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ORIGINAL ARTICLE

Role of Adult-Born Versus Preexisting Neurons Born at P0 in Olfactory Perception in a Complex Olfactory Environment in Mice

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Abstract

Olfactory perceptual learning is defined as an improvement in the discrimination of perceptually close odorants after passive exposure to these odorants. In mice, simple olfactory perceptual learning involving the discrimination of two odorants depends on an increased number of adult-born neurons in the olfactory bulb, which refines the bulbar output. However, the olfactory environment is complex, raising the question of the adjustment of the bulbar network to multiple discrimination challenges. Perceptual learning of 1 to 6 pairs of similar odorants led to discrimination of all learned odor pairs. Increasing complexity did not increase adult-born neuron survival but enhanced the number of adult-born neurons responding to learned odorants and their spine density. Moreover, only complex learning induced morphological changes in neurons of the granule cell layer born during the first day of life (P0). Selective optogenetic inactivation of either population confirmed functional involvement of adult-born neurons regardless of the enrichment complexity, while preexisting neurons were required for complex discrimination only.

Key words: adult neurogenesis, olfaction, optogenetic, perceptual learning, structural plasticity

Introduction

The rodent olfactory bulb (OB) is supplied during adulthood with new inhibitory interneurons (Altman 1969; Lois and Alvarez-Buylla 1994; Ming and Song 2005) that shape the message carried outside the OB by the mitral cells (Winner et al. 2002; Malvaut and Saghatelian 2016). These adult-born neurons play a central role in tuning the animal's discrimination abilities through perceptual learning (Mandairon et al. 2006b; Moreno et al. 2009). Perceptual learning is defined as a significant improvement in discrimination abilities induced by previous non-reinforced experiences (Gilbert et al. 2001), reflecting a dynamic adjustment by which animals discriminate relevant stimuli within their immediate environment. In the context of olfaction, mice can improve their performances of discrimination between perceptually similar odorants in a relative odor-specific manner thanks to passive enrichment with these odorants (Mandairon et al. 2006b; Moreno et al. 2009). This perceptual learning not only increases adult-born neuron survival and their spine density (Daroles et al. 2015; Mandairon et al. 2018) but requires their presence in the OB (Moreno et al. 2009). Importantly, in order to correctly guide the animal's behavior within a natural environment, the olfactory system needs to cope with its complexity, that is an environment in which multiple odorants are present within the same time window. What part do adult-born neurons play in perceptual learning when a complex olfactory environment challenges the network? In other words, does complex learning require more adult-born neurons and/or an enhanced structural plasticity of these neurons? Moreover, in order to understand the specificity of the role of adult-born neurons, it is crucial to compare their functional properties and structural plasticity with those of neurons born during the ontogenesis of the OB [labeled at P0 and so-called hereafter preexisting neurons (Bayer 1983)].

Newly integrated adult-born cells show specific electrophysiological properties compared with resident neurons (formed by a mix of older adult-born neurons and preexisting neurons) such as increased excitability, increased long-term potentiation (Carleton et al. 2003; Nissant et al. 2009), as well as a higher responsiveness to odorant stimulation (Livneh et al. 2014). Moreover, following olfactory enrichment in adulthood, adult-born neurons become more selective to the enriched odorants and thus acquire functional features distinct from those of older resident neurons (Magavi et al. 2005; Livneh et al. 2014).

The newly integrated adult-born neurons also show specific response properties compared specifically with preexisting neurons (Lemasson et al. 2005; Valley et al. 2013; Breton-Provencher et al. 2016). Recent studies using chemo- or optogenetic manipulations on adult-born versus preexisting neurons showed the differential involvement of preexisting versus adult-born neurons in reward-associated olfactory learning (Muthusamy et al. 2017; Grelat et al. 2018). However, the respective roles of adult-born compared with preexisting neurons in other learning contexts still remain unclear.

To answer these questions, we used a perceptual learning paradigm in different configurations in terms of complexity. More precisely, young adult mice were exposed to 1, 2, 3, or 6 pairs of odorants during the enrichment period. We assessed the effect of these different enrichment protocols on mice ability to discriminate. We also assessed adult-born cell survival, responsiveness to the learned odorants, and

structural plasticity. In addition, we analyzed the uniqueness of adult-born neuron plasticity by comparing it with that present in preexisting neurons. Finally, to determine the functional role of each population on discrimination performances after perceptual learning, we measured the effect of selective optogenetic inactivation of the populations of preexisting or adult-born neurons on learned discrimination.

Materials and Methods

Animals

A total of 103 inbred male C57BL/6J young adult mice born and raised in our laboratory were used in this study. Mice were housed in a controlled environment under a 12-h light/dark cycle with food and water *ad libitum*. All behavioral training (enrichment) was conducted between 8 AM and 8 PM, while behavioral testing was conducted in the afternoon (12 AM–7 PM) on young adult mice aged 2–3 months. 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 24 November 1986 (86/609/EEC). Protocols were approved by the French Ethical Committee [DR2013–48 (vM)]. Mice were obtained from multiple independent litters over several months, and mice from the same litter were assigned as much as possible to different experimental groups.

Overview of the Experiment

A total of 5 experimental groups of mice were formed based on the number of odorant pairs used for the enrichment (from 0 to 6 odorant pairs; Fig. 1). Using a habituation/cross-habituation test, we assessed each group for their discrimination performances, adult-born cell survival using Bromodeoxyuridine (BrdU) injections, and structural plasticity using a DsRed-expressing lentivirus injection at P0 for preexisting neuron labeling and a green fluorescent protein (GFP)-expressing lentivirus injection at P60 for adult-born neurons labeling followed by *ad hoc post mortem* fine neuronal morphology analysis. Mice were sacrificed 85 days after birth (Fig. 1).

Detailed Procedures

Behavioral Experiments

Enrichment. Odor enrichment consisted of exposure to odorant pairs (from 1 to 6, depending on the experimental group; Fig. 1) for 1 h per day over 10 consecutive days. For each odorant pair, the 2 odorants were presented simultaneously on 2 swabs containing 100 μ L of pure odorant placed in 2 tea balls hanging from the cover of the cage. For multiple enrichments, odorant pairs were presented with an interval of at least 1 hour. Odorant pairs were presented in the same order during the 10-day enrichment for a given cage, but this order was randomized between cages. The different experimental groups were as follows: Group 1 enriched with +lim/–lim; Group 2 with +lim/–lim and dec/dodec; Group 3 with +lim/–lim, dec/dodec, and acetic a./propionic a.; Group 4 with +lim/–lim, dec/dodec, acetic a./propionic a., buta/penta, propyl/butyl, and +terp/–terp (Fig. 1 and Supplementary Table 1). Control non-enriched (NE) mice were subjected to the same protocol and under the same conditions except that the tea balls contained only mineral oil.

Olfactory habituation/cross-habituation test. We used a habituation/cross-habituation test to assess olfactory discrimination

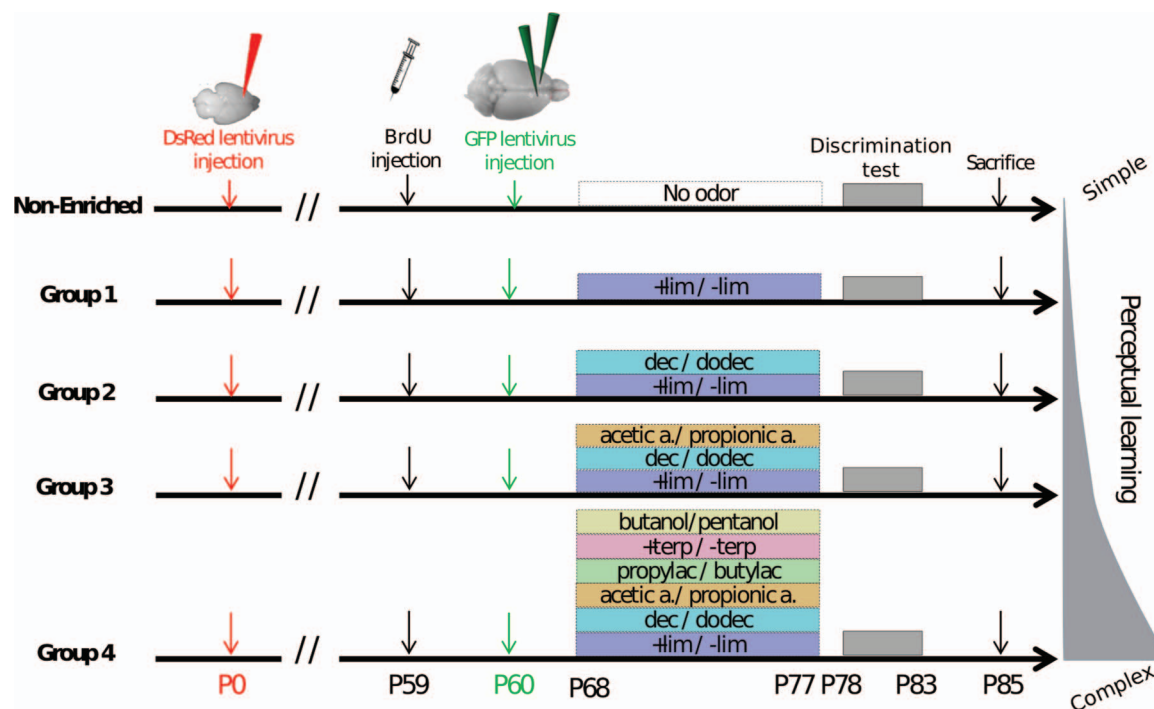


Figure 1. Experimental design. In mice, preexisting neurons (born during ontogenesis) were labeled by injecting DsRed-expressing lentivirus in the lateral ventricle at post-natal day 0 (P0). Adult-born neurons were labeled by injecting BrdU (i.p.) at P59 and GFP-expressing lentivirus in the subventricular zone at P60 (2 months old). Then, 5 groups of mice were formed depending on the number of odorant pairs used for the 10-day enrichment period (control NE: Group NE; enriched with 1 odor pair: Group 1; enriched with 2 odorant pairs: Group 2; enriched with 3 odorant pairs: Group 3; enriched with 6 odorant pairs: Group 4). Animals were enriched for 1 h per day with each odorant pair. After the enrichment period, mice were tested on their ability to discriminate the odorants of each odorant pair using an olfactory habituation/cross-habituation test. Mice were sacrificed 85 days after their birth.

(Moreno et al. 2009). Briefly, this task assessed whether mice were able to spontaneously discriminate between 2 odorants by habituating them to 1 odorant and measuring their cross-habituation to the second odorant. We tested the discrimination between the 2 odorants of each pair used for the enrichment (Group 1 was tested for discrimination between +lim/–lim; Group 2 +lim/–lim and dec/dodec; Group 3 +lim/–lim, dec/dodec, and acetic a./propionic a.; Group 4 +lim/–lim, dec/dodec, acetic a./propionic a., buta/penta, propyl/butyl, and +terp/–terp). All experiments took place in a clean cage similar to the home cage. Odorants were presented by placing 60 μ L of the diluted odorant (1 Pa) (see Supplementary Table 1) onto a filter paper (Whatman #1) that was put inside a tea ball hanging from the side of the cage. A behavioral session consisted of 1 presentation of mineral oil then 4 odorant presentations of the habituation odorant (Ohab1–4) followed by 1 presentation of the test odorant (Otest). Each odorant presentation lasted 50 s and was separated by 5 min. Each odorant of each pair was used alternatively as the habituation or test odorant. Investigation time (defined as the mouse being maximally 1 cm away from the tea ball and actively sniffing in its direction) of each odorant was manually recorded, and a difference between Ohab4 and Otest indicated discrimination.

Adult-born Neuron Quantification in the OB

BrdU injection. Mice were injected with BrdU (Sigma) (50 mg/kg in saline, 3 \times at 2-h intervals, i.p.) 9 days before the beginning of the enrichment period (27 days before sacrifice) in order to have a cohort of labeled adult-born cells in the OB at the beginning of

the enrichment period (Fig. 1) as previously described (Moreno et al. 2009).

Sacrifice. To investigate the expression of the immediate-early gene *Zif268* in response to the learned odorants, enriched mice were exposed to 100 μ L of pure learned odorants (odorants used for the enrichment presented simultaneously) for 1 h using tea balls. A control NE group (NE₁–NE₄) corresponding to each experimental group (group 1–4) was exposed to the same odorants on the day of sacrifice. These NE groups were composed of mice randomly selected from the NE group presented in Figure 1. Then, 1 h after the end of odorant stimulation, the mice were deeply anesthetized (pentobarbital, 0.25 mL/30 g) and killed by intracardiac perfusion of 40–50 mL of fixative (4% paraformaldehyde in phosphate buffer, pH 7.4). Their brains were removed, postfixed, cryoprotected in sucrose (20%), frozen rapidly, and then stored at –20 °C. Brains were sectioned with a cryostat (Reichert-Jung, NuBlock) at 14 μ m and 40 μ m (3 \times 14 μ m followed by 2 \times 40 μ m sectioning) for cell counts and morphological analysis, respectively.

BrdU immunohistochemistry. 14 μ m thick sections from mice randomly selected in each group were incubated in Target Retrieval Solution (Dako) for 20 min at 98 °C. After cooling, they were treated with pepsin (0.43 U/mL in 0.1 N HCl, Sigma) for 3 min. Sections were transferred to a blocking solution [5% normal horse serum (Sigma) with 5% Bovine Serum Albumine (BSA) and 0.125% Triton X-100] and were then incubated overnight at 4 °C in a mouse anti-BrdU antibody (1/100, Chemicon), followed by a biotylated anti-mouse secondary antibody (1/200, Vector Laboratories) for 2 h. The sections were then processed

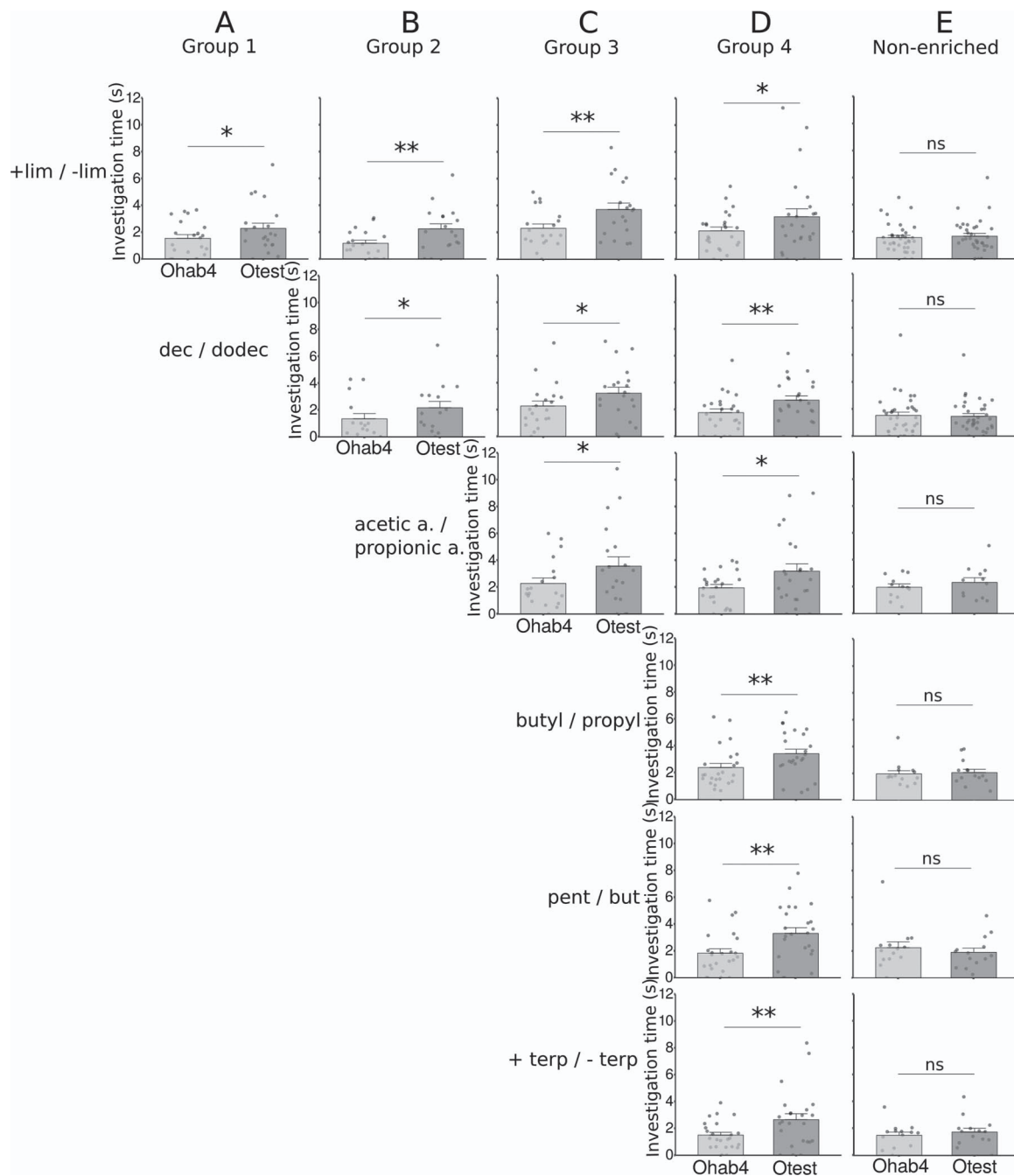


Figure 2. Increasing the complexity of perceptual learning leads to the discrimination of more odorant pairs. Only the last habituation trial (Ohab4) and the test trial (Otest) are represented to illustrate discrimination. (A) After enrichment with +lim and -lim (Group 1), the investigation time during the test trial is superior to that of the Ohab4 trial, indicating discrimination between these 2 odorants (see [Supplementary Table 2](#) for detailed statistics). (B) A similar result is observed after enrichment with +lim/-lim and dec/dodec (Group 2) indicating discrimination between the 2 odorants of the 2 pairs. (C) Enrichment with +lim/-lim, dec/dodec, and acetic a./propionic a. (Group 3) allows discrimination between the 2 odorants of the 3 pairs. (D) Enrichment with 6 pairs of odorants (Group 4), that is +lim/-lim, dec/dodec, acetic a./propionic a., propyl/butyl, pent/but, and +terp/-terp, allows discrimination between the 2 odorants in all 6 pairs. (E) In NE control animals (NE Group), no difference is observed between Ohab4 and Otest, for any of the 6 odorant pairs (+lim/-lim, dec/dodec, acetic a./propionic a., propyl/butyl, pent/but, and +terp/-terp) indicating no discrimination. * $P < 0.05$. ** $P < 0.01$. Data are expressed as mean \pm SEM, and all data points are represented. Each dot represents a trial.

through an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). After dehydration in graded ethanols, the sections were defatted in xylene and cover-slipped in DEPEX (Sigma).

Triple-labeling immunohistochemistry. To determine the phenotype of BrdU-positive cells in the OB and their functional involvement, triple labeling was performed using rat anti-BrdU (1:100, Harlan Sera Laboratory), mouse anti-NeuN (1:500, Chemicon),

and rabbit anti-Zif268 (1:1000, Santa Cruz Biotechnology) antibodies. Appropriate secondary antibodies coupled with Alexa 546 for revelation of BrdU, Alexa 633 for NeuN, and Alexa 488 for Zif268 were used (Molecular Probes).

Cell counting. All cell counts were conducted blind with regard to the experimental mouse group. BrdU data were collected with the help of a mapping software (Mercator Pro, Explora Nova), coupled with a Zeiss microscope. The BrdU-positive cells were counted in the entire granule cell layer of the OB on about 9 sections (14 μm thick, 216 μm intervals) of 5–8 mice/group. The number of positive cells was divided by the surface of the granule cell layer to yield the total density of labeled cells (labeled profiles/ μm^2).

Triple-labeled sections were examined using pseudo-confocal scanning microscopy equipped with an Aptomax (Zeiss). BrdU-positive cells were examined for co-labeling with NeuN and Zif268 (30 cells/animal, $n = 3$ –9 animals/group), yielding a percentage of activated neurons (Zif268⁺) over the whole adult-born neuron population (BrdU⁺ and NeuN⁺).

Neuronal Morphology Analysis

Preexisting neuron labeling. At P0, mice were anesthetized on ice and injected in the lateral ventricle with 1 μL of Lenti-PGK-DsRed lentivirus (6.11×10^8 UI/ml, Lenti-PGK-DsRed was generated by the Functional Neurogenetic and Optogenetic platform of the Lyon Neuroscience Research Center by excision of the GFP sequence from the Lenti-PGK-GFP and its replacement by a DsRed sequence). The injection was done using a glass micropipette linked to a Hamilton syringe connected to a programmable syringe controller (infusion rate: 0.2 $\mu\text{L}/\text{s}$).

Adult-born neuron labeling. Prior to surgery, the same mice previously injected at P0 were anesthetized at the age of 2 months with an intraperitoneal cocktail injection of 50 mg/kg ketamine and 7.5 mg/kg xylazine and secured in a stereotaxic instrument (Narishige Scientific Instruments). 100 nL of Lenti-PGK-GFP lentivirus was injected bilaterally (2×10^9 UI/ml, provided through Addgene #12252 by the Trono Lab) into the subventricular zone (with respect to the bregma: AP, +1 mm; ML, ± 1 ; DV, -2.3 mm) at a rate of 150 nL/min. Mice received analgesic ketoprofen (2 mg/kg) at the end of surgery and once every day until they regained the lost weight. Mice were allowed 8 days to recover from the surgery before behavioral testing.

Immunohistochemistry. Preexisting (DsRed-positive) and adult-born (GFP-positive) neurons activated by the learned odors were identified by Zif268 expression. Triple labeling was performed on 40 μm -thick sections by using chicken anti-GFP (1:1000, Anaspec), mouse anti-DsRed (1:200, Santa Cruz Biotechnology), and rabbit anti-Zif268 antibodies (1:1000, Santa Cruz Biotechnology). Appropriate secondary antibodies coupled with Alexa 546 for DsRed revelation, Alexa 488 for GFP, and Alexa 633 or Alexa 546 for Zif268 were used.

Morphological data analysis. For each neuron, we first determined whether it expressed Zif268 using images taken at both 40 \times and 63 \times magnification (lateral and z-axis resolutions were, respectively, 160 nm and 280 nm at 40 \times and 100 nm and 200 nm at 63 \times). Only Zif268-positive neurons were kept for further analysis. For the analysis of dendritic arborizations, images were taken with 20 \times (lateral and z-axis resolutions were 320 nm and 400 nm) and 40 \times objectives. The length of all the distal dendritic segments of the apical distal domain, of the proximal dendritic segment of the apical domain, or of all the dendritic segments of the basal domain was measured

based on previous reports (Kelsch et al. 2008). Then, spine densities on apical and basal dendrites were assessed on images taken with a 100 \times objective (lateral and z-axis resolutions were 60 nm and 200 nm, respectively). To calculate spine densities, apical distal and basal dendritic segments were randomly chosen from the apical distal and the basal dendritic arbor, respectively. The average length of dendrite used for the calculation was for the apical dendrites, 85.55 ± 1.98 μm [mean \pm standard error of the mean (SEM)] per adult-born neuron, 80.11 ± 1.97 (mean \pm SEM) per preexisting neuron and for the basal dendrites, 70.14 ± 2.62 μm (mean \pm SEM) per adult-born neuron and 43.59 ± 2.29 (mean \pm SEM) per preexisting neuron. The spine density of the proximal apical dendrite was calculated within the 100 μm from the soma. The average length of dendrite used for the calculation of the proximal spine density was 64.69 ± 0.93 μm (mean \pm SEM) in adult-born neurons and 53.98 ± 1.51 μm (mean \pm SEM) in preexisting neurons. All images were acquired using a Zeiss pseudo-confocal system (Mandairon et al. 2018).

Neuron stitching was performed using Vias (<http://research.mssm.edu/cnic/tools-vias.html>) and neuron reconstruction using NeuronStudio software (Wearne et al. 2005; Rodriguez et al. 2008) (<http://research.mssm.edu/cnic/tools-ns.html>). Neuronstudio allows 3D reconstructions of dendrites and spines from confocal z-series stacks on a spatial scale. Dendritic length was measured semi-automatically, and spines were manually identified with the help of the 3D reconstruction. All morphological analyses were conducted blind with regard to the mouse group. Number of animals, neurons per animals, total neurons analyzed, and number of dendrites per neurons for adult-born and preexisting neurons are reported in Supplementary Tables 3 and 4, respectively.

Optogenetics in Freely Behaving Mice

Experimental design. We used perceptual learning in its simplest (one pair of odorant) and most complex (6 pairs of odorants) configurations in order to unravel the specific contribution of preexisting versus adult-born neurons in the expression of the learned discrimination ($n = 39$; Fig. 3). Of the 39 mice, 14 were removed from the analysis either from optical fiber technical problems ($n = 3$), absence of halorhodopsin-expressing neurons in the OB ($n = 7$), or inappropriate optical fiber implantation site ($n = 4$).

Surgery. To be able to inhibit adult-born neurons or preexisting neurons in adult mice, we performed surgery either at P0 or at P60 in the same way as explained above for DsRed and GFP lentivirus injections except the injected virus was a Lenti-hSyn-eNpHR3.0-EYFP lentivirus (9.22×10^6 IU/ml, provided through Addgene #26775 by the Diesseroth Lab) (Kermen et al. 2016). Adult mice were then implanted with bilateral optical fibers (200 nm core diameter, 0.22 N.A., Doric Lenses) in the OB (with respect to the bregma: AP, +4.6 mm; ML, ± 0.75 mm; DV, -2 mm).

Behavior. Mice were light stimulated (crystal laser, 561 nm, 10–15 mW, continuous stimulation) during the test trial (Otest) of the habituation/cross-habituation task. More specifically, light stimulation was automatically triggered when the mouse's nose approached within 2.5 cm of the tea ball (VideoTrack, Viewpoint) and stopped automatically when the nose exited the zone.

Control of light-triggered inhibition. For the optogenetic experiment, mice were sacrificed as previously described with the exception that just before sacrifice they were randomly affected

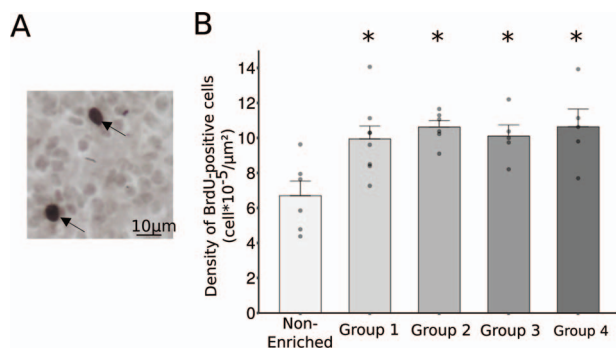


Figure 3. Perceptual learning increased adult-born granule cell density independently of the number of learned odorant pairs. (A) Example of BrdU-positive immunolabeled cells (arrows). Scale bar = 10 μm . (B) Groups enriched with 1 odorant pair (Group 1, $n = 8$), 2 odorant pairs (Group 2, $n = 6$), 3 pairs (Group 3, $n = 5$), or 6 pairs (Group 4, $n = 5$) have a significantly higher BrdU-positive cell density compared with the NE group (NE, $n = 6$). No difference in BrdU-positive cell density was observed between the 4 enriched groups. * $P < 0.05$ in comparison with the NE group. Data are expressed as mean \pm SEM, and all data points are represented. Each dot represents an animal.

to one of the following conditions: light stimulation associated with odorant stimulation or odorant stimulation alone. For the light stimulation group, this followed a pattern mimicking the average light stimulation pattern during the Otest session (0.5 s light ON, 2 s light OFF, pattern repeated over 1 h). After brain sectioning (see above), EYFP and Zif268 double immunohistochemistry was performed as described previously (Kermen et al. 2016). EYFP-positive and Zif268-positive cells were counted in the OB sampled on 1 to 3 sections (40 μm thick, interval 80 μm) located under the optic fiber implantation site to allow assessment of the inhibition efficiency.

In addition, we measured in the same area the percentage of transduced cells relative to the total granule cell layer population.

Statistical Analyses

All data were analyzed using in-house made scripts written in R and/or Python. The level of significance was set to $\alpha = 0.05$.

Behavioral data. Preliminary tests including Shapiro–Wilk test for normality checking (performed for each group, for each odorant, on each ensemble of habituation or test trial) and Mauchly’s test for sphericity were performed on the data. This resulted in the use of nonparametric tests as deviation from normality, and/or deviation from sphericity was observed. For each group, mice investigation time during habituation (Ohab1–4) of each odorant pair was analyzed using Friedman tests. Investigation time between Ohab4 and Otest for each group and each pair was compared using unilateral Wilcoxon tests. Only mice that investigated the odorant for at least 1 s at Ohab1 were included in the analysis (9 trials were excluded among 388 total trials).

Adult-born neurons quantification. BrdU-positive cell densities were compared between all experimental groups using a one-way analysis of variance (ANOVA) (group as factor) followed by Holm-corrected post hoc *t*-tests (normality of residuals and homoscedasticity were validated using Shapiro–Wilk and Levene tests, respectively). Triple-labeled sections were analyzed by two-way ANOVA (group and odorant stimulation

before sacrifice as factors, normality, and homoscedasticity were checked as above) followed by regression analysis and post hoc *t*-tests to compare the enriched groups with their respective controls.

Morphological data analysis. Morphological data (spine density and dendritic length) were compared using one-way ANOVA (with groups as factor) followed by post hoc *t*-tests using Holm–Bonferroni correction (Aickin and Gensler 1996) and using contrasts comparing enriched groups with the control group.

Results

Increasing the Complexity of Perceptual Learning Did Not Proportionally Increase Adult-born Cell Survival but Enhanced the Functional Recruitment of Adult-born Neurons

Groups of mice were submitted to different perceptual learning paradigms varying in the number of odorant pairs used during the olfactory enrichment period (from 1 to 6 pairs of odorants, Fig. 1). Enrichment consisted of successive presentations of odorant pairs during a day and the repetition of this process for over 10 days. After this enrichment period, we assessed whether mice were able to discriminate the odorants from each odorant pairs using a habituation/cross-habituation test. In this test, mice were submitted to 4 habituation trials (Ohab trials) with 1 odorant of the pair and a test trial (Otest) with the other odorant of the pair. Decreased investigation time of the odorant across Ohab trials reflected habituation to the odorant. Increased investigation time between Ohab4 and Otest reflected discrimination between the 2 odorants of the pair. A total of 5 experimental groups were constituted: control NE, enriched with 1 odorant pair (Group 1), enriched with 2 odorant pairs (Group 2), enriched with 3 odorant pairs (Group 3), and enriched with 6 odorant pairs (Group 4) (Fig. 1 and Supplementary Table 1).

Significant habituation was observed for all groups as evidenced by the reduction in investigation time across trials (Friedman tests, see Supplementary Table 2 and Supplementary Figure 1). Regarding discrimination, all enriched groups showed discrimination of the odorant pair(s) used for enrichment (see Supplementary Table 1 for abbreviations): +lim/–lim (Group 1) (Fig. 2A); +lim/–lim and dec/dodec (Group 2) (Fig. 2B). +lim/–lim, dec/dodec, and acetic a./propionic a. (Group 3) (Fig. 2C). It is worth noting that enrichment with +lim/–lim and dec/dodec did not improve discrimination of acetic a./propionic a. (see Supplementary Figure 2). Finally, the most complex enrichment (6 pairs: +lim/–lim, dec/dodec, acetic a./propionic a., propyl/butyl, penta/buta, and +terp/–terp) (Group 4) allowed mice to discriminate between the odorants of all 6 pairs (Fig. 2D). Importantly, the control NE group showed no improvement in discrimination abilities between any of the tested odorant pairs (Fig. 2E). These results showed that mice exposed to complex olfactory environment improved their performances of discrimination for at least 6 odorant pairs at a time.

We then asked whether increasing the number of odorant pairs mice passively learned to discriminate will increase the number of surviving adult-born neurons. Therefore, we labeled a cohort of adult-born cells integrating in the OB at the beginning of the enrichment, by injecting BrdU 9 days before the enrichment period. This delay allows neuroblasts to migrate from the subventricular zone to the OB. We assessed adult-born cells density in the granule cell layer since perceptual

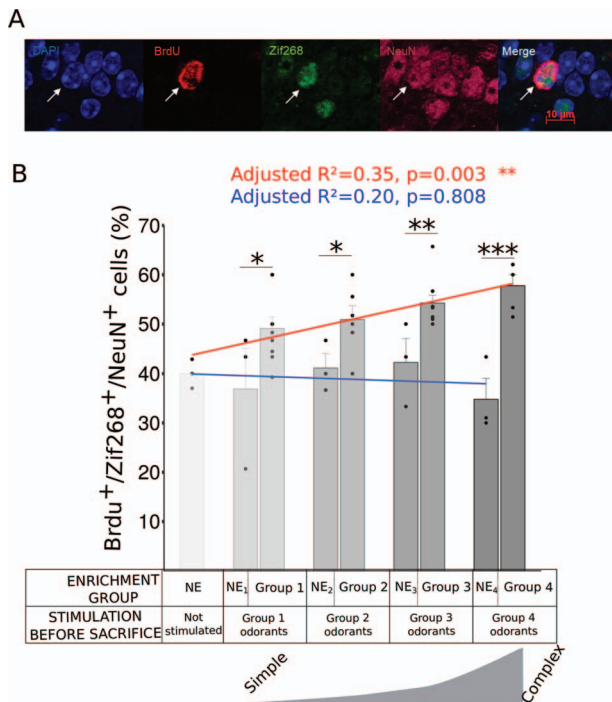


Figure 4. Increasing the complexity of perceptual learning increased the functional recruitment of adult-born neurons. (A) Example of a BrdU/Zif268/NeuN-positive neuron (arrows). (B) Enrichment with 1 (Group 1, $n=9$), 2 (Group 2, $n=6$), 3 (Group 3, $n=9$), or 6 (Group 4, $n=5$) pairs of odorants increased the percentage of adult-born neurons (BrdU/NeuN), responding to the learned odorants (Zif268) compared with their respective NE group. NE groups were stimulated on the day of sacrifice with 1 (NE1), 2 (NE2), 3 (NE3), or 6 (NE4) odorant pairs; $n=3$ for each NE group. Linear regression analysis indicated that the percentage of BrdU/NeuN/Zif268-positive cells is increasing with enrichment complexity (red line), while this is not the case for control groups (blue line). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. See Figure 1 for details on the odorants used. Data are expressed as mean \pm SEM, and all data points are represented. Each dot represents an animal.

learning had previously been shown to modulate the number of adult-born granule cells, whereas it was not the case for the number of adult-born glomerular interneurons (Moreno et al. 2009). We found a significant difference between experimental groups (one-way ANOVA, $F_{(4,25)} = 4.77$; $P = 0.005$; Fig. 3). More precisely, BrdU-positive cell density increased in all enriched groups compared with the control NE group (Holm-corrected post hoc t -tests—Group 1 vs. NE: $P = 0.025$; Group 2 vs. NE: $P = 0.011$; Group 3 vs. NE: $P = 0.036$; Group 4 vs. NE: $P = 0.014$). However, no significant difference between enriched groups was observed ($P > 0.9$ for all comparisons), indicating that the density of surviving adult-born cells is not dependent on the number of learned odorant pairs.

Since we found no increase in the density of surviving adult-born cells even when the enrichment became more complex, we hypothesized that a higher proportion of the surviving adult-born neurons could be recruited to process the learned odorants as the number of pairs mice were exposed to increased. To test this hypothesis, we assessed the percentage of adult-born granule cells expressing Zif268 in response to the learned odorants as an index of cellular activation (Moreno et al. 2012). Before sacrificing the animals, we exposed them to their respective learned odorants +lim/–lim for Group 1; +lim/–lim and dec/dodec for Group 2; +lim/–lim, dec/dodec, and acetic a./propionic a.

for Group 3; +lim/–lim, dec/dodec, acetic a./propionic a., propyl/butyl, buta/penta, and +terp/–terp for Group 6. We also tested an NE control for each experimental group that was stimulated with the same odorants on the day of sacrifice but that had not been previously enriched with the odorants (NE₁–NE₄).

First, we observed the effect of enrichment on the responsiveness of adult-born neurons to olfactory stimulation measured as the percentage of BrdU-positive cells co-expressing Zif268 and NeuN (two-way ANOVA, enrichment effect $F_{(4,35)} = 10.70$, $P = 0.000009$) (Fig. 4). Each group was different from its respective control (unilateral t -tests; Group 1 vs. its control: $t = -2.05$, $P = 0.034$; Group 2 vs. its control: $t = -2.18$, $P = 0.033$; Group 3 vs. its control: $t = -3.20$, $P = 0.005$; Group 4 vs. its control: $t = -5.30$, $P = 0.0009$). In addition, the more complex the enrichment, the more BrdU/Zif268/NeuN-positive cells were retrieved (linear regression: adjusted- $R^2 = 0.35$, $P = 0.003$). This was not seen in the control NE groups (linear regression: adjusted- $R^2 = -0.20$, $P = 0.81$). Thus, the correlation suggests that enrichment complexity and adult-born neuron responsiveness are linked, even though other factors may interact as well.

We further tested whether this result could be due to the different durations of odor enrichment between groups (1 h per day for Group 1 vs. 6 h per day for Group 4). To test this, we enriched a new group of mice with +lim/–lim 6 h per day for 10 days (similar duration of enrichment compared with Group 4). The percentage of BrdU/Zif268/NeuN-positive cells did not differ from that in Group 1 (see Supplementary Figure 3; unilateral t -test: $t = -1.00$, $P = 0.82$), suggesting that it was the diversity of the odorants presented during the enrichment and not the duration of the exposure that affected adult-born neuron recruitment.

Finally, to ensure that the increased BrdU/Zif268/NeuN co-labeling resulted from increased odor responsiveness and not from a change in the basal level of BrdU/Zif268/NeuN co-expression in the enriched groups, we assessed BrdU/Zif268/NeuN co-labeling in the basal condition (without odor stimulation) in animals enriched with 6 odorant pairs and compared it with that in NE animals. We found no difference between these 2 experimental groups (unilateral t -test: $t = 0.32$, $P = 0.38$; see Supplementary Figure 4), suggesting that the observed change in BrdU/Zif268/NeuN-positive levels was not due to an overall increase in adult-born granule cell activity in the enriched OB.

We observed no effect of enrichment or olfactory stimulation on the day of sacrifice on the rate of neuronal differentiation of adult-born cells (two-way ANOVA: enrichment effect $F_{(4,35)} = 1.32$; $P = 0.48$; stimulation effect: $F_{(4,35)} = 0.66$, $P = 0.62$; see Supplementary Figure 5A). Quantification of Zif268-positive cell density showed an effect of enrichment but no effect of odor stimulation before sacrifice (see Supplementary Figure 5B) (two-way ANOVA, enrichment effect: $F_{(4,35)} = 6.83$, $P = 0.00036$; stimulation effect: $F_{(4,35)} = 1.27$, $P = 0.3$; followed by unilateral t -tests: Group 1 vs. its control: $t = -2.46$, $P = 0.016$; Group 2 vs. its control: $t = -2.02$, $P = 0.041$; Group 3 vs. its control: $t = -1.90$, $P = 0.043$; Group 4 vs. its control: $t = -2.27$, $P = 0.032$), thus showing that enrichment increased overall granule cell activity independently of the number of learned odorants.

Together, these results indicate that even if the number of adult-born granule cells saved by learning reached a plateau, the pool of surviving granule cells are increasingly called upon to process the learned odorants as perceptual learning complexity increased in young adult mice.

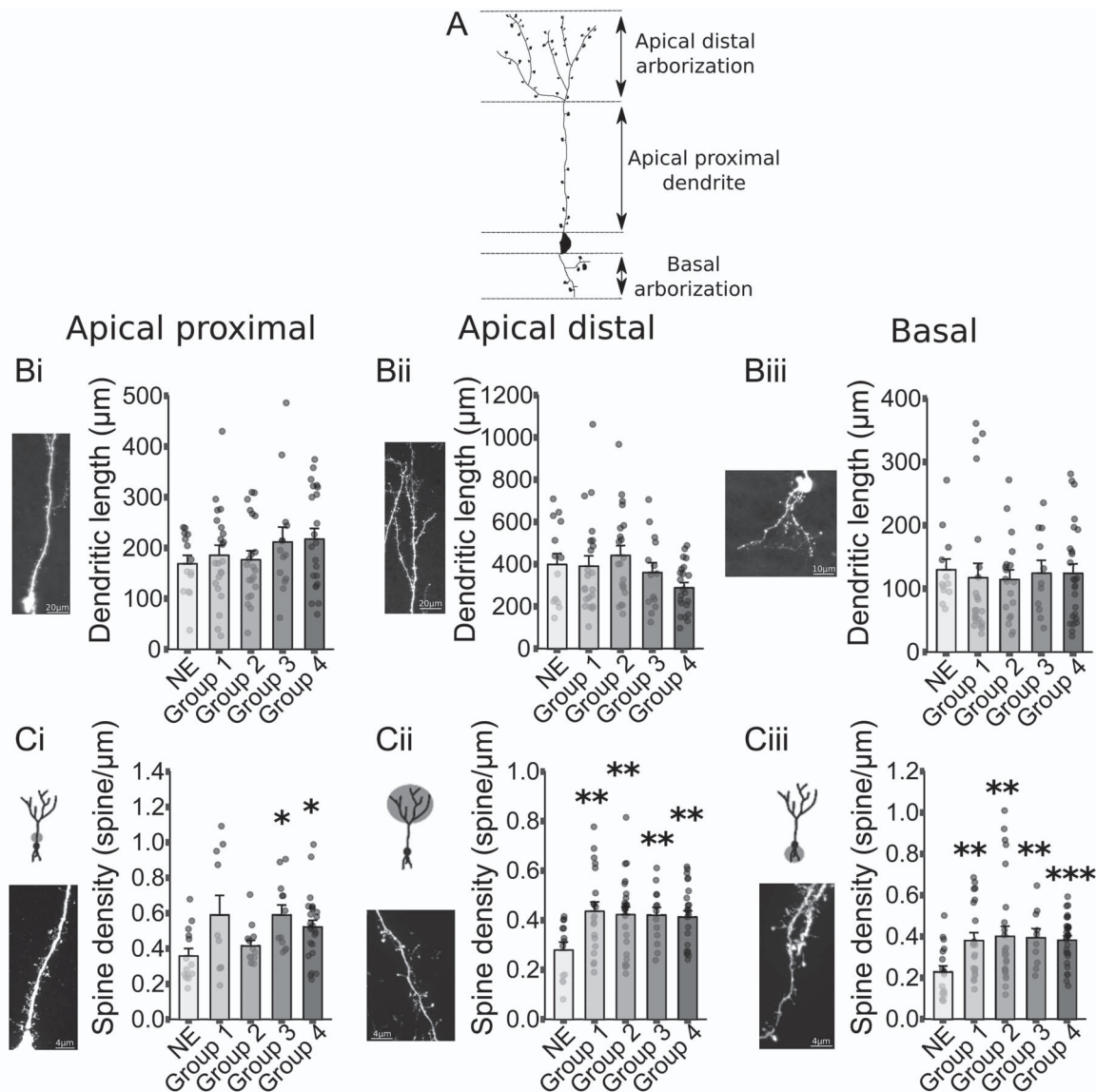


Figure 5. Increasing the complexity of perceptual learning increased structural plasticity of adult-born neurons. (A) Schematic representation of a granule cell and its dendritic domains. (B) No group differences for the length of apical proximal (Bi) (n_{neuron} from NE to Group 4 = 14, 24, 21, 14, 22), apical distal (Bii) (n_{neuron} from NE to Group 4 = 14, 23, 21, 14, 22), and basal dendritic arborization (Biii) (n_{neuron} from NE to Group 4 = 12, 23, 19, 11, 26). (C) Spine densities were increased after perceptual learning at the apical proximal (Ci) (n_{neuron} from NE to Group 4 = 14, 10, 13, 12, 25), apical distal (Cii) (n_{neuron} from NE to Group 4 = 13, 20, 25, 13, 24), and basal domains (Ciii) (n_{neuron} from NE to Group 4 = 18, 22, 27, 10, 29). Regarding the apical proximal domain, increased spine density was observed only in Groups 3 and 4, meaning after complex perceptual learning. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for comparison with the NE group, Holm-corrected t-tests. Data are expressed as mean \pm SEM, and all data points are represented. Each dot represents a neuron.

Increasing the Complexity of Perceptual Learning Increased Structural Plasticity of Adult-born Neurons and Induces Limited Morphological Changes in Preexisting Neurons

To better understand the increased functional recruitment of adult-born neurons with perceptual learning complexity, we looked for associated structural modifications in these neurons. To determine whether perceptual learning had an effect on the morphology of odor-responding adult-born neurons (Zif268-positive), we injected a lentivirus expressing GFP at postnatal day 60 (P60) in the subventricular zone 8 days before learning (Fig. 1). Animals were sacrificed 25 days post-injection,

and we analyzed dendritic arborization and spine density of the apical distal domain [the site of interactions with mitral/tufted (M/T) cells] as well as the apical proximal and basal domains (sites of centrifugal inputs) (Fig. 5A) (Mandairon et al. 2018) of Zif268-positive neurons.

Enrichment did not affect dendritic length of any domain (one-way ANOVAs, apical proximal, $F_{(4,89)} = 0.95$, $P = 0.44$; Fig. 5Bi; apical distal, $F_{(4,88)} = 1.92$, $P = 0.11$; Fig. 5Bii; basal, $F_{(4,86)} = 0.09$, $P = 0.98$; Fig. 5Biii).

However, we found that perceptual learning increased the spine density of all dendritic domains (one-way ANOVAs, apical proximal, $F_{(4,69)} = 3.48$, $P = 0.01$; Fig. 5Ci; apical distal, $F_{(4,90)} = 3.03$, $P = 0.022$; Fig. 5Cii; apical proximal basal, $F_{(4,101)} = 3.24$, $P = 0.015$;

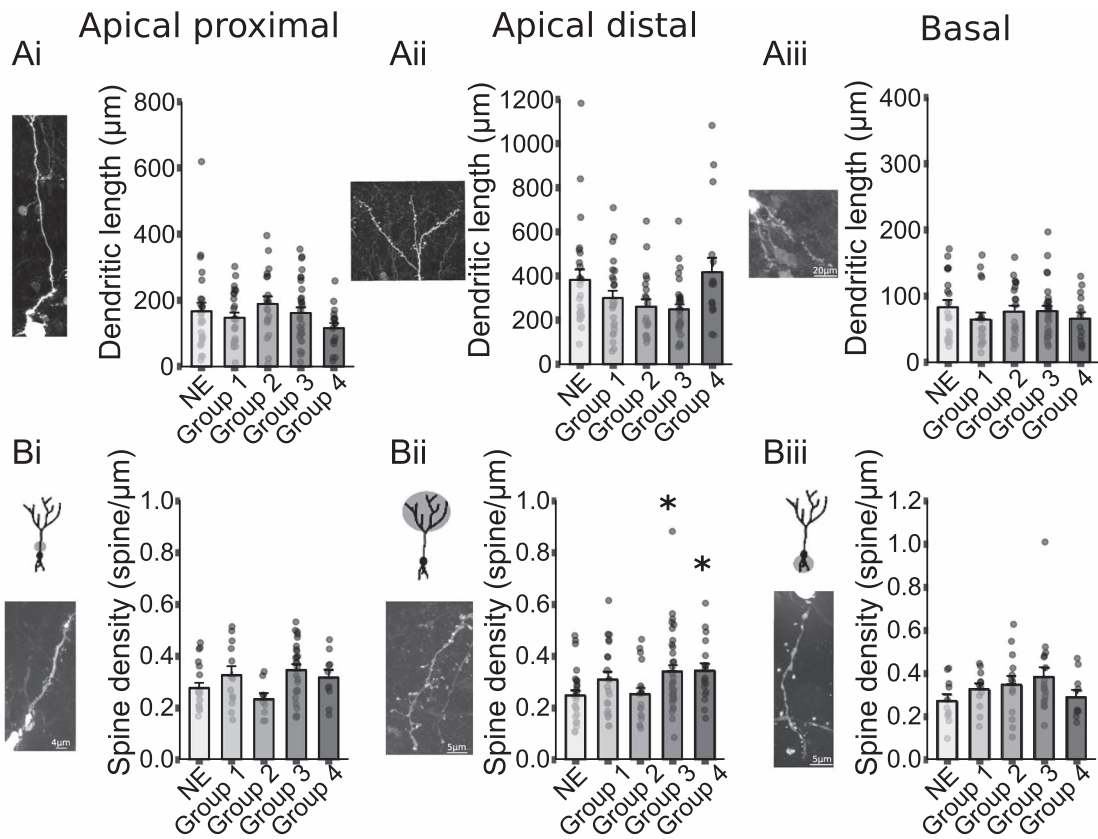


Figure 6. Perceptual learning induced limited morphological changes in preexisting neurons. (A) No effect of perceptual learning was observed on the length of the apical proximal dendrites (Ai) (n_{neuron} from NE to Group 4 = 25, 25, 20, 31, 17), the apical distal dendritic arborization (Aii) (n_{neuron} from NE to Group 4 = 25, 25, 20, 31, 17), or the basal dendritic arborization (Aiii) (n_{neuron} from NE to Group 4 = 20, 18, 21, 27, 14). (B) Spine densities after perceptual learning at the apical proximal (Bi) (n_{neuron} from NE to Group 4 = 21, 13, 9, 26, 10), apical distal (Bii) (n_{neuron} from NE to Group 4 = 27, 21, 18, 34, 18), and basal domains (Biii) (n_{neuron} from NE to Group 4 = 11, 12, 14, 18, 10). Increased spine density in the apical distal domain was seen after complex perceptual learning only (Groups 3 and 4). Regarding the apical proximal domain and basal domains, no increased spine densities were observed after learning. * $P < 0.05$ for comparison with the NE group. Data are expressed as mean \pm SEM, and all data points are represented. Each dot represents a neuron.

Fig. 5Ciii). A detailed analysis of the data revealed that, at the apical proximal domain, we observed an increased spine density on adult-born Zif268-positive neurons only in Groups 3 and 4 compared with NE (Holm-corrected post hoc *t*-tests; Group 3 vs. NE $P = 0.01$, Group 4 vs. NE $P = 0.02$; Fig. 5Ci). In the apical distal domain, the increased spine density is observed in all groups (Holm-corrected post hoc *t*-tests; Group 1 vs. NE $P = 0.011$, Group 2 vs. NE $P = 0.011$, Group 3 vs. NE $P = 0.011$, Group 4 vs. NE $P = 0.011$; Fig. 6Cii). The same global increase is observed for the basal domain (Holm-corrected post hoc *t*-tests; Group 1 vs. NE $P = 0.008$, Group 2 vs. NE $P = 0.008$, Group 3 vs. NE $P = 0.008$, Group 4 vs. NE $P = 0.0005$; Fig. 5Ciii).

These results suggested that when the environmental complexity was low (enrichment with 1 and 2 odorant pairs), only apical distal and basal domains showed morphological modifications (i.e. increased spine density), whereas when the environmental complexity increased (enrichment with 3 and 6 odorant pairs), morphological changes affected the apical proximal domain in addition to apical distal and basal domains. Thus as the environmental complexity increased, morphology changed in a non-linear manner and not uniformly across the different dendritic domains.

These same mice were beforehand injected with a lentivirus expressing DsRed at postnatal day 0 (P0) (Fig. 1). Because of that,

it was possible to investigate the structural plasticity induced in odor-responding preexisting neurons of the OB and evaluate the specificity of learning-induced morphological changes in adult-born neurons. We found no morphological difference between experimental groups (NE and Group 1–4) regarding the length of the apical proximal and basal dendritic domains (one-way ANOVAs; $F_{(4,113)} = 1.46$, $P = 0.22$; Fig. 6Ai; $F_{(4,95)} = 0.6$, $P = 0.66$; Fig. 6Aiii). Regarding the apical distal domain, the analysis revealed a group effect (one-way ANOVA, $F_{(4,113)} = 3.39$, $P = 0.012$), but there was no significant difference for comparisons between NE and enriched groups (Holm-corrected post hoc *t*-tests, $P > 0.06$; Fig. 6Aii).

An increase of spine density in the apical distal domain was observed in Groups 3 and 4 compared with the NE group (one-way ANOVA; $F_{(4,113)} = 3.12$, $P = 0.02$; Holm-corrected post hoc *t*-tests: Group 3 vs. NE $P = 0.027$, Group 4 vs. NE $P = 0.027$; Fig. 6Bii). For the apical proximal spine density, a group effect was found (one-way ANOVA, $F_{(4,74)} = 2.77$, $P = 0.03$), but there was no significant difference for comparisons between NE and enriched groups (Holm-corrected post hoc *t*-tests, $P > 0.08$; Fig. 6Bi). Spine density in the basal domain was not affected by enrichment (one-way ANOVA, $F_{(4,60)} = 1.39$, $P = 0.25$; Fig. 6Biii).

Taken together, these results suggest that the morphology of adult-born neurons is modified as soon as they are engaged in a

simple (single odorant pair) perceptual learning task, while the morphology of preexisting neurons is modified only when the perceptual learning becomes complex (3 or 6 odorant pairs).

Optogenetically Inhibiting Preexisting or Adult-born Neurons in Freely Moving Mice Reveals Their Functionally Distinct Involvement in Simple and Complex Perceptual Learning

Based on the previous morphological results, we hypothesized that inhibiting adult-born neurons after simple and complex perceptual learning would alter learned discrimination, while inhibiting preexisting neurons would only affect the expression of learned discrimination following the complex learning. To test this hypothesis, we used 4 new groups of mice (Fig. 7). The first group was infused with a halorhodopsin- and EYFP-expressing lentivirus in the subventricular zone at P60 to transduce and, later on, silence adult-born neurons. Half of the animals of this group were submitted to simple environmental enrichment (1 odorant pair; similar to Group 1) and the other half to complex environmental enrichment (6 odorant pairs; similar to Group 4). A second group of mice was infused with the same lentivirus in the lateral ventricle at P0 to transduce preexisting neurons and silence them later on in adulthood. This group was also divided in 2: 1 enriched with 1 odorant pair and 1 enriched with 6 odorant pairs (Fig. 7). As previously, mice discrimination was tested after enrichment, using habituation/cross-habituation tests assessing perceptual learning. Optogenetic inactivation of transduced neurons was achieved in freely behaving mice only during the test trial of the habituation/cross-habituation paradigm by automatically triggering the light stimulation (see methods) each time the animal approached the odor source. All mice were also tested in a light OFF condition.

As expected, since light was never on during this phase of the test, habituation proceeded normally (see Supplementary Figure 6 and Supplementary Table 5). Regarding discrimination, we found that inhibiting adult-born neurons during the test trials abolished the improvement of discrimination induced by simple and complex enrichment (Fig. 8; Supplementary Table 5). More specifically, animals enriched with +lim/–lim were able to discriminate these 2 odorants in the light OFF condition but not under light triggered inactivation of adult-born neurons (Fig. 8A and C; Supplementary Table 5). The same phenomenon was observed for complex enrichment; animals were able to discriminate the 6 learned odorant pairs in the light OFF condition, but this was no longer observed in the light ON condition (Fig. 8B and D; Supplementary Table 5).

We assessed the level of viral transduction by first analyzing the density of EYFP-positive cells in the OB under the optical fibers and found the same density of transduced neurons in the groups submitted to simple and complex enrichments (bilateral t-test: $P = 0.098$; see Supplementary Figure 7Ai). We also measured the proportion of transduced neurons (EYFP positive) relative to the whole cell population (DAPI positive). Groups injected at P0 showed higher percentages (simple $3.8\% \pm 0.21$, complex $2.8\% \pm 0.29$) than groups injected at P60 (simple $2.14\% \pm 0.23$, complex $1.59\% \pm 0.23$). Statistical analysis indicated that percentage of transduced neurons was superior in groups injected at P0 compared with groups injected at P60 (ANOVA, $F_{(1,21)} = 30.35$, $P < 0.0001$). Within the group injected at P0, the group tested in the simple paradigm exhibited a percentage of transduced cells higher than that of the complex group ($P = 0.029$, Holm-corrected t-test). It is worth noting that the group showing

the highest density of transduced cells is the group in which light has no behavioral effect. Thus, these differences can probably not account for the reported effects of light. Second, the effectiveness of light-mediated adult-born granule cell inhibition was assessed by counting Zif268-positive cells among EYFP-positive cells in the same region. A decrease in the percentage of EYFP/Zif268-positive cells was observed in the light ON condition compared with the light OFF confirming that light actually inhibited transduced adult-born neurons (unilateral t-test: simple learning, $P = 0.028$, complex learning, $P = 0.043$; see Supplementary Figure 7Aii).

In contrast to adult-born neurons, light-triggered inactivation of preexisting neurons did not alter discrimination of the learned odorants after simple enrichment (Fig. 9A and C, Supplementary Table 5). However, inhibiting preexisting neurons after complex enrichment impaired discrimination of most odorant pairs (Fig. 9B and D, Supplementary Table 5). The level of viral transduction was also analyzed in preexisting neurons. The density of EYFP-positive cells in the OB was similar between simple and complex learning groups (bilateral t-test, $P = 0.13$; see Supplementary Figure 7Bi), and a decrease in the percentage of EYFP/Zif268-positive cells was observed in the light ON compared with the light OFF condition (unilateral t-tests: simple learning, $P = 0.048$; complex learning, $P = 0.015$; see Supplementary Figure 7Bii).

As an additional control, the discrimination of dissimilar odorants was not altered by the inhibition of either adult-born or preexisting neurons in either the simple or complex enriched groups (see Supplementary Figure 8 and Supplementary Table 6).

These results reveal that adult-born and preexisting neurons can both undergo structural plasticity upon perceptual learning, but their involvement in the task is governed by distinct conditions. In this context, adult-born neurons are necessary for both simple and complex learned discrimination, while preexisting neurons are required only for the expression of complex learned discrimination.

Discussion

Perceptual learning contributes to the representation of the sensory environment that guides animal's behavior. An essential component of the plasticity of this representation in the olfactory system is adult neurogenesis, that is renewal of interneurons within a preexisting neuronal network. Perceptual learning requires the presence of OB adult-born neurons and increases their survival (Moreno et al. 2009). In this study, we did not find a higher level of surviving adult-born cells with increasing environmental complexity, suggesting that a plateau of adult-born cell survival is reached after simple perceptual learning. Furthermore, among the adult-born neurons surviving after perceptual learning, only some of them respond to the learned odorants (Moreno et al. 2009; Mandairon et al. 2011; Sultan et al. 2011). Our findings show a positive linear relationship between the proportion of adult-born neurons expressing Zif268 in response to olfactory stimulation and the number of discriminated pairs of odorants. Interestingly, the learning-dependent survival increase reported here is close to the rate of adult-born neurons saved by different olfactory learning paradigms [associative and perceptual learning tasks (Alonso et al. 2006; Mouret et al. 2008; Moreno et al. 2009; Kermen et al. 2010; Sultan et al. 2010; Mandairon et al. 2011)], suggesting that this survival increase might not be specific to a task but simply a correlate of environmental modifications. Thus learning type or

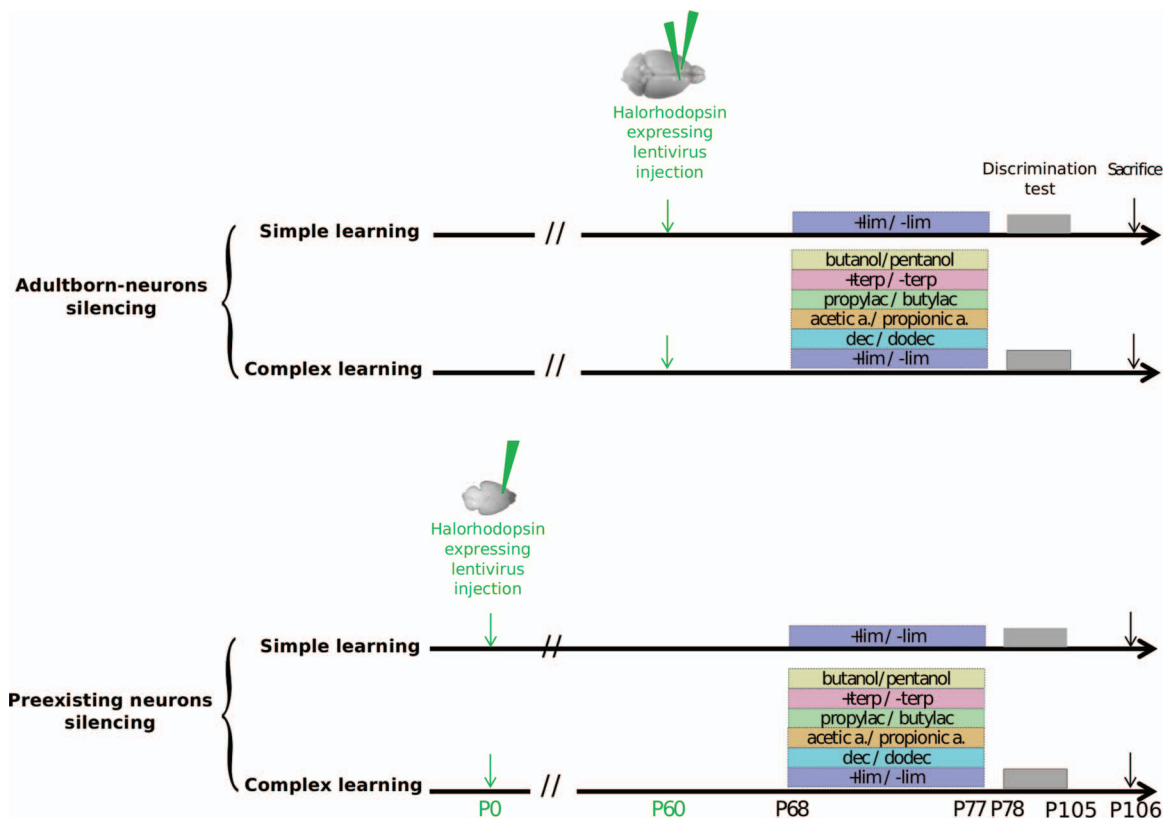


Figure 7. Design of the optogenetic experiment. Halorhodopsin-expressing lentivirus was injected in P0 mice to infect preexisting neurons or at P60 to infect adult-born neurons. 2 groups of mice were trained in a simple perceptual learning [1 injected at P0 ($n=6$) and 1 at P60 ($n=4$)], and 2 groups of mice were trained in a complex perceptual learning [1 injected at P0 ($n=8$) and 1 at P60 ($n=7$)]. After enrichment, mice were tested on their ability to discriminate the learned odorant pairs using an olfactory habituation/cross-habituation test. Light-stimulation was triggered in freely moving mice during the test trial of the habituation/cross-habituation task, when mice approached the odor source. The same groups of mice were also tested in a light OFF condition.

environmental change complexity is not coded by the survival rate of adult-born neurons. However, our results strongly suggest that the information about environmental complexity could be carried by functionally recruiting more adult-born neurons from the pool of available ones.

One limitation of our study comes from the fact that not all mice were tested on their ability to discriminate odorants of all 6 pairs. Previous studies showed that enrichment with +lim/-lim did not induce discrimination of dec/dodec, indicating that perceptual learning is relatively specific of the odorants used during exposure (Madaïron et al. 2006c; Moreno et al. 2009), in line with the specific activation patterns they evoke in the OB (Linster et al. 2001). Furthermore, we showed that enrichment with +lim/-lim and dec/dodec does not induce discrimination of acetic a./propionic a., strongly supporting the hypothesis that discrimination of more odorant pairs is actually allowed by a more complex environment. However, from 3 pairs of odorants onwards, the OB stimulation is broad and activates overlapping regions of the OB leading to a less specific improvement in discrimination. This is similar to what was observed following global odor enrichment (Rey et al. 2012) or pharmacological activation of the OB (Madaïron et al. 2006c).

Beyond the number of surviving adult-born cells or the proportion of them that is activated, another parameter of plasticity shown to be involved in improved discrimination is the structural plasticity of adult-born neurons (Daroles et al.

2015; Madaïron et al. 2018). Cell activity is a major regulator of neuronal morphology (Lu 2003; Kelsch et al. 2010). In line with this, we first found that simple and complex perceptual learning increased the density of dendritic spines at the apical distal, apical proximal, and basal domains of adult-born granule cells. Optogenetically inhibiting these neurons prevented discrimination after simple perceptual learning (in accordance with Moreno et al. 2009) and also after complex perceptual learning. We also report that simple olfactory perceptual learning does not modify the morphology of preexisting cells although an increase of spine density can be observed at the apical domain after complex perceptual olfactory learning. Importantly, these modifications seem to correlate with our observation that inhibition of preexisting neurons prevented discrimination of the learned odorants only after complex but not simple perceptual learning. Based on these observations, we can hypothesize that morphological modifications are the cause of the discrimination changes observed at the behavioral level. There are several ways they could underlie perceptual learning. First, apical dendrites are the site of reciprocal dendrodendritic synapses between granule and mitral cells (Price and Powell 1970; Shepherd 1972), thus a higher number of spines could elevate the previously reported inhibitory drive on mitral cells (Moreno et al. 2009; Madaïron et al. 2018) and explain why the absence or inactivity of these same cells prevents discrimination (Moreno et al. 2009). Second, the apical proximal and basal

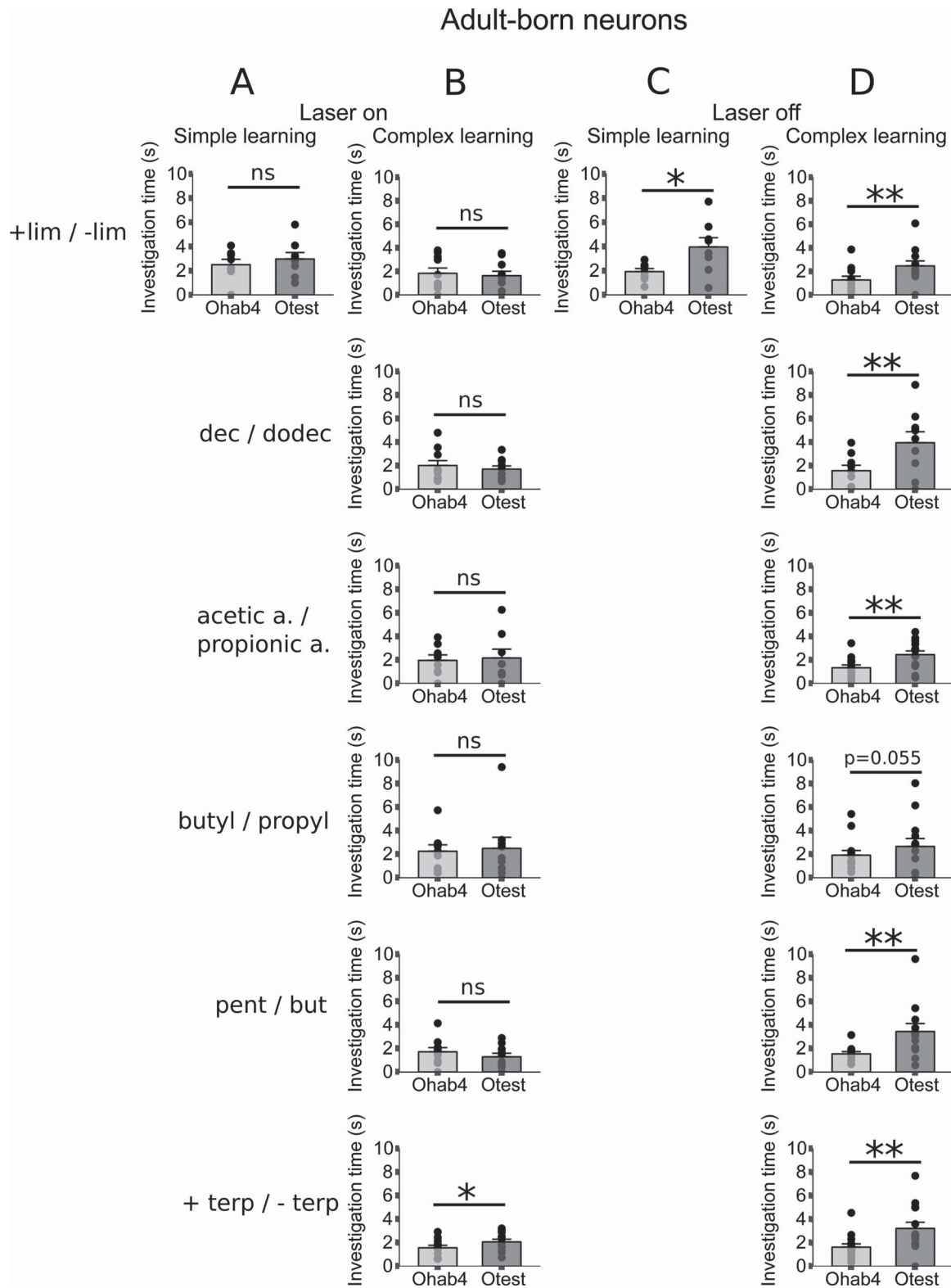


Figure 8. Optogenetically inhibiting adult-born neurons prevented discrimination after both simple and complex perceptual learning. In light On condition, mice were not able to discriminate learned odorant pairs during the discrimination test after both simple (A) and complex (B) learning. In light OFF conditions, the same mice were able to discriminate learned odorant pairs after both (C) simple and (D) complex learning. Data are expressed as mean \pm SEM, and all data points are represented. * $P < 0.05$, ** $P < 0.01$. Each dot represents a trial.

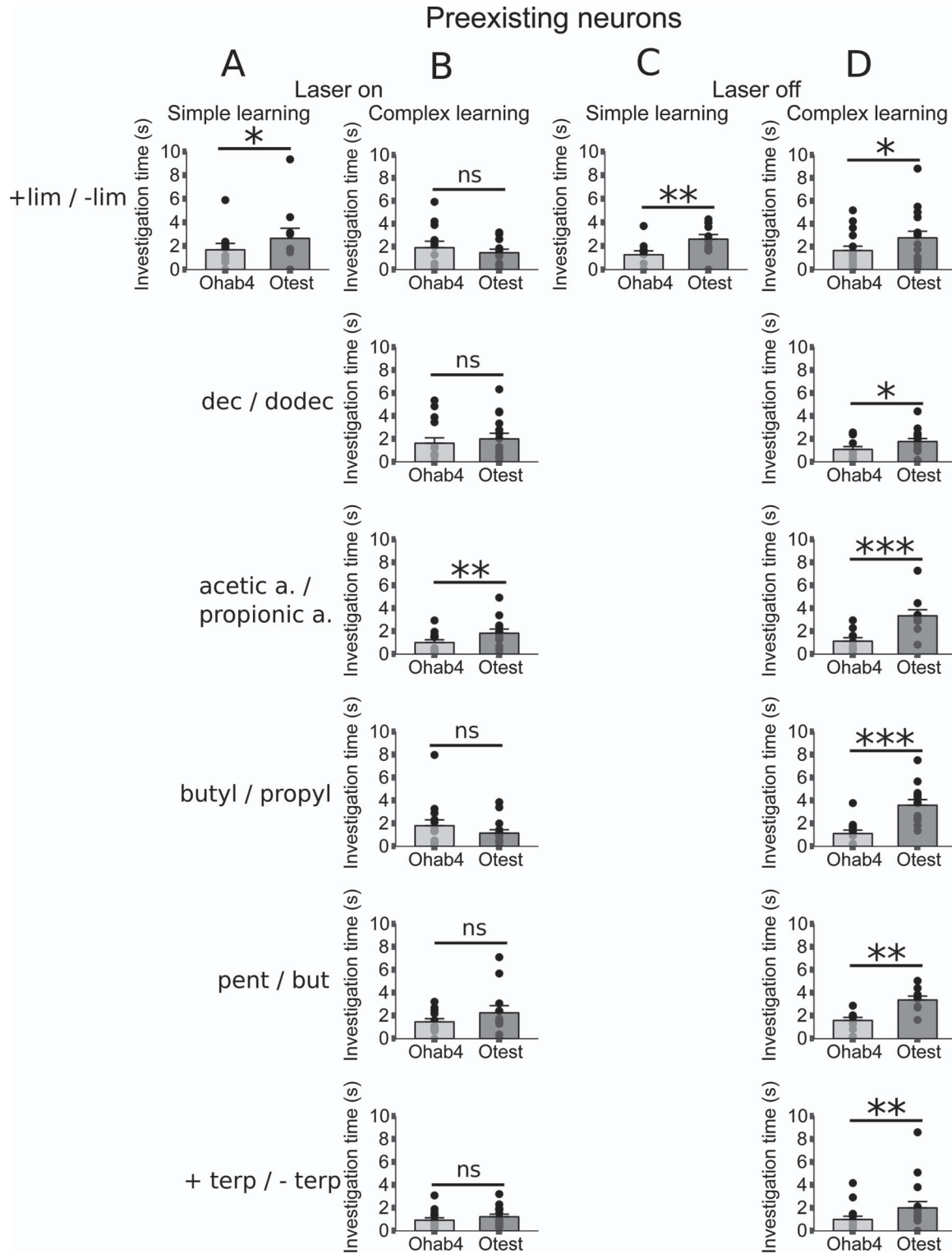


Figure 9. Optogenetically inhibiting preexisting neurons did not prevent discrimination after simple learning but did alter discrimination after complex learning. After simple perceptual learning (A), inhibiting preexisting neurons did not prevent discrimination, whereas (B) inhibiting them after complex perceptual learning altered the discrimination of most odorant pairs. In light OFF condition, (C) and (D), odorant pairs were discriminated after both types of learning. Data are expressed as mean \pm SEM, and all data points are represented. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Each dot represents a trial.

domains both receive cortico-bulbar projections from olfactory cortices (Nissant et al. 2009; Lepousez et al. 2014) as well as neuromodulatory inputs such as noradrenergic, cholinergic, and serotonergic (Fletcher and Chen 2010). Because the noradrenergic system acts on adult-born cells to allow olfactory perceptual learning (Moreno et al. 2012) and gates dendrodendritic inhibition onto mitral cells (Balu et al. 2007) while not being exclusive of other influences such as cholinergic (Ravel et al. 1994; Linster and Cleland 2002, 2016; Mandairon et al. 2006a; de Almeida et al. 2013), this increase in proximal and basal inputs could enhance noradrenergic influences on granule cell activity. Hence, as environmental complexity increases, spine density modifications (both apical, proximal, and basal) probably lead to a remapping of synaptic weights, thereby synergistically producing the linearly increased functional involvement of adult-born neurons as the environment gets more and more complex. Increased activation of adult-born interneurons together with increased inhibitory connectivity with mitral cells could serve as a means of decorrelation and mediate a refinement of the representations of each odorant in a pair.

The modifications of both basal and apical proximal spine densities observed here are similar to a previous study showing that associative olfactory learning can increase the excitatory drive from the olfactory cortex onto adult-born granule cells (Lepousez et al. 2014). However, we also observed changes in spine density at the apical distal domain. One possible explanation for this difference could simply be the nature of the task (reinforced vs. non-reinforced discrimination learning) as this has recently been shown to induce opposite changes in the apical connectivity of adult born cells (Mandairon et al. 2018).

It has been proposed that adult-born neurons confer two distinct but complementary forms of circuit plasticity to the OB network: an early form based on individual neuron survival and a later form based on the fine tuning of synaptic strength (Livneh et al. 2014). Our experimental data support this proposition by showing the absence of an increase in adult-born survival, regardless of task complexity, accompanied by an increase in the functional recruitment and structural plasticity of adult-born neurons. The present experiments further show that a third level of network plasticity is supported by preexisting neurons more particularly in complex learning when adjustments of the network supported by adult-born neurons may not be sufficient to adjust to the environmental context.

All the above as well as previous studies (see for review Hardy and Saghatelian 2017) indicate the existence of fundamental differences between adult-born and preexisting neurons. Preexisting neurons and their associated synapses seem more likely to be stable over time, whereas adult-born neurons constitute a more plastic and changing neuronal population that would first and foremost be the substrate of adaptive learning.

While the occurrence of adult neurogenesis in adult Human OB was subject to debate regarding its presence (Curtis et al. 2007), current consensus is that neurogenesis is absent beyond the age of 2 in the human OB (Sanai et al. 2007, 2011; Bergmann et al. 2012). This raises the interesting issue of how the human bulbar network adjusts to allow the plasticity of olfactory perception. Indeed humans improve their discrimination abilities with experience (Jehl et al. 1995). Our data indicating that neurons born during ontogenesis can also support structural plasticity and improved discrimination suggest that such plasticity could be at play in humans.

In conclusion, in this mice study, we have demonstrated both the necessity and sufficiency of adult-born neurons to simple perceptual learning as well as their necessity for complex perceptual learning. This reinforces their essential role in behavioral olfactory adaptation. For the first time, we have also shown that preexisting neurons do not participate in OB modifications associated with simple perceptual learning but can still be recruited both functionally and structurally to contribute to learning in cases of environmental complexity.

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