurons during adulthood, has been discovered in the sub-ventricular zone- main olfactory bulb (MOB) continuum and the dentate gyrus (DG) of the hippocampus (Abrous et al. 2005; Lepousez et al. 2015; Lledo et al. 2006; Ming and Song 2005). This neuroplasticity could provide an additional mechanism through which olfaction can contribute to the enhancement of maternal behavior and associated learning. In the olfactory system, neural stem cells function as primary precursors in the sub-ventricular zone located on the wall of the lateral ventricles. These cells produce transient amplifying cells which rapidly divide to produce neuroblasts. The neuroblasts migrate along the rostral migratory stream and after reaching the MOB, the majority of them mature into granular interneurons.

To date, the nature of the specific contributions of adult generated neurons to olfactory behavior is still under debate (Lazarini and Lledo 2011). Because the ability to process sensory information depends on the functional architecture and synaptic connectivity of the MOB, adult neurogenesis has a particular impact on fine tuning of information processing. Also, because adult-born olfactory neurons have unique properties of synaptic plasticity compared to early-born neurons (Nissant et al. 2009), they could provide a possible substrate for olfactory learning. However, in the context of social behavior which so importantly relies on olfaction, evidence for any function of olfactory neurogenesis remains sparse. In male hamsters, double immunohistochemistry labeling for c-Fos, a marker of cell activation, and Bromodeoxyuridine (BrdU), a marker of cell division, shows that olfactory bulb cells born in adulthood are activated by socio-sexual stimuli such as estrous female (Huang and Bittman 2002). This indicates that newly generated neurons might functionally integrate the olfactory network which processes social olfactory information. Moreover, the suppression of neurogenesis by an anti-mitotic agent prevents mate recognition (Oboti et al. 2011) and the display of preference for dominant males in female mice (Mak et al. 2007). Recently, it was found that a reduction of neuroblasts impaired social discrimination in an inducible transgenic mouse model of adult neurogenesis reduction (Garrett et al. 2015).

Although a regulation of neurogenesis by parturition and the onset of motherhood have been reported in mice, rats and sheep, few studies have explored the functional implication of olfactory neurogenesis in maternal behavior (Lévy et al. 2011). In mice, irradiation of the SVZ induces minor disturbances of maternal behavior

(Feierstein et al. 2010). However, infusion of an anti-mitotic agent, which transiently impairs both hippocampal and olfactory neurogenesis, affects maternal behavior but only when animals are tested in an anxiogenic environment (Larsen and Grattan 2010). Also, genetic manipulations inducing long-term alterations of neurogenesis impair nursing behavior in the home cage (Sakamoto et al. 2011). While we do not have direct evidence for the implication of olfactory neurogenesis in sheep, interactions with the lamb are specifically associated with a decrease in the number of olfactory adult-born neurons together with an acceleration of their maturation olfactory adult neurogenesis (Brus et al. 2014).

On the basis of these findings, we examined whether the processing of olfactory stimuli coming from neonates, specifically activates new interneurons of the MOB. We hypothesized that new olfactory interneurons will preferentially respond to exposure of any lamb in comparison with exposure to an adult conspecific. In addition, because sheep offer a unique possibility to scrutinize the mechanisms underlying olfactory memory, we also examined whether these new neurons are activated during the learning of the lambs' individual odor. We hypothesized that new olfactory interneurons will preferentially respond to exposure of their own lamb as compared to exposure of an unfamiliar lamb. To this end, BrdU was used in combination with two markers of neuronal maturation (DCX, an early maturation marker of neuronal differentiation and NeuN a marker of mature neuron), and two markers of neuronal activation (c-Fos and Zif268) to compare activation of adult-born neurons in the MOB between mothers exposed to their own lamb, an unfamiliar lamb or an adult female.

## Materials and methods

### Animals

Experiment was conducted on 34 primiparous parturient ewes *Ile de France* (2–3 years old) from the INRA Research Center in Nouzilly (Indre et Loire, France) approved by local authority (agreement No. E37-175-2). Animals were permanently housed indoors, with free access to water and were fed with lucerne, maize, straw and a supplement of vitamins and minerals. Animal care and experimental treatments complied with the guidelines of the French Ministry of Agriculture for animal experimentation and European regulations on animal experimentation (86/609/EEC). They were performed in accordance with the local animal regulation (authorization No. 006352 of the French Ministry of Agriculture in accordance with EEC directive) and with the ethical committee Val de Loire

(agreement No. 2011/05/03). Ewes were sacrificed by a licensed butcher in an official slaughterhouse (authorization No. A37801 E37-175-2 agreement UEPAO). All efforts were made to minimize the number of animals (8–9 animals per group).

### Bromodeoxyuridine injections

Three and a half months before sacrifice, ewes were housed in an individual pen (2X1 m) without preventing auditory, visual and olfactory contacts with conspecifics. Three months before sacrifice, ewes received four intravenous injections of BrdU within 24 h (20 mg/kg in 0.9 % saline per injection; Sigma-Aldrich, France), a thymidine analogue incorporated into the DNA during the S-phase of the mitotic division (Fig. 1). Dose of BrdU, timing between injections and sacrifice were based on a previous study reporting that maturation of adult-born cells is much longer than that of rodents and that a large proportion of neuroblasts and new mature neurons is found at 3 months after BrdU injections in the MOB (Brus et al. 2013). Ewes remained individually housed until lambing but they could interact with their conspecific housed in adjacent pens.

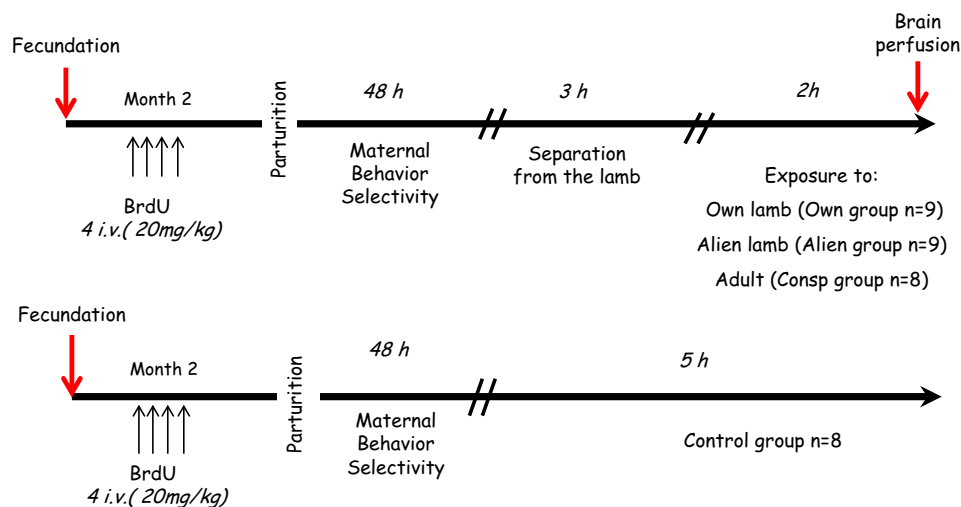
### Experimental design

Mating was synchronized by the use of vaginal sponges containing 45 mg of fluorogestone acetate for 14 days followed by an intra-muscular injection of pregnant-mare-

stimulating gonadotropins to induce ovulation. Lambing occurred within a period of gestation of  $149 \pm 4$  days.

Maternal behavior was observed for 20 min, at 1 and 2 h after parturition to ensure that maternal care was normally provided to lambs (data not shown; Fig. 1). Maternal selectivity was tested at 24 h postpartum by presenting an alien lamb to the mother and her rejection and acceptance behaviors were recorded for 3 min (data not shown). The alien lamb was then taken away and the ewe was observed with her own lamb for an additional 3 min (Keller et al. 2004, 2005). These tests indicated that all the mothers were maternal and selective.

Two days after parturition, lambs were separated from their mother for 3 h. To evaluate the activity-inducible immediate early genes (IEGs), after this period of separation, mothers were exposed for 2 h to either their own lamb (Own group,  $n = 9$ ), or an alien lamb (Alien group,  $n = 9$ ) or an unfamiliar ewe (Consp group,  $n = 8$ ). The time spent sniffing the own lamb or the alien lamb or the conspecific was video recorded during the first 15 min of exposure. Since it was reported that expression of IEGs return to basal levels after 2 h after exposure to a stimulus (Zangenehpour and Chaudhuri 2002), a Control group ( $n = 7$ ) was constituted of mothers separated from their lambs 2 days after parturition and staying with other mothers of this group for 5 h before sacrifice. For practical reasons, no behavioral observations were performed for this group. Mothers of the Consp and the Control groups were placed in a different barn to avoid any contact provided by lambs.



**Fig. 1** Time table and protocol design. All ewes received four intravenous injections of BrdU (20 mg/kg, 1 injection/day) 3 months before sacrifice. Ewes could interact with her lamb for 48 h and maternal behavior was observed for 20 min, 1 and 2 h after parturition and selectivity test was performed at 24 h postpartum. Two days after parturition, lambs were separated from their

mother for 3 h. Mothers were then exposed for 2 h to either their own lamb (Own group,  $n = 9$ ), or an alien lamb (Alien group,  $n = 9$ ) or a non-parturient ewe (Consp group,  $n = 8$ ). A Control group ( $n = 7$ ) was constituted of mothers separated from their lambs 2 days after parturition and stayed with other mothers of this group for 5 h before sacrifice

## Brain perfusion and immunohistochemistry

After the 2 h of lamb (Own and Alien groups) or conspecific exposure (Consp group) or 5 h after the removal of the lamb (Control group), ewes were deeply anesthetized with thiopental and decapitated by a licensed butcher. The head of the ewes was immediately perfused via carotid arteries with 2 L of 1 % sodium nitrite in phosphate buffer saline, followed by 4 L of ice-cold 4 % paraformaldehyde solution in 0.1 M phosphate buffer (pH 7.4). The brain was dissected out, cut into blocks and post-fixed in the same fixative for 48 h. The tissues were then stored in 30 % sucrose for at least 2 days until sectioned. Frontal sections of the MOB and the dorsal hippocampus were cut at a thickness of 30  $\mu$ m using a freezing microtome MICROM (HM430, MMFrance, Francheville). Sections were stored at  $-20^{\circ}\text{C}$  in a cryoprotectant solution until their use for immunostaining. Hippocampal neurogenesis was examined to evaluate whether activation of adult-born neurons induced by exposure to lambs is specific to olfactory neurons.

To evaluate the general activation of granular interneurons in the MOB a peroxidase single-immunostaining was performed against the activity-inducible IEG early growth factor response gene-1 (Zif268 or Egr-1) classically used to reveal brain activation occurring in memory formation (Dardou et al. 2006; Lonergan et al. 2010). To characterize activation of the population of 3-month-old newborn cells in the MOB double immunostaining was performed against the BrdU and Zif268. Another IEG, c-Fos, was also used to confirm this activation. To characterize the activation of the population of MOB neuroblasts, a double immunostaining was performed against the microtubule binding protein doublecortin (DCX) and Zif268 (Brown et al. 2003). A triple immunostaining against BrdU/DCX/Zif268 and BrdU/NeuN/Zif268 was performed to characterize activation of the population of 3-month-old neuroblasts or mature neurons, respectively. Finally, to determine specificity of activation of adult newborn neurons in the MOB a double immunostaining was performed against the BrdU and Zif-268 in the DG of the dorsal hippocampus.

## Peroxidase single-immunostaining

Free-floating sections from the MOB were left in a solution of  $\text{H}_2\text{O}_2$  1 %-TBS (Tris-Buffer Saline 0.025 M, pH = 7.4) at  $4^{\circ}\text{C}$  for 30 min, followed by TBS wash for 5 min. Sections were treated with a solution of TBS-T (Triton 0.3 %-A (Azide 0.1 %-)BSA (Bovine Serum Albumin 1 %) for 1 h and then incubated with the primary antibody rat anti-BrdU (1:300; AbC117-7513 AbCys, Paris, France) rabbit anti-Zif268 (1:1000; sc-189 Santa Cruz Biotechnology, Heidelberg, Germany) or mouse anti-cFos (1:2000;

sc-271243 Santa Cruz Biotechnology, Heidelberg, Germany) for 1 h at room temperature and left for 24 h (mouse anti-cFos) or 72 h (anti-Zif268) at  $4^{\circ}\text{C}$  in TBSTA-BSA. After 5 rinses in TBS, sections were incubated for Zif268 detection in SMAL (1:400; Serum de Mouton anti-lapin/sheep anti-rabbit serum, INRA, France) or for c-Fos detection in (1:500; Biotin sheep anti-mouse, Jackson Immunoresearch Laboratory, Europe Ltd) in TBS-BSA 0.1 % at  $4^{\circ}\text{C}$  for 3 h. After five rinses in TBS, sections were left overnight at  $4^{\circ}\text{C}$  in anti-rabbit PAP (1:80,000; peroxidase anti peroxidase complex Z0113 DAKO, Denmark) for Zif268 detection and in Streptavidin HRP (1:1000, Jackson Immunoresearch Laboratory, Europe Ltd) for c-Fos detection, in TBS-BSA 0.1 %. The last day, after 2 rinses in TBS and 3 rinses in Tris-HCl (0.05 M, pH 7.6) sections were reacted for peroxidase detection in a solution of 3,3'-diamineobenzidine tetrahydrochloride (DAB, 0.15 mg/mL; Sigma, USA) containing 0.001 %  $\text{H}_2\text{O}_2$  and nickel ammonium sulfate (0.33 %) for 12 min. All sections were mounted on gelatinized glass-coated slides, dehydrated and coverslipped with Depex.

## Fluorescence double/triple-immunostaining

After five rinses in TBS sections were treated for 1 h with a solution of TBS-TA-BSA or TBSTA-HS (Horse Serum 1 %, for DCX labeling). After one rinse in TBS, sections were treated with 2 N HCl in TBS for 30 min at  $25^{\circ}\text{C}$ . After 3 rinses in TBS, sections were incubated in two or three primary antibodies at the same time for 1 h at room temperature and left for 48 h at  $4^{\circ}\text{C}$  in TBSTA-BSA or TBSTA-HS using the following combinations: BrdU (1:300)/Zif268 (1:1000), BrdU (1:300)/Fos (1:2000), BrdU (1:300)/DCX (1:300)/Zif268 (1:500) and BrdU (1:300)/NeuN (1:1000)/Zif268 (1:500). When antibody against BrdU was not used, after TBSTA-HS (1 %) treatment, sections were incubated in the primary antibodies DCX (1:300)/Zif268 (1:500) in a solution of TBSTA-HS (1 %) for 1 h at room temperature and left for 48 h at  $4^{\circ}\text{C}$ . Primary antibodies used were: rat anti-BrdU (AbC117-7513 AbCys, Paris, France), rabbit anti-Zif268 (sc-189 Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-cFos (sc-271243 Santa Cruz Biotechnology, Heidelberg, Germany), goat anti-DCX (sc-8066 Santa Cruz Biotechnology, Heidelberg, Germany) and mouse anti-NeuN (MAB377 Chemicon Millipore, St Quentin en Yvelines, France). After 48 h of primary antibody incubation, sections were rinsed three times in TBS, and were incubated with two or three secondary antibodies simultaneously for 2 h in TBS-saponine (0.3 %-)BSA (1 %) for anti-rat 546 (1:1000)/anti-rabbit 488 (1:1000), TBS-BSA for anti-rat 546 (1:800)/anti-mouse 488 (1:800), TBS-saponine for anti-goat 546 (1:1000)/anti-rabbit 488 (1:1000), anti-rat CY3

(1:1000)/anti-goat 647 (1:1000)/anti-rabbit 488 (1:1000) and anti-rat CY3 (1:1000)/anti-mouse 647 (1:1000)/anti-rabbit 488 (1:1000). The secondary antibodies used were: goat anti-rat 546 (A11081 Jackson ImmunoResearch, UK), donkey anti-rat CY3 (712-165-153 Jackson ImmunoResearch, UK), donkey anti-rabbit 488 (711-546-152 Jackson ImmunoResearch, UK; A21206 Invitrogen, Cergy Pontoise, France), goat anti-mouse 488 (A11029 Invitrogen, Cergy Pontoise, France), donkey anti-mouse 647 (715-605-151 Jackson ImmunoResearch, UK), donkey anti-goat 546 (A11056 Invitrogen, Cergy Pontoise, France), donkey anti-goat 647 (705-605-147 Jackson ImmunoResearch, UK). After four rinses in TBS, sections were immersed in Hoechst (a nuclear staining) for 2 min (Hoechst 33258, 2 µg/ml in distilled water, Invitrogen, Cergy Pontoise, France), and washed twice in distilled water followed by four rinses in TBS. A nuclear intercalate staining (Hoechst) was used to control for artifact. All the sections were mounted on slides, allowed to dry and cover-slipped using Fluoromount-G (Southern Biotechnology, Birmingham, USA). The slides were stored at 4 °C in dark until confocal analysis.

### Quantification

Sections of the MOB and the DG through different levels along the rostro-caudal axis were examined. For the MOB the more rostral level is defined at the appearance of the olfactory ventricle and the more caudal level at the appearance of the accessory olfactory bulb. For the DG, the more rostral level is defined at the appearance of the CA3 and the more caudal level at the reunion of the dorsal and ventral hippocampus. The counter was blind to the experimental group.

#### Single immunostaining

The number of divided cells or activated cells within the granular cells (GC) of the MOB (7–8 sections/animal, 1 mm between sections) was evaluated by counting peroxidase/BrdU<sup>+</sup>, peroxidase/Zif268<sup>+</sup> or peroxidase/c-Fos stained sections using a light microscope on a magnification of X20 (Axioskope 2, Zeiss, Germany). Volumetric density of labelled cells was determined using unbiased stereology with the disector method and the Mercator software (computerized image analysis Mercator, Explora Nova, La Rochelle, France). The region of interest was traced and the reference volume was determined. Immunopositive cells were quantified by systematic random sampling using a counting frame of 300 × 200 µm at evenly spaced intervals of 20 × 20 µm. Cells that intersected the uppermost focal plane or the lateral exclusion borders of the counting frame were not quantified.

#### Double/triple immunostaining

All the BrdU<sup>+</sup> or DCX<sup>+</sup> cells were counted in the granular layer of the MOB. Each counted cell was analyzed in its entire z-axis, with 1 mm step intervals, through a 40× oil immersion objective, using a confocal laser-scanning microscope (LSM700, Zeiss, Germany) equipped of excitation wavelengths 405, 488, 555 and 639. Cells rotated in orthogonal planes to verify double/triple labeling with Zif268/c-Fos or DCX/Zif268, NeuN/Zif268. Double labeling was performed in eight sections per animal with 1 mm interval between sections. To calculate the proportion of either BrdU<sup>+</sup> or DCX<sup>+</sup> activated cells, we counted the number of IEGs<sup>+</sup> cells (Zif268 or c-Fos) among the population of either BrdU<sup>+</sup> or DCX<sup>+</sup> cells and we used the following formula: [(BrdU or DCX<sup>+</sup>/IEG<sup>+</sup> cell)/(total BrdU<sup>+</sup> or DCX<sup>+</sup> counted cells)] × 100. Triple immunostaining was performed in four sections of the MOB with 2 mm interval between sections. To calculate the proportion of BrdU<sup>+</sup> neuroblasts (DCX) or BrdU<sup>+</sup> mature neurons (NeuN) that were activated the number of Zif268<sup>+</sup> cells was counted among the population of DCX<sup>+</sup>/BrdU<sup>+</sup> or NeuN<sup>+</sup>/BrdU<sup>+</sup> cells. The following formula was used: [(DCX<sup>+</sup>/BrdU<sup>+</sup> or NeuN<sup>+</sup>/BrdU<sup>+</sup>/IEG<sup>+</sup> cell)/(total DCX<sup>+</sup>/BrdU<sup>+</sup> or NeuN<sup>+</sup>/BrdU<sup>+</sup> counted cells)] × 100.

#### Statistical analysis

As densities of Zif268<sup>+</sup> or c-Fos<sup>+</sup> cells and proportions of double/triple labelled cells were not normally distributed (Shapiro–Wilk Test), the data were analyzed with non-parametric tests. Inter-group comparisons were analyzed using two-tailed Kruskal–Wallis and Mann–Whitney tests. Statistical analyses were performed using the statistical package *Statistica 10* and the level of statistical significance was set at  $p < 0.05$ . All data are represented as median and interquartile ranges.

## Results

### Sniffing time during lamb or adult conspecific exposure

To estimate exposure time to odors of lamb or of adult conspecific, time spent sniffing was recorded during the first 15 min of the 2 h encounter. Time spent sniffing significantly differed between Own, Alien and Consp groups ( $H = 10.2$ ,  $p = 0.005$ ). Ewes spent significantly more time sniffing the familiar lamb [166 s (137–217),  $p = 0.003$ ] or the alien lamb [98 s (10–143),  $p = 0.048$ ] than the conspecific [26 s (0.5–34)]. However, the time spent by the mothers sniffing their own or the alien lamb was not significantly different.



### MOB activation by lamb or conspecific exposure

To evaluate cellular activation of the MOB promoted by the 2 h exposure, a single-immunostaining for Zif268 or for c-Fos was performed. For both markers, no significant differences in the volumetric density of positive cells were observed between the four groups (Fig. 2a, b).

### Neuroblasts in the MOB are preferentially activated by lamb exposure

Whether or not the whole population of neuroblasts showed a differential activation according to different exposures was investigated by assessing the percentage of DCX<sup>+</sup> cells that expressed Zif268. A statistical difference in this percentage was found between the four groups ( $H = 13$ ,  $p = 0.0038$ ; Fig. 3a, b). Mothers of the Own or the Alien groups showed the highest percentage compared to mothers of the Consp group ( $p = 0.006$ ,  $p = 0.03$ , respectively). In addition, the percentage of Zif268<sup>+</sup>/DCX<sup>+</sup> cells was significantly higher in mothers of the Own group than in mothers of the Control group ( $p = 0.02$ ).

### Three-month-old cells in the MOB are preferentially activated by lamb exposure

No significant differences in the volumetric density of BrdU<sup>+</sup> cells were observed between the 4 groups [Own: 340 cells/millimeter<sup>3</sup> (302–362); Alien: 350 cells/millimeter<sup>3</sup> (223–428); Consp: 318 cells/millimeter<sup>3</sup> (262–406); Control: 256 cells/millimeter<sup>3</sup> (210–290);  $H = 2.8$ ,  $p = 0.4$ ].

Because our previous study showed that the first newborn neurons were observed at 3 months after BrdU injections in the MOB of sheep, we quantified the

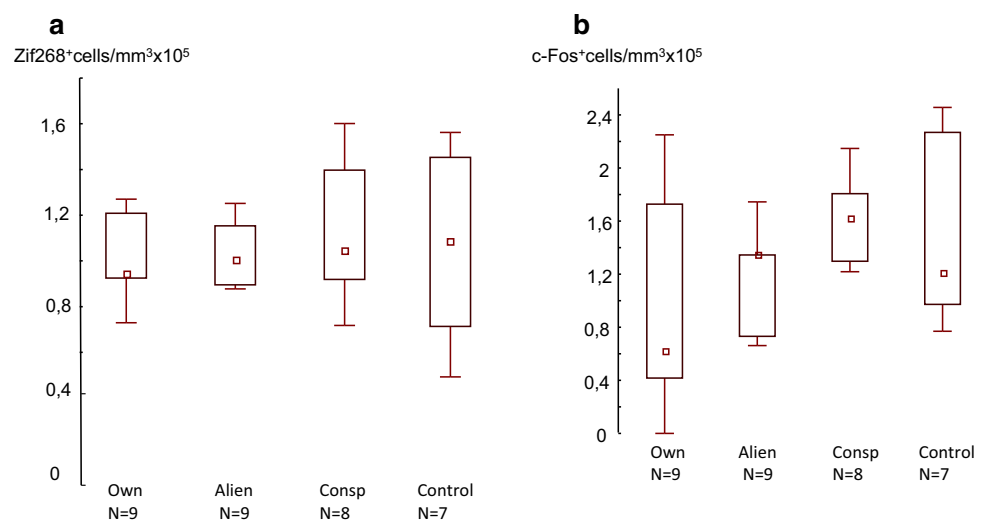
activation of this neuronal population. The percentage of Zif268<sup>+</sup>/BrdU<sup>+</sup> cells was significantly different between the four groups ( $H = 17.57$ ,  $p = 0.005$ , Fig. 4a, b). Mothers of the Own or Alien groups showed a significant higher percentage of Zif268<sup>+</sup>/BrdU<sup>+</sup> cells compared to mothers of the Control group ( $p = 0.003$ ,  $p = 0.01$ , respectively), or compared to mothers of the Consp group ( $p = 0.001$ ,  $p = 0.007$ , respectively). The proportion of Zif268<sup>+</sup>/BrdU<sup>+</sup> cells of the Consp group was significantly higher than the one of the Control group ( $p = 0.049$ ).

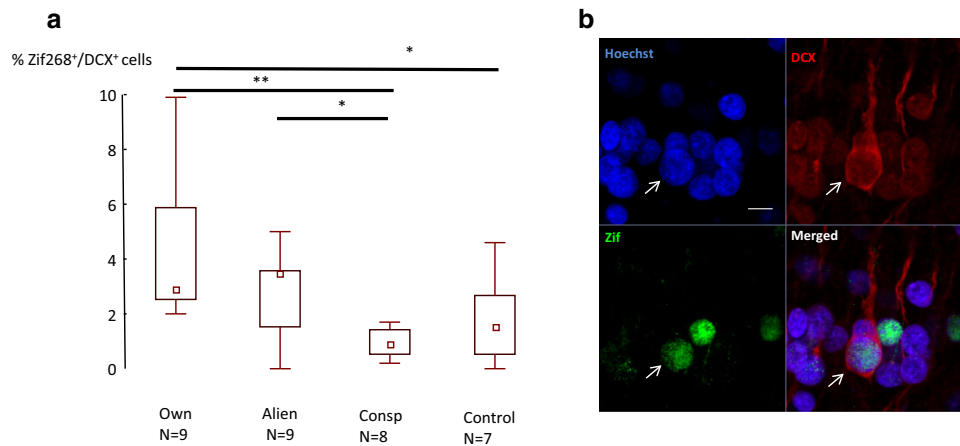
Similar differences were also observed using c-Fos (Fig. 4c, d). The percentage of c-Fos<sup>+</sup>/BrdU<sup>+</sup> was significantly different between the four groups ( $H = 12$ ,  $p = 0.0083$ ). Mothers of the Own groups showed a significant higher percentage of activated cells compared to mothers of the Control group ( $p = 0.004$ ,  $p = 0.04$ , respectively). However, only mothers of the Own group but not those of Alien group showed a significant higher activation compared to mothers of the Consp group ( $p = 0.01$ ).

### Neuroblasts and not mature neurons of 3-month-old are preferentially activated by lamb exposure

The proportion of activated cells (Zif268<sup>+</sup> cells) were assessed among the population of BrdU<sup>+</sup>/DCX<sup>+</sup> or BrdU<sup>+</sup>/NeuN<sup>+</sup> cells. A statistical difference in the percentage of Zif268<sup>+</sup>/DCX<sup>+</sup>/BrdU<sup>+</sup> was found among the four groups ( $H = 14.34$ ,  $p = 0.002$ ; Fig. 5a, b). The highest percentage of activation observed in the DCX<sup>+</sup>/BrdU<sup>+</sup> cells was observed in mothers of the Own or the Alien groups compared with mothers of the Control group ( $p = 0.002$ ). This percentage was only significantly higher in the Alien group than in the Consp group ( $p = 0.048$ ). Interestingly,

**Fig. 2** Activation of the MOB after exposure to lambs or conspecifics. Number of Zif268<sup>+</sup> cells (a) and c-Fos<sup>+</sup> cells (b) per millimeter<sup>3</sup> in the granular layer of the MOB. Both medians and interquartiles are presented. \*\*\* $p < 0.001$ , \*\* $p < 0.01$

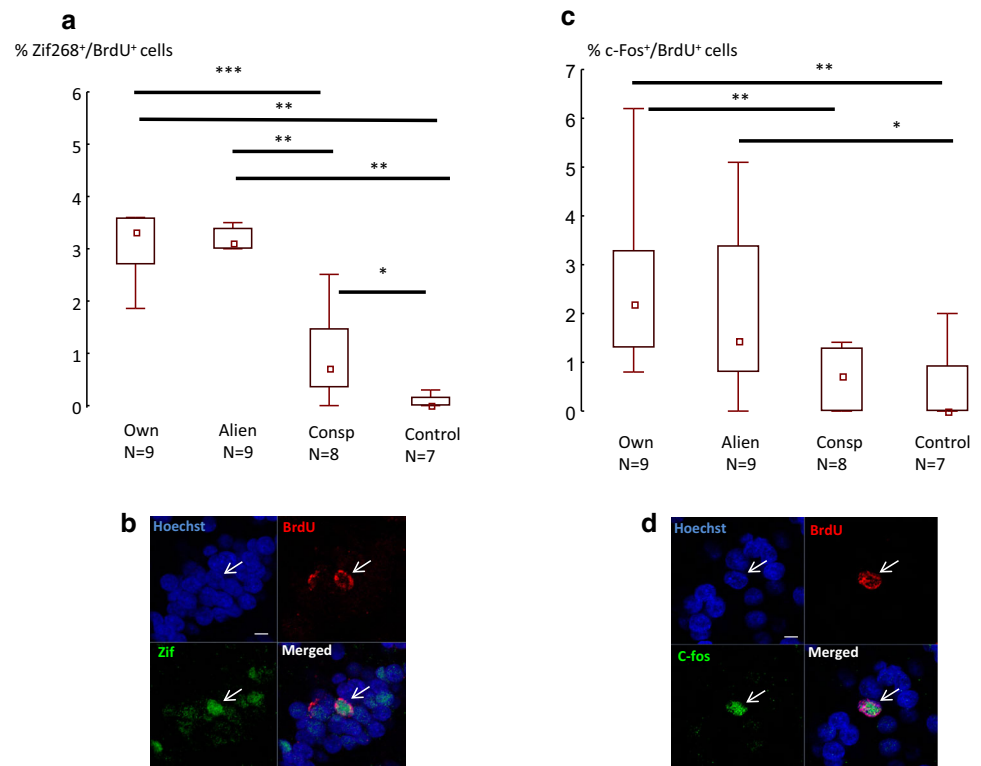




**Fig. 3** Neuroblasts in the MOB are preferentially activated by lamb exposure. **a** Percentage of DCX<sup>+</sup> cells expressing Zif268 in the granular layer of the MOB. Both medians and interquartiles are presented. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ . **b** Confocal

illustration showing coexpression of the neuroblast marker DCX (red) and the cellular activation marker Zif268 (vert) in the granular layer of the MOB. Cell nuclei are labeling with Hoechst (blue). Scale bar 5  $\mu\text{m}$

**Fig. 4** Three-month-old cells of the MOB are preferentially activated by lamb exposure. **a** Percentage of BrdU<sup>+</sup> cells expressing Zif268 in the granular layer of the MOB. **b** Confocal illustration showing co-expression of the cell division marker BrdU (red) and the cellular activation marker Zif268 (vert) in the granular layer of the MOB. Cell nuclei are labeling with Hoechst (blue). Scale bar 5  $\mu\text{m}$ . **c** Percentage of BrdU<sup>+</sup> cells expressing c-Fos in the granular layer of the MOB. Both medians and interquartiles are presented. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ . **d** Confocal illustration showing co-expression of the cell division marker BrdU (red) and the cellular activation marker c-Fos (vert) in the granular layer of the MOB. Cell nuclei are labeling with Hoechst (blue). Scale bar 5  $\mu\text{m}$



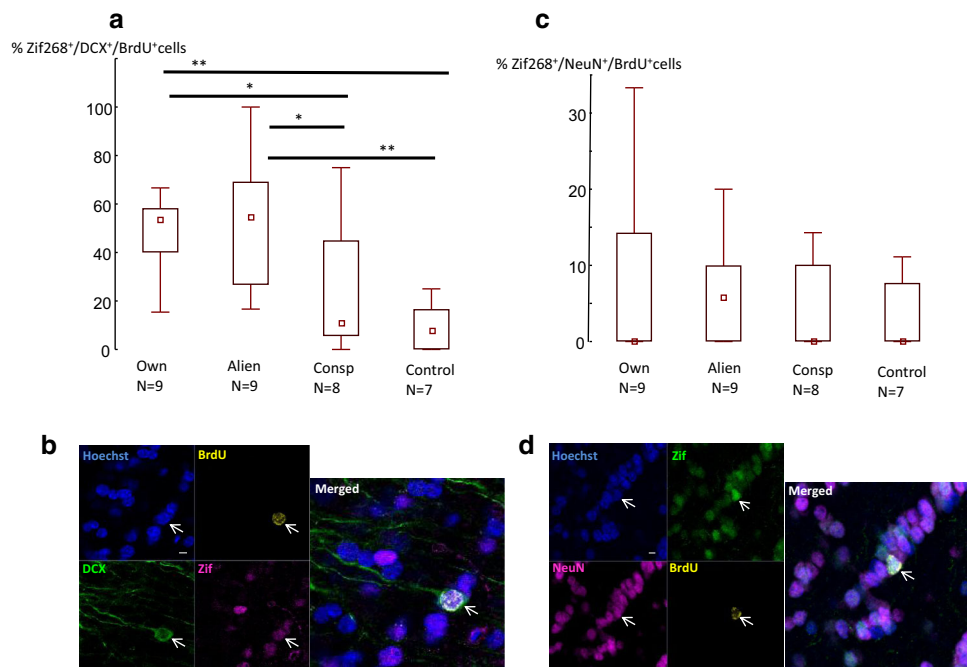
the percentage of BrdU<sup>+</sup>/DCX<sup>+</sup> did not significantly differ across the groups [Own 17 (12–19), Alien 14 (9–17), Consp 10 (6–12), Control 9 (8–17);  $H = 7.7$ ,  $p > 0.05$ ].

By contrast, the percentage of Zif268<sup>+</sup>/NeuN<sup>+</sup>/BrdU<sup>+</sup> cells was low across groups and no statistical difference were observed between the four groups ( $H = 1.321$ ,  $p = 0.724$ ; Fig. 5c, d). The percentage of BrdU<sup>+</sup>/NeuN<sup>+</sup> did not significantly differ across the groups [Own 9

(7–11), Alien 11 (2–18), Consp 10 (8–16), Control 10 (9–12);  $H = 1.1$ ,  $p = 0.7$ ].

#### Newborn cells in the MOB are not preferentially activated by own lamb exposure

The percentage of Zif268<sup>+</sup>/BrdU<sup>+</sup>, c-Fos<sup>+</sup>/BrdU<sup>+</sup>, Zif268<sup>+</sup>/DCX<sup>+</sup>, Zif268<sup>+</sup>/DCX<sup>+</sup>/BrdU<sup>+</sup> or Zif268<sup>+</sup>/



**Fig. 5** Neuroblasts and not mature neurons of 3-month-old are preferentially activated by lamb exposure. Percentage of activated cells (Zif268<sup>+</sup> cells) among the population of **a** BrdU<sup>+</sup>/DCX<sup>+</sup> or **c** BrdU<sup>+</sup>/NeuN<sup>+</sup> cells in the granular layer of the MOB. Both medians and interquartiles are presented. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ . **b** Confocal illustration showing BrdU<sup>+</sup> cells (yellow) co-

expressing the neuroblast marker DCX (green) and the cellular activation marker Zif268 (pink) in the granular layer of the MOB. Scale bar 5  $\mu$ m. **d** Confocal illustration showing BrdU<sup>+</sup> cells (yellow) co-expressing the neuronal marker NeuN (pink) and the cellular activation marker Zif268 (green) in the granular layer of the MOB. Scale bar 5  $\mu$ m

NeuN<sup>+</sup>/BrdU<sup>+</sup> did not significantly differ between mothers exposed to the own lamb and mothers exposed to the alien lamb ( $p > 0.05$  for all comparisons).

### The 3-month-old cells in the DG are not preferentially activated after lamb exposure

To evaluate whether activation of newborn cells after lamb exposure was specific to the MOB, the percentage of Zif268<sup>+</sup>/BrdU<sup>+</sup> cells was assessed in the DG of the dorsal hippocampus. No significant differences in the volumetric density of BrdU<sup>+</sup> cells were observed between the 4 groups [Own: 0 cells/millimeter<sup>3</sup> (0–0.9); Alien: 0 cells/millimeter<sup>3</sup> (0–0); Consp: 0 cells/millimeter<sup>3</sup> (0–406); Control: 0 cells/millimeter<sup>3</sup> (0–0);  $H = 7.6$ ,  $p = 0.4$ ].

## Discussion

This study addresses the recruitment of olfactory adult-born neurons when exposed to a biologically meaningful stimulus, such as offspring, in a socially relevant context, such as motherhood. Using different markers of neuronal maturation and activation, we report that the entire population of olfactory neuroblasts and in particular, the

3-month-old neuroblasts, is preferentially activated by lamb exposure. The preferential activation is specific to olfactory neurogenesis since virtually no activation was observed in newborn neurons of the DG. Second, neither neuroblasts nor mature neurons of the MOB differentiate between familiar and unfamiliar lamb exposure.

### Exposure to lambs preferentially recruits olfactory neuroblasts

In a previous experiment, we found that interactions with the young during the first 2 days postpartum reduced the survival of olfactory neuroblasts and enhanced their maturation suggesting their involvement in the processing of infantile odors (Brus et al. 2014). This study goes one step further by showing that olfactory neuroblasts preferentially respond to lamb exposure in comparison to adult conspecific exposure. Because the first mature adult-born neurons can only be observed 3 months after BrdU injection in the MOB of ewes (Brus et al. 2013), we explored activation of this population. We found that 3-month-old cells were more activated (i.e. Zif labelling) when exposed to either the familiar lamb or an unknown lamb in comparison to exposure to an unfamiliar ewe. To broaden the validity of our data, we used another neuronal marker of



activation, c-Fos, and found similar results. The effects observed using both immediate early genes could be the consequence of a longer period of olfactory activation before sacrifice, suggested by the higher sniffing time spent by the ewe toward either the familiar or the unfamiliar lamb than toward a conspecific. However, general activation of the MOB was not enhanced by lamb exposure, as Zif268 and c-Fos immunohistochemistry alone revealed no differences in general cell activation in the MOB among groups. Hence the difference in activation between lamb and conspecific exposure was specific to newborn cells.

Activation of adult newborn neurons following lamb exposure could have been induced by any odor. However, the greater Zif activation after lamb exposure than after adult conspecific exposure indicates that the increase in responsiveness of adult-born neurons is specific to lambs. Data showing that after birth, when attraction to lambs is a priority, lamb odors are a potent olfactory stimulus producing enhanced mitral cell activity supports this view (Kendrick et al. 1992; Keverne et al. 1993; Lévy et al. 1993, 1995a). Because adult-born neurons mainly target mitral cells and are specifically responsive to lamb odors, they may participate in coding this odor in the MOB.

Similar increased activation after exposure to lambs was observed when considering the whole population of neuroblasts and the population of 3-month-old cells. We then examined the phenotype of the activated population and found that 3-month-old neuroblasts (Zif268<sup>+</sup>/DCX<sup>+</sup>/BrdU<sup>+</sup> cells) were more responsive to lamb exposure than to conspecific exposure. By contrast, virtually no mature neurons (Zif268<sup>+</sup>/NeuN<sup>+</sup>/BrdU<sup>+</sup> cells) were activated in any exposure condition. Consistent with this result, our previous study reports that the survival of neuroblasts, but not post-mitotic neurons in the MOB, is influenced by early interactions with the lamb together with an enhancement of their dendritic maturation (Brus et al. 2014). This data is reminiscent to other reports, showing that in mice, immature olfactory neurons are particularly sensitive to sensory experience (Mouret et al. 2008; Yamaguchi and Mori 2005). In addition, other reports show a preferential recruitment of immature neurons over more mature ones by odor stimulation (Belnoue et al. 2011; Magavi et al. 2005). At the functional level, electrophysiological studies report that immature neurons of the MOB or the DG are highly excitable (Nissant et al. 2009; Schmidt-Hieber et al. 2004), rendering them ideally suited for processing specific experience-related inputs. Thus, together with the literature, our results indicate that early postpartum period specifically primes immature bulbar newborn neurons to be sensitive to and recruited by lamb odors. It is possible that this priming participates in the coding of lamb odors and one would predict that once mature these neurons bear a trace of this olfactory coding.

### Exposure to the own lamb does not preferentially recruit olfactory adult-born cells

During the first hours postpartum, mothers learn the olfactory signature of their lamb, allowing the development of discriminative maternal care (Lévy and Keller 2009; Lévy et al. 2004). At the level of the MOB, electrophysiological recordings of mitral cells reveal a preferential response of some neurons to the familiar lamb odor (Kendrick et al. 1992). Interestingly, a growing body of evidence indicates that olfactory neurogenesis is required for various olfactory learning (Lazarini and Lledo 2011). Recent data suggests that adult-born neurons are critical for discriminating between similar odorants (Alonso et al. 2012). We, therefore, hypothesized that adult-born neurons generated when mothers interacted with their young will preferentially respond to the familiar lamb odor. However, neither the entire population of olfactory neuroblasts nor the 3-month-old cells showed an enhanced activation to own lamb exposure in comparison to unfamiliar lamb exposure. Previous studies showed that recruitment of olfactory adult-born neurons is age-dependent: associative learning based on odor discrimination activates specifically mature new neurons but not younger ones (Belnoue et al. 2011). Thus, it is possible that older neurons over 3 months at the time of the test would be preferentially activated by the familiar lamb's odor. Another possibility accounting for the absence of differential activation at familiar or unfamiliar lamb exposure is that adult olfactory neurogenesis could be specifically required for long-term olfactory memory (Lazarini et al. 2009; Sultan et al. 2010). Also consistent with this hypothesis is the fact that mice fathers, interacting with their pups for 2 days after birth, showed a preferential activation of olfactory adult-born neurons when exposed to 6-week-old offspring odors in comparison to non-offspring odors (Mak and Weiss 2010). Hence, specific activation of olfactory adult-born neurons to own lamb exposure could have been detected after a longer separation time with the own lamb.

Altogether our results highlight the existence of neural plasticity that occurs with motherhood, which could constitute an adaptive response to the associated high olfactory perceptual demand at that time. More specifically, they show that adult-born olfactory neurons are activated by lamb odors, contributing to the processing of infantile odors, which are involved in the attraction to any young expressed by the mother in the early postpartum period. In rodents, deficits in expression of maternal behavior have been reported after impairment of both olfactory and hippocampal neurogenesis (Larsen and Grattan 2010; Sakamoto et al. 2011). Thus, future ablation studies specifically targeting population of newborn olfactory neurons are needed in sheep to better understand the involvement of neurogenesis in maternal behavior.

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