Neurogenic correlates of an olfactory discrimination task in the adult olfactory bulb

Nathalie Mandairon,1,2 Joëlle Sacquet,1,2 Samuel Garcia,1,2 Nadine Ravel,2,3 François Jourdan1,2 and Anne Didier1,2
1Laboratoire de Neurosciences et Systèmes Sensoriels, CNRS UMR 5020, Université Claude Bernard Lyon1, 50 avenue Tony Garnier, 69366, LYON cedex 07, France
2Institut Fédératif des Neurosciences de Lyon, IFNL, France
3Institut des Sciences Cognitives, UMR CNRS 5015, 67 Boulevard Pinel, 69675 Bron Cedex, France

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Abstract
In the main olfactory bulb, stimuli are coded within the spatio-temporal pattern of mitral cells’ activity. Granule cells are interneurons that shape the mitral cells’ activity, and are continuously generated in the adult main olfactory bulb. However, the role of granule cell renewal remains elusive. We show here that an associative olfactory discrimination task reduces the survival of newborn neurons. However, when the olfactory task involves perceptually related odorants, the learning process is slower and does not induce such a reduction in the number of new neurons. Mapping newborn cells within the granule cell layer of the main olfactory bulb reveals a clustered distribution that evolves with learning as a function of odorant similarity and partly overlaps with the immediate-early gene Zif268 expression pattern. These data provide insight into the functional mechanisms underlying olfactory discrimination learning, and promote the importance of neurogenesis as a cellular basis for the restructuring of odor images in the main olfactory bulb.

Introduction
The olfactory inputs to the main olfactory bulb (MOB) are spatially segregated based on the expression of molecular odorant receptors by sensory neurons (Buck, 1996), imparting a chemotopic and dynamic activation map on the MOB on the sensory input stage (glomeruli) (Kauer & White, 2001; Korsching, 2002). When a similar glomerular map is obtained for two odorants, these two odorants are perceptually similar, attesting to the perceptual correlates of glomerular activation maps (Rubin & Katz, 1999; Linster et al., 2001; Schaefer et al., 2001a). Sensory information is then transmitted to a population of mitral cells (MCs), the output neurons of the MOB. Within the MOB, MCs activate granule cells (GRs), interneurons that, in turn, inhibit MCs through dendro-dendritic reciprocal synapses (Shepherd & Greer, 1990). These dendro-dendritic interactions mediate lateral inhibition and are thought to contribute to the synchronization of MC firing. GRs are thought to play a key role in olfactory coding by shaping the temporal aspects of spatially distributed MC responses to odors (Lowe, 2003; Schoppa & Urban, 2003; Lledo et al., 2005).

In the MOB of rodents (Lois & Alvarez-Buylla, 1994) and primates (Kornack & Rakic, 2001), new GRs are formed throughout life from neural stem cells located in the subventricular zone (SVZ) of the lateral ventricle (Alvarez-Buylla & Garcia-Verdugo, 2002), which migrate from the SVZ to the MOB and functionally integrate into the neuronal network (Carlen et al., 2002; Belluzzi et al., 2003; Carleton et al., 2003; Magavi et al., 2005). The survival of newborn GRs is dependent on sensory activity (Petraneu & Alvarez-Buylla, 2002; Rochefort et al., 2002; Mandairon et al., 2003, 2006; Yamaguchi & Mori, 2005), but the function of GRs renewal remains largely speculative (Lledo et al., 2005). A modeling study based on the activity-dependent survival of newborn GRs (Cecchi et al., 2001) suggests that they could adjust the tuning of the odor-specific spatio-temporal pattern of bulbar activity for better discrimination. This view was reinforced by data linking a low rate of neurogenesis to an impairment of olfactory discrimination (Gheusi et al., 2000; Enwere et al., 2004). Better survival of newly formed neurons is also associated with enhanced short-term olfactory memory (Rochefort et al., 2002). As is the case in other neurogenic regions (Nottebohm, 2002; Shors et al., 2002; Kempermann et al., 2004), bulbar neurogenesis may thus subserve learning-related functions, but there is no direct evidence for such a role.

In the present study, we asked whether olfactory learning modulates the rate of neurogenesis within the MOB. A discrimination learning task involving perceptually similar or dissimilar odorants was used to test the hypothesis that modulation of neurogenesis may be prominent when the task is made more difficult. Neurogenesis was assessed by counting and mapping newborn GRs in the MOB of mice submitted to the learning task. We show that: (i) learning reduces the survival of newborn neurons only when dissimilar odorants are used; (ii) the density of the newborn neurons is inversely correlated with the behavioral performances; and (iii) mapping bromodeoxyuridine (BrdU)-positive cells within the granule cell layer (GrL) of the MOB reveals clusters of newborn cells whose positions within the GrL are at least partly odor-driven and modified by the learning process. These data provide the first evidence for the influence of olfactory learning on the rate and distribution within the GrL of bulbar neurogenesis, pointing to newborn neurons as important cellular targets of olfactory learning-induced plasticity.
Materials and methods

Animals

Adult male C57Bl/6J mice (Charles River, L’Arbresles, France), aged 8 weeks at the beginning of the experiments, were housed under a 12 h light : dark cycle in an environmentally controlled room. All behavioral training was conducted in the afternoon (14:00–17:00). All efforts were made to minimize the number of animals used, and the experimental procedures were in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC), and the French Ethical Committee.

Behavioral experiments

Experimental set up

All behavioral experiments were conducted in a specially designed computer-assisted two-hole-board apparatus (40 × 40 cm), allowing detection of the beginning of each trial (when the mouse is placed in the start area facing the holes), and monitoring of the sequence and duration of nose poking into the holes (3 cm diameter, 4.5 cm deep). A polypropylene swab embedded in fine plastic mesh was placed at the bottom of the hole, covered with bedding. For trials involving odors, the swab was impregnated with 20 μL of pure odorant. The bedding was replaced after every trial.

Odorant set

The odorant set consisted of the (+) and (–) enantiomers of limonene [(+)L and (–)L] and propionic acid (PA) (Sigma-Aldrich, Saint Louis, MO, USA). Purity reported by the manufacturers was > 95% for (–)L, >97% for (+)L and 99% for PA.

Experiment 1: olfactory habituation/dishabituation

Food and water were continuously available during the course of the experiment. Mice (n = 30) were first familiarized with the experimental set up by a 3-min trial on the hole-board with no odorant. Habituation/dishabituation was assessed for two pairs of odorants: (+)L/(–)L and (+)L/PA. Each training session consisted of four successive trials (3-min duration, 15-min interval). In the first three trials, one hole was odorized with one odor of the pair. On the fourth trial, animals were exposed to the other odorant of the pair.

All data analyses on time spent sniffing during odor presentation trials were performed with Statist software (SSI, Richmond, CA, USA). Only mice that investigated the odorized hole for at least 8 s during its first presentation were included in the analysis. Data obtained for each pair of odors were averaged across animals and analysed by ANOVA followed by Tukey post hoc tests to determine whether the investigation time elicited by the test odor was significantly different from that elicited by the habituated odor during its fourth presentation. The level of significance was set to 0.05.

Experiment 2: olfactory discrimination

Shaping. Naïve mice (n = 60), different from the mice used in Experiment 1, were first trained to retrieve a reward (small bit of sweetened cereal, Kelloggs, Battle Creek, MI, USA) by digging through the bedding. The mouse was put in the start area and was allowed to dig for 2 min. During the first few trials the reward was placed on the top of the bedding of one of the holes. After several successful retrievals, the reward was buried deeper into the bedding. Shaping was considered to be complete when a mouse could successfully retrieve a reward that was deeply buried in the bedding (8–12 trials).

Conditioning. During the discrimination learning experiments, water was continuously available, but the mice were food-deprived for 8 h before the sessions. We determined whether adult mice could learn to discriminate between (+)L and (–)L [(+)L/(–)L, n = 15], which are known to elicit overlapping glomerular patterns of activity and, thus, are difficult to discriminate (Linster et al., 2001). For comparison, we tested the ability of mice to discriminate between (+)L and PA [(+)L/PA, n = 15]. Discrimination learning consisted of six sessions (one per day) of four trials (total number of trials = 24). Each of the two holes was randomly odorized with one odorant of the pair, and the reward was systematically associated with (+)L. The same pairs of odorants were used in two pseudo-conditioning groups (n = 15 each) in which the reinforcement was randomly associated with either odorant of the pair. The number and duration of visits to the holes (nose pokes) were recorded. The latency (time to find the reward) was measured as an index of learning.

Data analysis

Mice that did not perform the task (no visit to any hole for one entire session (four consecutive trials) were excluded from the experiment. For the remaining animals [conditioning (+)L/PA n = 15; (+)L/(–)L n = 10; pseudo-conditioning (+)L/(–)L n = 11; (+)L/PA n = 10], ANOVA for repeated measures were applied to the latency values to assess the learning process (time effect) and between-groups differences. For each pair of odorants, conditioned and pseudo-conditioned groups were compared by bilateral Student’s t-tests (Systat software). The level of significance was set to 0.05.

Newborn cells mapping in the MOB

BrdU administration

Mice to be used for conditioning or pseudo-conditioning were injected with BrdU (Sigma; 50 mg/kg in saline three times daily at 2-h intervals) 30 days before behavioral tests and 45 days before death.

Histology

Under deep anesthesia (pentobarbital, 0.2 mL/30 g), five mice randomly selected from each of the four behavioral groups were killed by intracardiac perfusion of 50 mL of cold fixative (paraformaldehyde 4% in phosphate-buffered saline, pH 7.4). Brains were removed, post-fixed, frozen rapidly and then stored at −20 °C before sectioning with a cryostat (Jung).

BrdU immunocytochemistry

The protocol has been previously described (Mandaion et al., 2003). Briefly, sections were incubated in Target Retrieval Solution (Dako, Glostrup, Denmark) for 20 min at 98 °C. After cooling, they were treated with pepsin (0.43 U/mL in 020 min.1-N HCl, Sigma) for 3 min. Sections were transferred to a blocking solution [5% normal horse serum (Sigma) with 5% bovine serum albumin (BSA) and 0.125% Triton X-100], and were then incubated overnight in a mouse anti-BrdU antibody (1/100, Chemicon, Temecula, CA, USA) at 4 °C followed by a biotinylated anti-mouse secondary antibody (1/200, Vector Laboratories, Burlingame, CA, USA) for 2 h. The sections were then processed through an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). Following dehydration in graded ethanol, the sections were defatted in xylene and coverslipped in DPX (Fluka, Sigma).
BrdU-positive cell mapping
All cell counts were conducted blind with regards to the mouse status. In each animal, every fifth section was analysed (thickness = 14 µm, sampling interval = 70 µm). Within each section analysed, every BrdU-positive cell was counted in the GrL of the left MOB using mapping software (Mercator, Explora Nova, La Rochelle, France) coupled to a Zeiss microscope. Maps of BrdU-positive cells were constructed as follows, inspired by the work of Schaefer et al. (2001b). The GrL was divided into 36 sectors of 10° with a reference axis drawn parallel to the most ventral aspect of the subependymal layer of the MOB. The cell density (number of labeled profiles/µm²) was calculated for each sector. Measurements were then merged into arrays of 10° × 70-µm bins (Fig. 5A and B). A z-score (normalization of the matrix to a mean = 0 and SD = 1) was calculated for each animal. The most rostral aspect of the accessory olfactory bulb served as an anatomical landmark to align the sections across animals (Matlab v.6). For visualization of the BrdU-positive cell density maps, arrays were averaged across animals within each group, and a colored image plot of the data was constructed in Matlab v.6.

Data analysis
The mean BrdU-positive cell density of each array was calculated and averaged within each experimental group. Between-groups comparisons were performed by bilateral Student’s t-tests.

Comparisons of the distribution of BrdU-positive cell density between the pseudo-conditioned groups exposed to the related and dissimilar pairs of odorants were achieved by a t-test performed on z-scored individual arrays and using a sliding area successively centered on each bin and covering 25 adjacent bins. To compare the magnitude of the changes induced by conditioning with the (+)L/(-)L. vs. the (+)L/PA pair of odorants, differences between the pseudo-conditioned and conditioned patterns needed to be normalized with regards to the pseudo-conditioned pattern. The pseudo-conditioned array was subtracted from the conditioned array for each pair (Excel software). In the resulting matrices, the number of bins having a value of BrdU-positive cell density greater than 1 SD was counted and divided by the number of bins exhibiting a BrdU-positive cell density greater than 1 SD in the corresponding pseudo-conditioned array. The 1 SD criteria was chosen on the basis of the matrices distribution histograms (not shown), as it includes populations of bins above mean values that represent a measure of the high-density areas. This ratio indicates the proportion of high-density foci found in the conditioning groups that were not present in the pseudo-conditioning groups. This index of change induced by learning was similarly calculated for the number of bins with values less than −1 SD in the subtraction-generated matrices, thus indicating the proportion of high-density foci present in the pseudo-conditioned group that were not retrieved in the conditioned group. Statistical comparisons were performed by t-tests for comparison of proportions.

Double-labeling BrdU-NeuN
Sections were treated as described above except that they were incubated simultaneously with rat anti-BrdU (1:100, Harlan Sera lab, Loughborough, UK) and a mouse anti-NeuN (1:500, Chemicon) overnight at 4°C. Immunoreactivities were revealed with a goat anti-rat antibody coupled to Texas Red (Vector Laboratories) and a horse anti-mouse coupled to FITC (DAKO). In three–four animals of each experimental group, 20–30 BrdU-positive cells were examined for NeuN double-labeling. Double-labeling was analysed by confocal scanning microscopy (Zeiss, at the Centre Commun de Quantimétrie, Université Claude Bernard-Lyon1). All labeled cells were examined along the z-axis to ensure proper detection of double-labeled cells. A percentage of double-labeled cells was calculated for each group and compared using ANOVA. The number of newly formed neurons in the GrL was extrapolated from the percentage of double-labeled cells, the BrdU-positive cell density and the volume of the GrL.

Zif268 expression mapping
Odorant stimulation
Naïve animals, different from the ones used in the behavioral procedure, were submitted to (+)L (n = 5) or PA (n = 5) stimulation 1 h before death (Inaki et al., 2002). Briefly, mice were put in a 1-L hard plastic chamber. Deodorized air was generated by compressed air passing through activated charcoal and distilled water. The deodorized air was applied at a rate of 5 L/min to the chamber from the top inlet. For odorant stimulation, the deodorized air was passed through undiluted odorant solution. The odorant-containing air was then diluted with the deodorized air (1:100) and the outlet of the flow-meter was connected to the chamber. The odorant-containing air was applied for 5 min followed by 5 min application of deodorized air. This step was repeated three times. One hour after the last odor exposure, mice were killed by perfusion as described above.

Zif268 immunocytochemistry
The MOB of odorant-stimulated mice was coronally sectioned (14 µm). Every fifth section was processed for Zif268 immunostaining. Sections were transferred to 10% normal goat serum (Sigma) with 2% BSA and 0.1% Triton X-100 for 1 h to block non-specific binding and were then incubated overnight in a rabbit anti-Zif268 antibody (1/1000, Santa Cruz Biotechnology, Santa-Cruz, CA, USA) at room temperature for 16 h. Sections were then incubated in a biotinylated anti-rabbit secondary antibody (1/200, Vector Laboratories) for 2 h. The remaining treatments were similar to those for the BrdU labeling.

Zif268 expression mapping
Zif268-positive cells were counted automatically in the GrL (Mercator Pro, Explora Nova, La Rochelle, France). Zif268-positive cell density maps were constructed using the same procedure as for BrdU-positive cell density.

The overlap between BrdU-positive cell maps and Zif268 expression maps was assessed as the number of bins exhibiting values within 15% of the highest values in both the Zif268 and BrdU-positive maps. This threshold was set as best fitted to the clusters delineated by visual inspection of the Zif268 expression maps. Because the absolute value of overlap depends on the threshold, relative overlap should be considered. T-tests for comparisons of proportions were used to compare the overlap between groups.

Results
Experiment 1: olfactory habituation/dishabituation
Two pairs of odorants were selected, one perceptually similar and the other perceptually dissimilar, based on the ability of mice to discriminate the two odorants of the pair in a habituation–dishabituation task (Linstner et al., 2001). Mice for this experiment were divided into two groups. In the first group, we tested if they were able to spontaneously discriminate between the two enantiomers of limonene (n = 13), and in the second one we tested if they were able to discriminate between (+)L and PA (n = 17).
Habituation was evident, because in the two groups there was a significant effect of trial number (Fig. 1): (+)L/–(−)L, $F_{3,24} = 14.03$, $P < 0.0001$; (+)L/PA, $F_{3,52} = 5.76$, $P = 0.002$), and the investigation response during trial 1 was significantly higher than the response during trial 3 ($P < 0.05$ for all odor pairs, Tukey) (Fig. 1). Multiple comparisons testing showed that the response to test odor was significantly higher than the response to habituated odor for the dissimilar odorants group ($P = 0.03$), but not for the similar odorants group ($P = 0.93$, Tukey), indicating that while mice discriminated between (+)L and PA, they did not discriminate between the enantiomers of limonene (Fig. 1).

Experiment 2: olfactory discrimination

Two groups of food-restricted mice ($n = 30$) were submitted to a discrimination learning task using the pair of perceptually similar odorants, the (+)L/–(−)L, or the pair of distinct odorants (+)L and PA. (+)L was positively reinforced in both groups by a food reward. Two control groups (one for each pair of odorants, $n = 30$) were subjected to the same procedure, but the reinforcement was randomly associated with either odor of the pair (pseudo-conditioning). The evolution of latency across the learning sessions is presented for conditioned and pseudo-conditioned animals with the pair of different odorants or the pair of similar odorants (Fig. 2). An ANOVA analysis using the group (conditioning vs. pseudo-conditioning) and the pair of odorants [(+L)/(−)L vs. (+)L/PA] as independent factors, and the session of learning as a dependent factor indicates a difference between conditioned and pseudo-conditioned animals (group effect, $F_{1,42} = 16.57$, $P < 0.0005$), which is modulated by the pair of odorants used (group × pair of odorants interaction, $F_{1,42} = 5.57$, $P = 0.023$). The latency decreased with learning (day effect $F_{3,210} = 7.57$, $P < 0.0005$), and this effect depends on the group (day × group interaction, $F_{3,210} = 4.68$, $P < 0.0005$). Indeed, a simple comparison between the conditioning and pseudo-conditioning groups indicates that latency decreased across sessions in the conditioning ($F_{5,115} = 10.851$, $P < 0.0005$) but not in the pseudo-conditioning group ($F_{5,95} = 0.99$, $P = 0.42$). This decrease in latency is also influenced by the pair of odorants used (day × pair of odorants, $F_{5,201} = 2.50$, $P = 0.03$). Taken together these data indicate that latency decreased selectively across learning for the conditioned animals and that this decrease is different for the two pairs of odorants used. Comparison of pseudo-conditioned vs. conditioned animals for each pair of odorants further confirms the latter conclusion. Latency in the conditioning group differed from pseudo-conditioning on Day 1 for the animals learning the pair of different odorants, and only on Day 5 for animals conditioned with the similar pair (bilateral t-tests, $*P < 0.05$, $**P < 0.001$, $***P < 0.0005$).

**Discrimination learning affects newborn cell survival as a function of odorant proximity**

Under physiological conditions, many GRs in the MOB die between 15 and 45 days after their birth in the SVZ (Petreanu & Alvarez-Buylla, 2002; Winner et al., 2002; Mandairon et al., 2006). This phenomenon is enhanced in olfactory-deprived mice (Petreanu & Alvarez-Buylla, 2002; Yamaguchi & Mori, 2005; Mandairon et al., 2006), suggesting that the long-term survival of newborn cells is dependent upon activity. To test the influence of olfactory discrimination learning on the fate of newly formed cells during this timeframe, the DNA synthesis marker BrdU was injected 30 days before behavioral training. Five days after the last training session, five animals from each experimental group were randomly selected, killed (Fig. 3A), and then subjected to BrdU-positive cells counts in GrL (see Materials and methods). BrdU-positive cell density was reduced in the...
A group conditioned with (+)L/P compared with the corresponding pseudo-conditioned group (bilateral t-test, \( P = 0.008 \)). In contrast, no such reduction was observed in the group conditioned with the pair of closely related odorants (+)L/(-)L (same test, \( P = 0.19 \)) (Fig. 3B). These changes in cell density occurred with no significant change in GrL volumes (not shown). A confocal analysis of the double-labeling of BrdU-positive cells with the neuronal marker NeuN (Fig. 3D) showed no statistical difference among the experimental groups (ANOVA, \( F_{3,8} = 2.5, P = 0.13 \)), and averaged 84 ± 7.6% (mean ± SEM). Extrapolation of the number of neurons based on the percentage of double-labeled cells obtained in each group yielded a reduction in neuronal survival in the (+)L/PA conditioned group (bilateral t-test, \( P = 0.007 \)) (Fig. 3C), which was not observed in the (+)L/(-)L conditioned group (bilateral t-test, \( P = 0.11 \)).

In order to further characterize the relationship between changes in neurogenesis and learning, we correlated latencies in the discrimination learning task of the individual animals with their respective number of BrdU-positive cells. We found a correlation on Day 3 of learning, large reductions in BrdU-labeled cell survival being associated with short latencies (Pearson correlation, \( r = -0.66, P = 0.037, n = 10 \)) (Fig. 3E). The number of BrdU-positive cells retrieved in the OB thus correlates with performance measured in the course of the learning process (Day 3), but not with the initial (Day 1, \( r = 0.02 \)) or the final level of performance (Day 6, \( r = 0.14 \)).

To assess possible differences in the sampling of the odorants due to different appetence of the mice towards the odorants used, we measured the time spent exploring each odorant of each pair. In the pseudo-conditioned groups (Fig. 4A and B), all odors were explored to the same level (\( F_{3,38} = 0.88, P = 0.45 \)). In the conditioned groups (Fig. 4C and D), the reinforced odor was explored more than the other odor of the pair (\( F_{1,28} = 49.23, P < 0.0005 \) for the similar pair; \( F_{1,28} = 56.18, P < 0.0005 \) for the different pair) (Fig. 4C and D). Comparisons between the two conditioned groups indicated that exploration times for (+)L were higher in the group learning the different pair than in the group learning the similar pair.
(F_{1,23} = 19.48, P < 0.0005). The same was true for PA vs. (-)L (F_{1,23} = 4.86, P = 0.038).

The exploration time for (+)L or for the non-reinforced odors, averaged across the six sessions, did not correlate with the number of BrdU-positive cells (r = -0.06, P = 0.85; r = 0.24, P = 0.5, respectively).

**Spatial distribution of BrdU-positive cells and learning-induced changes**

BrdU-positive cells were counted on a series of sections taken from the GrL (sampling interval = 70 μm), and each section was divided into 36 sectors of 10 ° with the reference axis drawn on the ventral aspect of the subependymal layer of the MOB (Schafer et al., 2001b) (Fig. 5A, and Materials and methods). The density of BrdU-positive cells was calculated for each sector. These data were combined for each animal in an array where each bin has the value of the labeled cell density measured in one sector. One column of the data array represents the 36 sectors of one section and the x-axis represents the rostro-caudal distance (Fig. 5B). The individual arrays were normalized by calculating a z-score and averaged within groups, before representations as color-code maps (Matlab). In all groups, BrdU-positive cells showed uneven densities within the GrL (Fig. 5D, E, G and H). An example of BrdU-positive cells labeling in two different regions of the GrL is shown in Fig. 5C. In the (+)L/(-)L pseudo-conditioned group, two main areas of the GrL showed a high density of BrdU-positive cells: one anterior, ventro-lateral; and one posterior, ventro-medial (Fig. 5D). The BrdU-positive cell map obtained from the (+)L/PA pseudo-conditioned group also showed accumulation of BrdU-positive cells in the anterior ventro-lateral and posterior ventro-medial GrL and, in addition, small clusters were scattered along the rostro-caudal axis of the dorso-medial GrL (Fig. 5E). These two maps thus exhibited both similar features and significant differences as revealed by t-test comparisons (Fig. 5F).

In the two conditioning groups, maps of clustered BrdU-positive cells (Fig. 5G and H, respectively) were also different (Fig. 5I), and these differences were not at the same locations as in pseudo-conditioning groups, suggesting that the pattern of BrdU-positive cell clustering has evolved with learning differently for the two pairs. Differences of the conditioning maps with the corresponding pseudo-conditioning maps can be visualized on color-coded maps resulting from the point-to-point subtraction of the pseudo-conditioning maps from the corresponding conditioning maps [Fig. 5J and K for (+)L/(-)L and (+)L/PA, respectively]. High-density foci of BrdU-positive cells were present after learning, but they were not found in pseudo-conditioning groups (red areas). For instance, an increased density of BrdU-positive cells could be observed in the medial and anterior part of the MOB in the (+)L/(-)L conditioned map. Conversely, some high-density foci that were present in the pseudo-conditioned groups were not present after learning (dark blue areas). A prominent difference of this type was the almost complete disappearance, in the group conditioned with (+)L/(-)L, of the posterior ventro-medial accumulation of BrdU-positive cells observed in the pseudo-conditioned group.

![Fig. 4. Mean time spent each day exploring the odorants. The two odorants of each pair are explored to the same level during pseudo-conditioning (A and B). In conditioned animals, the reinforced odorant [(+)]limonene (L)] is more explored than the other odor of the pair [(-)L or propionic acid (PA)] (C and D). Animals exposed to the (+)L/PA pair spent more time exploring the odorants than the animals exposed to (+)L/(-)L. See text for statistics.](image-url)
To address the functional significance of the learning-specific patterning of newly formed GRs, we then asked whether these changes in BrdU-positive cell distribution were quantitatively different for the two pairs of odorants. We calculated an index of learning-induced changes in BrdU-positive cell maps in which positive differences as given by the subtracted map (high-density bins present in the conditioned map and not in the pseudo-conditioned map) were normalized with regards to the number of high-density bins observed in the corresponding pseudo-conditioned map (see Materials and methods). The same was done for negative differences between conditioned and pseudo-conditioned maps (high-density foci present in pseudo-conditioned but not conditioned maps). Positive and
negative differences were considered only if they reached a value = 1 SD or = -1 SD, respectively. For both odorant pairs, the bins exhibiting such changes represented about 20% of the total number of bins forming the BrdU-positive cell pseudo-conditioned maps (22.6% and 20.1%, respectively, $P = 0.13$, t-test for comparison of proportions). The same amount of spatial reorganization thus occurred with learning for both pairs of odorants. However, within the ~20% of bins exhibiting learning-induced changes, more high-density bins appeared upon conditioning in the group trained with (+)L/(-)L than in the group trained with (+)L/PA (induction, Fig. 5L). In contrast, high-density foci present in the pseudo-conditioned group, although absent after conditioning (suppression, Fig. 5L), were about 40% more abundant for the (+)L/PA pair than for the (+)L/(-)L pair.

Acquisition of an olfactory task is thus able to shape the distribution of newborn Grs in a way that is different from exposure to the odor pairs with random reinforcement and depends on the difficulty of the discrimination task.

**Relationship between the distribution of BrdU-positive cells and the chemotopic organization of the MOB**

The clustered distribution of BrdU-positive cells in the pseudo-conditioned and conditioned groups raised the possibility of odor-driven survival of newborn cells.

Initial evidence of such an odor-related pattern is the significant differences observed between the maps obtained for the two pairs (Fig. 5F and I). Because the only difference between these groups was exposure to different pairs of odorants [(+)L/(-)L vs. (+)L/PA], the differential patterning of the BrdU-positive cells strongly suggests that the survival of newly formed cells is odor-dependent.

To further substantiate this hypothesis, BrdU-positive maps need to be compared with a 3D map of odorant-induced activity in the GrL. Such maps were not available from the literature. In order to visualize the neuronal network involved in (+)L and PA processing in the GrL, naïve mice were exposed to (+)L or PA 1 h before death, and Zif268 expression was mapped in the GrL by immunocytochemistry, using the same mapping method as for BrdU-positive cell density. We found a patterned expression of Zif268 in response to exposure to (+)L or PA (Fig. 6A and B). t-test comparisons indicated significant differences between the (+)L- and PA-induced Zif268 maps (Fig. 6C), strongly arguing in favor of an odor-specific and activity-dependent component of the expression of Zif268 in the GrL.

We then analysed the overlap between these activation maps and maps of BrdU-positive cell obtained in pseudo-conditioned and conditioned animals (see Materials and methods). The overlap between the BrdU-positive cell maps obtained in the group pseudo-conditioned or conditioned with (+)L/(-)L was greater with the (+)L-induced than with the PA-induced Zif268 map (Fig. 6D). We also analysed the overlap between the BrdU-positive cell maps obtained in the (+)L/PA groups and the (+)L- or PA-induced Zif268 maps. We found that the pseudo-conditioned BrdU-positive cell map overlapped more with the (+)L- than with the PA-induced Zif268 maps. In the learning condition, the BrdU-positive cell map showed a similar overlap with the (+)L- and PA-induced Zif268 maps (Fig. 6E).

**Discussion**

The present study addresses the influence of an olfactory discrimination task on the survival and spatial distribution of newborn neurons in the MOB. We also look at the influence of the task difficulty on learning and on the modulation of neurogenesis.

We first focused on the characterization of the discrimination performances of adult mice. Despite the fact that the two enantiomers of limonene were not spontaneously discriminated, adult mice could learn to discriminate them as shown by their ability to perform the associative task when (+)L and (-)L had to be discriminated.

The learning curve obtained in the group conditioned with the pair of related odorants exhibited a 4-day lag compared with the learning curve obtained when two distinct odors were used. This time lag was probably due to the poor ability of the animals to effectively discriminate the two enantiomers of limonene, which are perceptually similar.

Neurogenesis is a complex process, which includes proliferation, differentiation and survival or death of the newborn cells (Alvarez-Buylla & Garcia-Verdugo, 2002). In the present study, the behavioral procedure took place 30 days after labeling of dividing cells with BrdU. This time window was chosen because in a previous study (Mandairon et al., 2006), we have shown that adult-born neurons survival is dependent upon sensory inputs from 15 to 45 days after birth (Mandairon et al., 2006). In addition, this paradigm of BrdU administration prevents any effect of conditioning or pseudo-conditioning on proliferation or migration of labeled cells. Indeed, although round divisions of previously marked progenitors occur during the days following DNA synthesis marker administration, changes in the number of BrdU-positive cells induced 30 days post-BrdU can only reflect changes in survival and not in proliferation rate or migration of labeled progenitors along the rostral migratory stream, which is achieved in 10–15 days (Lois & Alvarez-Buylla, 1994; Carleton et al., 2003). Our data, however, do not exclude an effect of learning on
proliferation or migration of unlabeled progenitors (young cells formed after BrdU administration).

For the learning task involving the (+)L/PA pair, newborn cell density was lower in the conditioned group than the pseudo-conditioned group (i.e. in animals exposed to the odorants with no pairing of the reinforcement with one odorant of the pair). In contrast, animals conditioned with the pair (+)L/(-)L did not show any significant reduction in newborn neuron survival compared with the pseudo-conditioned group. As shown by the behavioral data, discrimination was more difficult for the pair of similar odorants than for the pair of distinct odorants, resulting in a slower learning process for the similar pair. A functional relationship between newborn cell survival and learning is substantiated by the correlation found between the behavioral performances in the course of the learning process, and the rate of survival of newborn cells.

All together, these data suggest an effect of the associative discrimination learning process on newborn GRs survival and the influence of the perceptual proximity of olfactory cues used in the discrimination task on these learning-induced changes in neurogenesis. One interpretation could be that more newborn cells are required to learn the more difficult discrimination task. Thus, a better survival of newborn cells may be critical for shaping MOB responses during a complex discrimination task. This is consistent with the report that any condition reducing the number of newborn neurons in the MOB alters olfactory discrimination ability (Gheusi et al., 2000; Ambrogini et al., 2004). It has been proposed that learning favors the survival of recently born cells, while older cells undergo increased death. Our data from the MOB are consistent with this view of the complex influence of learning on neuronal turnover. Therefore, an alternative interpretation of our data could be that the reduced survival of 30-day-old neurons observed following learning of the distinct odorants reflects an increased neuronal turnover that could facilitate learning.

The measure of the time spent sniffing the odors revealed that within each pair the two odorants were similarly explored by pseudo-conditioned animals, suggesting they have similar levels of appetite. In the two groups of conditioned animals, as can be expected, the learned odor was more explored. More surprising was the finding that the sniffing times were higher in the group learning the different pairs of odorants than in the group learning the similar pair. Although the reason for this difference remains unclear, a putative explanation could be that the easier task creates a higher level of motivation to do the task, inducing more time spent exploring the odors. Because olfactory stimulation influences newborn cell survival, these data raised the question of the influence of these differences in exploration time on the rate of neuronal turnover. Such influence is not supported by the absence of correlation between the time spent exploring the odors and the rate of neurogenesis. In addition, it is worth noting that only lifelong (Petreanu & Alvarez-Buylla, 2002) or long-term (several weeks) odor deprivation (Mandairon et al., 2003, 2006) or stimulation (Rochefort et al., 2002) have been reported to affect the rate of neurogenesis, while in our study the exploration of odors was in the order of magnitude of 1–3 s per session. In the context of our study, a significant effect of the exploration time on neurogenesis cannot thus be favored. Rather, our data support the hypothesis that short exposures to odors may affect neurogenesis if they are associated with a positive reinforcement.

Zif268 is an immediate-early gene (IEG) with neuronal expression, driven by activity-dependent plasticity (Knapskaja & Kaczmarek, 2004). The expression of IEGs is widely used to map neural activity in the brain, particularly in sensory systems, including the glomerular

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**Fig. 6. Zif268 expression maps in response to odorant stimulation partly overlap with BrdU-positive cell maps.**

- **(A)** Map of Zif268 expression 1 h after stimulation by (+)limonene (L).
- **(B)** Map of Zif268 expression 1 h after stimulation by PA.
- **(C)** Comparison of the (+)L- and propionic acid (PA)-induced Zif268 expression maps by t-test. The right scale gives the P-values.
- **(D)** Overlap of high-density foci of the BrdU maps obtained in the pseudo-conditioned (PC) or conditioned (C) groups exposed to (+)L/(-)L, with (+)L-induced (gray bars) or PA-induced (black bars) Zif268 expression maps. ***P < 0.005, t-test for comparison of proportions.
- **(E)** Same analysis as in (D) for the groups exposed to the (+)L/PA pair of odorants.
layer of the MOB (Inaki et al., 2002), where it maps the odor-induced pattern of activation. We found that Zif268 expression in naive animals was also a reliable marker to map odor-related activity in the GrL. We then reasoned that if BrdU-positive cells clustered in areas of the GrL involved in processing the odors to which the animals were exposed, the BrdU-positive cell map resulting from pseudo-conditioning or conditioning with the pair (+)L/–L should show more overlap with the (+)L-induced than with the PA-activation maps, assuming that (+)L and –L elicited very similar bulbar patterns of activation (Linster et al., 2001). We found this to be exactly the case, providing support to the hypothesis of a regulation of neurogenesis in areas of odor activation. For BrdU maps obtained from animals conditioned with the pair (+)L–PA, our prediction was that they would have similar overlap with the (+)L- and the PA-induced Zif268 expression pattern. This was true for conditioned but not pseudo-conditioned animals. This discrepancy may arise from overlap and/or interactions of activity-driven survival induced by the two dissimilar odors (Luo & Katz, 2001; Linster & Cleland, 2004), obscuring the relationships of the BrdU-positive cell map with the Zif268 maps of each single odor. Finally, the partial overlap between Zif268- and BrdU-positive cell maps also suggests that by recruiting new inhibitory interneurons to the OB network, learning may induce changes in the activation maps.

The functional implications of changes in the number and spatial distribution of newborn cells might be important for olfactory coding and learning. It has been proposed that olfactory coding relies on the temporal coherence of spatially distributed activity in the MOB, largely under the control of GRs (Laurent et al., 2001; Lledo et al., 2005), and it is important to note that the spatio-temporal output of the MOB is affected by learning (Kaye & Laurent, 1999; Fletcher & Wilson, 2003; Ravel et al., 2003; Martin et al., 2004). Any change in the set of GRs responding to olfactory stimulation may thus be expected to affect the functional image of the odorant within the MOB. The learning-induced changes in neurogenesis reported here provide a cellular substrate for modulating odorant representation within the MOB, which should be considered in further studies of bulbar functional plasticity.

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Abbreviations
BrdU, bromodeoxyuridine; BSA, bovine serum albumin; GRs, granule cells; GRL, granule cell layer; IEG, immediate-early gene; L, limonene; MCs, mitral cells; MOB, main olfactory bulb; PA, propionic acid; SVZ, subventricular zone.

References


