

Olfactory perceptual learning requires adult neurogenesis

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Perceptual learning is required for olfactory function to adapt appropriately to changing odor environments. We here show that newborn neurons in the olfactory bulb are not only involved in, but necessary for, olfactory perceptual learning. First, the discrimination of perceptually similar odorants improves in mice after repeated exposure to the odorants. Second, this improved discrimination is accompanied by an elevated survival rate of newborn inhibitory neurons, preferentially involved in processing of the learned odor, within the olfactory bulb. Finally, blocking neurogenesis before and during the odorant exposure period prevents this learned improvement in discrimination. Olfactory perceptual learning is thus mediated by the reinforcement of functional inhibition in the olfactory bulb by adult neurogenesis.

discrimination | mice | enrichment | olfactory bulb

Perceptual learning is an implicit (nonassociative) form of learning in which discrimination between sensory stimuli is improved by previous experience (1). For instance, animals trained on a tactile discrimination task improve their behavioral performances and in parallel, the neural representation of the stimuli is sharpened (2, 3). In the olfactory modality, perceptual learning has been shown to occur in humans (4), and an experimental model of olfactory perceptual learning has recently been proposed in rats (5). Olfactory perceptual learning is crucial for basic olfactory functions because it sets the degree of discrimination between stimuli, and thus contributes to the perceptual representation of the environment, which guides the animal's behavior. However, neural mechanisms underlying such changes of perception remain elusive. We here show that a modulation of newborn cell survival in the olfactory bulb (OB) underlies olfactory perceptual learning. We show that neurogenesis is not only involved in, but necessary for perceptual learning to occur.

We have shown that odor enrichment enhances rats' ability to discriminate between chemically similar odorants in a relatively odor-unspecific manner (5, 6). Indeed, the discrimination of a pair of similar odorants is improved by enrichment with the same odorants or with other odorants that activate regions of the OB partially overlapping with the regions activated by the discriminated pair. Even if the mechanisms underlying this learning remain unclear, it has been shown that infusions of NMDA into the OB improves odor discrimination in a manner similar to odor enrichment indicating that changes in OB processing contribute at least partially to the perceptual plasticity (5). A computational model proposed that activation of OB neurons produces widespread changes in inhibitory processing, which can underlie the observed improvement of odor discrimination (5). In support to this model, odor exposure has been shown to increase inhibition of mitral cells (7) and to increase the responsiveness of the inhibitory granule cells to odorants, as measured by expression of an immediate early gene (8).

Inhibitory neurons in the OB are continuously generated in adulthood. They are formed from neural stem cells located in the subventricular zone (SVZ) of the lateral ventricle (9), and migrate from the SVZ to the OB where they functionally

integrate into the neuronal network as granule and periglomerular interneurons (10–13). Modulation of the rate of newborn neurons formation parallels changes in discrimination induced by sensory experience (14) but the exact role of OB neuronal renewal is still unclear (15).

In this work, we studied the neural mechanisms underlying perceptual learning in mice. The experiments reported here showed that (i) daily odor enrichment improves behavioral discrimination between odorants when there is spatial overlap between the bulbar areas activated by enrichment and test odors; (ii) odor enrichment increases the survival of the newborn neurons involved in processing of experienced odors; (iii) inhibition in the OB network increases in response to odor enrichment; and (iv) newborn cells are necessary for the improvement of olfactory discrimination in response to odor enrichment. Our results reveal increased network inhibition in the OB due to OB neurogenesis as the cellular mechanism underlying olfactory perceptual learning.

Results

Perceptual Learning in Response to Enrichment Improves Discrimination. In this experiment, we used a habituation/dishabituation test (four habituation trials with the same odorant followed by a test trial with a different odorant, see *Materials and Methods*) to assess olfactory discrimination between three pairs of chemically similar odorants: +/–limonene, pentanol/butanol, and decanal/dodecanone. Discrimination was assessed before and after a 10-day enrichment period (Fig. 1A). Enrichment consisted of exposure to a pair of similar odorants (+/–limonene or decanal/dodecanone) for 1 h daily in the home cage. At the end of the enrichment period, mice were tested for discrimination between the two odorants of each of the three tested pairs. Tested pairs of odorants (+/–limonene, pentanol/butanol, and decanal/dodecanone) exhibit various degree of response overlap (as measured by 2-deoxyglucose activation maps; <http://leonlab.bio.uci.edu>) with enrichment odors (6) (Fig. 1A). Overlaps of the activation pattern of +limonene and pentanol (pairwise correlation coefficient, $r = 0.28$) are larger than overlaps between +limonene and decanal (pairwise correlation coefficient, $r = -0.06$) (5).

Significant habituation, evidenced by the reduction in investigation of the odorant across repeated presentations, was observed in preenrichment and postenrichment conditions (Fig. S1). We confirmed that before enrichment none of the three test odor pairs (+/–limonene, pentanol/butanol, and decanal/dodecanone) was discriminated by the mice ($P > 0.05$ for difference between Hab4 and Otest, Fig. 1Bi).

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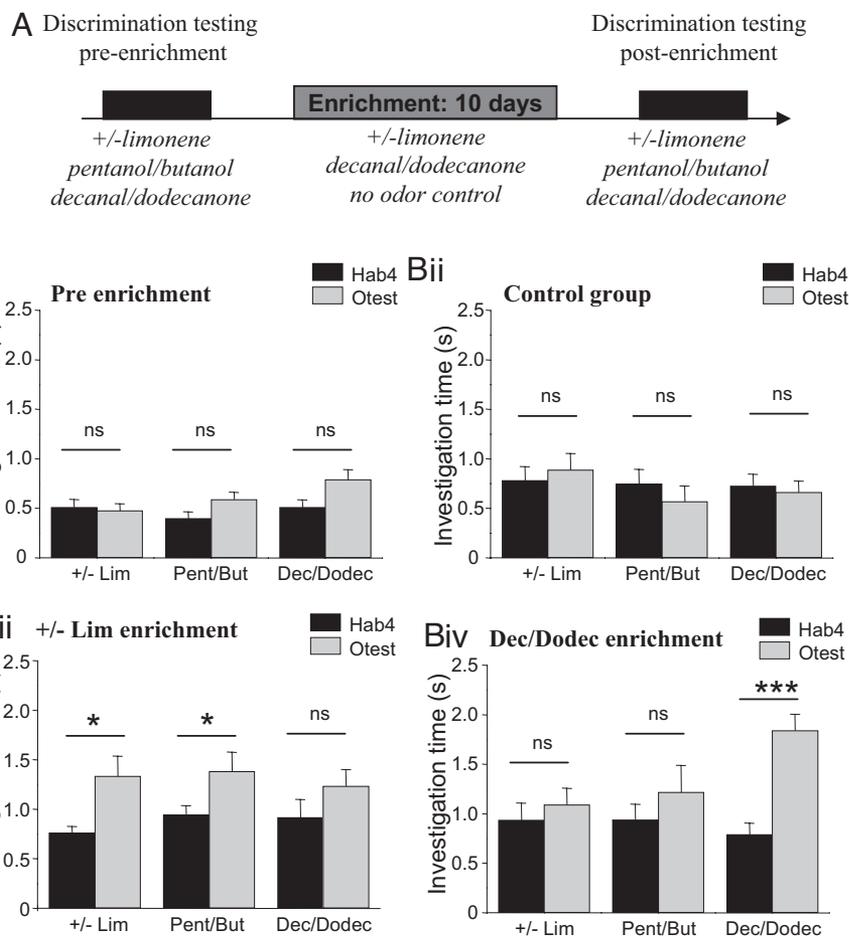


Fig. 1. Olfactory enrichment improves discrimination. (A) Design of the experiment. Spontaneous discrimination between +/-limonene, pentanol/butanol, and decanal/dodecanone was tested before and after an odor enrichment period. Experimental groups were enriched by introducing +/-limonene or decanal/dodecanone into the home cage for 1-h periods over 10 days. A control group was not enriched (no odor). (B) Behavioral discrimination before (Bi) and after (Bii-Biv) enrichment. The two odors of each pair are confused before the enrichment period (Bi), and in the control nonenriched group (Bii). After enrichment with +/-limonene, +/-limonene and pentanol/butanol are discriminated (Biii), and after enrichment with decanal/dodecanone, only this pair of odorants is discriminated (Biv). [**P* < 0.05, ****P* < 0.0001, in response magnitude between trials 4 (Hab4) and 5 (Otest)]. The data are expressed as mean values ± SEM.

Mice were then exposed to +/-limonene (*n* = 10), decanal/dodecanone (*n* = 10), or no odor (Control group, *n* = 10) and subsequently tested on the same discrimination task again. Significant differences were found between experimental groups [F (2, 475) = 15.33, *P* < 0.0001]. As expected, control mice showed no improvement of discrimination after the enrichment phase (exposure to mineral oil only) (ANOVA followed by Fisher post hoc comparison between Hab4 and Otest; *P* > 0.05 for all three odor pairs) (Fig. 1Bii). In contrast, the +/-limonene enriched group displayed a significant improvement of discrimination between +limonene and -limonene or between pentanol and butanol (*P* < 0.05), but not between decanal and dodecanone (*P* > 0.05; Fig. 1Biii). In the decanal/dodecanone enriched group the discrimination between decanal and dodecanone was improved (*P* < 0.001) but not the discrimination between the enantiomers of limonene or between pentanol and butanol (*P* > 0.05; Fig. 1Biv).

These results show that odor enrichment improves olfactory discrimination in adult mice, when the OB activation patterns of enrichment odors and test odors partially overlap.

Newborn Cell Survival Is Increased During Perceptual Learning. To test the effect of olfactory enrichment on OB neurogenesis, the DNA synthesis marker Bromodeoxyuridine (BrdU) was injected

8 days before behavioral training and 25 days before sacrifice (Fig. 2A). Because newborn neurons reach the OB ≈8–10 days after their birth and then start to differentiate (15, 16), this injection protocol allows to label the newborn neurons arriving in the OB at the beginning of the enrichment period and integrating in the network during the enrichment phase.

In the granule cell layer (GrL), the density of newborn cells differed significantly between experimental groups (+/-limonene or decanal/dodecanone enriched and no odor group) [F (2, 10) = 5.344; *P* = 0.026]. Enrichment with +/-limonene (Fisher; *P* < 0.05) and decanal/dodecanone (Fisher; *P* < 0.05) increased the density of BrdU-positive cells compared to control (Fig. 2B). In contrast, there was no effect of enrichment on BrdU-positive cell density in the glomerular layer (GL) [ANOVA, F (2, 8) = 0.753, *P* = 0.502] (Fig. 2B). Enrichment had no effect on the GrL [F (2, 9) = 0.669; *P* = 0.452] or the GL volume [F (2, 8) = 1.511; *P* = 0.277] (Fig. S2).

By using BrdU/NeuN double-labeling in the GrL (Fig. 2C and Fig. S3A) and BrdU/Calbindin double-labeling in the GL (Fig. 2D and Fig. S3B), we did not observe any effect of enrichment on the level of neuronal differentiation of newborn cells neither in the GrL (*P* > 0.05, Fig. 2C) nor in the GL (*P* > 0.05, Fig. 2D).

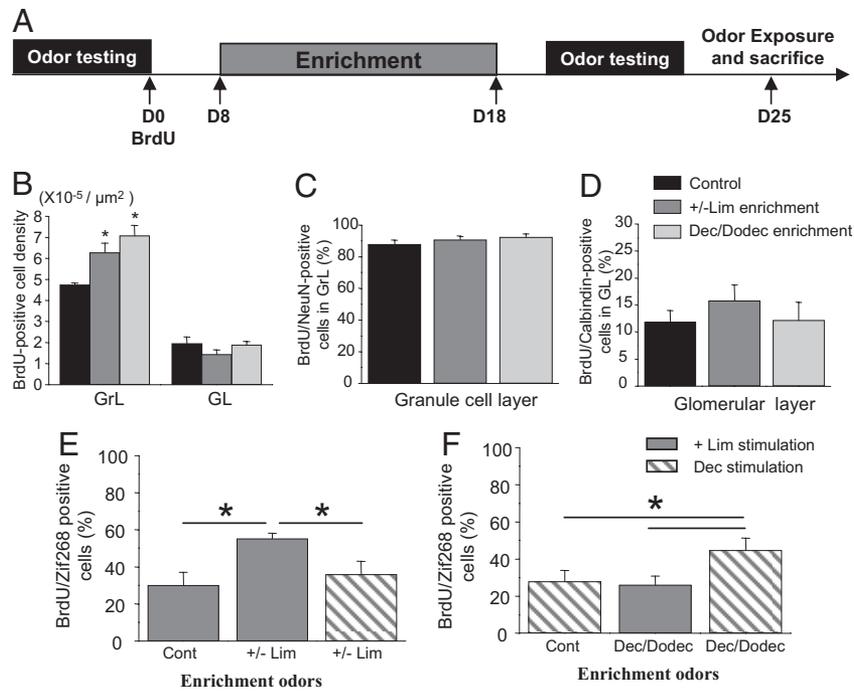


Fig. 2. Olfactory enrichment improves the survival of newborn cells involved in odor processing. (A) Experimental paradigm. BrdU was administered 8 days before the enrichment period and mice were killed 25 days after administration of BrdU. Animals were exposed to + limonene or decanal 1 h before sacrifice. (B) Olfactory enrichment induces an increase of BrdU-positive cell density in the granule (GrL) but not glomerular (GL) cell layers of the OB (*, $P < 0.05$). (C) Quantification of BrdU/NeuN double-labeling in the GrL showed no effect of the enrichment on the neuronal fate of newborn cells. (D) By using BrdU/Calbindin double-labeling in the GL, no effect of the enrichment on the phenotype of newborn cells in the GL was found. (E) Enrichment with +/- limonene increases the percentage of newborn neurons responding to +limonene compared to newborn neurons responding to decanal or to newborn neurons that respond to +limonene in control non enriched animals. (F) Similarly, enrichment with decanal/dodecanone increases the percentage of newborn neurons responding to decanal compared to newborn neurons responding to +limonene or to newborn neurons that respond to decanal in control non enriched animals. (*, $P < 0.05$). The data are expressed as mean values \pm SEM.

Newborn Neurons Are Preferentially Involved in Processing of the Learned Odor. Because the density of newborn granule but not periglomerular cells is increased by perceptual learning, we further examine the involvement of newborn granule cells in the plasticity induced by odor enrichment. We assessed the percentage of newborn granule cells expressing Zif268 as an index of cellular activation in response to odor stimulation (Fig. 2E and F and Fig. S3C) (8).

Control and enriched animals (+/-limonene or decanal/dodecanone) were stimulated with +limonene or decanal on the day of sacrifice (Table 1) to assess the responsiveness of newborn neurons to the odorant used for the enrichment or to a different odorant. +/-limonene enriched animals displayed more newborn neurons expressing Zif268 in response to +limonene than in response to decanal more Zif268-positive cells than nonenriched animals stimulated with +limonene [$F(5, 24) = 3.026$, $P = 0.029$, Fisher $P < 0.05$] (Fig. 2E). Similarly, in the decanal/dodecanone enriched animals, there was an increase of the percentage of BrdU/Zif268 double-labeled

cells after decanal stimulation compared to +limonene stimulation and to nonenriched animals stimulated with decanal (Fisher $P < 0.05$, Fig. 2F).

Taken together, these results indicate that adult-born granule neurons are preferentially recruited in the processing of the enrichment odor.

Perceptual Learning Increases GAD65/67 Expression. A computational model proposed that perceptual learning produces widespread increase in inhibitory processing that could underlie the observed increase in odor discrimination (5). To test this hypothesis, we assessed the level of GAD65/67 expression, the GABA synthesizing enzymes, by using optical density measurements on OB sections treated for immunohistochemistry of GAD65/67 and calculated a labeling index (Fig. 3A, see Materials and Methods). In both groups of enriched animals, the labeling index was increased compared to control animals [$F(2, 21) = 10.885$, $P = 0.001$; Fisher $P = 0.001$; Fig. 3B] indicating that olfactory enrichment increases GAD65/67 expression in the OB. These data suggest that odor enrichment induces an increase in GABA synthesis in the OB and could thereby increase the network inhibition.

Inhibition in the Olfactory Bulb Is Increased During Perceptual Learning. To confirm that the increase of GAD65/67 induced by perceptual learning is accompanied by an actual increase of inhibitory activity in the OB, in a separate group of mice we used paired-pulse stimulation of the lateral olfactory tract (LOT) to estimate the relative strength of granule-to-mitral inhibition in the OB (17, 18) after enrichment with +/-limonene and in control mice (Fig. 3C). ANOVA with experimental group (control and enriched) and Inter Stimulus Interval (ISI: 10, 20, 40,

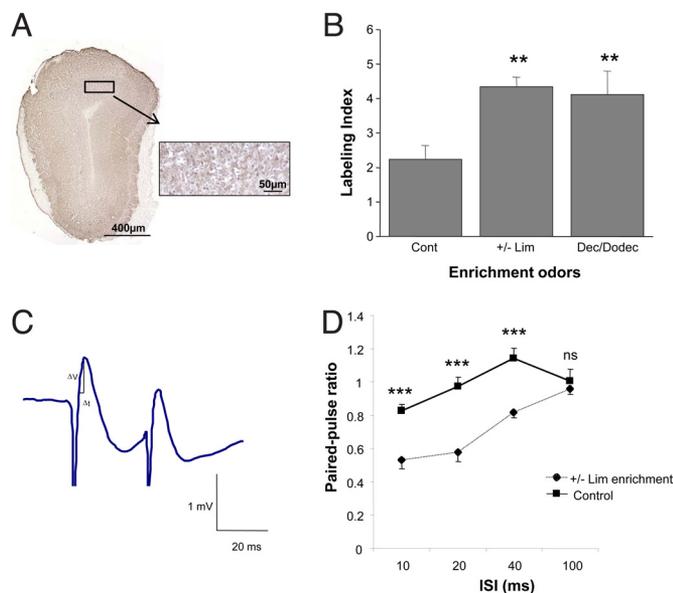


Fig. 3. Olfactory enrichment increases inhibition in the olfactory bulb. (A) Example of GAD65/67 immunolabeling in the OB. (B) Enrichment with +/-limonene or decanal/dodecanone increases the expression of GAD65/67 in the granule cell layer of the OB compared to control nonenriched animals. (**, $P < 0.001$ for difference from control group). (C) Paired-pulse stimulation. The graph shows an example of population EPSPs in the granule cell layer in response to two LOT stimulations separated by 20 ms. The response to the second pulse is significantly decreased. (D) Paired-pulse inhibition quantified by the ratio between the rising slopes of responses to the second and first LOT pulse in control and +/-limonene enriched mice. The data are expressed as mean values \pm SEM.

and 100 ms) as main factors showed a significant effect of group [$F(1, 142) = 39.296, P < 0.001$], ISI [$F(3, 142) = 9.895, P < 0.001$] and a significant interaction between group and ISI [$F(3, 142) = 5.188, P < 0.01$]. Paired-pulse inhibition was significantly larger in enriched as compared to control mice at 10, 20, and 40-ms delay ($P < 0.001$) but not at 100-ms delay ($P > 0.7$) (Fig. 3 C and D) indicating stronger inhibitory processes in the enriched OB. Before the electrophysiology experiment, we verified that enriched mice used in this experiment were able to discriminate the +/-limonene [$F(1, 72) = 6.864, P < 0.02$] whereas control mice were not [$F(1, 32) = 2.393, P > 0.05$]. Significant habituation for both groups was observed (difference between the first and the last habituation trial; $P < 0.05$ in all cases).

These data strengthen previous results on GAD65/67 expression and show an increase of functional inhibitory activity accompanying the improvement of olfactory discrimination after enrichment.

Neurogenesis Is Necessary for Perceptual Learning. Perceptual learning induces an increase of neurogenesis and an increased level of inhibition in the OB. Next, we tested how essential neurogenesis is for perceptual learning. For that purpose, we infused the mitotic blocker cytosine arabinoside (AraC) in the SVZ, which has been shown to block the division of constitutively proliferating cells (19) and as a consequence to inhibit neurogenesis. The AraC treatment was started 10 days before the enrichment and was maintained during the whole enrichment period (Fig. 4A).

After +/-limonene enrichment, AraC-treated mice had a strong reduction of BrdU-positive cell density in the OB compared to saline injected control mice [$F(1, 5) = 8.838, P = 0.031$] (Fig. 4B). A nonsignificant trend toward a reduction of BrdU-positive cell density was observed in the dentate gyrus of the hippocampus in AraC-treated, +/-limonene enriched mice

(Fig. S4). The volume of the OB was not significantly modified by the treatment (Fig. S5). The strong reduction of OB neurogenesis induced by the AraC treatment had no effect on habituation memory, as evidenced by the habituation curves obtained in both groups (Fig. S6 Ai and Aii). However, inhibition of OB neurogenesis blocks perceptual learning (Fig. 4C). Indeed, Saline and AraC-treated groups behaved differently [$F(1, 177) = 14.89, P < 0.0001$]. Enriched mice treated with AraC did not discriminate any of the two odor pairs tested after the enrichment period [$F(4, 47) = 5.97, P < 0.0001$, Fisher between Hab4 and Otest, $P = 0.87$ for +/-limonene; $F(4, 39) = 2.71, P < 0.05$, Fisher $P = 0.48$ for pentanol/butanol; Fig. 4Ci], whereas saline treated enriched mice did [$F(4, 45) = 11.89, P < 0.0001$ Fisher $P = 0.004$ for +/-limonene; $F(4, 51) = 3.69; P < 0.001$; Fisher $P = 0.025$ for pentanol/butanol; Fig. 4Cii]. This result indicates that neurogenesis is required for perceptual learning. Saline and AraC treated mice significantly discriminated between mineral oil and limonene (Fig. S6B) indicating the absence of nonspecific effects of AraC. In addition, AraC treatment did not affect locomotor's activity as measured during a 2-min trial on an open field [$F(1, 15) = 1.429, P = 0.25$].

To further investigate whether newborn neurons support the increase of inhibition in the OB, we analyzed the expression of GAD65/67 in the OB of enriched mice after neurogenesis inhibition. GAD65/67 expression is lower in the AraC group compared to saline [$F(1, 14) = 15.789, P = 0.001$, Fig. 4D] indicating that increased neurogenesis contributes to the enhancement of bulbar inhibition during perceptual learning.

Discussion

Perceptual learning is evidenced by an improvement of discrimination due to prior experience and is a crucial feature of sensory system, which adjusts the level of discrimination between stimuli to a changing environment (4). We show here that the integration of newborn cells into the OB neural network is necessary for perceptual learning to occur in the olfactory system. The increase in newborn granule cell survival observed after enrichment is necessary for the increase in inhibitory activity in the OB, as evidenced by GAD65/67 immunohistochemistry and electrophysiology, and ultimately leads to better discrimination of highly similar odorants.

We confirmed that, similar to our previous observations in rat (5, 6), odor enrichment in mice affects the perception of odorants that activate at least partially overlapping regions of the OB.

Our computational model of the OB predicted that the perceptual effects of olfactory enrichment depend on strengthening of inhibitory inputs onto mitral cells. Because mitral cells are modulated by granule and periglomerular interneurons in the OB, which are the target of adult neurogenesis, we assessed the effect of a 10-day odor enrichment on OB neurogenesis. We found an increase in the number of BrdU-positive cells in the granule cell layer of odor enriched groups compared to the control group. Due to our BrdU protocol, this increase likely reflects an enhancement of cell survival rather than an increase in proliferation or migration (20, 21). Furthermore, newborn neurons respond preferentially to the odorant used for the enrichment. This result is in agreement with a previous study showing that the response of newborn granule cells is altered by olfactory experience in a stimulus-specific manner (13) and further suggests that newborn neurons support the learning-induced improvement of discrimination.

Correlative studies have suggested a role of neurogenesis in associative discrimination learning (14, 22, 23), as well as an impairment of spontaneous discrimination due to a low rate of neurogenesis (19, 24). However, a recent study reported no effect of blockade of neurogenesis on olfactory discrimination learning (25). Those contradictory results may be related to the

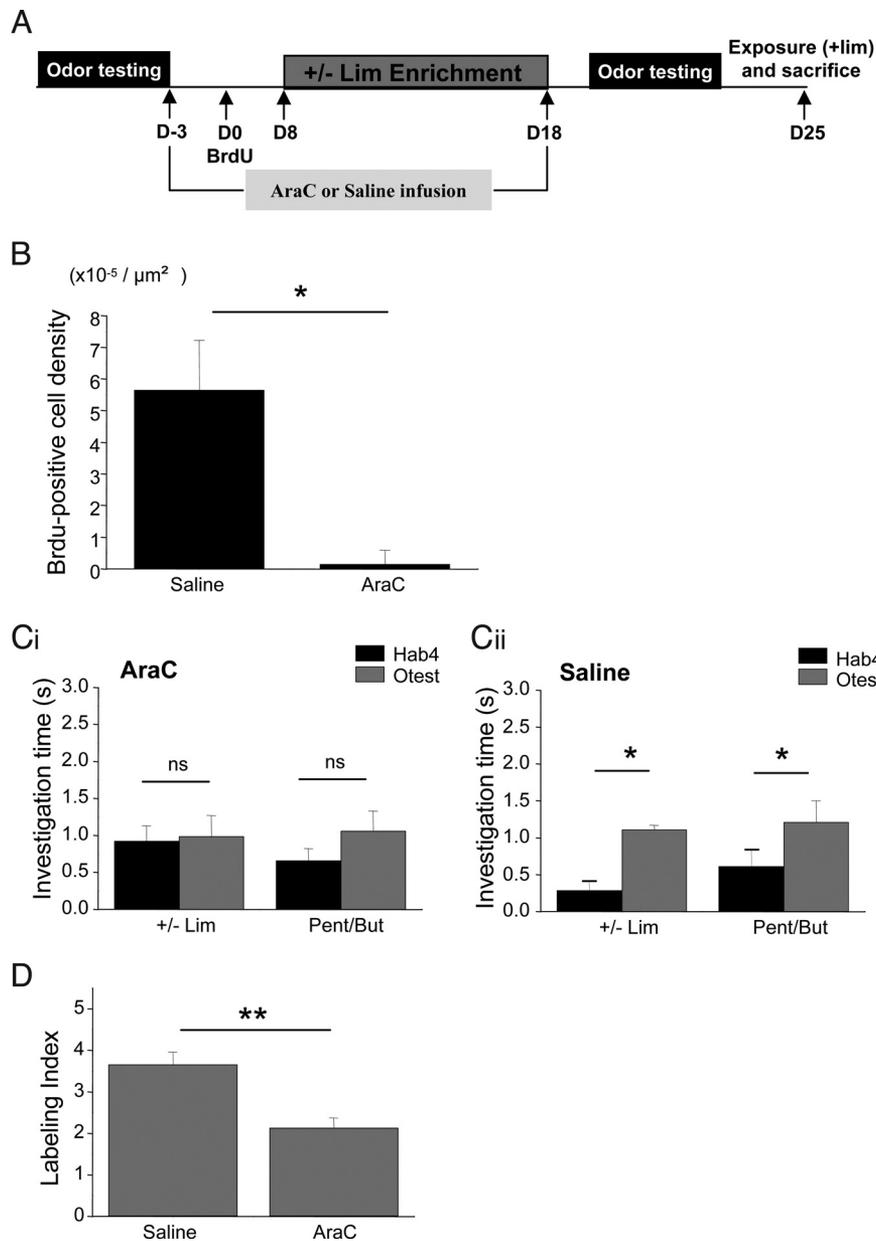


Fig. 4. Neurogenesis is necessary for perceptual learning. (A) Experimental design. Saline or AraC was locally infused 3 days before the administration of BrdU and lasting for 21 days. (B) +/- limonene enriched mice that received AraC have a significant reduction of BrdU-positive cell density in the granule cell layer of the OB. (C) The strong reduction of bulbar neurogenesis in the AraC group blocks the enrichment-induced improvement of discrimination that occurs in the saline group (*, $P < 0.05$; **, $P < 0.001$, ***, $P < 0.0001$). (D) The expression of GAD65/67 in the granule cell layer of the OB is decreased in the AraC group compared to the saline group. (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). The data are expressed as mean values \pm SEM.

difficulty of the discrimination task used. Indeed, the more difficult the task, the more it seems to require modulation of newborn neuron survival (14). An easy task (25) is performed with a low level of neurogenesis, in accordance with our data in AraC treated animals. Our experiment, by using an ecological model of learning and testing discrimination of odorants with differential degrees of similarity, allows a fine and precise evaluation of olfactory discrimination and unravels the important role of neurogenesis in olfactory learning and perception. We further found that perceptual learning is accompanied by increased inhibition evidenced by increased in GAD65/67 expression and paired-pulse inhibition. Paired-pulse inhibition and GAD65/67 expression assessment suggest an overall increase of inhibition within the granule cell layer. This observation is

compatible with the specific improvement of discrimination for the enriched odorants: the network involved in processing of individual odorants is specific but largely distributed across the OB (26) due to 1- activation of several glomeruli and 2- lateral dendrites of mitral cells running several hundred micrometers across the granule cell layer (27). As a consequence, even relatively localized changes in granule cell activation can create widespread changes in inhibitory inputs to mitral cells, as shown by computational modeling (5). This is in line with previous work showing a widespread increase in responsiveness of granule cells after perceptual learning (8).

We now propose that continuous integration of new GABAergic interneurons in the OB provides a way by which learning increases the inhibitory activity by increasing the density of

newborn interneurons. Modulation of neurogenesis thus adjusts bulbar odor representation, which in turn allows an improvement of olfactory discrimination.

In conclusion, relevant sensory input during learning allows an odor-specific increase of survival of newborn neurons. Neurogenesis sustains the ability of the adult network to adapt information processing to relevant ethologic needs.

Materials and Methods

Animals. Sixty adult male C57BL/6J mice (8 weeks old, Charles River, L'Arbresles, France) were used in this study. Thirty mice were involved in the first experiment including behavioral testing, quantification of newborn cells and assessment of GAD65/67 expression. A second cohort of 10 mice was used in the electrophysiology experiment. Finally, 20 more mice were used in the AraC experiment. All behavioral training was conducted in the afternoon (14:00–17:00). All efforts were made to minimize the number of animals used and their suffering during the experimental procedure in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the French Ethical Committee.

Behavioral Experiments

Enrichment. Swabs containing 100 μ L of pure odorant were placed in tea balls hanging from the cover of the cage. Odorants were presented daily for 1 h during 10 days. Ten mice were enriched with +/-limonene and 10 other mice with decanal/dodecanone. Control mice were housed under the same conditions except that tea balls contained mineral oil ($n = 10$).

Discrimination Testing. We tested the discrimination of +/-limonene, pentanol/butanol, and decanal/dodecanone by using an olfactory habituation/dishabituation task (Table S1). A test session consisted of one presentation of mineral oil then four odor presentations of the habituation odor, followed by one presentation of test odor. Investigation time of the Otest significantly different from that of Hab4 indicated discrimination (SI Methods).

Newborn Cells in the OB. Bromodeoxyuridine (BrdU) administration. Mice were injected with BrdU (Sigma) (50 mg/kg in saline, 3 \times at 2-h intervals), 8 days before the beginning of the enrichment period (25 days before sacrifice).

Sacrifice and BrdU immunohistochemistry. Mice taken randomly from each experimental group were stimulated with 100 μ L of pure +limonene ($n = 5$) or decanal ($n = 5$) before sacrifice and BrdU immunohistochemistry was carried on as described in SI Methods and (28).

Morphometry and BrdU-positive cell quantification. BrdU-positive cell densities were assessed in the GL and GrL of 24 sections of the left OB in all experimental groups and on five sections of the dentate gyrus. Cell counting procedures are detailed in SI Methods.

Double Labeling Immunohistochemistry and Analysis. BrdU, NeuN, calbindin, and Zif268-positive cells were detected by using rat anti-BrdU (1:100, Harlan Sera lab), mouse anti-NeuN (1:500; Chemicon), rabbit anti-calbindin (1:500; Chemicon), and rabbit anti-Zif268 antibody (1:1000; Santa Cruz Biotechnology) (SI Methods).

GAD65/67 Expression in the GrL. GAD65/67 immunohistochemistry (anti-rabbit GAD65/67 (1/750, Chemicon), was performed as described in SI Methods, by using optical density measurements (Morpho Expert, Explora Nova).

Paired-Pulse Inhibition. Electrophysiology was performed on control and +/-limonene enriched mice ($n = 2$ per group). Bipolar stimulation electrodes (5–10 MOHms, A-M Systems) were placed in the LOT (4.7 mm anterior; 3.4 mm lateral; 5.2 ventral from bregma). For recording of field potentials in the OB, a monopolar tungsten electrode (5–10 MOHms) was lowered into the approximate center of the OB until the population EPSP typical in the granule cell layer in response to LOT stimulation was observed. For paired-pulse stimulation, two 0.3-ms pulses were delivered at interstimulus intervals of 10, 20, 50, and 100 ms. Paired-pulse stimulation was quantified by measuring the ratio of the rising slope of the evoked potentials in response to the second and first pulse. Analysis was done on the paired pulse ratio of individual stimulations with experimental group (control and enriched) and interstimulus interval (10, 20, 50, and 100 ms) as main factors. For more details see SI Methods.

Neurogenesis Blockade. Mice were stereotaxically implanted with an osmotic pump (Alzet; anteroposterior, +1.2 mm; lateral, +0.9 mm, dorsoventral, -3 mm). Mice were injected in the SVZ with cytosine arabinoside AraC (4% in 0.9% saline, Sigma) ($n = 10$) or saline solution ($n = 10$) at a flow rate of 0.25 μ L/h (SI Methods).

Statistical Analysis. Results were expressed as mean \pm SEM. Differences between groups were assessed by using ANOVAs followed by *posthoc* comparisons with Fisher least significant difference test when appropriate (Systat statistical software). For all comparisons, values of $P < 0.05$ were considered significant.

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