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Role of adult-born versus preexisting neurons born at P0 in olfactory perception in a complex olfactory environment in mice.

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26 Abstract

Olfactory perceptual learning is defined as an improvement in the discrimination of perceptually close 27 28 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 29 30 in the olfactory bulb, which refines the bulbar output. However, the olfactory environment is complex 31 raising the question of the adjustment of the bulbar network to multiple discrimination challenges. Perceptual learning of one to six pairs of similar odorants led to discrimination of all learned odor 32 pairs. Increasing complexity did not increase adult-born neuron survival but enhanced the number of 33 34 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 35 of life (P0). Selective optogenetic inactivation of either population confirmed functional involvement 36 of adult-born neurons regardless of the enrichment complexity while preexisting neurons were 37 required for complex discrimination only. 38

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41 Keywords:

42 adult neurogenesis, olfaction, optogenetic, perceptual learning, structural plasticity

43 Introduction

The rodent olfactory bulb (OB) is supplied during adulthood with new inhibitory interneurons 44 (Altman 1969; Lois and Alvarez-Buylla 1994; Ming and Song 2005) that shape the message carried 45 46 outside the OB by the mitral cells (Winner et al. 2002; Malvaut and Saghatelyan 2016). These adultborn neurons play a central role in tuning the animal's discrimination abilities through perceptual 47 learning (Mandairon et al. 2006; Moreno et al. 2009). Perceptual learning is defined as a significant 48 improvement in discrimination abilities induced by previous non-reinforced experiences (Gilbert et 49 al. 2001), reflecting a dynamic adjustment by which animals discriminate relevant stimuli within their 50 immediate environment. In the context of olfaction, mice can improve their performances of 51 52 discrimination between perceptually similar odorants in a relative odor-specific manner thanks to passive enrichment with these odorants (Mandairon et al. 2006; Moreno et al. 2009). This perceptual 53 54 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 55 order to correctly guide the animal's behavior within a natural environment, the olfactory system 56 needs to cope with its complexity, i.e. an environment in which multiple odorants are present whithin 57 the same time window. What part do adult-born neurons play in perceptual learning when a complex 58 olfactory environment challenges the network? In other words, does complex learning require more 59 60 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 61 properties and structural plasticity to those of neurons born during the ontogenesis of the OB (labeled 62 63 at P0 and so-called hereafter preexisting neurons (Bayer 1983).

Newly integrated adult-born cells show specific electrophysiological properties compared to 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 to preexisting neurons (Lemasson et al. 2005; Valley et al. 2013; Breton-Provencher et al. 2016). Recent studies using chemo or optogenetic manipulation adult born versus preexisting neurons showed the differential involvement of preexisting versus adult-born neurons in rewardassociated olfactory learning (Muthusamy et al. 2017; Grelat et al. 2018). However, the respective roles of adult-born compared to preexisting neurons in other learning contexts still remain unclear.

77 To answer these questions, we used a perceptual learning paradigm in different configurations in term of complexity. More precisely, young adult mice were exposed to one, two, three or six pairs of 78 79 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 80 81 learned odorants and structural plasticity. In addition, we analyzed the uniqueness of adult-born 82 neuron plasticity by comparing it to that present in preexisting neurons. Finally, to determine the functional role of each population on discrimination performances after perceptual learning, we 83 measured the effect of selective optogenetic inactivation of the populations of preexisting or adult-84 85 born neurons on learned discrimination.

86

87 Materials and Methods

88 <u>Animals</u>

64 inbred male C57BL/6J young adult mice born and raised in our laboratory were used in the first 89 part of this study. Mice were housed in a controlled environment under a 12h light/dark cycle with 90 91 food and water ad libitum. All behavioral training (enrichment) was conducted between 8am and 8pm 92 while behavioral testing was conducted in the afternoon (12am-7pm) on young adult mice aged 2-3 93 months. All efforts were made to minimize the number of animals used and their suffering during the 94 experimental procedure in accordance with the European Community Council Directive of November 95 24, 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 96 97 same litter were assigned as much as possible to different experimental groups.

98

99 Overview of the experiment.

Five experimental groups of mice were formed based on the number of odorant pairs used for the enrichment (from 0 to 6 odorant pairs; Figure 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 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 (Figure 1).

107

108 **Detailed procedures**

109 Behavioral experiments

Enrichment. Odor enrichment consisted of exposure to odorant pairs (from one to six depending on
the experimental group; Figure 1) for 1 hour per day over 10 consecutive days. For each odor pair,
the two odorants were presented simultaneously on two swabs containing 100 µl of pure odorant

placed in two tea balls hanging from the cover of the cage. For multiple enrichments, odorant pairs 113 were presented with an interval of at least 1 hour. Odorant pairs were presented in the same order 114 115 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/-116 117 lim and dec/dodec ; Group 3 with +lim/-lim, dec/dodec and acetic a./propionic a. ; Group 4 with 118 +lim/-lim, dec/dodec, acetic a./propionic a, buta/penta, propyl/butyl, +terp/-terp (Figure 1 and 119 Supplementary Table 1). Control non-enriched mice were subjected to the same protocol and under 120 the same conditions except that the tea balls contained only mineral oil.

121 Olfactory habituation/cross habituation test. We used a habituation/cross habituation test to assess olfactory discrimination (Moreno et al. 2009). Briefly, this task assessed whether mice were able to 122 spontaneously discriminate between two odorants by habituating them to one odorant and measuring 123 their **cross** habituation to the second odorant. We tested the discrimination between the two odorants 124 of each pair used for the enrichment (Group 1 was tested for discrimination between +lim/–lim; Group 125 126 2 +lim/-lim and dec/dodec; Group 3 +lim/-lim, dec/dodec and acetic a./propionic a.; Group 4 +lim/-127 lim, dec/dodec, acetic a./propionic a, buta/penta, propyl/butyl, +terp/-terp). All experiments took place in a clean cage similar to the home cage. Odorants were presented by placing 60µL of the 128 diluted odorant (1Pa) (Supplementary Table 1) onto a filter paper (Whatman #1) which was put inside 129 a tea ball hanging from the side of the cage. A behavioral session consisted of one presentation of 130 mineral oil then four odorant presentations of the habituation odorant (Ohab1-4) followed by one 131 presentation of the test odorant (Otest). Each odorant presentation lasted 50 sec and was separated by 132 133 5 min. Each odorant of each pair was used alternatively as the habituation or test odorant. 134 Investigation time (defined as mice 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 135 indicated discrimination. 136

138 <u>Adult-born neuron quantification in the OB</u>

BrdU injection. Mice were injected with BrdU (Sigma) (50 mg/kg in saline, three times at 2h 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 (Figure 1) as previously described (Moreno et al. 2009).

143 Sacrifice. To investigate the expression of the immediate-early-gene Zif268 in response to the learned 144 odorants, enriched mice were exposed to 100 µl of pure learned odorants (odorants used for the enrichment presented simultaneously) for 1h using tea balls. A control non-enriched group (NE1-NE4 145 146 on Figure 4) 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 147 group presented in Figure 1. Then, 1h after the end of odorant stimulation, the mice were deeply 148 anesthetized (pentobarbital, 0.25 ml/30 g) and killed by intracardiac perfusion of 40-50 ml of fixative 149 (4% paraformaldehyde in phosphate buffer, pH 7.4). Their brains were removed, postfixed, 150 151 cryoprotected in sucrose (20%), frozen rapidly, and then stored at -20°C. Brains were sectioned with a cryostat (Reichert-Jung, NuBlock, Germany) at 14µm and 40µm (3*14µm followed by 2*40µm 152 153 sectioning) for cell counts and morphological analysis respectively.

154 BrdU immunohistochemistry. 14µm 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 155 with pepsin (0.43 U/mL in 0.1N HCl, Sigma) for 3 min. Sections were transferred to a blocking 156 solution (5% normal horse serum (Sigma) with 5% BSA and 0,125% Triton X-100), and were then 157 incubated overnight at 4°C in a mouse anti-BrdU antibody (1/100, Chemicon), followed by a 158 159 biotylated anti-mouse secondary antibody (1/200, Vector Laboratories) for 2h. The sections were then processed through an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). After 160 dehydration in graded ethanols, the sections were defatted in xylene and cover-slipped in DEPEX 161 162 (Sigma).

163 *Triple labeling immunohistochemistry.* To determine the phenotype of BrdU-positive cells in the 164 OB and their functional involvement, triple labeling was performed using rat anti-BrdU (1:100, 165 Harlan Sera Laboratory), mouse anti-NeuN (1:500, Chemicon) and rabbit anti-Zif268 (1:1000, Santa 166 Cruz Biotechnology) antibodies. Appropriate secondary antibodies coupled with Alexa 546 for 167 revelation of BrdU, Alexa 633 for NeuN and Alexa 488 for Zif268 were used (Molecular Probes).

168 *Cell counting*. All cell counts were conducted blind with regard to the experimental mouse group. 169 BrdU data were collected with the help of mapping software (Mercator Pro, Explora Nova), coupled 170 with a Zeiss microscope. BrdU-positive cells were counted in the entire granule cell layer of the OB 171 on about 9 sections (14 μ m thick, 216 μ m intervals) of 5-8 mice/group. The number of positive cells 172 was divided by the surface of the granule cell layer to yield the total density of labeled cells (labeled 173 profiles/ μ m²).

Triple labeled sections were examined using pseudo-confocal scanning microscopy equipped with an Apotome (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⁺).

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179 <u>Neuronal morphology analysis</u>

Preexisting neuron labeling. At P0, mice were anesthetized on ice and injected in the lateral ventricle with 1 μ l of Lenti-PGK-DsRed lentivirus (6.11 x 10⁸ UI/ml Lenti-PGK-DsRed which 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 μ L/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, Tokyo, Japan). 100 nl of Lenti-PGK-GFP lentivirus (2 x 10^9 UI/ml, provided through Addgene #12252 by the Trono Lab) were injected bilaterally into the subventricular zone (with respect to the bregma: AP, +1 mm; ML, ± 1; DV, -2.3 mm) at a rate of 150nL/min. Mice received antalgic 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 odorants were identified by Zif268 expression. Triple labeling was performed on 40µm 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 200 201 images taken at both 40x and 63x magnification (lateral and z-axis resolutions were respectively 160 202 and 280 nm at 40x and 100 and 200 nm at 63x). Only Zif268-positive neurons were kept for further analysis. Then, spine densities on apical and basal dendrites were assessed on images taken with a 203 204 100x objective (lateral and z-axis resolutions were 60 and 200 nm respectively). For the analysis of dendritic arborizations, images were taken with 20x (lateral and z-axis resolutions were 320 and 400 205 nm) and 40x objectives. The length of all the distal dendritic segments of the apical distal domain, of 206 the proximal dendritic segment of the apical domain or of all the dendritic segments of the basal 207 domain were measured based on previous reports (Kelsch et al. 2008). All images were acquired 208 209 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)
(<u>http://research.mssm.edu/cnic/tools-ns.html</u>). Neuronstudio allows three-dimensional (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 Table 3 and 4 respectively.

218

219 <u>Optogenetics in freely behaving mice.</u>

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; Figure 7). Fourteen of the 39 mice 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, +4.6mm, ML, ± 0.75 mm, DV, -2mm).

Behavior. Mice were light stimulated (crystal laser, 561nm, 10-15mW, 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 and at P106, and with the exception that just before sacrifice they were randomly

affected to one of the following conditions: light stimulation associated with odorant stimulation or 238 odorant stimulation alone. For the light stimulation group, this followed a pattern mimicking the 239 240 average light stimulation pattern during the Otest session (0.5s light ON, 2s light OFF, pattern repeated over 1h). After brain sectioning (see above), EYFP and Zif268 double 241 immunohistochemistry was performed as described previously (Kermen et al. 2016). EYFP-positive 242 243 and Zif268-positive cells were counted in the OB sampled on one to 3 sections (40µm thick, interval 80 µm) located under the optic fiber implantation site to allow assessment of the inhibition efficiency. 244 In addition, we measured in the same area the percentage of transduced cells relative to the total 245 246 granule cell layer population.

247

248 Statistical Analyses.

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

251 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 252 sphericity were performed on the data. This resulted in the use of non-parametric tests as deviation 253 254 from normality and/or deviation from sphericity were observed. For each group, mice investigation time during habituation (Ohab1-4) of each odorant pair was analyzed using Friedman tests. 255 Investigation time between Ohab4 and Otest for each group and each pair was compared using 256 unilateral Wilcoxon tests. Only mice that investigated the odorant for at least 1s at Ohab1 were 257 included in the analysis (9 trials were excluded among 388 total trials). 258

Adult-born neurons quantification. BrdU-positive cell densities were compared between all experimental groups using a one-way ANOVA (group as factor) followed by Holm-corrected posthoc t-tests (normality of residuals and homoscedasticity were validated using Shapiro-Wilk and Levene tests respectively). Triple labelled section 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 to the control
 group.

270

271 Results

272 Increasing the complexity of perceptual learning did not proportionally increase adult-born cell

273 <u>survival but enhanced the functional recruitment of adult-born neurons.</u>

Groups of mice were submitted to different perceptual learning paradigms varying in the number of 274 odorant pairs used during the olfactory enrichment period (from 1 to 6 pairs of odorants, Figure 1). 275 Enrichment consisted of successive presentations of odorant pairs during a day and the repetition of 276 this process over 10 days. After this enrichment period, we assessed whether mice were able to 277 discriminate the odorants from each odorant pairs using a habituation/cross habituation test. In this 278 test, mice were submitted to four habituation trials (Ohab trials) with one odorant of the pair and a 279 280 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 281 Otest reflected discrimination between the two odorants of the pair. Five experimental groups were 282 constituted: control non-enriched (NE), enriched with 1 odorant pair (Group 1), enriched with 2 283 odorant pairs (Group 2), enriched with 3 odorant pairs (Group 3) and enriched with 6 odorant pairs 284 (Group 4) (Figure 1 and Supplementary Table 1). 285

Significant habituation was observed for all groups as evidenced by the reduction in investigation
time across trials (Friedman tests, Supplementary Table 2; Supplementary Figure 1). Regarding

discrimination, all enriched groups showed discrimination of the odor pair(s) used for enrichment 288 (see Supplementary Table 1 for abbreviations): +lim/-lim (Group 1) (Figure 2A); +lim/-lim and 289 290 dec/dodec (Group 2) (Figure 2B). +lim/-lim, dec/dodec and acetic a./propionic a. (Group 3) (Figure 291 2C). It is worth noting that enrichment with +lim/-lim and dec/dodec did not improve discrimination 292 of acetic a./propionic a (Supplementary Figure 2). Finally the most complex enrichment (6 pairs: 293 +lim/-lim, dec/dodec, acetic a./propionic a., propyl/butyl, penta/buta and +terp/-terp)(Group 4) 294 allowed mice to discriminate between the odorants of all 6 pairs (Figure 2D). Importantly, the control NE group showed no improvement in discrimination abilities between any of the tested odorant pairs 295 296 (Figure 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. 297

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We then asked whether increasing the number of odorant pairs mice passively learned to discriminate 299 will increase the number of surviving adult-born neurons. Therefore, we labeled a cohort of adult-300 301 born cells integrating in the OB at the beginning of the enrichment, by injecting BromodeoxyUridine (BrdU) 9 days before the enrichment period. This delay allows neuroblasts to migrate from the 302 subventricular zone to the OB. We assessed adult-born cells density in the granule cell layer since 303 304 perceptual 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. 305 2009). We found a significant difference between experimental groups (one-way-ANOVA: F(4,25)= 306 4.77; p=0.005; Figure 3). More precisely, BrdU-positive cell density increased in all enriched groups 307 compared to the control non-enriched group (Holm-corrected post hoc t-tests - Group 1 versus NE: 308 p=0.025; Group 2 versus NE: p=0.011; Group 3 versus NE: p=0.036; Group 4 versus NE: p=0.014). 309 However, no significant difference between enriched groups was observed (p>0.9 for all 310 comparisons) indicating that the density of surviving adult-born cells is not dependent on the number 311 312 of learned odorant pairs.

Since we found no increase in the density of surviving adult-born cells even when the enrichment 313 became more complex, we hypothesized that a higher proportion of the surviving adult-born neurons 314 315 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 316 317 Zif268 in response to the learned odorants as an index of cellular activation(Moreno et al. 2012). 318 Before sacrificing the animals we exposed them to their respective learned odorants +lim/-lim for 319 Group 1; +lim/-lim and dec/dodec for Group 2, +lim/-lim, dec/dodec, acetic a./propionic a. for Group 3; +lim/-lim, dec/dodec, acetic a./propionic a., propyl/butyl, buta/penta, +terp/-terp for Group 6. We 320 321 also tested a Non-Enriched 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 to 322 NE₄). 323

First, we observed the effect of enrichment on the responsiveness of adult-born neurons to olfactory 324 stimulation measured as the percentage of BrdU-positive cells co-expressing Zif268 and NeuN (two-325 326 way ANOVA, enrichment effect $F_{(4,35)}=10.70$, p=0.000009) (Figure 4). Each group was different from its respective control (unilateral t-tests; Group 1 versus its control: t=-2.05, p=0.034; Group 2 327 versus its control: t=-2.18, p=0.033 ; Group 3 versus its control: t=-3.20, p=0.005 ; Group 4 versus 328 its control: t=-5.30, p=0.0009). In addition, the more complex the enrichment, the more 329 BrdU/Zif268/NeuN-positive cells were retrieved (linear regression: adjusted-R²=0.35, p=0.003). This 330 was not seen in the control NE groups (linear regression: adjusted-R²=-0.20, p=0.81). Thus, the 331 correlation suggests that complexity and adult born neuron responsiveness are linked, even though 332 other factors may interact as well. 333

We further tested whether this result could be due to the different durations of odor enrichment between groups (1 hour per day for Group 1 versus 6 hours per day for Group 4). To test this, we enriched a new group of mice with +lim/-lim 6 hours per day for 10 days (similar duration of enrichment compared to Group 4). The percentage of Brdu/Zif268/NeuN-positive cells did not differ from that in Group 1 (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 six odorant pairs and compared it to that in non-enriched animals. We found no difference between these two experimental groups (unilateral t-test: t=0.32, p=0.38; 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 348 neuronal differentiation of adult-born cells (two way ANOVA: enrichment effect F_(4,35)=1.32; p=0.48 349 ; stimulation effect: F_(4,35)=0.66, p=0.62; Supplementary Figure 5A). Quantification of Zif268-350 351 positive cell density showed an effect of enrichment but no effect of odor stimulation before sacrifice (Supplementary Figure 5B) (two way ANOVA, enrichment effect: F_(4,35)=6.83, p=0.00036; 352 stimulation effect: F_(4,35)=1.27, p=0.3; followed by unilateral t-tests : Group 1 versus its control: t=-353 2.46, p=0.016; Group 2 versus its control: t=-2.02, p=0.041; Group 3 versus its control: t=-1.90, 354 p=0.043 ; Group 4 versus its control: t=-2.27, p=0.032), thus showing that enrichment increased 355 overall granule cell activity independently of the number of learned odorants. 356

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.

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361 Increasing the complexity of perceptual learning increased structural plasticity of adult-born

362 <u>neurons and induces limited morphological changes in preexisting neurons</u>

To better understand the increased functional recruitment of adult-born neurons with perceptual 363 learning complexity, we looked for associated structural modifications in these neurons. To determine 364 365 whether perceptual learning had an effect on the morphology of odor-responding adult-born neurons (Zif268-positive), we injected a lentivirus expressing GFP at post-natal day 60 (P60) in the 366 subventricular zone 8 days before learning (Figure 1). 25 days post-injection, animals were sacrificed 367 368 and we analyzed dendritic arborization and spine density of the apical distal domain (the site of 369 interactions with mitral/tufted (M/T) cells) as well as the apical proximal and basal domains (sites of centrifugal inputs) (Figure 5A)(Mandairon et al. 2018) of Zif268-positive neurons. 370

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

However, we found that perceptual learning increased the spine density of all dendritic domains (one-374 way ANOVAs, apical distal $F_{(4,90)}=3.03$, p=0.022; Figure 5Ci; apical proximal $F_{(4,69)}=3.48$, p=0.01; 375 376 Figure 5Cii; basal F_(4,101)=3.24, p=0.015; Figure 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 377 neurons only in Group 3 and 4 compared to NE (Holm-corrected post-hoc t-tests; Group 3 versus NE 378 379 p=0.01, Group 4 versus NE p=0.02; Figure 5Ci). In the apical distal domain, the increased spine density is observed in all groups (Holm-corrected *post-hoc* t-tests; Group 1 versus NE p=0.011, Group 380 2 versus NE p=0.011, Group 3 versus NE p=0.011, Group 4 versus NE p=0.011; Figure 5Cii). The 381 same global increase is observed for the basal domain (Holm-corrected post-hoc t-tests; Group 1 382 versus NE p=0.008, Group 2 versus NE p=0.008, Group 3 versus NE p=0.008, Group 4 versus NE 383 384 p=0.0005; Figure 5Ciii).

These results suggested that when the environmental complexity was low (enrichment with one and two 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 4 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.

391

These same mice were beforehand injected with a lentivirus expressing DsRed at post-natal day 0 392 393 (P0) (Figure 1). Because of that, it was possible to investigate the structural plasticity induced in odor-394 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 395 396 experimental groups (NE and Group 1-4) regarding length of the apical proximal and basal dendritic domains (one-way ANOVAs, respectively $F_{(4,113)}=1.46$, p=0.22; Figure 6Ai; $F_{(4,95)}=0.6$, p=0.66; 397 Figure 6Aiii). Regarding the apical distal domain, the analysis revealed a group effect (one-way 398 ANOVA F_(4,113)=3.39, p=0.012) but there was no significant difference for comparisons between NE 399 and enriched groups (Holm-corrected *post-hoc* t-tests, p>0.06; Figure 6Aii). 400

An increase of spine density in the apical distal domain was observed in Group 3 and Group 4 compared to the NE group (one-way ANOVA; $F_{(4,113)}=3.12$, p=0.02; Holm-corrected *post-hoc* t-tests: Group 3 versus NE p=0.027, Group 4 versus NE p=0.027; Figure 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* ttests, p>0.08; Figure 6Bi). Spine density in the basal domain was not affected by enrichment (oneway ANOVA $F_{(4,60)}=1.39$, p=0.25; Figure 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).

413 Optogenetically inhibiting preexisting or adult-born neurons in freely moving mice reveals their

414 <u>functionally distinct involvement in simple and complex perceptual learning.</u>

Based on the previous morphological results, we hypothesized that inhibiting adult-born neurons after 415 416 simple and complex perceptual learning would alter learned discrimination while inhibiting preexisting neurons would only affect the expression of learned discrimination following the complex 417 learning. To test this hypothesis, we used four new groups of mice. The first group was infused with 418 a halorhodopsin- and EYFP-expressing lentivirus in the subventricular zone at P60 to transduce and 419 later on silence adult-born neurons. Half of the animals of this group were submitted to simple 420 421 environmental enrichment (one odorant pair; similar to Group 1) and the other half to complex 422 environmental enrichment (six 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 423 424 silence them later on in adulthood. This group was also divided in two; one enriched with one odorant pair and one enriched with six odorant pairs (Figure 7). As previously, mice discrimination was tested 425 after enrichment, using habituation/cross habituation tests assessing perceptual learning. Optogenetic 426 inactivation of transduced neurons was achieved in freely behaving mice only during the test trial of 427 the habituation/cross habituation paradigm by automatically triggering the light stimulation (see 428 methods) each time the animal approached the odor source. All mice were also tested in a light OFF 429 condition. 430

As expected, since light was never on during this phase of the test, habituation proceeded normally (Supplementary Figure 6, 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 (Figure 8; Supplementary Table 5). More specifically, animals enriched with +lim/-lim were able to discriminate these two odorants in the light OFF condition but not when light triggered inactivation of adult-born neurons (Figure 8A and 8C; Supplementary Table 5). The same phenomenon was observed for complex enrichment: animals were
able to discriminate the six learned odorant pairs in the light OFF condition but this was no longer
observed in the light ON condition (Figure 8B and 8D; Supplementary Table 5).

440 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 441 442 submitted to simple and complex enrichments (bilateral t-test: p=0.098; Supplementary Figure 7Ai). We also measured the proportion of transduced neurons (EYFP-positive) relative to the whole cell 443 population (DAPI-positive). Groups injected at P0 showed higher percentages (simple $3.8\% \pm 0.21$, 444 complex 2.8% \pm 0.29) than groups injected at P60 (simple 2.14% \pm 0.23, complex 1.59 \pm 0.23). 445 446 Statistical analysis indicated that percentage of transduced neurons was superior in groups injected at P0 compared to groups injected at P60 (ANOVA $F_{(1,21)} = 30,35$, p<0.0001). Within the group injected 447 448 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 449 the highest density of transduced cells is the group in which light has no behavioral effect. Thus, these 450 differences can probably not account for the reported effects of light. Second, the effectiveness of 451 452 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 453 454 cells was observed in the light ON condition compared to light OFF confirming that light actually inhibited transduced adult-born neurons (unilateral t-test: simple learning p=0.028, complex learning 455 p=0.043 ; Supplementary Figure 7Aii). 456

In contrast to adult-born neurons, light-triggered inactivation of preexisting neurons did not alter discrimination of the learned odorants after simple enrichment (Figure 9A and 9C, Supplementary Table 5). However, inhibiting preexisting neurons after complex enrichment impaired discrimination of most odorant pairs (Figure 9B and 9D, 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; Supplementary Figure 7Bi) and
a decrease in the percentage of EYFP/Zif268-positive cells was observed in the light ON compared
to the light OFF condition (unilateral t-tests: simple learning p=0.048, complex learning: p=0.015,
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
(Supplementary Figure 8, 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.

473

474 Discussion

475 Perceptual learning contributes to the representation of the sensory environment which guides 476 animal's behavior. An essential component of the plasticity of this representation in the olfactory system is adult neurogenesis, i.e. renewal of interneurons within a preexisting neuronal network. 477 478 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 479 increasing environmental complexity, suggesting that a plateau of adult-born cell survival is reached 480 481 after simple perceptual learning. Furthermore, of the adult-born neurons surviving after perceptual 482 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 483 adult-born neurons expressing Zif268 in response to olfactory stimulation and the number of 484

discriminated pairs of odorants. Interestingly, the learning-dependent survival increase reported here 485 is close to the rate of adult-born neurons saved by different olfactory learning paradigms (associative 486 487 and perceptual learning tasks (Alonso et al. 2006; Mouret et al. 2008; Moreno et al. 2009; Kermen et 488 al. 2010; Sultan et al. 2010; Mandairon et al. 2011)) suggesting that this survival increase might not 489 be specific to a task but simply a correlate of environmental modifications. Thus learning type or 490 environmental change complexity is not coded by the survival rate of adult-born neurons. However 491 our results strongly suggest that the information about environmental complexity could be carried by 492 functionally recruiting more adult-born neurons from the pool of available ones.

493 One limitation of our study comes from the fact that not all mice were tested on their ability to 494 discriminate odorants of all six 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 495 odorants used during exposure (Mandairon, Stack, et al. 2006; Moreno et al. 2009), in line with the 496 specific activation patterns they evoke in the OB (Linster et al. 2001). Furthermore, we showed that 497 enrichment with +lim/-lim and dec/dodec does not induce discrimination of acetic a./propionic a, 498 strongly supporting the hypothesis that discrimination of more odor pairs is actually allowed by a 499 more complex environment. However from three pairs of odorants onwards, the OB stimulation is 500 broad and activates overlapping regions of the OB leading to a less specific improvement in 501 502 discrimination. This is similar to what was observed following global odor enrichment (Rey et al. 2012) or pharmacological activation of the OB (Mandairon, Stack, et al. 2006). 503

Beyond the number of surviving adult-born cells or the proportion of them that are activated, another parameter of plasticity shown to be involved in improved discrimination is the structural plasticity of adult-born neurons (Daroles et al. 2015; Mandairon 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 509 prevented discrimination after simple perceptual learning (in accordance with Moreno and colleagues 510 (Moreno et al. 2009)) and also after complex perceptual learning. We also report that simple olfactory 511 perceptual learning does not modify the morphology of preexisting cells although an increase of spine 512 density can be observed at the apical domain after complex perceptual olfactory learning. Importantly, 513 these modifications seem to correlate with our observation that inhibition of preexisting neurons 514 prevented discrimination of the learned odorants only after complex but not simple perceptual 515 learning. Based on these observations, we can hypothesize that morphological modifications are the 516 cause of the discrimination changes observed at the behavioral level. There are several ways they 517 could underlie perceptual learning. First, apical dendrites are the site of reciprocal dendrodendritic 518 synapses between granule and mitral cells (Price and Powell 1970; Shepherd 1972), thus a higher 519 number of spines could elevate the previously reported inhibitory drive on mitral cells (Moreno et al. 520 2009; Mandairon et al. 2018) and explain why the absence or inactivity of these same cells prevents 521 discrimination (Moreno et al. 2009). Second, the apical proximal and basal domains both receive 522 cortico-bulbar projections from olfactory cortices (Nissant et al. 2009; Lepousez et al. 2014) as well 523 as neuromodulatory inputs such as noradrenergic, cholinergic and serotoninergic (Fletcher and Chen 524 2010). Because the noradrenergic system acts on adult-born cells to allow olfactory perceptual 525 learning (Moreno et al. 2012) and gates dendrodendritic inhibition onto mitral cells (Balu et al. 2007) 526 while not being exclusive of other influences such as cholinergic (Ravel et al. 1994; Linster and 527 Cleland 2002, 2016; Mandairon, Ferretti, et al. 2006; de Almeida et al. 2013), this increase in 528 proximal and basal inputs could enhance noradrenergic influences on granule cell activity. Hence, as 529 530 environmental complexity increases, spine density modifications (both apical, proximal and basal) probably lead to a remapping of synaptic weights thereby synergically producing the linearly 531 532 increased functional involvement of adult-born neurons as the environment gets more and more 533 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 versus 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 543 544 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 545 546 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 547 adult-born neurons. The present experiments further show that a third level of network plasticity is 548 549 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. 550

All the above as well as previous studies (see for review (Hardy and Saghatelyan 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.

556 While the occurrence of adult neurogenesis in adult Human olfactory bulb was subject to debate 557 regarding its presence (Curtis et al. 2007), current consensus is that neurogenesis is absent beyond the age of 2 in the human OB. 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 human.

In conclusion, in this mice study, we have demonstrated both the necessity and sufficiency of adultborn 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 olfactory bulb modifications associated with simple perceptual learning but, can still be recruited both functionally and structurally to contribute to learning in cases of environmental complexity.

569

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574

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709 Captions

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711 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 712 neurons were labeled by injecting BrdU (i.p.) at P59 and GFP expressing lentivirus in the 713 714 subventricular zone at P60 (2-months old). Then, 5 groups of mice were formed depending on the 715 number of odorant pairs used for the 10-day enrichment period (control non-enriched: Group NE; 716 enriched with one odor pair: Group 1; enriched with two odor pairs: Group 2, enriched with 3 odor 717 pairs: Group 3; enriched with 6 odor pairs: Group 4). Animals were enriched for one hour per day with each odorant pair. After the enrichment period, mice were tested on their ability to discriminate 718 the odorants of each odorant pair using an olfactory habituation/cross habituation test (post-test). 719 Mice were sacrificed 85 days after their birth. 720

721

722 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 723 illustrate discrimination. A. After enrichment with +lim and -lim (Group 1), the investigation time 724 725 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 726 enrichment with +lim/-lim and dec/dodec (Group 2) indicating discrimination between the 2 odorants 727 of the 2 pairs. C. Enrichment with +lim/-lim, dec/dodec and acetic a./propionic a. (Group 3) allows 728 discrimination between the 2 odorants of the 3 pairs. D. Enrichment with six pairs of odorants (Group 729 730 4), i.e. +lim/-lim, dec/dodec, acetic a./propionic a., propyl/butyl, pent/but, +terp/-terp, allows discrimination between the 2 odorants in all six pairs. E. In non-enriched control animals (NE group), 731 no difference is observed between Ohab4 and Otest, for any of the six odorant pairs (+lim/-lim ; 732 dec/dodec ; acetic a./propionic a. ; propyl/butyl ; pent/but ; +terp/-terp) indicating no discrimination. 733

*p<0.05. **p<0.01. Data are expressed as mean±sem and all data points are represented. Each dot
represent a trial.

736

Figure 3: Perceptual learning increased adult-born granule cell density independently of the 737 number of learned odorant pairs. A. Example of BrdU-positive immunolabeled cells (arrows). 738 739 Scale bar = $10\mu m$. B. Groups enriched with one odorant pair (Group 1, n=8), two odorant pairs (Group 740 2, n=6), three pairs (Group 3, n=5) or six pairs (Group 4, n=5) have a significantly higher BrdUpositive cell density compared to the non-enriched group (Non-enriched, n=6). No difference in 741 742 BrdU-positive cell density was observed between the 4 enriched groups. *p<0.05 in comparison with the non-enriched group. Data are expressed as mean±sem and all data points are represented. Each 743 dot represent an animal. 744

745

746 Figure 4: Increasing the complexity of perceptual learning increased the functional

747 recruitment of adult-born neurons.

A. Example of a BrdU/Zif268/NeuN-positive neuron (arrows). B. Enrichment with one (group 1, 748 n=9), two (group 2, n=6), three (group 3, n=9) or six (group 4, n=5) pairs of odorants increased the 749 750 percentage of adult-born cells (Brdu/NeuN) responding to the learned odorants (Zif268) compared to their respective non-enriched (NE) group. NE groups were stimulated on the day of sacrifice with 1 751 (NE1), 2 (NE2), 3 (NE3) or 6 (NE4) odorant pairs; n=3 for each NE group. Linear regression analysis 752 indicated that the percentage of BrdU/NeuN/Zif268-positive cells is increasing with enrichment 753 complexity (red line) while this is not the case for control groups (blue line). *p<0.05, **p<0.01, 754 ***p<0.001. See Figure 1 for details on the odorants used. Data are expressed as mean±sem and all 755 data points are represented. Each dot represent an animal. 756

Figure 5: Increasing the complexity of perceptual learning increased structural plasticity of 758 759 adult-born neurons A. Schematic representation of a granule cell and its dendritic domains. B. No 760 group differences for the length of apical proximal (**Bi**) (n_{neuron} from NE to Group 4 = 14, 24, 21, 14, 22), of the apical distal (**Bii**) (n_{neuron} from NE to Group 4 = 14, 23, 21, 14, 22) and basal 761 762 dendritic arborization (Biii) (n_{neuron} from NE to Group 4 = 12, 23, 19, 11, 26). C. Spine densities 763 were increased after perceptual learning at the apical proximal (Ci) (n_{neuron} from NE to Group 4 = 764 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 765 766 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 767 corrected t-tests. Data are expressed as mean±sem and all data points are represented. Each dot 768 769 represent a neuron.

770

771 Figure 6: Perceptual learning induced limited morphological changes in preexisting neurons. A. No effect of perceptual learning was observed on the length of apical proximal dendrites (Ai) 772 (n_{neuron} from NE to Group 4 = 25, 25, 20, 31, 17), the apical distal dendritic arborization (Aii) (n_{neuron} 773 from NE to Group 4 = 25, 25, 20, 31, 17) or the basal dendritic arborization (Aiii) (n_{neuron} from NE to 774 Group 4 = 20, 18, 21, 27, 14). **B.** Spine densities after perceptual learning at the apical proximal (**Bi**) 775 (n_{neuron} from NE to Group 4 = 21, 13, 9, 26, 10), apical distal (**Bii**) (n_{neuron} from NE to Group 4 = 27, 776 21, 18, 34, 18) and basal domains (**Biii**) (n_{neuron} from NE to Group 4 = 11, 12, 14, 18, 10). Increased 777 spine density in the apical distal domain was seen after complex perceptual learning only (groups 3 778 779 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 780 mean±sem and all data points are represented. Each dot represent a neuron. 781

Figure 7: Design of the optogenetic experiment. Halorhodopsin expressing lentivirus was injected 783 in P0 mice to infect preexisting neurons or at P60 to infect adult-born neurons. Two groups of mice 784 785 were trained in a simple perceptual learning (one injected at P0 (n=6) and one at P60 (n=4)) and two groups of mice were trained in a complex perceptual learning (one injected at P0 (n=8) and one at 786 787 P60 (n=7)). After enrichment, mice were tested on their ability to discriminate the learned odorant 788 pairs using an olfactory habituation/cross habituation test. Light-stimulation was triggered in freely 789 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 ligh OFF condition. 790

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Figure 8: Optogenetically inhibiting adult-born neurons prevented discrimination after both simple and complex perceptual learning. After A. simple and B. complex perceptual learning tasks, inhibiting adult-born neurons prevented discrimination. In light off conditions, the same mice were able to discriminate learned odorant pairs when there was no inhibition during the discrimination test after both C. simple and D. complex learning. Data are expressed as mean±sem and all data points are represented. *: p<0.05, **: p<0.01. Each dot represent a trial.

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Figure 9: Optogenetically inhibiting preexisting neurons did not prevent discrimination after
simple learning but did alter discrimination after complex learning, i.e. preventing
discrimination of most odorant pairs. 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±sem and all data
points are represented. *: p<0.05, **: p<0.01, ***: p<0.001. Each dot represent a trial.















Carebral Cortex neurons



- 52
- 53







Supplementary Figure 2: Behavioral experiment. Effect of enrichment with 2 odor pairs (+lim/-lim and dec/dodec) Ihr per day during 10 days on the performances of discrimination of +lim/-lim, dec/dodec and acetic a./propionic a. Protocol of enrichment and discrimination tests are similar as described previously. After verifying the normality (Kolmogorov-Smirnov), paired parametric tests (ANOVA for repeated measures and unilateral paired t-tests) were used. Mice that investigated the odorant less than 1s at the first trial (OHab1) and mice that were immobile for at least 3 trials were removed. A. Mice showed significant habituation (Trial effect, F(3,51)=4.74, p=0.005 and difference between OHab1 and OHab4 p=0.0003; n=18) and a significant discrimination (p=0.02 for difference between OHab4 and OTest). B. Mice showed significant habituation (Trial effect, F(3,45)=3.80, p=0.016 and difference between OHab1 and OHab4 p=0.005; n=17) and a significant discrimination (p=0.006 for difference between OHab4 and OTest). C. Mice showed significant habituation (Trial effect, F(3,45)=5.9, p=0.001 and difference between OHab1 and OHab4 p=0.22; n=16) and no discrimination (p=0.42 for difference between OHab4 and OTest). All data points are represented.



Supplementary Figure 3: Multiple enrichments with the same pair of odorants had a similar impact on the functional recruitment of adult-born neurons as did simple enrichment (Group 1). A. Protocol B. The percentage of BrdU/Zif268/NeuN similar between the group enriched with +lim/-lim once a day over 10 days (n=9) and the group enriched six times a day over days with +lim/-lim (n=3). Data are expressed as mean±sem and all data points are represented.





Supplementary Figure 4: Enrichment had no impact on the basal expression of Zif268 in adult-born neurons. In the absence of odorant stimulation, the percentage of BrdU/Zif268/NeuN was similar between non enriched animals (n=3) and animals enriched with 6 pairs of odorants (n=4). Data are expressed as mean±sem and all data points are represented.



20 Supplementary Figure 5: Enrichment, regardless of complexity did not alter the fate of adult-born neurons

(BrdU/NeuN-positive cells) and increased the density of activated (Zif268-positive) neurons. A. Enrichment with one 22 odorant pair (Group 1), two odorant pairs (Group 2), three odorant pairs (Group 3) or six odorant pairs (Group 4) does not 23 change the percentage of BrdU/NeuN positive cells. B. The density of Zif268-positive cells is superior in enriched groups 24 compared to their respective controls. Zif268-positive cell density is similar between all enriched groups. *p<0.05. Data are 25 expressed as mean±sem and all data points are represented. Number of animals is the same as reported in Figure 4.



Sopplementary Figure 6: Optogenetic experiment – habituation of all groups. Almost all groups showed habituation behavior **3**/4th repeated odorant exposure (see table 3 for details). Data are expressed as mean±sem and all data points are represented.



Supplementary Figure 7: Assessment of viral transfection and light-induced inhibition in the olfactory bulb. Density of cells expressing halorhodopsin (EYFP-positive cells) in the OB when transducing Ai. adult-born (simple n=4, complex n=7) or Bi. preexisting neurons (simple n=6, complex n=8). Percentage of Aii. adult-neurons (simple: OFF n=6 and ON n=3, complex: OFF n=2 and ON n=5) or Bii. preexisting neurons (simple: OFF n=3 and ON n=3, complex: OFF n=3 and ON n=5) being both EYFP-positive and Zif268-positive. Shining light at the OB before sacrifice reduces Zif268 expression in the transfected cell populations both for adult-born and preexisting neurons. C. Example of DAPI/EYFP/Zif268 immunohistochemistry in an halorhodopsin transfected OB. *p<0.05. Data are expressed as mean±sem and all data points are represented.



Cerebral Cortex

	Odorant pair	Dilution (vol/vol %)	Abbreviation
1	(+)limonene	0.203	+lim
3	(-)limonene	0.203	-lim
4	decanal	3.521	dec
6	2-dodecanone	27.741	dodec
/ 8	acetic acid	0.007	acetic a.
9	propionic acid	0.041	propionic a.
10	propyl-acetate	0.006	propyl
11	butyl-acetate	0.022	butyl
13	pentanol	0.026	penta
14	butanol	0.044	buta
15 16	(+)terpinen-2-ol	6.632	+terp
17 18	(-)terpinen-2-ol	6.632	-terp
19			

Supplementary Table 1: Odorants table. All odorants were diluted in mineral oil in order to reach the vapor pressure of 4 a. All odorant abbreviations are described here for future references.

Page 53 of 57			Cerebral Cortex Habituation Discrimination						
1	Group	Odorant pair(s)	n	Friedman	Difference	Wilcoxon		Difference	
1 2 3 4 5		+lim/-lim	35	X=30.58; df=3	P=0.000001	***	V=260.5	p=0.36	ns
		dec / dodec	35	X=18.22; df=3	P=0.0004	***	V=293	P=0.47	ns
	Non Enriched	acetic a. / propionic a.	12	X=12.4; df=3	P=0.0061	***	V=26	P=0.17	ns
6		butyl / propyl	14	X=16.6; df=3	P=0.00086	***	V=64	P=0.77	ns
7 8 9		pent / but	14	X=16.46; df=3	P=0.000909	***	V=50	P=0.45	ns
		+terp / -terp	14	X=22.17; df=3	P=0.00006	***	V=42.5	P=0.28	ns
10 11 12	Group 1	+lim / -lim	21	X=16.72; df=3	P=0.0008	***	V=57	P=0.022	*
	Group 2	+lim / -lim	18	X=24.39; df=3	P=0.000021	***	V=18	P=0.0052	**
13		dec / dodec	15	X=19.88; df=3	P=0.00018	***	V=56	P=0.028	*
14		+lim / -lim	19	X=12.03; df=3	P=0.0073	***	V=36	P=0.008	**
15 16	Group 3	dec / dodec	20	X=23.76; df=3	P=0.000028	***	V=53	P=0.027	*
17 18		acetic a. / propionic a.	19	X=20.05; df=3	P=0.00017	***	V=52	P=0.044	*
19		+lim / -lim	24	X=14.55; df=3	P=0.0022	***	V=80	P=0.023	*
20 21		dec / dodec	24	X=20.95; df=3	P=0.0001	***	V=39	P=0.0024	**
22	Group 4	acetic a. / propionic a.	23	X=12.03; df=3	P=0.0073	***	V=65	P=0.024	*
24		butyl / propyl	24	X=26.063; df=3	P=0.0000093	***	V=65.5	0.0082	**
25		pent / but	24	X=19.75; df=3	P=0.00019	***	V=55	P=0.0061	**
26 27		+terp / -terp	24	X=28.21; df=3	P=0.0000033	***	V=45.5	P=0.0045	**
28 29	pplementary T	able 2: Statistics or	n habitu	ation and discrimi	ination performa	ances.			

Cerebral Cortex								
Adult-born neurons	Group	Animals	Neurons/animal	Total number of neurons				
	NE	4	1-5	14				
	1	6	1-8	24				
Apical lenght	2	4	1-10	21				
	3	4	1-5	14				
	4	3	3-8	22				
	NE	4	3	12				
	1	6	1-8	23				
Basal length	2	4	1-6	19				
	3	4	2-3	11				
	4	5	1-8	26				
	NE	4	2-5	13				
	1	4	1-7	20				
Apical spine density	2	5	1-5	25				
	3	4	2-8	13				
	4	4	1-8	24				
	NE	3	4-6	14				
	1	3	1-5	10				
Proximal spine density	2	5	1-4	13				
	3	3	3-5	12				
	4	4	1-9	25				
	NE	4	3-6	18				
	1	6	1-5	22				
Basal spine density	2	5	1-9	27				
	3	4	1-4	10				
	4	5	1-11	29				

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Supplementary Table 3: Details of the numbers underlying the morphological analysis in adult-born neurons. 35

Pag)Protex Raing neurons	Group	Animals	Cerebrali	Total number of neurons		
		NE	7	1-7	25		
1 2 3 4 5	Apical lenght	1	5	2-4	25		
		2	4	1-6	20		
		3	6	1-7	31		
6		4	4	1-6	17		
7		NE	7	1-7	20		
8 9		1	5	1-4	18		
10	Basal length	2	5	1-8	21		
11 12 13 14 15 16		3	5	1-6	27		
		4	4	1-4	14		
		NE	7	1-8	27		
	Apical spine density	1	5	1-4	21		
17		2	5	1-6	18		
18 19		3	5	1-7	34		
20		4	4	1-7	18		
21		NE	7	1-8	21		
23		1	3	1-4	13		
24 25	Proximal spine density	2	2	2-5	9		
26		3	5	2-6	26		
27 28		4	3	1-5	10		
29		NE	5	1-5	11		
30 21		1	5	1-3	12		
32	Basal spine density	2	4	1-5	14		
33		3	4	1-5	18		
34 35		4	2	2-4	10		
36 37 38	5 10 10 6 7 applementary Table 4: Details on the numbers underlying the morphological analysis in preexisting ne 8						

1	Silenced	Looming	Odoront nair(s)	Lase	e _	Cerebral Cort ^{Exbituation}			Discrimination age 56 of 52					
	neurons	Learning	Odorant pair(s)	r	п	Friedman	Differ	rence	Wilcoxon	Diffe	rence			
1 2 3 4 5 6 7 8 9 10 11				ON	8	X=13.94; df=3	P=0.003	**	V=11	P=0.19	ns			
		Simple	+lim/-lim	OFF	8	X=12.6; df=3	P=0.005	**	V=3	P=0.019	*			
				ON	10	X=13.08; df=3	P=0.0045	**	V=30	P=0.62	ns			
			+lim/-lim	OFF	12	X=20.24; df=3	P=0.00015	***	V=3	P=0.0044	**			
			d (d. d	ON	10	X=9.55; df=3	P=0.023	*	V=35	P=0.78	ns			
			dec / dodec	OFF	9	X=7.18; df=3	P=0.066	ns	V=0	P=0.0071	**			
12 13	Adult-born		acetic a. /	ON	8	X=10.81; df=3	P=0.013	*	V=16	P=0.42	ns			
14 15 16 17 18 19 20 21 22 23 24 25 26	neurons	Complex	propionic a.	OFF	14	X=14.31; df=3	P=0.0025	**	V=13	P=0.0072	**			
		1	hated (and and	ON	9	X=8.2; df=3	P=0.042	*	V=31	P=0.85	ns			
			butyi / propyi	OFF	13	X=9.47; df=3	P=0.024	*	V=22	P=0.055	ns			
			nent / hut	ON	10	X=15.24; df=3	P=0.0016	**	V=38	P=0.86	ns			
			pena, cui	OFF	12	X=14.4; df=3	P=0.0024	**	V=4	P=0.0017	**			
			terp+ / terp-	ON	12	X=5.87; df=3	P=0.12	ns	V=15	P=0.033	*			
			torp / torp	OFF	14	X=20.01; df=3	P=0.00017	***	V=8	P=0.0015	**			
26 27		Simple	Simple	+lim / -lim	ON	10	X=16.32; df=3	P=0.001	***	V=8	P=0.048	*		
28 29				OFF	11	X=24.30; df=3	P=0.00002	***	V=1	P=0.004	**			
30 31		3				+lim / -lim	ON	12	X=16.15; df=3	P=0.001	***	V=41	P=0.78	ns
32				OFF	16	X=17.04; df=3	P=0.0007	***	V=25	P=0.012	*			
33 34					dec / dodec	ON	15	X=20.31; df=3	P=0.00015	***	V=29	P=0.13	ns	
35 36					OFF	15	X=23.29; df=3	P=0.00004	***	V=22.5	P=0.018	*		
37 38	Preexisting		acetic a. /	ON	13	X=24.05; df=3	P=0.00002	***	V=5	P=0.0012	**			
39 40	neurons	Complex	propionic a.	OFF	10	X=15.12; df=3	P=0.0017	**	V=0	P=0.00098	***			
41			butul / propul	ON	15	X=15.24; df=3	P=0.0016	**	V=68	P=0.84	ns			
42			outyr/ propyr	OFF	13	X=20.54; df=3	P=0.00013	***	V=0	P=0.00012	***			
44 45	4 5 6		<i>n</i> + (1	ON	12	X=14.6; df=3	P=0.0022	**	V=21	P=0.15	ns			
46 47						pent / out	OFF	9	X=12.33; df=3	P=0.0063	**	V=1	P=0.0039	**
48 ⊿q			tern+/tern-	ON	15	X=17.05; df=3	P=0.00069	***	V=34	P=0.076	ns			
50			terp. / terp-	OFF	16	X=23.82; df=3	P=0.00007	***	V=5	P=0.0016	**			
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Sapplementary Table 5: Statistics on habituation and discrimination performances of similar odorants during the optogenetic experiment 55

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10 11	Learning	Odorant				Habituation	Discrimination			
Neurons 12 _{labelled} 13			Laser	n	Fried	man	Difference	Wilc	oxon	Difference
14 15	Circula		ON	8	X=8,55; df=3	P=0,036	*	V=4	P=0,027	*
16 17Adult-born	Simple	carv+ / lim+	OFF	8	X=10,95; df=3	P=0,012	*	V=2	P=0,012	*
18 ^{neurons} 19 20	Complex	x carv+/lim+	ON	9	X=13,93; df=3	P=0,003	**	V=5	P=0,02	*
			OFF	9	X=15; df=3	P=0,0018	**	V=1	P=0,004	**
21 22	Simple	comut / line t	ON	10	X=15; df=3	P=0,0018	**	V=4	P=0,0068	**
23 24 _{neurons} 25		carv+7 lim+	OFF	10	X=20,25; df=3	P=0,00015	***	V=6	P=0,014	*
	Complex	comut / limt	ON	14	X=15,99; df=3	P=0,0011	**	V=8	P=0,0015	**
26 27	Complex	carv+ / lim+	OFF	15	X=21,96; df=3	P=0,000066	***	V=19	P=0,009	**

Supplementary Table 6: Statistics on habituation and discrimination performances of dissimilar odorants ((+)limonene / (+)carvone) during the optogenetic experiment