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

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26 **Abstract**

27 Olfactory perceptual learning is defined as an improvement in the discrimination of perceptually close
28 odorants after passive exposure to these odorants. In mice, simple olfactory perceptual learning
29 involving the discrimination of two odorants depends on an increased number of adult-born neurons
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.
32 Perceptual learning of one to six pairs of similar odorants led to discrimination of all learned odor
33 pairs. Increasing complexity did not increase adult-born neuron survival but enhanced the number of
34 adult-born neurons responding to learned odorants and their spine density. Moreover, only complex
35 learning induced morphological changes in neurons of the granule cell layer born during the first day
36 of life (P0). Selective optogenetic inactivation of either population confirmed functional involvement
37 of adult-born neurons regardless of the enrichment complexity while preexisting neurons were
38 required for complex discrimination only.

39

40

41 **Keywords:**

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

43 **Introduction**

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

64 Newly integrated adult-born cells show specific electrophysiological properties compared to resident
65 neurons (formed by a mix of older adult-born neurons and preexisting neurons) such as increased
66 excitability, increased long-term potentiation (Carleton et al. 2003; Nissant et al. 2009) as well as a

67 higher responsiveness to odorant stimulation (Livneh et al. 2014). Moreover, following olfactory
68 enrichment in adulthood, adult-born neurons become more selective to the enriched odorants and thus
69 acquire functional features distinct from those of older resident neurons (Magavi et al. 2005; Livneh
70 et al. 2014).

71 The newly integrated adult-born neurons also show specific response properties compared
72 specifically to preexisting neurons (Lemasson et al. 2005; Valley et al. 2013; Breton-Provencher et
73 al. 2016). Recent studies using chemo or optogenetic manipulation adult born versus preexisting
74 neurons showed the differential involvement of preexisting versus adult-born neurons in reward-
75 associated olfactory learning (Muthusamy et al. 2017; Grelat et al. 2018). However, the respective
76 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
78 of complexity. More precisely, young adult mice were exposed to one, two, three or six pairs of
79 odorants during the enrichment period. We assessed the effect of these different enrichment protocols
80 on mice ability to discriminate. We also assessed adult-born cell survival, responsiveness to the
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
83 functional role of each population on discrimination performances after perceptual learning, we
84 measured the effect of selective optogenetic inactivation of the populations of preexisting or adult-
85 born neurons on learned discrimination.

86

87 **Materials and Methods**

88 **Animals**

89 64 inbred male C57BL/6J young adult mice born and raised in our laboratory were used in the first
90 part of this study. Mice were housed in a controlled environment under a 12h light/dark cycle with
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
96 (vM)). Mice were obtained from multiple independent litters over several months and mice from the
97 same litter were assigned as much as possible to different experimental groups.

98

99 **Overview of the experiment.**

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

107

108 **Detailed procedures**

109 **Behavioral experiments**

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

113 placed in two tea balls hanging from the cover of the cage. For multiple enrichments, odorant pairs
114 were presented with an interval of at least 1 hour. Odorant pairs were presented in the same order
115 during the 10 day-enrichment for a given cage but this order was randomized between cages. The
116 different experimental groups were as follows: Group 1 enriched with +lim/-lim ; Group 2 with +lim/-
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
122 olfactory discrimination (Moreno et al. 2009). Briefly, this task assessed whether mice were able to
123 spontaneously discriminate between two odorants by habituating them to one odorant and measuring
124 their **cross** habituation to the second odorant. We tested the discrimination between the two odorants
125 of each pair used for the enrichment (Group 1 was tested for discrimination between +lim/-lim; Group
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
128 place in a clean cage similar to the home cage. Odorants were presented by placing 60µL of the
129 diluted odorant (1Pa) (Supplementary Table 1) onto a filter paper (Whatman #1) which was put inside
130 a tea ball hanging from the side of the cage. A behavioral session consisted of one presentation of
131 mineral oil then four odorant presentations of the habituation odorant (Ohab1-4) followed by one
132 presentation of the test odorant (Otest). Each odorant presentation lasted 50 sec and was separated by
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
135 in its direction) of each odorant was manually recorded and a difference between Ohab4 and Otest
136 indicated discrimination.

137

138 Adult-born neuron quantification in the OB

139 **BrdU injection.** Mice were injected with BrdU (Sigma) (50 mg/kg in saline, three times at 2h
140 intervals, i.p.), 9 days before the beginning of the enrichment period (27 days before sacrifice) in
141 order to have a cohort of labeled adult-born cells in the OB at the beginning of the enrichment period
142 (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 μ l of pure learned odorants (odorants used for the
145 enrichment presented simultaneously) for 1h using tea balls. A control non-enriched group (NE₁-NE₄
146 on Figure 4) corresponding to each experimental group (group 1-4) was exposed to the same odorants
147 on the day of sacrifice. These NE groups were composed of mice randomly selected from the NE
148 group presented in Figure 1. Then, 1h after the end of odorant stimulation, the mice were deeply
149 anesthetized (pentobarbital, 0.25 ml/30 g) and killed by intracardiac perfusion of 40-50 ml of fixative
150 (4% paraformaldehyde in phosphate buffer, pH 7.4). Their brains were removed, postfixed,
151 cryoprotected in sucrose (20%), frozen rapidly, and then stored at -20°C. Brains were sectioned with
152 a cryostat (Reichert-Jung, NuBlock, Germany) at 14 μ m and 40 μ m (3*14 μ m followed by 2*40 μ m
153 sectioning) for cell counts and morphological analysis respectively.

154 **BrdU immunohistochemistry.** 14 μ m thick sections from mice randomly selected in each group were
155 incubated in Target Retrieval Solution (Dako) for 20 min at 98°C. After cooling, they were treated
156 with pepsin (0.43 U/mL in 0.1N HCl, Sigma) for 3 min. Sections were transferred to a blocking
157 solution (5% normal horse serum (Sigma) with 5% BSA and 0,125% Triton X-100), and were then
158 incubated overnight at 4°C in a mouse anti-BrdU antibody (1/100, Chemicon), followed by a
159 biotylated anti-mouse secondary antibody (1/200, Vector Laboratories) for 2h. The sections were then
160 processed through an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). After
161 dehydration in graded ethanols, the sections were defatted in xylene and cover-slipped in DEPEX
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).

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

178

179 Neuronal morphology analysis

180 **Preexisting neuron labeling.** At P0, mice were anesthetized on ice and injected in the lateral ventricle
181 with 1 μ l of Lenti-PGK-DsRed lentivirus (6.11 x 10⁸ UI/ml Lenti-PGK-DsRed which was generated
182 by the Functional Neurogenetic and Optogenetic platform of the Lyon Neuroscience Research Center
183 by excision of the GFP sequence from the Lenti-PGK-GFP and its replacement by a DsRed
184 sequence). The injection was done using a glass-micropipette linked to a Hamilton syringe connected
185 to a programmable syringe controller (infusion rate: 0.2 μ L/s).

186 **Adult-born neuron labeling.** Prior to surgery, the same mice previously injected at P0, were
187 anesthetized at the age of 2 months with an intraperitoneal cocktail injection of 50 mg/kg ketamine

188 and 7.5 mg/kg xylazine and secured in a stereotaxic instrument (Narishige Scientific Instruments,
189 Tokyo, Japan). 100 nl of Lenti-PGK-GFP lentivirus (2×10^9 UI/ml, provided through Addgene
190 #12252 by the Trono Lab) were injected bilaterally into the subventricular zone (with respect to the
191 bregma: AP, +1 mm; ML, ± 1 ; DV, -2.3 mm) at a rate of 150nL/min. Mice received analgic
192 ketoprofen (2 mg/kg) at the end of surgery and once every day until they regained the lost weight.
193 Mice were allowed 8 days to recover from the surgery before behavioral testing.

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

200 ***Morphological data analysis.*** For each neuron, we first determined whether it expressed Zif268 using
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
203 analysis. Then, spine densities on apical and basal dendrites were assessed on images taken with a
204 100x objective (lateral and z-axis resolutions were 60 and 200 nm respectively). For the analysis of
205 dendritic arborizations, images were taken with 20x (lateral and z-axis resolutions were 320 and 400
206 nm) and 40x objectives. The length of all the distal dendritic segments of the apical distal domain, of
207 the proximal dendritic segment of the apical domain or of all the dendritic segments of the basal
208 domain were measured based on previous reports (Kelsch et al. 2008). All images were acquired
209 using a Zeiss pseudo-confocal system (Mandairon et al. 2018).

210 Neuron stitching was performed using Vias (<http://research.mssm.edu/cnic/tools-vias.html>) and
211 neuron reconstruction using NeuronStudio software (Wearne et al. 2005; Rodriguez et al. 2008)
212 (<http://research.mssm.edu/cnic/tools-ns.html>). Neuronstudio allows three-dimensional (3D)

213 reconstructions of dendrites and spines from confocal z-series stacks on a spatial scale. Dendritic
214 length was measured semi-automatically and spines were manually identified with the help of the 3D
215 reconstruction. All morphological analyses were conducted blind with regard to the mouse group.
216 Number of animals, neurons per animals, total neurons analyzed and number of dendrites per neurons
217 for adult-born and preexisting neurons are reported in Supplementary Table 3 and 4 respectively.

218

219 Optogenetics in freely behaving mice.

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

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

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

236 **Control of light-triggered inhibition.** For the optogenetic experiment, mice were sacrificed as
237 previously described and at P106, and with the exception that just before sacrifice they were randomly

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

247

248 **Statistical Analyses.**

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

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

259 *Adult-born neurons quantification.* BrdU-positive cell densities were compared between all
260 experimental groups using a one-way ANOVA (group as factor) followed by Holm-corrected post-
261 hoc t-tests (normality of residuals and homoscedasticity were validated using Shapiro-Wilk and
262 Levene tests respectively). Triple labelled section were analyzed by two-way ANOVA (group and

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

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

270

271 **Results**

272 Increasing the complexity of perceptual learning did not proportionally increase adult-born cell
273 survival but enhanced the functional recruitment of adult-born neurons.

274 Groups of mice were submitted to different perceptual learning paradigms varying in the number of
275 odorant pairs used during the olfactory enrichment period (from 1 to 6 pairs of odorants, Figure 1).

276 Enrichment consisted of successive presentations of odorant pairs during a day and the repetition of
277 this process over 10 days. After this enrichment period, we assessed whether mice were able to
278 discriminate the odorants from each odorant pairs using a habituation/cross habituation test. In this

279 test, mice were submitted to four habituation trials (Ohab trials) with one odorant of the pair and a
280 test trial (Otest) with the other odorant of the pair. Decreased investigation time of the odorant across

281 Ohab trials reflected habituation to the odorant. Increased investigation time between Ohab4 and
282 Otest reflected discrimination between the two odorants of the pair. Five experimental groups were

283 constituted: control non-enriched (NE), enriched with 1 odorant pair (Group 1), enriched with 2
284 odorant pairs (Group 2), enriched with 3 odorant pairs (Group 3) and enriched with 6 odorant pairs
285 (Group 4) (Figure 1 and Supplementary Table 1).

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

288 discrimination, all enriched groups showed discrimination of the odor pair(s) used for enrichment
289 (see Supplementary Table 1 for abbreviations): +lim/-lim (Group 1) (Figure 2A); +lim/-lim and
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
295 NE group showed no improvement in discrimination abilities between any of the tested odorant pairs
296 (Figure 2E). These results showed that mice exposed to complex olfactory environment improved
297 their performances of discrimination for at least 6 odorant pairs at a time.

298

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

313 Since we found no increase in the density of surviving adult-born cells even when the enrichment
314 became more complex, we hypothesized that a higher proportion of the surviving adult-born neurons
315 could be recruited to process the learned odorants as the number of pairs mice were exposed to
316 increased. To test this hypothesis, we assessed the percentage of adult-born granule cells expressing
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
320 3; +lim/-lim, dec/dodec, acetic a./propionic a., propyl/butyl, buta/penta, +terp/-terp for Group 6. We
321 also tested a Non-Enriched control for each experimental group, that was stimulated with the same
322 odorants on the day of sacrifice but that had not been previously enriched with the odorants (NE₁ to
323 NE₄).

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

334 We further tested whether this result could be due to the different durations of odor enrichment
335 between groups (1 hour per day for Group 1 versus 6 hours per day for Group 4). To test this, we
336 enriched a new group of mice with +lim/-lim 6 hours per day for 10 days (similar duration of
337 enrichment compared to Group 4). The percentage of Brdu/Zif268/NeuN-positive cells did not differ

338 from that in Group 1 (Supplementary Figure 3; unilateral t-test: $t=-1.00$, $p=0.82$) suggesting that it
339 was the diversity of the odorants presented during the enrichment and not the duration of the exposure
340 that affected adult-born neuron recruitment.

341 Finally, to ensure that the increased BrdU/Zif268/NeuN co-labeling resulted from increased odor
342 responsiveness and not from a change in the basal level of BrdU/Zif268/NeuN co-expression in the
343 enriched groups, we assessed BrdU/Zif268/NeuN co-labeling in the basal condition (without odor
344 stimulation) in animals enriched with six odorant pairs and compared it to that in non-enriched
345 animals. We found no difference between these two experimental groups (unilateral t-test: $t=0.32$,
346 $p=0.38$; Supplementary Figure 4) suggesting that the observed change in BrdU/Zif268/NeuN-positive
347 levels was not due to an overall increase in adult-born granule cell activity in the enriched OB.

348 We observed no effect of enrichment or olfactory stimulation on the day of sacrifice on the rate of
349 neuronal differentiation of adult-born cells (two way ANOVA: enrichment effect $F_{(4,35)}=1.32$; $p=0.48$
350 ; stimulation effect: $F_{(4,35)}=0.66$, $p=0.62$; Supplementary Figure 5A). Quantification of Zif268-
351 positive cell density showed an effect of enrichment but no effect of odor stimulation before sacrifice
352 (Supplementary Figure 5B) (two way ANOVA, enrichment effect: $F_{(4,35)}=6.83$, $p=0.00036$;
353 stimulation effect: $F_{(4,35)}=1.27$, $p=0.3$; followed by unilateral t-tests : Group 1 versus its control: $t=-$
354 2.46 , $p=0.016$; Group 2 versus its control: $t=-2.02$, $p=0.041$; Group 3 versus its control: $t=-1.90$,
355 $p=0.043$; Group 4 versus its control: $t=-2.27$, $p=0.032$), thus showing that enrichment increased
356 overall granule cell activity independently of the number of learned odorants.

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

360

361 Increasing the complexity of perceptual learning increased structural plasticity of adult-born
362 neurons and induces limited morphological changes in preexisting neurons

363 To better understand the increased functional recruitment of adult-born neurons with perceptual
364 learning complexity, we looked for associated structural modifications in these neurons. To determine
365 whether perceptual learning had an effect on the morphology of odor-responding adult-born neurons
366 (Zif268-positive), we injected a lentivirus expressing GFP at post-natal day 60 (P60) in the
367 subventricular zone 8 days before learning (Figure 1). 25 days post-injection, animals were sacrificed
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
370 centrifugal inputs) (Figure 5A)(Mandairon et al. 2018) of Zif268-positive neurons.

371 Enrichment did not affect dendritic length of any domain (one-way ANOVAs, apical proximal,
372 $F_{(4,89)}=0.95$, $p=0.44$; Figure 5Bi; apical distal, $F_{(4,88)}=1.92$, $p=0.11$; Figure 5Bii; basal, $F_{(4,86)}=0.09$,
373 $p=0.98$; Figure 5Biii).

374 However, we found that perceptual learning increased the spine density of all dendritic domains (one-
375 way ANOVAs, apical distal $F_{(4,90)}=3.03$, $p=0.022$; Figure 5Ci; apical proximal $F_{(4,69)}=3.48$, $p=0.01$;
376 Figure 5Cii; basal $F_{(4,101)}=3.24$, $p=0.015$; Figure 5Ciii). A detailed analysis of the data revealed that,
377 at the apical proximal domain, we observed an increased spine density on adult-born Zif268-positive
378 neurons only in Group 3 and 4 compared to NE (Holm-corrected *post-hoc* t-tests; Group 3 versus NE
379 $p=0.01$, Group 4 versus NE $p=0.02$; Figure 5Ci). In the apical distal domain, the increased spine
380 density is observed in all groups (Holm-corrected *post-hoc* t-tests; Group 1 versus NE $p=0.011$, Group
381 2 versus NE $p=0.011$, Group 3 versus NE $p=0.011$, Group 4 versus NE $p=0.011$; Figure 5Cii). The
382 same global increase is observed for the basal domain (Holm-corrected *post-hoc* t-tests; Group 1
383 versus NE $p=0.008$, Group 2 versus NE $p=0.008$, Group 3 versus NE $p=0.008$, Group 4 versus NE
384 $p=0.0005$; Figure 5Ciii).

385 These results suggested that when the environmental complexity was low (enrichment with one and
386 two odorant pairs), only apical distal and basal domains showed morphological modifications (i.e.
387 increased spine density) whereas when the environmental complexity increased (enrichment with 3

388 and 4 odorant pairs), morphological changes affected the apical proximal domain in addition to apical
389 distal and basal domains. Thus as the environmental complexity increased, morphology changed in a
390 non-linear manner and not uniformly across the different dendritic domains.

391

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

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

408 Taken together, these results suggest that the morphology of adult-born neurons is modified as soon
409 as they are engaged in a simple (single odorant pair) perceptual learning task while the morphology
410 of preexisting neurons is modified only when the perceptual learning becomes complex (3 or 6
411 odorant pairs).

412

413 Optogenetically inhibiting preexisting or adult-born neurons in freely moving mice reveals their
414 functionally distinct involvement in simple and complex perceptual learning.

415 Based on the previous morphological results, we hypothesized that inhibiting adult-born neurons after
416 simple and complex perceptual learning would alter learned discrimination while inhibiting
417 preexisting neurons would only affect the expression of learned discrimination following the complex
418 learning. To test this hypothesis, we used four new groups of mice. The first group was infused with
419 a halorhodopsin- and EYFP-expressing lentivirus in the subventricular zone at P60 to transduce and
420 later on silence adult-born neurons. Half of the animals of this group were submitted to simple
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
423 infused with the same lentivirus in the lateral ventricle at P0 to transduce preexisting neurons and
424 silence them later on in adulthood. This group was also divided in two; one enriched with one odorant
425 pair and one enriched with six odorant pairs (Figure 7). As previously, mice discrimination was tested
426 after enrichment, using habituation/cross habituation tests assessing perceptual learning. Optogenetic
427 inactivation of transduced neurons was achieved in freely behaving mice only during the test trial of
428 the habituation/cross habituation paradigm by automatically triggering the light stimulation (see
429 methods) each time the animal approached the odor source. All mice were also tested in a light OFF
430 condition.

431 As expected, since light was never on during this phase of the test, habituation proceeded normally
432 (Supplementary Figure 6, Supplementary Table 5). Regarding discrimination, we found that
433 inhibiting adult-born neurons during the test trials abolished the improvement of discrimination
434 induced by simple and complex enrichment (Figure 8; Supplementary Table 5). More specifically,
435 animals enriched with +lim/-lim were able to discriminate these two odorants in the light OFF
436 condition but not when light triggered inactivation of adult-born neurons (Figure 8A and 8C;

437 Supplementary Table 5). The same phenomenon was observed for complex enrichment: animals were
438 able to discriminate the six learned odorant pairs in the light OFF condition but this was no longer
439 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
441 the OB under the optical fibers and found the same density of transduced neurons in the groups
442 submitted to simple and complex enrichments (bilateral t-test: $p=0.098$; Supplementary Figure 7Ai).

443 We also measured the proportion of transduced neurons (EYFP-positive) relative to the whole cell
444 population (DAPI-positive). Groups injected at P0 showed higher percentages (simple $3.8\% \pm 0.21$,
445 complex $2.8\% \pm 0.29$) than groups injected at P60 (simple $2.14\% \pm 0.23$, complex 1.59 ± 0.23).

446 Statistical analysis indicated that percentage of transduced neurons was superior in groups injected at
447 P0 compared to groups injected at P60 (ANOVA $F_{(1,21)} = 30,35$, $p < 0.0001$). Within the group injected
448 at P0, the group tested in the simple paradigm exhibited a percentage of transduced cells higher than
449 that of the complex group ($p=0.029$, Holm corrected t-test). It is worth noting that the group showing
450 the highest density of transduced cells is the group in which light has no behavioral effect. Thus, these
451 differences can probably not account for the reported effects of light. Second, the effectiveness of
452 light-mediated adult-born granule cell inhibition was assessed by counting Zif268-positive cells
453 among EYFP-positive cells in the same region. A decrease in the percentage of EYFP/Zif268-positive
454 cells was observed in the light ON condition compared to light OFF confirming that light actually
455 inhibited transduced adult-born neurons (unilateral t-test: simple learning $p=0.028$, complex learning
456 $p=0.043$; Supplementary Figure 7Aii).

457 In contrast to adult-born neurons, light-triggered inactivation of preexisting neurons did not alter
458 discrimination of the learned odorants after simple enrichment (Figure 9A and 9C, Supplementary
459 Table 5). However, inhibiting preexisting neurons after complex enrichment impaired discrimination
460 of most odorant pairs (Figure 9B and 9D, Supplementary Table 5). The level of viral transduction

461 was also analyzed in preexisting neurons. The density of EYFP-positive cells in the OB was similar
462 between simple and complex learning groups (bilateral t-test $p=0.13$; Supplementary Figure 7Bi) and
463 a decrease in the percentage of EYFP/Zif268-positive cells was observed in the light ON compared
464 to the light OFF condition (unilateral t-tests: simple learning $p=0.048$, complex learning: $p=0.015$,
465 Supplementary Figure 7Bii).

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

469 These results reveal that adult-born and preexisting neurons can both undergo structural plasticity
470 upon perceptual learning but their involvement in the task is governed by distinct conditions. In this
471 context, adult-born neurons are necessary for both simple and complex learned discrimination while
472 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
477 system is adult neurogenesis, i.e. renewal of interneurons within a preexisting neuronal network.
478 Perceptual learning requires the presence of OB adult-born neurons and increases their survival
479 (Moreno et al. 2009). In this study, we did not find a higher level of surviving adult-born cells with
480 increasing environmental complexity, suggesting that a plateau of adult-born cell survival is reached
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.
483 2011; Sultan et al. 2011). Our findings show a positive linear relationship between the proportion of
484 adult-born neurons expressing Zif268 in response to olfactory stimulation and the number of

485 discriminated pairs of odorants. Interestingly, the learning-dependent survival increase reported here
486 is close to the rate of adult-born neurons saved by different olfactory learning paradigms (associative
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
495 induce discrimination of dec/dodec indicating that perceptual learning is relatively specific of the
496 odorants used during exposure (Mandairon, Stack, et al. 2006; Moreno et al. 2009), in line with the
497 specific activation patterns they evoke in the OB (Linster et al. 2001). Furthermore, we showed that
498 enrichment with +lim/-lim and dec/dodec does not induce discrimination of acetic a./propionic a,
499 strongly supporting the hypothesis that discrimination of more odor pairs is actually allowed by a
500 more complex environment. However from three pairs of odorants onwards, the OB stimulation is
501 broad and activates overlapping regions of the OB leading to a less specific improvement in
502 discrimination. This is similar to what was observed following global odor enrichment (Rey et al.
503 2012) or pharmacological activation of the OB (Mandairon, Stack, et al. 2006).

504 Beyond the number of surviving adult-born cells or the proportion of them that are activated, another
505 parameter of plasticity shown to be involved in improved discrimination is the structural plasticity of
506 adult-born neurons (Daroles et al. 2015; Mandairon et al. 2018). Cell activity is a major regulator of
507 neuronal morphology (Lu 2003; Kelsch et al. 2010). In line with this, we first found that simple and
508 complex perceptual learning increased the density of dendritic spines at the apical distal, apical

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

534 connectivity with mitral cells could serve as a means of decorrelation and mediate a refinement of the
535 representations of each odorant in a pair.

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

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

551 All the above as well as previous studies (see for review (Hardy and Saghatelian 2017) indicate the
552 existence of fundamental differences between adult-born and preexisting neurons. Preexisting
553 neurons and their associated synapses seem more likely to be stable over time whereas adult-born
554 neurons constitute a more plastic and changing neuronal population that would first and foremost be
555 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

558 the age of 2 in the human OB. This raises the interesting issue of how the human bulbar network
559 adjusts to allow the plasticity of olfactory perception. Indeed, humans improve their discrimination
560 abilities with experience (Jehl et al. 1995). Our data indicating that neurons born during ontogenesis
561 can also support structural plasticity and improved discrimination suggest that such plasticity could
562 be at play in human.

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

569

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574

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709 **Captions**

710

711 **Figure 1: Experimental design.** In mice, preexisting neurons (born during ontogenesis) were labeled
712 by injecting DsRed expressing lentivirus in the lateral ventricle at post-natal day 0 (P0). Adult-born
713 neurons were labeled by injecting BrdU (i.p.) at P59 and GFP expressing lentivirus in the
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
718 with each odorant pair. After the enrichment period, mice were tested on their ability to discriminate
719 the odorants of each odorant pair using an olfactory habituation/cross habituation test (post-test).
720 Mice were sacrificed 85 days after their birth.

721

722 **Figure 2: Increasing the complexity of perceptual learning leads to the discrimination of more**
723 **odorant pairs.** Only the last habituation trial (Ohab4) and the test trial (Otest) are represented to
724 illustrate discrimination. **A.** After enrichment with +lim and -lim (Group 1), the investigation time
725 during the test trial is superior to that of the Ohab4 trial indicating discrimination between these 2
726 odorants (see Supplementary Table 2 for detailed statistics). **B.** A similar result is observed after
727 enrichment with +lim/-lim and dec/dodec (Group 2) indicating discrimination between the 2 odorants
728 of the 2 pairs. **C.** Enrichment with +lim/-lim, dec/dodec and acetic a./propionic a. (Group 3) allows
729 discrimination between the 2 odorants of the 3 pairs. **D.** Enrichment with six pairs of odorants (Group
730 4), i.e. +lim/-lim, dec/dodec, acetic a./propionic a., propyl/butyl, pent/but, +terp/-terp, allows
731 discrimination between the 2 odorants in all six pairs. **E.** In non-enriched control animals (NE group),
732 no difference is observed between Ohab4 and Otest, for any of the six odorant pairs (+lim/-lim ;
733 dec/dodec ; acetic a./propionic a. ; propyl/butyl ; pent/but ; +terp/-terp) indicating no discrimination.

734 * $p < 0.05$. ** $p < 0.01$. Data are expressed as mean \pm sem and all data points are represented. Each dot
735 represent a trial.

736

737 **Figure 3: Perceptual learning increased adult-born granule cell density independently of the**
738 **number of learned odorant pairs. A.** Example of BrdU-positive immunolabeled cells (arrows).
739 Scale bar = 10 μ 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 BrdU-
741 positive cell density compared to the non-enriched group (Non-enriched, n=6). No difference in
742 BrdU-positive cell density was observed between the 4 enriched groups. * $p < 0.05$ in comparison with
743 the non-enriched group. Data are expressed as mean \pm sem and all data points are represented. Each
744 dot represent an animal.

745

746 **Figure 4: Increasing the complexity of perceptual learning increased the functional**
747 **recruitment of adult-born neurons.**

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

757

758 **Figure 5: Increasing the complexity of perceptual learning increased structural plasticity of**
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,
761 14, 22), of the apical distal (**Bii**) (n_{neuron} from NE to Group 4 = 14, 23, 21, 14, 22) and basal
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
765 domains (**Ciii**) (n_{neuron} from NE to Group 4 = 18, 22, 27, 10, 29). Regarding the apical proximal
766 domain, increased spine density was observed only in groups 3 and 4 meaning after complex
767 perceptual learning. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ for comparison with the NE group, Holm
768 corrected t-tests. Data are expressed as mean \pm sem and all data points are represented. Each dot
769 represent a neuron.

770
771 **Figure 6: Perceptual learning induced limited morphological changes in preexisting neurons.**
772 **A.** No effect of perceptual learning was observed on the length of apical proximal dendrites (**Ai**)
773 (n_{neuron} from NE to Group 4 = 25, 25, 20, 31, 17), the apical distal dendritic arborization (**Aii**) (n_{neuron}
774 from NE to Group 4 = 25, 25, 20, 31, 17) or the basal dendritic arborization (**Aiii**) (n_{neuron} from NE to
775 Group 4 = 20, 18, 21, 27, 14). **B.** Spine densities after perceptual learning at the apical proximal (**Bi**)
776 (n_{neuron} from NE to Group 4 = 21, 13, 9, 26, 10), apical distal (**Bii**) (n_{neuron} from NE to Group 4 = 27,
777 21, 18, 34, 18) and basal domains (**Biii**) (n_{neuron} from NE to Group 4 = 11, 12, 14, 18, 10). Increased
778 spine density in the apical distal domain was seen after complex perceptual learning only (groups 3
779 and 4). Regarding the apical proximal domain and basal domains, no increased spine densities were
780 observed after learning. *: $p < 0.05$ for comparison with the NE group. Data are expressed as
781 mean \pm sem and all data points are represented. Each dot represent a neuron.

782

783 **Figure 7: Design of the optogenetic experiment.** Halorhodopsin expressing lentivirus was injected
784 in P0 mice to infect preexisting neurons or at P60 to infect adult-born neurons. Two groups of mice
785 were trained in a simple perceptual learning (one injected at P0 (n=6) and one at P60 (n=4)) and two
786 groups of mice were trained in a complex perceptual learning (one injected at P0 (n=8) and one at
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
790 odor source. The same groups of mice were also tested in a light OFF condition.

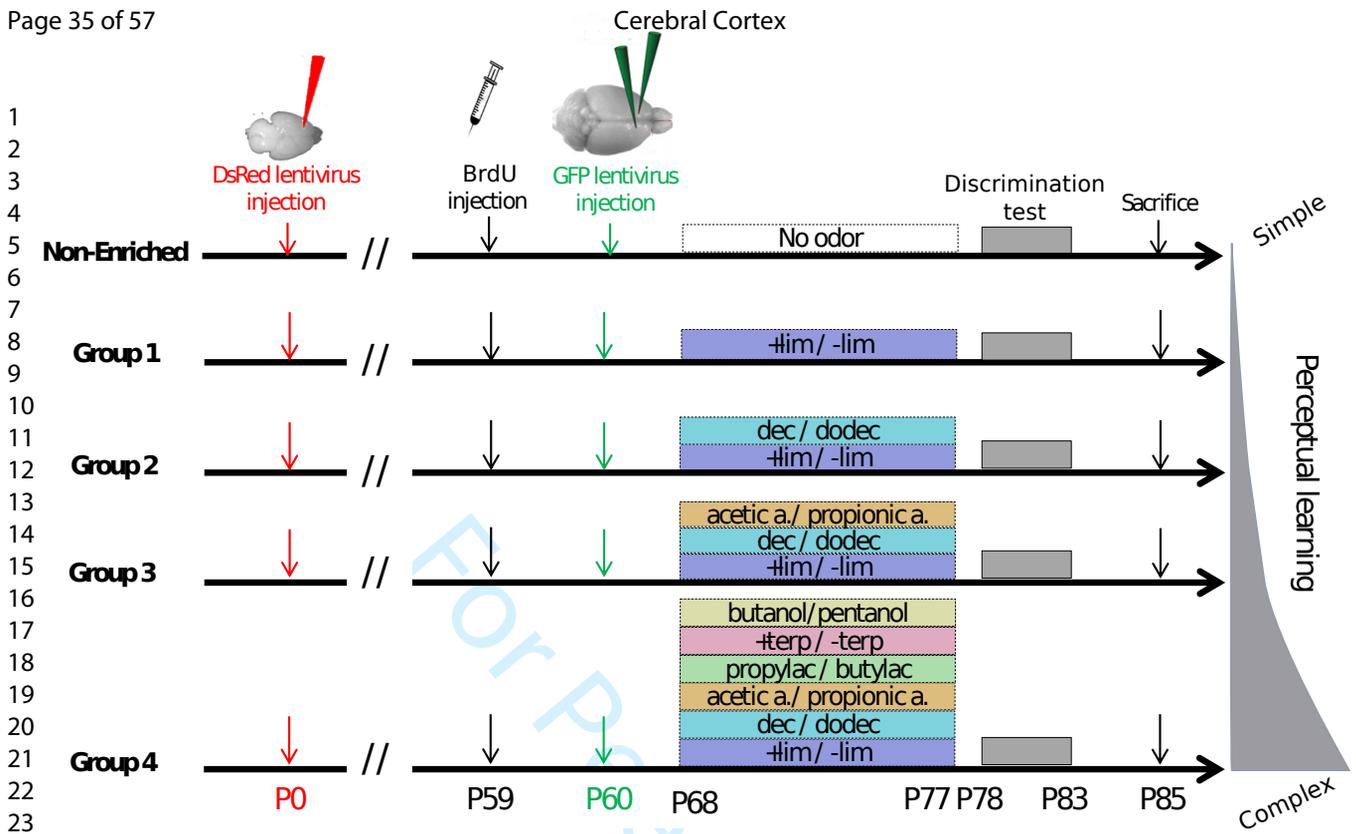
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792 **Figure 8: Optogenetically inhibiting adult-born neurons prevented discrimination after both**
793 **simple and complex perceptual learning.** After **A.** simple and **B.** complex perceptual learning tasks,
794 inhibiting adult-born neurons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

798

799 **Figure 9: Optogenetically inhibiting preexisting neurons did not prevent discrimination after**
800 **simple learning but did alter discrimination after complex learning, i.e. preventing**
801 **discrimination of most odorant pairs.** After simple perceptual learning **A.**, inhibiting preexisting
802 neurons did not prevent discrimination whereas **B.** inhibiting them after complex perceptual
803 learning altered the discrimination of most odorant pairs. In light OFF condition, **C.** and **D.**, odorant
804 pairs were discriminated after both types of learning. Data are expressed as mean±sem and all data
805 points are represented. *: p<0.05, **: p<0.01, ***: p<0.001. Each dot represent a trial.

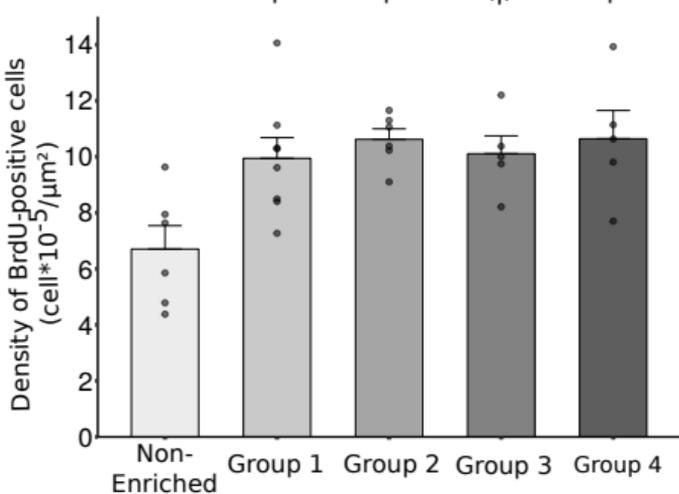
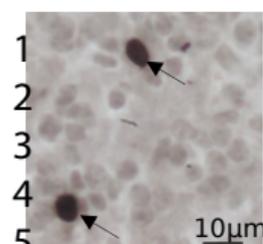
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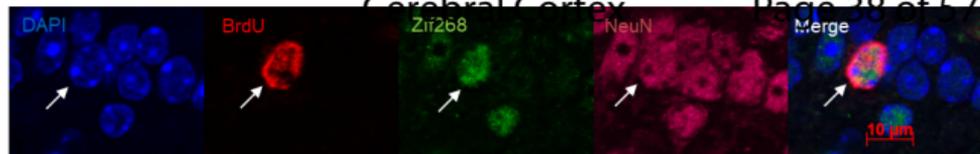
For Peer Review

B Cerebral Cortex



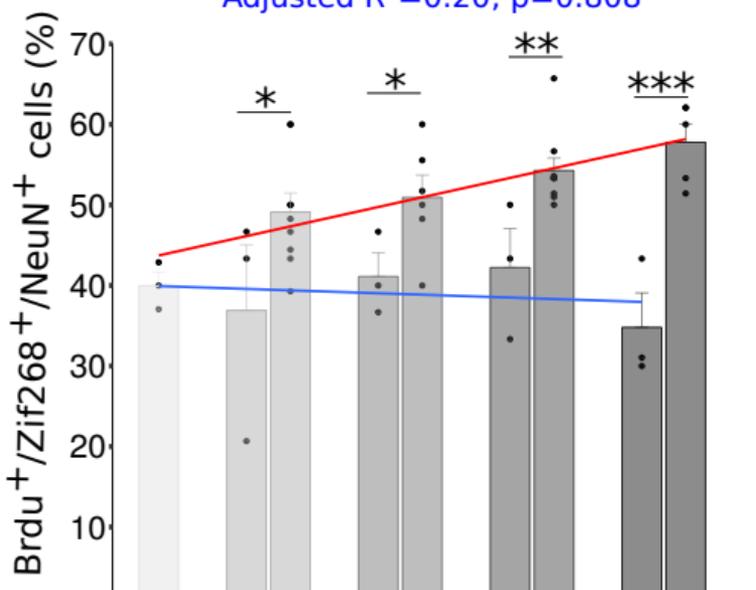
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Adjusted $R^2=0.35$, $p=0.003$ **
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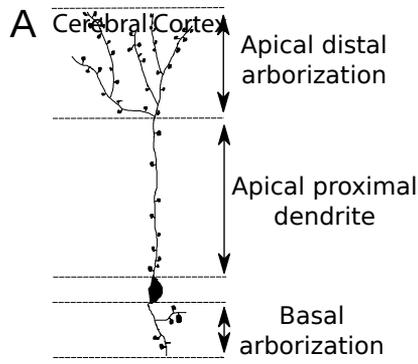


ENRICHMENT GROUP	NE	NE ₁ Group 1	NE ₂ Group 2	NE ₃ Group 3	NE ₄ Group 4
STIMULATION BEFORE SACRIFICE	Not stimulated	Group 1 odorants	Group 2 odorants	Group 3 odorants	Group 4 odorants

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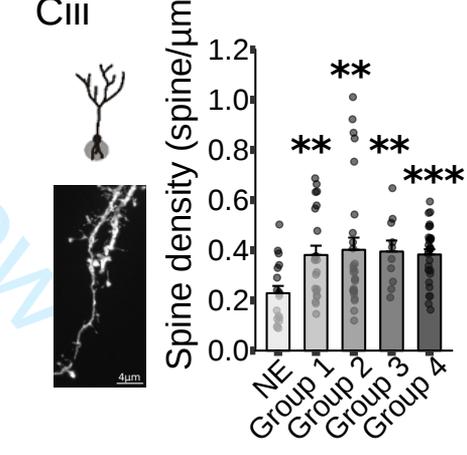
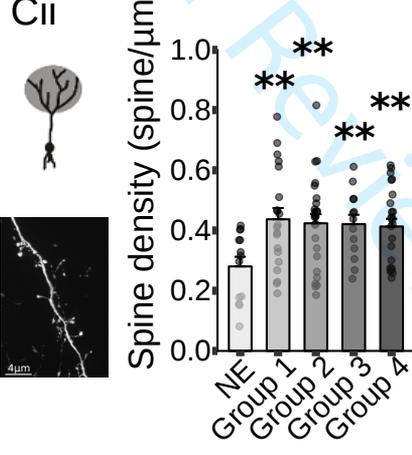
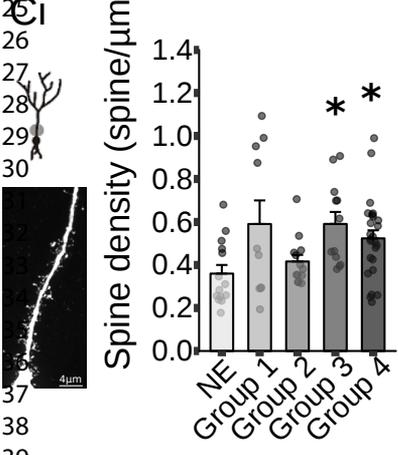
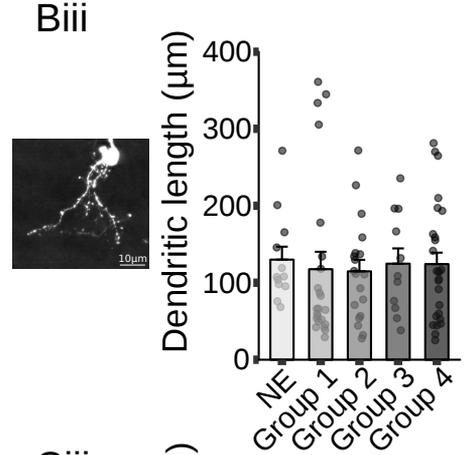
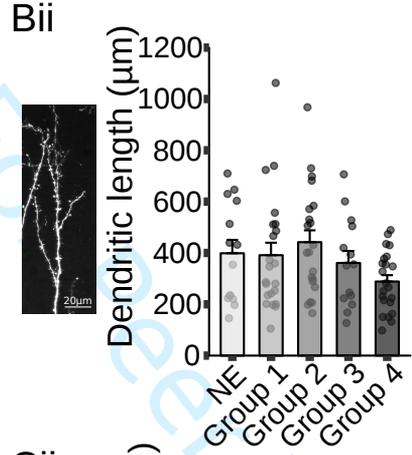
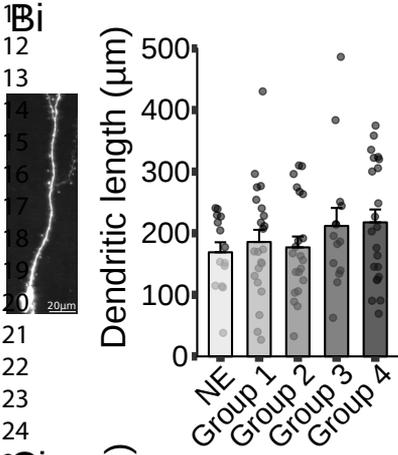
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Apical proximal

Apical distal

Basal



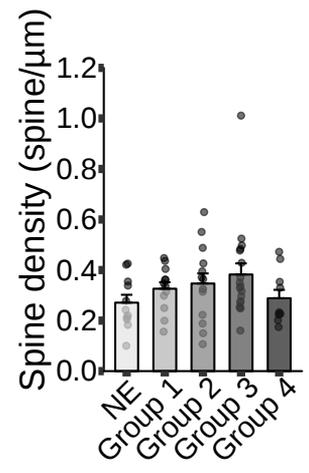
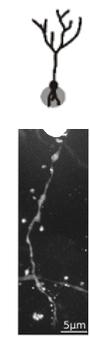
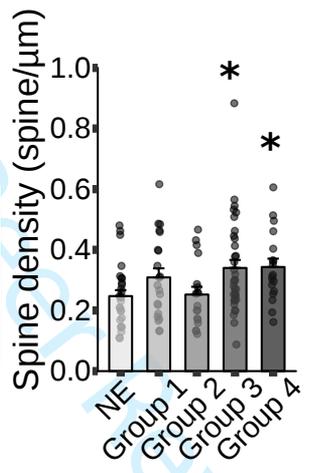
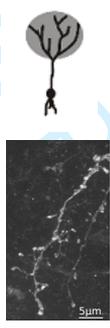
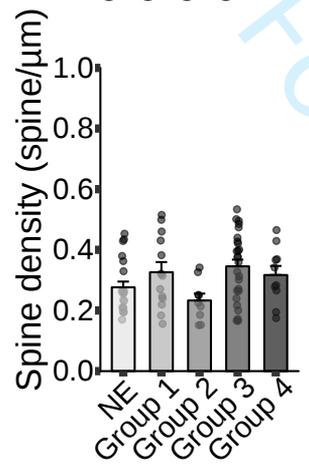
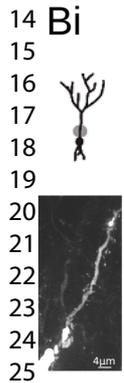
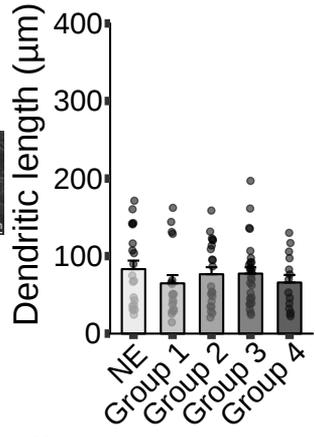
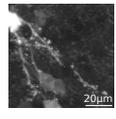
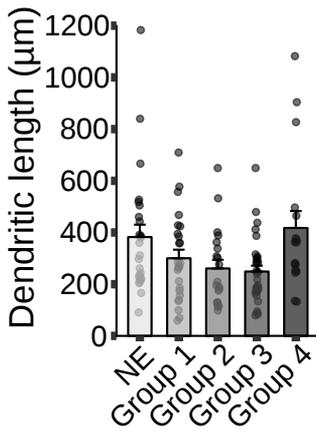
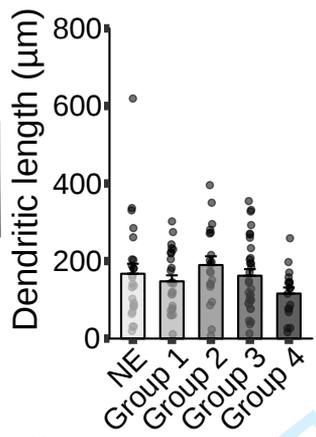
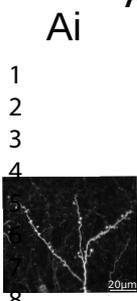
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Cerebral Cortex

Apical proximal

Apical distal

Basal



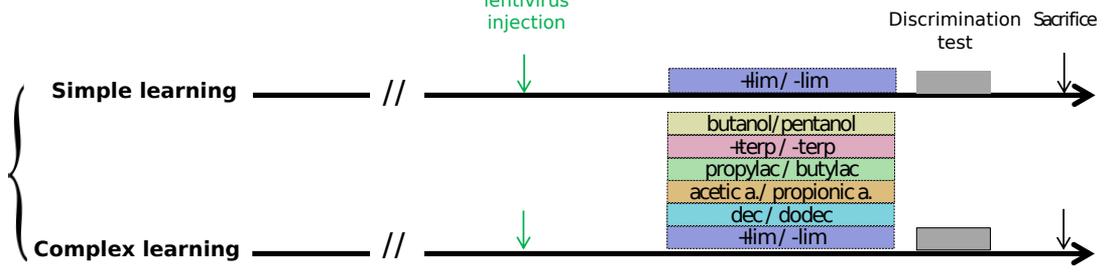
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Cerebral Cortex



Halorhodopsin
expressing
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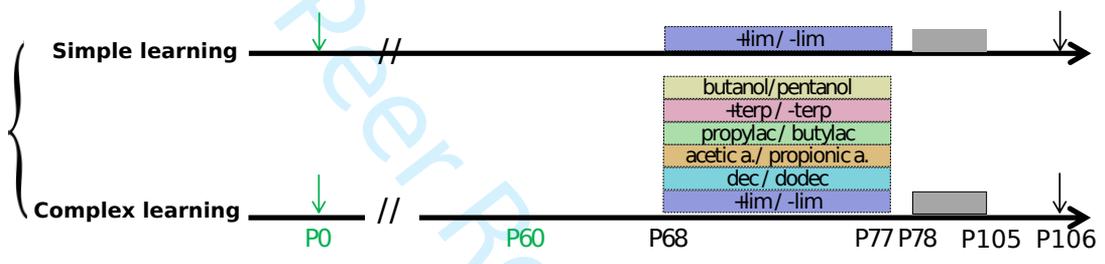
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7 **Adultborn-neurons**
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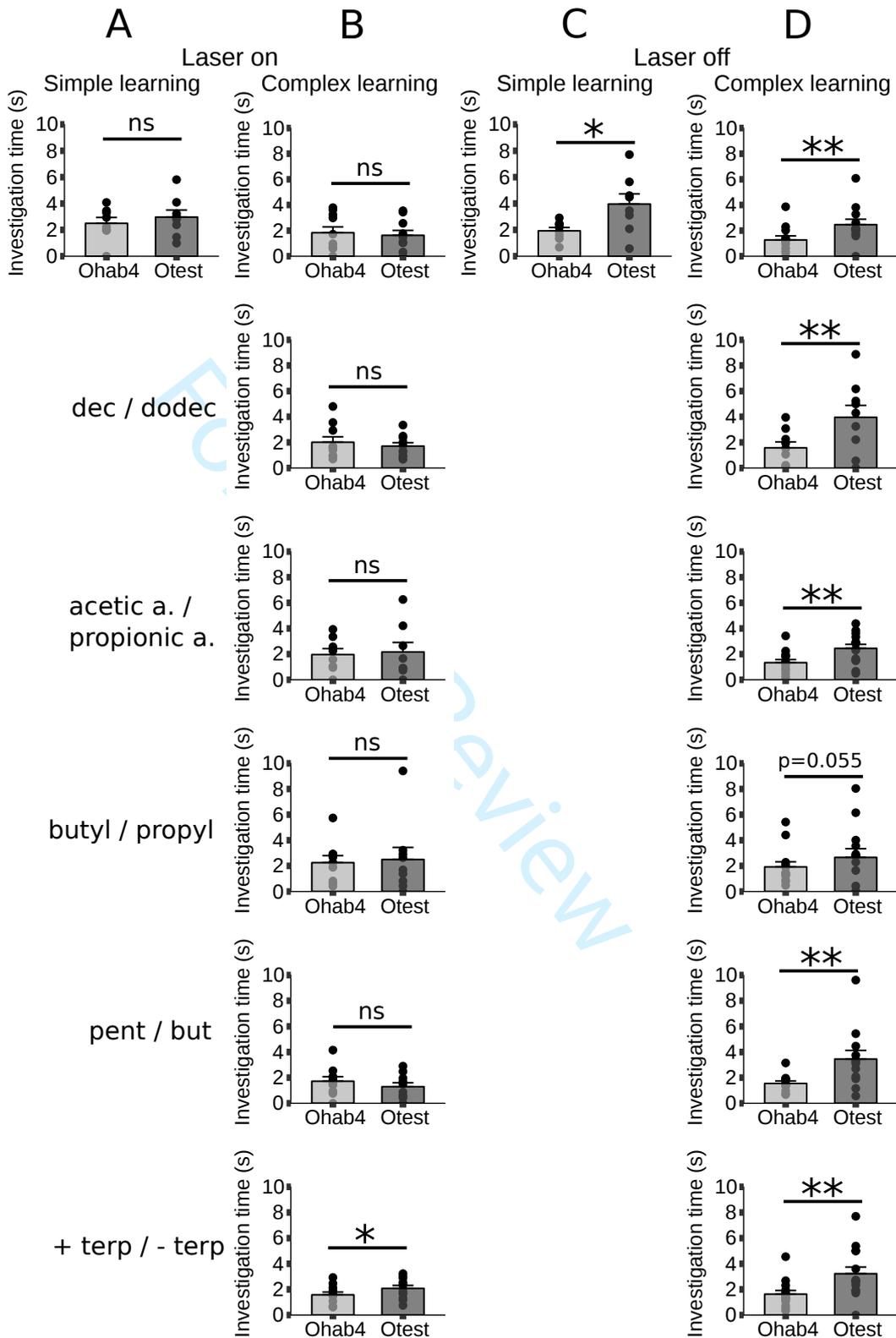


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22 **Preexisting neurons**
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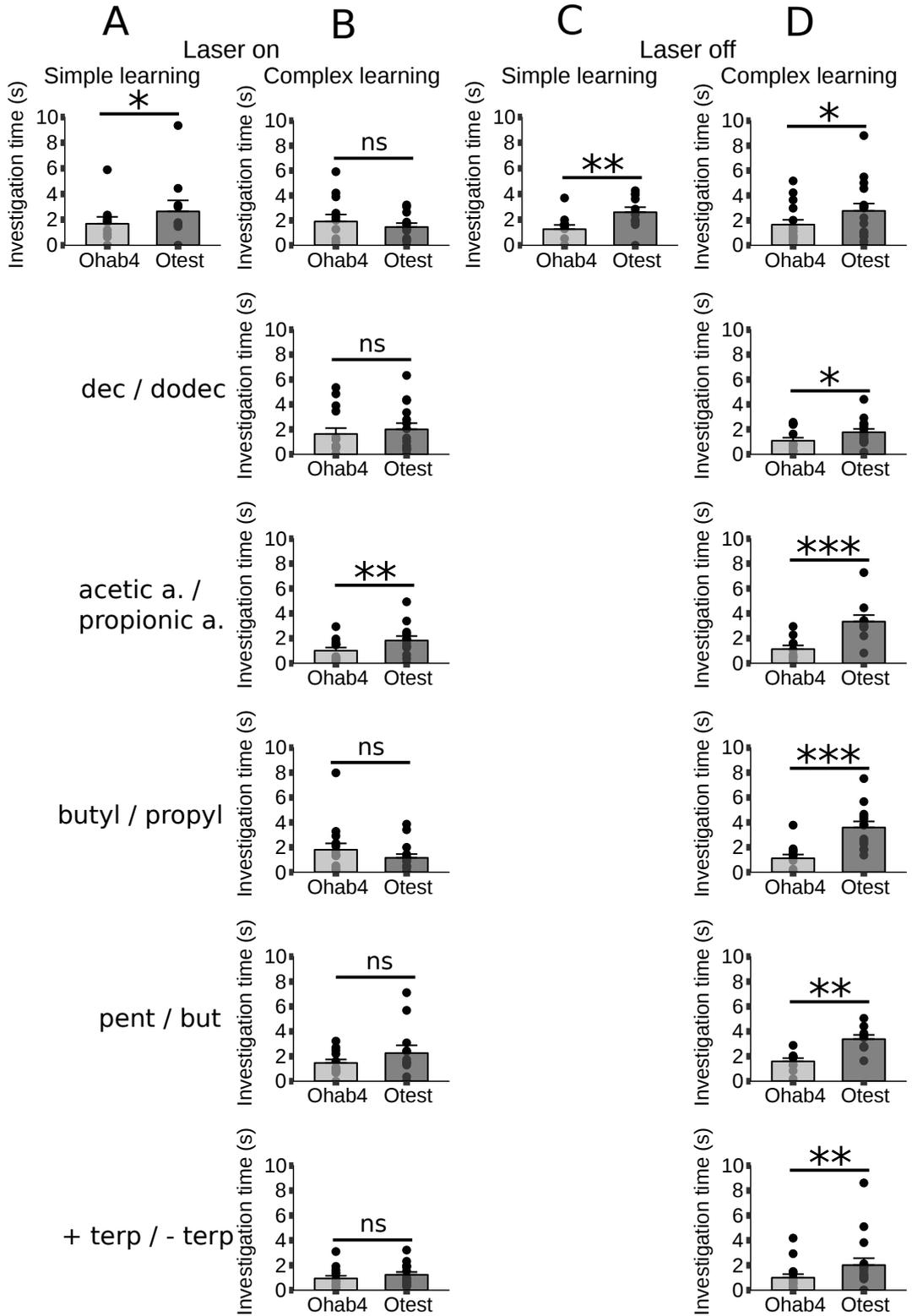
Halorhodopsin
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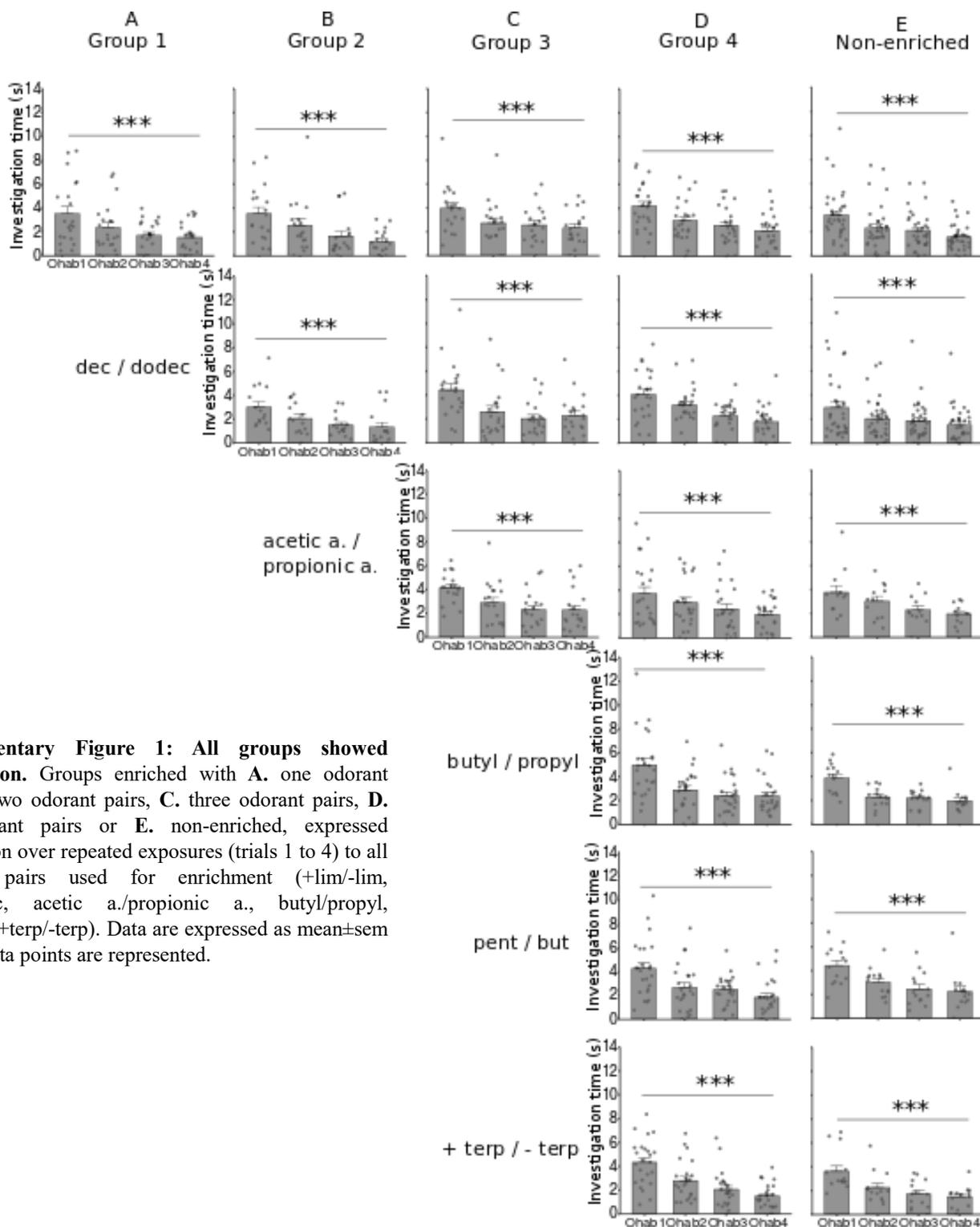


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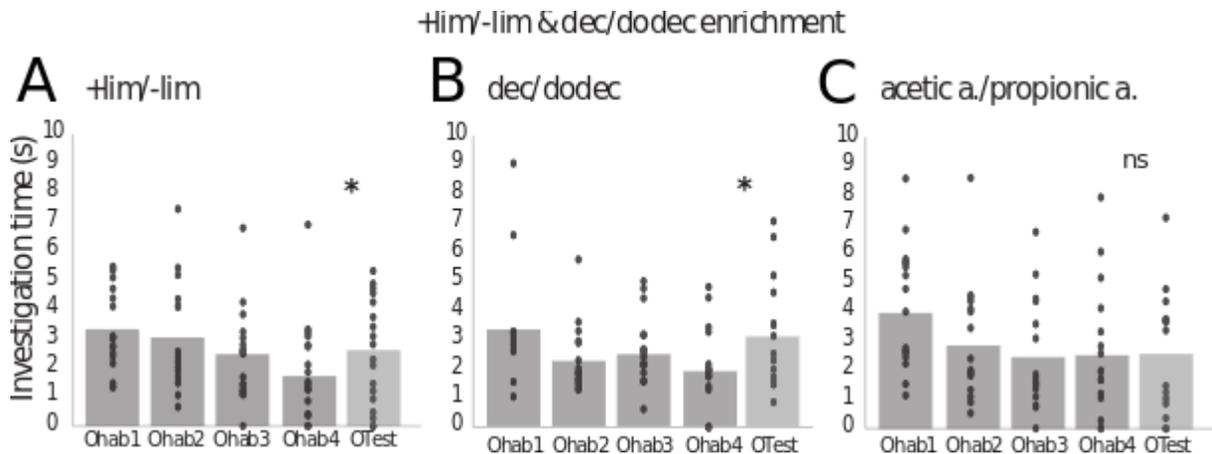
Cerebral Cortex
Preexisting neurons



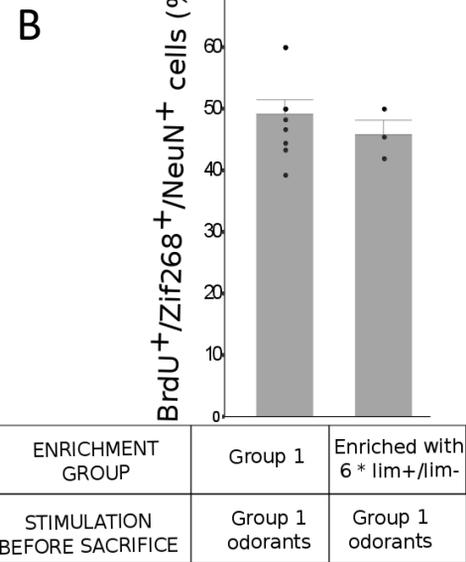
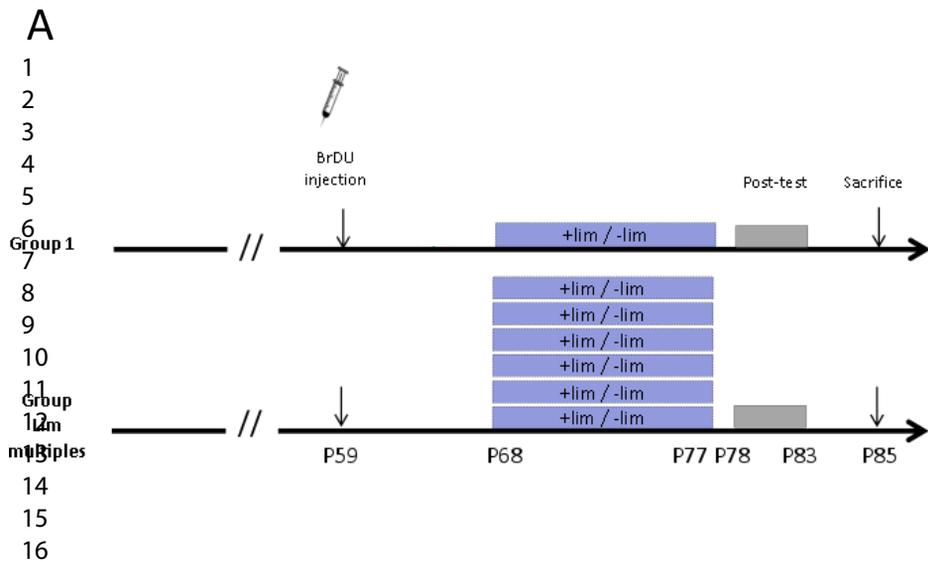
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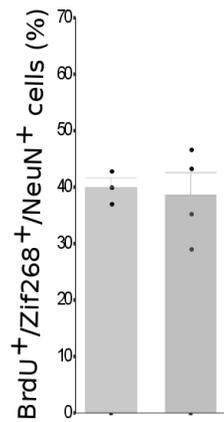
Supplementary Figure 1: All groups showed habituation. Groups enriched with **A.** one odorant pair, **B.** two odorant pairs, **C.** three odorant pairs, **D.** six odorant pairs or **E.** non-enriched, expressed habituation over repeated exposures (trials 1 to 4) to all odorant pairs used for enrichment (+lim/-lim, dec/dodec, acetic a./propionic a., butyl/propyl, pent/but, +terp/-terp). Data are expressed as mean±sem and all data points are represented.



Supplementary Figure 2: Behavioral experiment. Effect of enrichment with 2 odor pairs (+lim/-lim and dec/dodec) 1hr 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.

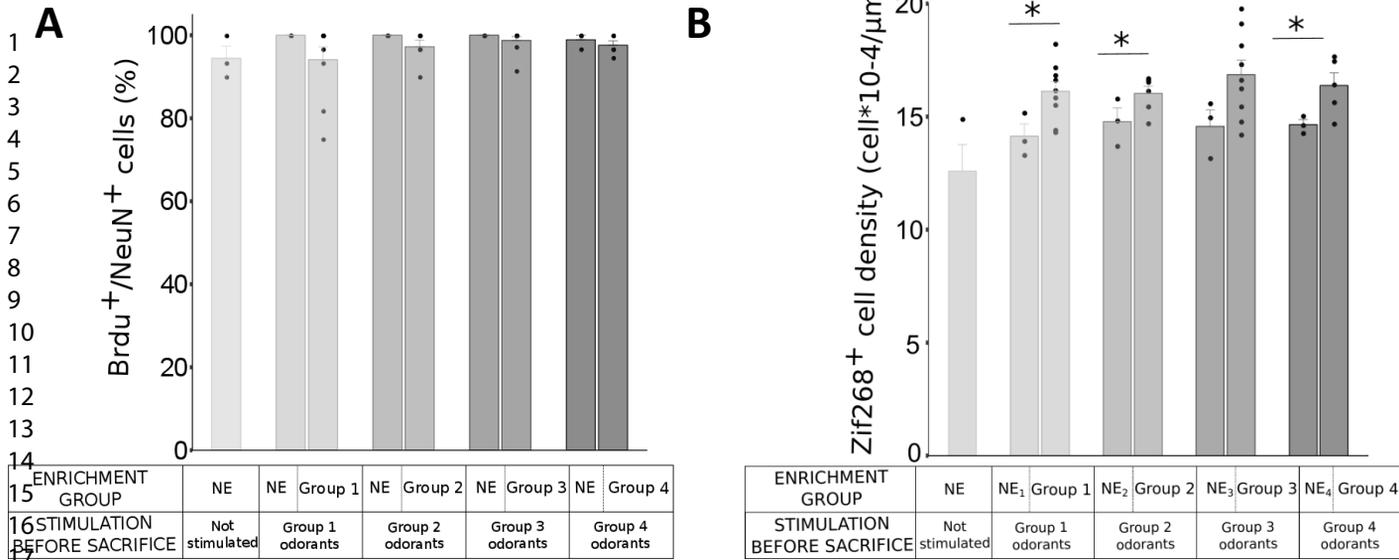


Supplementary Figure 3: Multiple enrichments with the same pair of odorants had a similar impact on the functional recruitment of adult-born neurons as did simple enrichment (Group 1). **A.** Protocol **B.** The percentage of BrdU/Zif268/NeuN is similar between the group enriched with +lim/-lim once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



ENRICHMENT GROUP	Non enriched	Enriched with 6 pairs
STIMULATION BEFORE SACRIFICE	Not stimulated	Not stimulated

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.



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20 **Supplementary Figure 5: Enrichment, regardless of complexity did not alter the fate of adult-born neurons**
21 **(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 \pm sem and all data points are represented. Number of animals is the same as reported in Figure 4.

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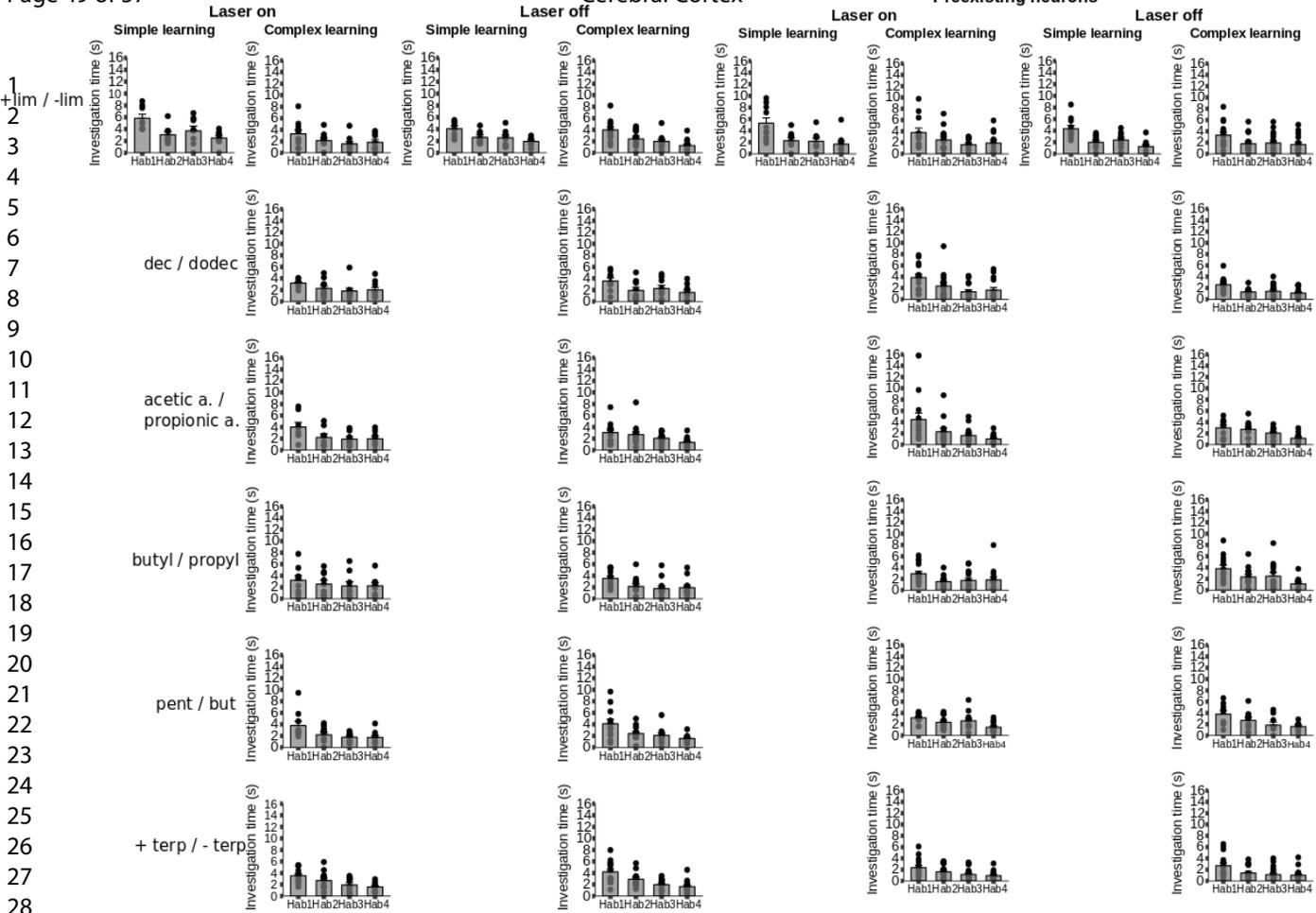
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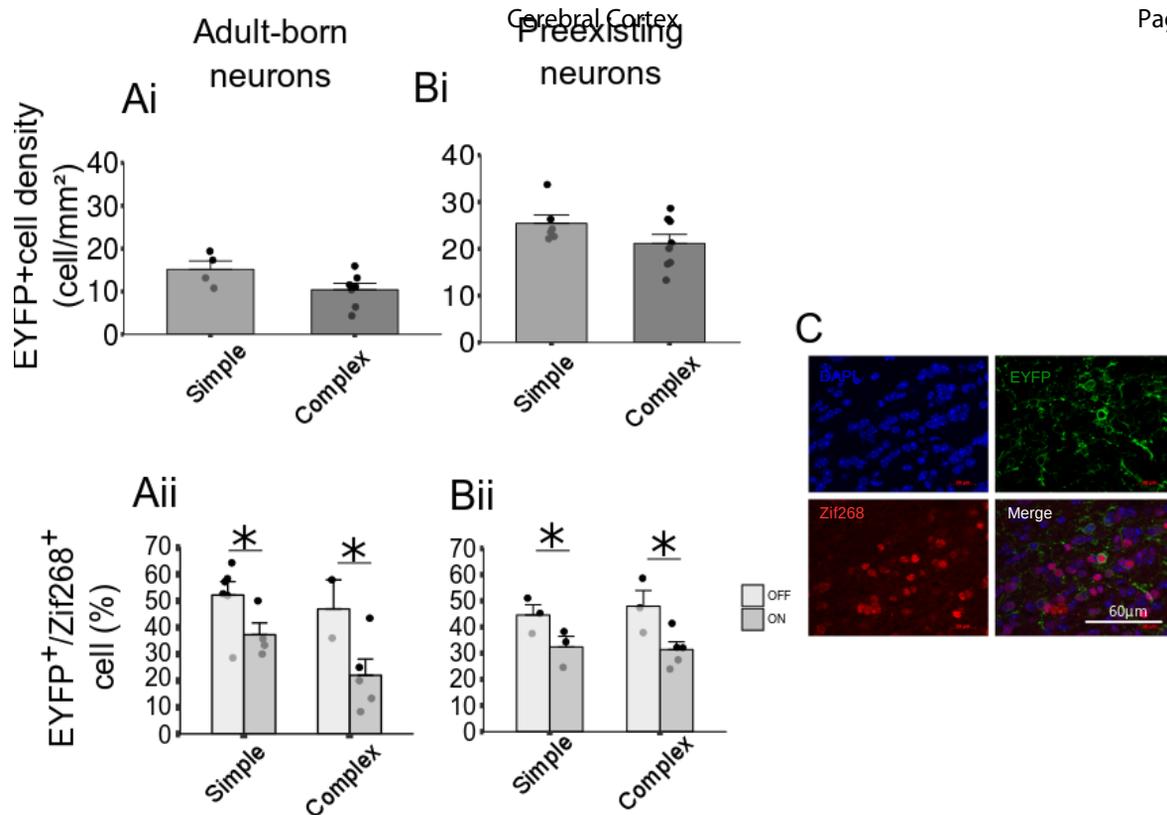
Adult-born neurons Cerebral Cortex

Preexisting neurons

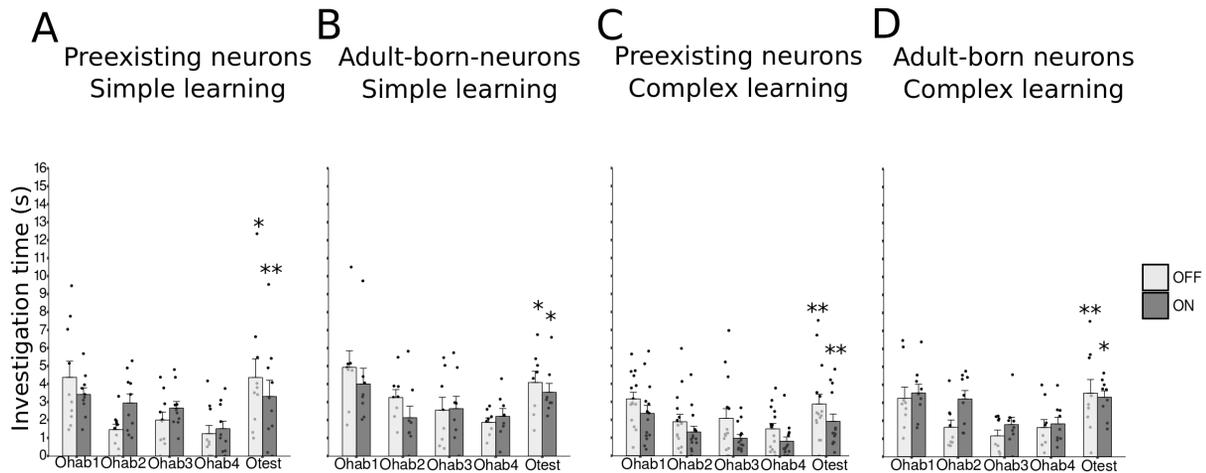


Supplementary Figure 6: Optogenetic experiment – habituation of all groups. Almost all groups showed habituation behavior with repeated odorant exposure (see table 3 for details). Data are expressed as mean±sem and all data points are represented.

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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.



24 **Supplementary Figure 8: Optogenetically inhibiting preexisting or adult-born neurons did not alter the**
 25 **discrimination of perceptually dissimilar odorants ((+)limonene/(+)carvone).** Optogenetic inhibition was performed
 26 during the test trial (Otest) when the mouse approached the odor source. All groups expressed habituation over repeated
 27 odorant exposure (Ohab trials 1 to 4). In all cases, light-triggered inhibition of preexisting neurons after **A.** simple or **C.**
 28 complex learning or adult-born neurons after **B.** simple or **D.** complex learning did not impair the discrimination of
 29 (+)limonene from (+) carvone. * $p < 0.05$, ** $p < 0.01$. Data are expressed as mean \pm sem and all data points are represented.

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Odorant pair	Dilution (vol/vol %)	Abbreviation
(+)limonene	0.203	+lim
(-)limonene	0.203	-lim
decanal	3.521	dec
2-dodecanone	27.741	dodec
acetic acid	0.007	acetic a.
propionic acid	0.041	propionic a.
propyl-acetate	0.006	propyl
butyl-acetate	0.022	butyl
pentanol	0.026	penta
butanol	0.044	buta
(+)terpinen-2-ol	6.632	+terp
(-)terpinen-2-ol	6.632	-terp

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

Cerebral Cortex

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

Supplementary Table 2: Statistics on habituation and discrimination performances.

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Cerebral Cortex

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

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

Preexisting neurons	Group	Animals	Cerebral Cortex Neurons/animal	Total number of neurons
1 2 3 4 5 6	NE	7	1-7	25
	1	5	2-4	25
	2	4	1-6	20
	3	6	1-7	31
	4	4	1-6	17
7 8 9 10 11 12 13	NE	7	1-7	20
	1	5	1-4	18
	2	5	1-8	21
	3	5	1-6	27
	4	4	1-4	14
14 15 16 17 18 19 20	NE	7	1-8	27
	1	5	1-4	21
	2	5	1-6	18
	3	5	1-7	34
	4	4	1-7	18
21 22 23 24 25 26 27 28	NE	7	1-8	21
	1	3	1-4	13
	2	2	2-5	9
	3	5	2-6	26
	4	3	1-5	10
29 30 31 32 33 34 35	NE	5	1-5	11
	1	5	1-3	12
	2	4	1-5	14
	3	4	1-5	18
	4	2	2-4	10

Supplementary Table 4: Details on the numbers underlying the morphological analysis in preexisting neurons.

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	Silenced neurons	Learning	Odorant pair(s)	Laser	n	Cerebral Cortex		Habituation			Discrimination	
						Friedman	Difference	Wilcoxon	Difference			
1	Adult-born neurons	Simple	+lim/-lim	ON	8	X=13.94; df=3	P=0.003	**	V=11	P=0.19	ns	
2				OFF	8	X=12.6; df=3	P=0.005	**	V=3	P=0.019	*	
3			+lim/-lim	ON	10	X=13.08; df=3	P=0.0045	**	V=30	P=0.62	ns	
4				OFF	12	X=20.24; df=3	P=0.00015	***	V=3	P=0.0044	**	
5		dec / dodec	ON	10	X=9.55; df=3	P=0.023	*	V=35	P=0.78	ns		
6			OFF	9	X=7.18; df=3	P=0.066	ns	V=0	P=0.0071	**		
7		acetic a. / propionic a.	ON	8	X=10.81; df=3	P=0.013	*	V=16	P=0.42	ns		
8			OFF	14	X=14.31; df=3	P=0.0025	**	V=13	P=0.0072	**		
9		butyl / propyl	ON	9	X=8.2; df=3	P=0.042	*	V=31	P=0.85	ns		
10			OFF	13	X=9.47; df=3	P=0.024	*	V=22	P=0.055	ns		
11		pent / but	ON	10	X=15.24; df=3	P=0.0016	**	V=38	P=0.86	ns		
12			OFF	12	X=14.4; df=3	P=0.0024	**	V=4	P=0.0017	**		
13		terp+ / terp-	ON	12	X=5.87; df=3	P=0.12	ns	V=15	P=0.033	*		
14			OFF	14	X=20.01; df=3	P=0.00017	***	V=8	P=0.0015	**		
15		Simple	+lim / -lim	ON	10	X=16.32; df=3	P=0.001	***	V=8	P=0.048	*	
16				OFF	11	X=24.30; df=3	P=0.00002	***	V=1	P=0.004	**	
17		+lim / -lim	ON	12	X=16.15; df=3	P=0.001	***	V=41	P=0.78	ns		
18			OFF	16	X=17.04; df=3	P=0.0007	***	V=25	P=0.012	*		
19		dec / dodec	ON	15	X=20.31; df=3	P=0.00015	***	V=29	P=0.13	ns		
20			OFF	15	X=23.29; df=3	P=0.00004	***	V=22.5	P=0.018	*		
21		acetic a. / propionic a.	ON	13	X=24.05; df=3	P=0.00002	***	V=5	P=0.0012	**		
22			OFF	10	X=15.12; df=3	P=0.0017	**	V=0	P=0.00098	***		
23		butyl / propyl	ON	15	X=15.24; df=3	P=0.0016	**	V=68	P=0.84	ns		
24			OFF	13	X=20.54; df=3	P=0.00013	***	V=0	P=0.00012	***		
25	pent / but	ON	12	X=14.6; df=3	P=0.0022	**	V=21	P=0.15	ns			
26		OFF	9	X=12.33; df=3	P=0.0063	**	V=1	P=0.0039	**			
27	terp+ / terp-	ON	15	X=17.05; df=3	P=0.00069	***	V=34	P=0.076	ns			
28		OFF	16	X=23.82; df=3	P=0.00007	***	V=5	P=0.0016	**			
29	Preexisting neurons	Simple	+lim / -lim	ON	10	X=16.32; df=3	P=0.001	***	V=8	P=0.048	*	
30				OFF	11	X=24.30; df=3	P=0.00002	***	V=1	P=0.004	**	
31	+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	dec / dodec	ON	15	X=20.31; df=3	P=0.00015	***	V=29	P=0.13	ns			
34		OFF	15	X=23.29; df=3	P=0.00004	***	V=22.5	P=0.018	*			
35	acetic a. / propionic a.	ON	13	X=24.05; df=3	P=0.00002	***	V=5	P=0.0012	**			
36		OFF	10	X=15.12; df=3	P=0.0017	**	V=0	P=0.00098	***			
37	butyl / propyl	ON	15	X=15.24; df=3	P=0.0016	**	V=68	P=0.84	ns			
38		OFF	13	X=20.54; df=3	P=0.00013	***	V=0	P=0.00012	***			
39	pent / but	ON	12	X=14.6; df=3	P=0.0022	**	V=21	P=0.15	ns			
40		OFF	9	X=12.33; df=3	P=0.0063	**	V=1	P=0.0039	**			
41	terp+ / terp-	ON	15	X=17.05; df=3	P=0.00069	***	V=34	P=0.076	ns			
42		OFF	16	X=23.82; df=3	P=0.00007	***	V=5	P=0.0016	**			

Supplementary Table 5: Statistics on habituation and discrimination performances of similar odorants during the optogenetic experiment

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Neurons labelled	Learning	Odorant	Laser	n	Habituation			Discrimination		
					Friedman	Difference		Wilcoxon	Difference	
Adult-born neurons	Simple	carv+ / lim+	ON	8	X=8,55; df=3	P=0,036	*	V=4	P=0,027	*
			OFF	8	X=10,95; df=3	P=0,012	*	V=2	P=0,012	*
	Complex	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	**
Preexisting neurons	Simple	carv+ / lim+	ON	10	X=15; df=3	P=0,0018	**	V=4	P=0,0068	**
			OFF	10	X=20,25; df=3	P=0,00015	***	V=6	P=0,014	*
	Complex	carv+ / lim+	ON	14	X=15,99; df=3	P=0,0011	**	V=8	P=0,0015	**
			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

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