12 months is a pivotal age for olfactory perceptual learning and its underlying neuronal plasticity in aging mice

Juliette Greco-Vuilloud, Maëllie Midroit, Claire Terrier, Jérémy Forest, Joëlle Sacquet, Nathalie Mandairon, Anne Didier, Marion Richard

12 months is a pivotal age for olfactory perceptual learning and its underlying neuronal plasticity in aging mice

Juliette Greco-Vuilloud [1,2,3*], Maëllie Midroit [1,2,3*], Claire Terrier [1,2,3], Jérémy Forest [1,2,3], Joëlle Sacquet [1,2,3], Nathalie Mandairon [1,2,3], Anne Didier [1,2,3] and Marion Richard [1,2,3].

[1] INSERM, U1028; CNRS, UMR5292; Lyon Neuroscience Research Center, Neuroplasticity and Neuropathology of Olfactory Perception Team, Lyon, F-69000, France;
[2] University of Lyon, F-69000, France;
[3] University Lyon 1, Villeurbanne, F-69000, France.

[*] equal contributions

Corresponding author
Marion Richard, Ph.D.
Centre de Recherche en Neurosciences de Lyon
Centre Hospitalier Le Vinatier - Bâtiment 462 - Neurocampus Michel Jouvet
95 boulevard Pinel
69675 Bron Cedex
France

Tel.: +33 4 81 10 65 20
marion.richard@univ-lyon1.fr

ACKNOWLEDGMENTS

This work was supported by CNRS, Inserm, Lyon 1 University, Fondation de France-Fondation Roudnitska (PhD fellowships to M.M and J.G-V), Région Auvergne-Rhone-Alpes (PhD fellowship to C.T) and the French Ministry of Research (PhD fellowship to J.F). We would like to thank C. Benetollo of the Neurogenetic and Optogenetic Platform of the Lyon Neuroscience Research Center (CRNL) for lentiviral production, and G. Froment, D. Nègre and C. Costa from the lentivector production facility /SFR BioSciences de Lyon (UMS3444/US8).

DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

VERIFICATION

The submitted manuscript has not been published previously and is not under consideration for publication elsewhere. This manuscript is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out. If accepted, it will not be published elsewhere in the same form.

AUTHOR CONTRIBUTIONS

Juliette Greco-Vuilloud: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. Maellie Midroit: Conceptualization, Investigation, Formal analysis, Writing –review & editing. Claire Terrier: Investigation, Writing –review & editing. Jérémy Forest: Methodology, Writing – review & editing. Joëlle Sacquet: Investigation. Nathalie Mandairon: Conceptualization, Methodology, Resources, Writing – review & editing. Anne Didier: Conceptualization, Methodology, Resources, Writing – review & editing, Supervision. Marion Richard: Conceptualization, Methodology, Resources, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Supervision.

ABSTRACT

Normal brain aging is associated with deficits in cognitive and sensory processes, due to subtle impairment of synaptic contacts and plasticity. Impairment may be discrete in basal conditions but is revealed when cerebral plasticity is involved, such as in learning contexts. We used olfactory perceptual learning, a non-associative form of learning in which discrimination between perceptually similar odorants is improved following exposure to the odorants, to better understand the cellular bases of olfactory aging in mice. We first evaluated learning ability and memory retention in 2-, 6-, 12- and 18-month-old mice, and identified 12 months as a pivotal age when memory retention subtly declines before learning becomes totally impaired at later ages. We then showed that learning-induced structural plasticity of adult-born granule cells is specific to cells responding to the learned odorants in the olfactory bulb of young adult mice and loses its specificity in 12-month-old mice, in parallel with to memory impairment. Taken together, our data refine our understanding of aging-related impairment of plasticity mechanisms in the olfactory bulb and consequent induction of olfactory learning and memory deficits.

1. INTRODUCTION

Age-induced impairment in brain function was long considered as resulting from massive neuronal cell death. However, the current literature reports remarkable conservation of the number of neurons in the aged brain, and suggests that structural impairment is restricted to specific brain regions, due to subtle decreases in synaptic contacts and synaptic plasticity (Burke and Barnes, 2006). This is also true in the olfactory system, where olfactory functional impairment is accompanied by fine histological modifications in the peripheral and central regions of the olfactory pathway, including reduced neuronal replacement in the olfactory epithelium (Weiler and Farbman, 1997; Ueha et al., 2018), and reduced adult neurogenesis and synaptic density in the olfactory bulb (Mirich et al., 2002; Enwere, 2004; Luo et al., 2006; Richard et al., 2010; Rey et al., 2012; Moreno et al., 2014).

In addition, age-induced impairment can be slight in basal conditions and revealed only when the system is submitted to a challenging environment requiring cerebral plasticity. In the olfactory system, several studies, including ours, support this hypothesis, reporting age-related decrease in olfactory detection and discrimination performances as well as specific deficits in olfactory learning and memory (*e.g.* Roman et al., 1996; Enwere, 2004; Kraemer and Apfelbach, 2004; Dardou et al., 2008; Patel and Larson, 2009; Rey et al., 2012; Moreno et al., 2014; Yoder et al., 2017). Notably, in 18-month-old mice, we reported disruption of olfactory perceptual learning (Moreno et al., 2014), a non-associative form of learning in which discrimination between perceptually similar odorants is acquired following exposure to these odorants (Mandairon et al., 2006a, 2006c). However, we still do not know when this learning deficit appears during the mouse's lifetime or whether it is a progressive or sudden degradation of learning and/or retention ability.

Olfactory perceptual learning and long-term retention are dependent on adult neurogenesis in the olfactory bulb (OB), the first cortical relay in the olfactory pathway. Neuroblasts that are continuously generated in the adult subventricular zone (SVZ) migrate to the OB, where 95% differentiate into GABAergic interneurons known as granule cells (Ming and Song, 2011). Progressive integration of adult-born granule cells is observed in the bulbar network for approximately 6 weeks after their birth (Whitman and Greer, 2007; Panzanelli et al., 2009; Kelsch et al., 2010; Sailor et al., 2016). During this period, migration, survival and synaptic integration of adult-born neurons are modulated by olfactory stimulation from the environment as well as by different forms of learning involving an olfactory stimulus (*e.g.* Rochefort et al., 2002; Alonso et al., 2006; Mandairon et al., 2006b, 2011; Livneh et al., 2009; Sultan et al.,

2010; Belnoue et al., 2011; Lepousez et al., 2014). Notably, olfactory perceptual learning increases the survival and the number of adult-born neurons responding to the learned odorant in young adult mice (Moreno et al., 2009), and also induces specific structural plasticity (increased spine density) in these neurons, which is not seen in granule cells born during ontogeny (Daroles et al., 2016; Mandairon et al., 2018; Forest et al., 2020). Experimental strategies aiming at blocking adult neurogenesis, interfering with the structural plasticity of adult-born neurons or inhibiting their activity, revealed their crucial involvement in olfactory perceptual learning (Moreno et al., 2009; Daroles et al., 2016; Forest et al., 2020). Notably, presence and survival of adult-born OB neurons are required for the maintenance of olfactory memory after olfactory perceptual learning (Forest et al., 2019).

Olfactory perceptual learning and the underlying structural plasticity of adult-born granule cells are thus well described in young adult mice, as is reduced adult neurogenesis in aging mice and the inability of 18-month-old mice to achieve such learning. However, it is not known when this learning deficit appears during the mouse's lifetime or how this behavioral impairment relates to deterioration of neuronal plasticity within the OB. The present study took used the olfactory perceptual learning paradigm to better understand the functional and structural changes occurring during brain aging. We characterized the lifetime kinetics of olfactory perceptual learning impairment. The learning and retention performances of 2-, 6-, 12- and 18-month-old mice were evaluated in the short and long term after learning and analyzed in the light of the learning-induced structural plasticity of adult-born granule cells within the OB.

2. MATERIAL AND METHODS

2.1. Experimental design

In order to characterize the kinetics of olfactory perceptual learning impairment over lifetime, four independent groups of mice aged 2, 6, 12 and 18 months at the beginning of the experiment were constituted and tested for olfactory discrimination (pre-test) (Fig.1A). Each age group was subdivided into a "control" and a "learning" subgroup and tested twice after a 10-day olfactory enrichment period (short- and long-term post-tests), in order to evaluate the efficiency of learning and the duration of memory retention after learning. Half of the mice were sacrificed at short-term after learning and the other half at long-term, to identify structural changes underlying short-and long-term memory retention. For the structural analysis of adult-born neurons, mice received an intracerebral injection of lentivirus expressing GFP at D0 to label a cohort of adult-born granule cells starting their integration in the OB at the beginning of

learning (Fig.1A). Since the maturation of adult-born neurons lasts around 6 weeks (Whitman and Greer, 2007; Kelsch et al., 2010), adult-born neurons analyzed at the short-term time point were aged 25 days and thus still rather immature, while those analyzed at the long-term time point were aged 60 days and had reached maturity. Finally, a group of mice aged 12 months received an injection of a lentivirus expressing DsRed 3 months before the beginning of the experiment, to label a cohort of pre-existing neurons that were fully mature when enrichment started, and the effect of learning on their morphology was analyzed (Supp.Fig.2A).

2.2. Mice and housing

159 male C57Bl6/J mice (Charles River, L'Arbresle, France) were used in the study. Mice arrived at 8 weeks and aged in our animal facility in order to be able to control their olfactory environment during their entire life. Mice were housed in groups in a controlled environment with food and water *ad libitum,* under a normal light cycle (light phase from 6am to 6pm). The protocols were approved by the Lyon 1 University ethical review board and registered with the French Ministry of Research (protocol number DR2013-48). All efforts were made to reduce the number and suffering of mice used in the experiments, in accordance with European Directive 2010/63/UE.

2.3. Olfactory perceptual learning

2.3.1. Learning

Olfactory perceptual learning consisted in olfactory enrichment by 1 hour's exposure per day for 10 days to (+) Limonene and (–) Limonene, a pair of odorants that is not spontaneously discriminated by mice (CAS number (+) Lim : 5989-27-5, (-) Lim: 5989-54-8, purity > 97%, Sigma-Aldrich), as previously described (Mandairon et al., 2006c, 2006a; Moreno et al., 2009, 2014; Vinera et al., 2015). Odorants were presented in 2 separate tea balls (100µl pure odorant spotted on a filter paper, 1 odorant per tea ball) hanging from the cover of the home cage. Control non-enriched mice were exposed to 2 tea balls, each containing 100µl odorless mineral oil (Sigma-Aldrich). Discrimination performance between (+) Limonene and (–) Limonene was assessed before (pre-test) and twice after the enrichment period (post-tests), using a habituation/dishabituation task described in section 2.3.2. The post-tests were performed 5 days after the enrichment period (D22-D23, short-term post-test) to evaluate perceptual learning and at 40 days after enrichment (D58-59, long-term post-test) to test long-term retention (Fig.1A).

2.3.2. Discrimination testing

Behavioral experiments were performed in the afternoon. A habituation/dishabituation task was

used to evaluate discrimination ability. This experiment was performed in a clean cage, similar to the animal's home cage. (+) Limonene and (–) Limonene were presented in tea balls (60µl odorant at 1Pa, diluted in mineral oil and spotted on a filter paper), hanging from the cover of the cage. Each session consisted in one 50-second presentation of mineral oil, then four 50-second presentations of the first odorant ("habituation odorant": OHab1-4), followed by one 50-second presentation of the second odorant of the pair ("test odorant": OTest). The inter-trial interval was 5 minutes. Our group previously reported that a 5 minute interval between two odorant presentations effectively avoided interference by age-induced olfactory memory impairment (Rey et al., 2012). The amount of time that the mice spent investigating the odorant was recorded manually. Investigation was defined as active sniffing within 5 cm of the tea ball. The experimenter recording the animal's behavior was blind to the status of the mouse (control versus learning group).

Investigation time in response to the first odorant of the pair is expected to decrease across the four successive presentations (OHab1 to OHab 4), representing habituation to repeated presentation of the same stimulus. Comparing investigation time between OHab4 and OTest measures discrimination between the two odorants of the pair. Longer investigation time in OTest than OHab4 indicates that the mouse discriminates between the two odorants; similar or shorter investigation time in OTest than OHab4 indicates that the mouse does not discriminate between the two odorants.

(+) Limonene and (–) Limonene were alternatively used as habituation and as test odorants. For each mouse, the data analyzed correspond to the mean of both configurations ((+) and (-) Limonene as OTest). Another pair of odorants (Dodecanone/Carvone), known to be perceptually dissimilar, was used in the pre-test to ensure that aging mice were still able to discriminate an easy pair of odorants (Supp.Fig.1B).

2.3.3. Statistical analysis

Sessions in mice that explored less than 1s during the first odor presentation (OHab1) were excluded from analysis: 120 out of a total 992 sessions. For each mouse, investigation time was averaged between the two sessions ((+) and (-) Limonene as OTest) for each trial. Investigation time was then averaged within groups for each trial. Data were analyzed using R software (stats package). As the behavioral data did not show normal distribution and variance equality (assessed on Shapiro-Wilk normality test on residues and Bartlett test of homogeneity of variances), non-parametric tests were used: Friedman's ANOVA to test for trial effect (OHab1-4, OTest) followed by unilateral paired Wilcoxon comparison (OHab1 *vs* OHab4 to test for odor

habituation and OHab4 *vs* OTest to test for odor discrimination). The significance threshold was set at p=0.05. Detailed statistical results are provided in Supp. Table 1.

2.4. Evaluation of adult-born granule cell morphology

2.4.1. Lentivirus injection protocol

Granule cells were transduced at their birth with a lentivirus coding for a fluorescent protein under the ubiquitous phosphoglycerate kinase promoter (Lenti-PGK-GFP, Fig.1A), provided through Addgene #12252 by the Trono Lab. The lentivirus was bilaterally injected in the SVZ at a rate of 150nl/min at the following stereotaxic coordinates (from bregma: AP: +1 mm; ML: ±1 mm; DV: -2.3 mm. Narishige Scientific Instruments, Japan) under anesthesia (50mg/kg ketamine and 7.5mg/kg xylazine, i.p.). The virus titer was 2 x 10^9 IU/ml and the injected volume was adapted to obtain an exploitable density of labeled neurons in the different age groups (100nl/side for 2-and 6-month-olds, 150nl/side for 12-month-olds and 300nl/side for 18-month-olds). After surgery, mice received i.p. injection of analgesic (Ketoprofen 2mg/kg in 0.9% NaCl). This treatment was administered for 2-3 days after surgery to facilitate recovery. Mice were allowed to recover from surgery for 8 days before the beginning of the olfactory enrichment period. Only the 12-month-old group was used for morphological analysis and compared to 2-month-old mice, based on behavioral results identifying this age as a critical time point for learning and memory degradation.

2.4.2. Olfactory stimulation before sacrifice

In order to differentiate neurons responding or not responding to the learned odorants, we used expression of the immediate early gene Zif268 as a marker of odor-induced neuronal activity (Inaki et al., 2002; Mandairon et al., 2008; Moreno et al., 2009, 2012, 2014; Forest et al., 2020). Mice were housed individually in a clean cage for 1 hour. Then two tea balls containing (+) Limonene and (–) Limonene, as during enrichment, were introduced for 1 hour. Mice were then placed in a new clean cage in order to reach a maximal Zif268 expression level before being sacrificed. Mice were deeply anesthetized (pentobarbital 400mg/kg) and killed by intracardiac perfusion of 4% paraformaldehyde (in phosphate buffer PBS, pH=7.4). Brains were dissected, post-fixed in 4% paraformaldehyde, cryoprotected in 20% sucrose for 4 days and frozen in cooled isopentane. 40µm-thick OB sections were made with a Microm HM550 cryostat.

2.4.3. Immunohistochemistry

We performed GFP/Zif268 double immunostaining to distinguish adult-born neurons responding to the learned odorants from non-responding neurons. Sections were rehydrated in 1X Phosphate Buffer Saline (PBS), then permeabilized for 30 min in Triton X-100 (0.5% in 1X PBS) and rinsed for 5 min in 1X PBS at room temperature. To block non-specific binding, sections were incubated at room temperature for 2h in a solution containing goat serum (5%) and Triton X-100 (0.3%) in 1X PBS. They were then incubated with primary antibodies diluted in the blocking solution for 48h at 25°C: chicken anti-GFP (1:1000, Anaspec/Tebu, ref 55423 RRID:AB_1657428) and rabbit anti-Zif268 (1:1000, Erg1, Santa Cruz Biotechnology, ref sc-189 RRID:AB_2231020). After rinsing, sections were incubated for 2h at room temperature in a solution containing goat serum (5%), biotinylated goat anti-rabbit (1:200, Vector, ref BA-1000) and goat anti-chicken Alexa 488 (1:250, Molecular Probes, ref A11039) antibodies in 1X PBS. Sections were rinsed and incubated for 90 min with Streptavidin Alexa 546 (1:1000, Molecular Probes, ref S11225). Finally, after rinsing, sections were coverslipped with a Vectashield medium containing DAPI (Vector Lab).

2.4.4. Image acquisition

Image acquisition was performed by an experimenter blind to the age and enrichment status of the animal and to the post-learning interval. Image acquisition was performed with a Zeiss pseudo-confocal microscope (AxioImager.ZI), equipped with an Apotome system and Axio Vision 4.8 software. Neurons were randomly chosen within the granule cell layer along the entire antero-posterior axis of the OB. The selection criterion was that the neuron appeared complete with its cell body and dendritic arborization. Z-stacks were acquired through the region of interest (1µm step for the 20x lens, 0.28µm for the 40x oil immersion lens and 0.2µm for the 100x oil immersion lens).

2.4.5. Morphological analysis

Morphological analysis was performed by an experimenter blind to the age and enrichment status of the animal, the post-learning interval and the neuron's immunoreactivity for Zif268. Morphological analysis used NeuronStudio software (Wearne et al., 2005; Rodriguez et al., 2008), to measure the total length of the dendritic arborization (sum of all dendritic branches, starting from the first branch point), number of branch points (including the first branch point) and spine density (number of spines per µm dendritic length) in both apical and basal dendritic compartments (Fig.2A and Fig.3A), as previously described (Mandairon et al., 2018; Forest et al., 2020).

2.4.6. Statistics

Data were analyzed using R software (stats package and car package for ANOVAs). The sampling unit used in the morphological analysis is the number of neurons, acknowledging the limitation regarding the independence of the data coming from the same animal (the numbers of neurons and of animals analyzed are mentioned in each figure caption). When the morphological data showed normal distribution and variance equality (assessed on Shapiro-Wilk normality test on residues and Bartlett test of homogeneity of variances), parametric statistical tests were used: 3- or 4-way ANOVA (type III ANOVA, with animal age, post-learning interval, learning status and Zif status as factors) with *post-hoc* bilateral unpaired t-tests, corrected for selected multiple comparisons by FDR (Fig.2D, Fig.4B). Otherwise, non-parametric statistical tests were used: Wilcoxon bilateral unpaired tests, corrected for multiple comparisons by FDR (Fig.2B-C, Fig.3, Fig.4C). Detailed statistical results are provided in Supp. Tables 2, 3 and 4.

2.5. Evaluation of pre-existing granule cell morphology

In order to label a cohort of pre-existing granule cells in 12-month-old mice, a group of mice received an injection of a lentivirus expressing DsRed 3 months before the beginning of the experiment (Supp.Fig.2A). These pre-existing neurons were aged 3 months and thus fully mature when learning started. Viral injection was performed as explained above with a Lenti-PGK-DsRed virus (titer 6.1 x 10^8 UI/ml), the generation of which was described previously (Forest et al., 2020). DsRed immunostaining followed a protocol similar to that described above. Blocking solution contained goat serum (5%), bovine serum albumin (2%) and Triton X-100 (0.3%) in 1X PBS. Chicken anti-DsRed antibody (EnCor, 1/5000, ref ABIN1842222) was diluted in the blocking solution and incubated for 48h at 25°C. Goat anti-chicken Alexa 546 antibody (1:250, Molecular Probes, ref A11040) was diluted in goat serum (5%) in 1X PBS and applied for 2h at room temperature. Image acquisition and analysis of neuronal morphology were performed as described for adult-born neurons. For statistical analysis, the sampling unit used is the number of neurons (as for the morphological analysis of adult-born neurons). As only two groups were compared, unpaired bilateral Student t-tests were used when data showed normal distribution and variance equality (Supp.Fig.2B); otherwise, unpaired bilateral Wilcoxon tests were used (Fig.2E, Fig. 3E, Supp.Fig.2C-E). Detailed statistical results are provided in Supp. Tables 2, 3 and 5.

3. RESULTS

3.1. Evolution of olfactory perceptual learning performance over lifetime.

In each age group (2, 6, 12 and 18 months), olfactory discrimination performances were tested using a habituation/dishabituation task, once before (pre-test) and twice (short- and long-term post-tests) after an olfactory enrichment period triggering olfactory perceptual learning (Fig.1A). Short- and long-term testing respectively assessed the ability to perform olfactory perceptual learning (i.e., acquired discrimination of (+) Limonene / (-) Limonene) and long-term retention of the learning.

Significant habituation was observed in all groups, as revealed by the decrease in investigation time from OHab1 to OHab4 in the pre-enrichment tests, and in the short- and long-term post-tests (Supp.Fig.1 and Supp. Table 1 for complete statistical report). One exception was the long-term post-test in the 6-month-old control group, which showed a strong trend but without reaching statistical significance ($p=0.082$).

Discrimination results are depicted in Fig.1B for each age group (2, 6, 12 and 18 months), comparing odorant investigation time at OHab4 versus OTest (see Supp. Table 1 for complete statistical report). During the pre-tests with (+) and (-) Limonene, investigation times were similar between OHab4 and OTest (Wilcoxon unilateral paired tests, $p>0.05$; Fig.1B), indicating that these two similar odorants were not spontaneously discriminated, regardless of age. In contrast, all age groups spontaneously discriminated Dodecanone from Carvone (OHab4 *vs* OTest, $p<0.001$; Supp.Fig.1B), indicating that easy olfactory discrimination of dissimilar odorants was not impaired by aging.

On post-tests, control animals displayed no discrimination between (+) Limonene and (–) Limonene, regardless of post-learning interval (short or long-term) and age (Fig.1B, controls, OHab4 *vs* OTest $p>0.05$). On the other hand, the data revealed learned discrimination in young adult mice, with deterioration in performance with age (Fig.1B, learning group). Two- and 6-month-old animals learned to discriminate the initially similar odorants (short-term OHab4 *vs* OTest, 2-months $p=0.013$, 6-months $p=0.0007$) and still remembered this in the long term (long-term OHab4 *vs* OTest, 2-months $p=0.031$, 6-months $p = 0.007813$). In contrast, 18-month-old mice were unable to learn to discriminate (short-term OHab4 *vs* OTest, $p>0.05$) and consequently could not show memory (long term OHab4 *vs* OTest, $p>0.05$). Interestingly, 12-month-old mice showed an intermediate pattern: they discriminated (+) Limonene from (–) Limonene shortly after odor enrichment (short-term OHab4 *vs* OTest, $p=4.269e-05$), indicating olfactory perceptual learning; in the longer term, however, OHab4 and OTest showed similar

investigation times (long-term OHab4 *vs* OTest, $p>0.05$), indicating that 12-month-old mice had forgotten the learned discrimination.

In conclusion, our results showed that 12-month-old mice were still able to learn to discriminate between two similar odorants, in contrast to 18-month-old mice, but learning retention was shorter than in younger mice (2-and 6-months-old). 12 months is thus a critical time point in mouse lifetime, when memory retention after olfactory perceptual learning begins to decline before learning becoming totally impaired at later ages.

3.2. Integration of adult-born granule cells in the aging OB in basal conditions and after olfactory perceptual learning.

As adult-born granule cells are required for olfactory perceptual learning and retention (Moreno et al., 2009; Daroles et al., 2016; Mandairon et al., 2018; Forest et al., 2019, 2020), we analyzed the morphology of this neuronal population in the short and long term after olfactory perceptual learning, to identify structural changes that might underly behavioral performance. Our team previously identified learning failure in 18-month-old mice, associated with a strong reduction in the number of adult-born neurons at this age and failure of odor enrichment to increase adult-born neuron survival (Moreno et al., 2014). In the present study, 12 months emerged as a critical time point when acquisition is still possible but long-term memory is impaired. We therefore focused our morphological analysis on 12-month-old compared to 2-month-old mice, to study the cellular basis of reduced retention. Since maturation of adult-born granule cells lasts around 6 weeks (Whitman and Greer, 2007; Kelsch et al., 2010), neurons analyzed at the short-term time point were aged 25 days and thus still rather immature, and neurons analyzed at the long-term time point were aged 60 days and had reached maturity. We analyzed apical and basal dendrites of adult-born granule cells, supporting synaptic outputs and inputs respectively (Fig.2A and 3A).

3.2.1. Apical dendrites

In the apical compartment of adult-born granule cells (Fig.2A), learning (control *vs* learning), age (2 *vs* 12 months) or post-learning interval (D25 *vs* D60) showed no significant impact on total dendritic length and number of branch points (Fig.2B-C, see Supp. Table 2 for complete statistical report on Fig.2). These first results indicated 1) that adult-born granule cells integrated in a 12-month-old OB developed apical ramification similar to that of cells born in a

2-month-old OB, and 2) that learning did not modify the development of apical ramification, whatever the age of the animal.

Data for apical spine density (Fig.2D) showed a significant effect of learning ($F_{(1,136)}$= 8.7775; p= 0.0036010), which depended on age and post-learning interval (interaction animal age * post-learning interval * learning, $F_{(1,136)}$=14.4096; p=0.0002207), and a trend for an age effect ($F_{(1,136)}$=3.7090; p=0.0562051). *Post-hoc* tests of the effect of learning revealed that, in 2-month-old mice, apical spine density was higher in D25 neurons in the learning group compared to controls (Fig.2D, p=0.025), confirming previous findings of learning-induced structural plasticity in adult-born granule cells of young adult mice shortly after learning (Daroles et al., 2016; Mandairon et al., 2018; Forest et al., 2020). This effect of learning was no longer visible in D60 neurons, also confirming our previous report (Mandairon et al., 2018). In 12-month-old mice, no effect of learning was visible in D25 neurons (Fig.2D), but their spine density was equivalent to that of the 2-month-old learning group, and spine density in 12-month-old controls tended to be higher than in 2-month-old controls (p=0.07). Thus, granule cells born in a 12-month-old OB might display an increased synaptic integration in control conditions but showed no effect of learning at D25. Only a late effect of learning was found in 12-month-old mice: apical spine density was significantly higher in D60 neurons of the learning group than in controls (Fig.2D, p=0.0025). Furthermore, in the 12-month-old learning group, apical spine density was higher in D60 neurons than in D25 neurons (Fig.2D, p=0.045), suggesting an extended period of spine growth after learning in middle age. The spine density reached by D60 neurons of 12-month-old mice after learning also exceeded that of D60 neurons of 2-month-old mice after learning (Fig.2D, p=0.005).

Taken together, these data revealed that the early structural plasticity induced by perceptual learning in the apical dendrites of immature adult-born granule cells immediately after learning (D25) was impaired by middle age. Granule cells born in a 12-month-old OB did not display such early plasticity, but rather delayed plasticity at long-term post-learning (D60), when mice had forgotten. Early structural plasticity was thus concomitant with the ability to learn only in young adult mice. However, 12-month-old mice were still able to perform perceptual learning, although retention was shortened. We thus hypothesized that other cellular mechanisms were involved in learning at 12 months. In addition to the above observations suggesting that the high spine density observed in control conditions at 12 months could be sufficient to enable learning, we tested an alternative hypothesis that pre-existing mature granule cells could be a cellular substrate of perceptual learning at this age. At the age of 12 months, pre-existing granule cells present in the OB are either neurons born during ontogeny or neurons born earlier

during adulthood. However, given the high rate of renewal of granule cells (approx. 50% in 12 months) enabled by adult neurogenesis (Imayoshi et al., 2008), few granule cells born during the perinatal period remain present in the OB at 12 months. Therefore, we chose to label and study a cohort of pre-existing granule cells that were born during adulthood and that had reached full maturity (3-month-old neurons) at the beginning of learning in 12-month-old mice (Supp.Fig.2A). Morphological analysis was performed for the short-term time point after learning, when 12-month-old mice displayed the learned discrimination. The apical spine density of pre-existing granule cells appeared similar between the control and 12-month-old learning groups (Fig.2E; Supp. Table 2), as did their apical dendritic arborization (Supp.Fig.2B-C; Supp. Table 5). Thus, this population of pre-existing mature granule cells of 12-month-old mice did not display learning-induced structural plasticity in their apical dendrites, suggesting that these were unlikely to contribute to learning.

3.2.2. Basal dendrites

In the basal compartment of adult-born granule cells (Fig.3A), total dendritic length and number of basal dendrites showed no significant changes with learning (control *vs* learning), age (2 *vs* 12 months) or post-learning interval (D25 *vs* D60) (Fig.3B-C, see Supp. Table 3 for complete statistical report on Fig.3). These results indicated 1) that adult-born granule cells integrated in a 12-month-old OB developed basal dendrites similarly to cells born in a 2-month-old OB, and 2) that learning did not modify the development of the basal dendrites, whatever the age of the animal.

Data for basal spine density and the effect of aging in control mice revealed that basal spine density was significantly higher in D60 neurons of 12-month-old than 2-month-old mice (Fig.3D, $p=0.019$), suggesting greater synaptic input to the basal dendrites of adult-born granule cells integrated in a middle-aged OB. Regarding the effect of learning, basal spine density was higher in D60 neurons of the 2-month-old learning group compared to controls (Fig.3D, $p=0.019$), revealing structural plasticity induced by learning in adult-born granule cells in 2-month-old mice in the long term post-learning. In contrast, 12-month-old mice displayed no learning-induced changes in the basal spine density of adult-born granule cells, either at short- or long-term post-learning (D25 or D60, Fig.3D).

Taken together, these data revealed that granule cells born in a 12-month-old OB displayed different integration than neurons born in a 2-month-old OB, suggesting increased synaptic input to basal dendrites with aging. In addition, the structural plasticity induced by perceptual learning in the basal dendrites of adult-born granule cells at long-term post-learning (D60) in

2-month-old mice was absent in 12-month-old mice, when memory impairments appeared. Similar to our analysis of apical dendrites, we investigated whether pre-existing mature granule cells might be a cellular substrate of perceptual learning in 12-month-old mice. The basal spine density of pre-existing granule cells appeared similar between control and 12-month-old learning groups (Fig.3E; Supp. Table 3), as did their basal dendritic arborization (Supp. Fig.2D-E; Supp. Table 5). These pre-existing mature granule cells of 12-month-old mice thus did not display structural plasticity induced by learning in their basal dendrites, suggesting that they are unlikely to contribute to learning in middle-aged mice.

3.3. Structural plasticity of adult-born granule cells is specific to cells responding to the learned odorants in young adult mice but becomes non-specific with aging.

We then hypothesized that learning would mainly affect the morphology of adult-born granule cells that were responsive to the learned odorants. Mice were exposed to the learned odorants 1h before sacrifice, and we used expression of the immediate early gene Zif268 to identify neurons responding to the learned odorants (Inaki et al., 2002; Mandairon et al., 2008; Moreno et al., 2009, 2012, 2014; Forest et al., 2020). GFP/Zif268 double staining identified adult-born granule cells responding to the learned odorants (ZIF+ neurons), and their morphology was compared to that of adult-born granule cells that did not respond (ZIF- neurons) (Fig.4A). A complete statistical report on Fig.4 is provided in Supp. Table 4.

Data for apical spine density showed a significant effect of learning ($F_{(1,128)}$=5.0582; p=0.02622), expression of ZIF ($F_{(1,128)}$=5.4402; p=0.02124) and the interactions between age * learning ($F_{(1,128)}$=6.5911; p=0.01140) and between age * learning * expression of ZIF ($F_{(1,128)}$= 6.2562; p=0.01364). This indicated that the effect of learning on apical spine density depended on ZIF expression and on the age of the animal. In 2-month-old mice, ZIF+ neurons displayed higher apical spine density at D25 in the learning group than in controls (Fig.4B top panel, p=0.006), confirming our previous report (Forest et al., 2020). This difference was no longer present at long-term post-learning (D60). On the other hand, ZIF- neurons of 2-month-old mice had similar apical spine densities regardless of learning status (learning *vs* control) at both post-learning intervals (D25 *vs* D60) (Fig.4B bottom panel). In addition, apical spine density in control 2-month-old mice was lower in ZIF+ than in ZIF- neurons at the short-term interval (D25 ZIF+ vs ZIF- p=0.014). Although unexpected, this finding suggested that, in adult-born cells, less mature neurons, exhibiting less connectivity, may show greater excitability and thus higher ZIF expression, in line with previous reports (Nissant et al., 2009). Basal spine density was higher in ZIF+ neurons in the 2-month-old learning group than in controls (D25 p=0.036

and D60 p=0.036, Fig 4C top panel), while no such difference was observed for ZIF- neurons (Fig.4C bottom panel). These results indicated that the learning-induced structural plasticity identified in 2-month-old mice was specific to adult-born granule cells responding to the learned odorants.

In 12-month-old mice, both ZIF+ and ZIF- D60 granule cells displayed greater apical spine densities in the learning group than in controls (Fig.4B, ZIF+ p=0.068, ZIF- p=0.006). There were no learning-induced changes in the basal spine density of adult-born granule cells in 12-month-old mice, whatever the post-learning interval or expression of ZIF (Fig.4C). This comparison of the effect of learning on subpopulations of adult-born granule cells responding or not to the learned odorants demonstrated that the effect of olfactory perceptual learning on the morphology of adult-born granule cells in 12-month-old mice lost specificity compared to 2-month-old mice: at 12 months, learning-induced plasticity affected adult-born granule cells whether responding or not to the learned odorants.

Altogether, morphological data revealed that aging impaired the structural plasticity induced by olfactory perceptual learning in adult-born granule cells. In 2-month-old mice, learning induced an increase in spine density in both apical and basal dendritic compartments specifically in adult-born granule cells responding to the learned odorants. With aging, this plasticity disappeared in basal spines, is delayed and lost its specificity in apical spines, also affecting cells that did not respond to the learned odorants, suggesting that impaired plasticity could contribute to the decreased memory capacity of middle-aged mice.

4. DISCUSSION

4.1. 12 months is a decisive time point in the progressive deterioration in olfactory perceptual learning and its underlying plasticity with aging.

Olfactory perceptual learning is a non-associative form of learning in which discrimination between perceptually similar odorants is improved following exposure to these odorants (Moreno et al., 2009). We provide here the first report that middle-aged mice (12-month-old) are still able to acquire this discrimination capacity but that their memory is shorter than younger mice (retention <40 days in 12-month-old mice *versus* >40 days in 2- and 6-month-old mice). This memory impairment is accompanied by deterioration of the structural plasticity induced by olfactory perceptual learning in adult-born granule cells. In 2-month-old mice, perceptual learning increases spine density in both apical and basal dendritic compartments,

specifically in adult-born granule cells responding to the learned odorants. In 12-month-old mice, this plasticity has disappeared in basal spines and lost its specificity in apical spines: the plasticity is delayed and also affects cells that do not respond to the learned odorants.

In 2-month-old mice, the presence and activity of adult-born granule cells are necessary for olfactory perceptual learning (Moreno et al., 2009; Forest et al., 2020), but also their structural plasticity, as blocking this plasticity interferes with learning (Daroles et al., 2016). A greater number of apical spines induced by learning increases the inhibition exerted by adult-born granule cells on mitral cells (Moreno et al., 2009; Mandairon et al., 2018) via the reciprocal dendro-dendritic synapses (Price and Powell, 1970; Shepherd et al., 2007). The fact that this increased inhibition is specifically exerted by granule cells responding to the learned odorants (this study), associated with our previous reports that an increased proportion of adult-born neurons responds to the learned odorants after learning (Moreno et al., 2009, 2014), suggests that there are two levels of refinement of the representation of these odorants within the OB after learning. Not only are more adult-born granule cells involved in the network activated by the learned odorants, but these cells also make more inhibitory synaptic contacts. These two phenomena combine to improve pattern separation and thereby discrimination in 2-month-old mice (Chu et al., 2016). In contrast, in 12-month-old mice, apical spine density increases at a later time point after learning and in adult-born granule cells both responding and not responding to the learned odorants, when aging mice have forgotten. Interestingly, the spine density reached by D60 granule cells of 12-month-old mice after learning exceeded that of the same neuronal population in 2-month-old mice after learning. We do not know whether perceptual learning increases the recruitment of adult-born neurons at this age like in 2-month-old mice (Moreno et al., 2014). However, the broad increase in inhibitory contacts, regardless of the involvement of granule cells in the network coding for the learned odorants, suggests a deficit in synapse pruning, affecting pattern separation and contributing to impaired retention of the learned discrimination, as observed behaviorally. More generally, the loss of specificity of structural plasticity in the aging OB may be related to the reduced specificity of activation patterns that is often observed in the aging brain (Grady, 2012; Cabeza et al., 2018). Whether the delay and loss of specificity of structural plasticity is an intrinsic property of granule cells born in a 12-month-old OB and/or results from changes in the availability of molecular cues inducing plasticity remains to be determined.

The decline of memory at 12 months preceded the complete impairment of olfactory perceptual learning in 18-month-old mice, in agreement with our previous study (Moreno et al., 2014). Reduced memory duration in 12-month-old mice could be due to several processes:

discrimination performance reached immediately after learning is weaker than in 2-month-old mice and is therefore more rapidly lost over time, causing early forgetting, or similar acquired discrimination performance but associated impairment of the maintenance mechanisms allowing memory to endure. Another hypothesis could be impaired reconsolidation of memory upon reactivation. We report here longer memory duration after olfactory perceptual learning in 2-month-old mice (>40 days) than in previous reports where this acquired discrimination capacity was spontaneously lost between 30 and 40 days post-learning (Mandairon et al., 2018; Forest et al., 2019). This apparent discrepancy could be explained by the fact that, in the present protocol, mice were tested twice after learning (5 and 40 days post-learning). This result suggests that reactivation of the memory trace by retesting increases memory duration in young adult mice. The memory deficit seen in 12-month-old mice might also be explained by impaired reconsolidation upon reactivation.

4.2. Neuronal bases of memory retention after olfactory perceptual learning in 2-month-old mice.

Our results suggest that reactivation of adult-born granule cells that are responsive to the learned odorants can extend memory duration in young adult mice, presumably by enabling adult-born neuron survival. This is consistent with other data from our group showing that maintaining the stimulus in the environment beyond the initial 10 days' training promoted the survival of these neurons and extended the duration of conserved discrimination capacity (Forest et al., 2019). Interestingly, basal spine density was higher in granule cells of 2-month-old mice in the learning group than in controls at D60, indicating long-term persistence of structural plasticity in adult-born granule cells. This persistence was not seen in a previous study (Mandairon et al., 2018), in which discrimination was lost 40 days after learning. This suggests that high basal spine density and correspondingly high corticofugal and neuromodulatory control inputs, such as glutamatergic, noradrenergic, cholinergic and serotoninergic inputs (Fletcher and Chen, 2010; Linster and Escanilla, 2019), could be one of the neuronal bases of memory maintenance in 2-month-old mice. Among the neuromodulatory inputs received in basal dendrites of granule cells, noradrenaline is required for olfactory perceptual learning and regulates adult-born granule cell survival in young adult mice (Moreno et al., 2012). A specific increase in this innervation, restricted to adult-born granule cells responding to the learned odorants, could increase their survival compared to all adult-born granule cells, thereby explaining the increased recruitment of adult-born neurons involved in the network coding for the learned odorants (Moreno et al., 2009; Forest et al., 2020) and contributing to memory maintenance. Conversely,

adult-born granule cells in 12-month-old mice that did not show any structural plasticity in basal spines would show lower survival, and this lack of plasticity could contribute to reduced retention of discrimination memory at this age.

4.3. How do 12-month-old mice learn to discriminate?

Despite the decrease in the number of adult-born granule cells (Enwere, 2004; Luo et al., 2006; Rey et al., 2012; Moreno et al., 2014) and in their structural plasticity (current study), middle-aged mice are still able to acquire fine olfactory discrimination following enrichment. Two mechanisms may underly the short-lasting perceptual learning taking place at 12 months. Firstly, the number of adult-born neurons, although small, may still be sufficient for task acquisition. This first study investigating how granule cells born in an aging OB integrate in control conditions suggests increased integration of granule cells born in a 12-month-old OB, in terms of both synaptic output to mitral cells and synaptic inputs received from mitral cells in apical spines (increased apical spine density at a relatively immature stage) and from centrifugal fibers in basal spines (increased basal spine density at a more mature stage). This increased integration within the bulbar network may compensate for the small number of adult-born granule cells produced at middle age (Enwere et al., 2004; Luo et al., 2006; Rey et al., 2012) and maintain the remodeling of the bulbar network allowed by adult neurogenesis. We show here that the spine density of adult-born granule cells in 12-month-old mice after learning, although not different from control conditions, was elevated and equivalent to that of 2-month-old mice after learning. These neurons thus may exert sufficient inhibition to enable acquisition of perceptual learning at 12 months, without needing to display structural plasticity after learning. This inhibition could also be reinforced by functional synaptic plasticity in the spines of adult-born neurons. Our results suggest that this form of plasticity is short-lasting, enabling learning acquisition but not long-term maintenance of the memory in 12-month-old mice. The involvement of adult-born neurons in memory in aged animals despite their small number has also been reported in the hippocampus. Adult-born neurons in the aged dentate gyrus maintain capacity for functional integration (Marrone et al., 2012) and their recruitment during a complex task is associated with resilience to cognitive aging (Montaron et al., 2020). Secondly, adult-born granule cells may not contribute to perceptual learning in middle-aged mice and other cellular mechanisms could be active in the OB network to support learning, but are short-lasting and/or non-reactivable. In young adult mice, increasing complexity of olfactory perceptual learning induces additional recruitment and structural plasticity of granule cells born during the perinatal period (Forest et al., 2020). The present data suggest that recruitment of pre-existing

granule cells (3-month-old neurons) is not one of these compensatory mechanisms in middle-aged mice. However, we cannot exclude recruitment of neurons born perinatally, although, given the renewal of granule cells (approx. 50%) by adult neurogenesis (Imayoshi et al., 2008), the number of granule cells born during the perinatal period that remain present in the OB at 12 months would be small.

In conclusion, this study identified middle age (12 months) as a pivotal age, when memory retention after olfactory perceptual learning subtly declines before learning becomes totally impaired at later ages. Structural plasticity is induced by learning specifically in adult-born granule cells responding to the learned odorants in young adult mice, a process that loses its specificity in middle age. These results enhance our understanding of the network remodeling induced by olfactory perceptual learning in young adult mice and provide new insights on how aging impairs plasticity mechanisms in the olfactory bulb and thereby induces progressive olfactory learning and memory deficits.

REFERENCES

Alonso M, Viollet C, Gabellec M-M, Meas-Yedid V, Olivo-Marin J-C, Lledo P-M (2006) Olfactory Discrimination Learning Increases the Survival of Adult-Born Neurons in the Olfactory Bulb. Journal of Neuroscience 26:10508–10513.

Belnoue L, Grosjean N, Abrous DN, Koehl M (2011) A Critical Time Window for the Recruitment of Bulbar Newborn Neurons by Olfactory Discrimination Learning. Journal of Neuroscience 31:1010–1016.

Burke SN, Barnes CA (2006) Neural plasticity in the ageing brain. Nature Reviews Neuroscience 7:30–40.

Cabeza R, Albert M, Belleville S, Craik FIM, Duarte A, Grady CL, Lindenberger U, Nyberg L, Park DC, Reuter-Lorenz PA, Rugg MD, Steffener J, Rajah MN (2018) Maintenance, reserve and compensation: the cognitive neuroscience of healthy ageing. Nat Rev Neurosci 19:701–710.

Chu MW, Li WL, Komiyama T (2016) Balancing the Robustness and Efficiency of Odor Representations during Learning. Neuron 92:174–186.

Dardou D, Datiche F, Cattarelli M (2008) Memory is differently impaired during aging according to the learning tasks in the rat. Behavioural Brain Research 194:193–200.

Daroles L, Gribaudo S, Doulazmi M, Scotto-Lomassese S, Dubacq C, Mandairon N, Greer CA, Didier A, Trembleau A, Caillé I (2016) Fragile X Mental Retardation Protein and Dendritic Local Translation of the Alpha Subunit of the Calcium/Calmodulin-Dependent Kinase II Messenger RNA Are Required for the Structural Plasticity Underlying Olfactory Learning. Biological Psychiatry 80:149–159.

Enwere E (2004) Aging Results in Reduced Epidermal Growth Factor Receptor Signaling, Diminished Olfactory Neurogenesis, and Deficits in Fine Olfactory Discrimination. Journal of Neuroscience 24:8354–8365.

Enwere E, Shingo T, Gregg C, Fujikawa H, Ohta S, Weiss S (2004) Aging Results in Reduced Epidermal Growth Factor Receptor Signaling, Diminished Olfactory Neurogenesis, and Deficits in Fine Olfactory Discrimination. Journal of Neuroscience 24:8354–8365.

Fletcher ML, Chen WR (2010) Neural correlates of olfactory learning: Critical role of centrifugal neuromodulation. Learning & Memory 17:561–570.

Forest J, Chalençon L, Midroit M, Terrier C, Caillé I, Sacquet J, Benetollo C, Martin K, Richard M, Didier A, Mandairon N (2020) Role of Adult-Born Versus Preexisting Neurons Born at P0 in Olfactory Perception in a Complex Olfactory Environment in Mice. Cereb Cortex 30:534–549.

Forest J, Moreno M, Cavelius M, Chalençon L, Ziessel A, Sacquet J, Richard M, Didier A, Mandairon N (2019) Short-term availability of adult-born neurons for memory encoding. Nat Commun 10:5609.

Grady C (2012) The cognitive neuroscience of ageing. Nature Reviews Neuroscience 13:491–505.

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 11:1153–1161.

Inaki K, Takahashi YK, Nagayama S, Mori K (2002) Molecular-feature domains with posterodorsal-anteroventral polarity in the symmetrical sensory maps of the mouse olfactory bulb: mapping of odourant-induced Zif268 expression. Eur J Neurosci 15:1563–1574.

Kelsch W, Sim S, Lois C (2010) Watching Synaptogenesis in the Adult Brain. Annual Review of Neuroscience 33:131–149.

Kraemer S, Apfelbach R (2004) Olfactory sensitivity, learning and cognition in young adult and aged male Wistar rats. Physiology & Behavior 81:435–442.

Lepousez G, Nissant A, Bryant AK, Gheusi G, Greer CA, Lledo P-M (2014) Olfactory learning promotes input-specific synaptic plasticity in adult-born neurons. Proceedings of the National Academy of Sciences 111:13984–13989.

Linster C, Escanilla O (2019) Noradrenergic effects on olfactory perception and learning. Brain Research 1709:33–38.

Livneh Y, Feinstein N, Klein M, Mizrahi A (2009) Sensory Input Enhances Synaptogenesis of Adult-Born Neurons. Journal of Neuroscience 29:86–97.

Luo J, Daniels SB, Lennington JB, Notti RQ, Conover JC (2006) The aging neurogenic subventricular zone. Aging Cell 5:139–152.

Mandairon N, Didier A, Linster C (2008) Odor enrichment increases interneurons responsiveness in spatially defined regions of the olfactory bulb correlated with perception. Neurobiol Learn Mem 90:178–184.

Mandairon N, Kuczewski N, Kermen F, Forest J, Midroit M, Richard M, Thevenet M, Sacquet J, Linster C, Didier A (2018) Opposite regulation of inhibition by adult-born granule cells during implicit versus explicit olfactory learning. Elife 7.

Mandairon N, Sacquet J, Garcia S, Ravel N, Jourdan F, Didier A (2006a) Neurogenic correlates of an olfactory discrimination task in the adult olfactory bulb. Eur J Neurosci 24:3578–3588.

Mandairon N, Sacquet J, Jourdan F, Didier A (2006b) Long-term fate and distribution of newborn cells in the adult mouse olfactory bulb: Influences of olfactory deprivation. Neuroscience 141:443–451.

Mandairon N, Stack C, Linster C (2006c) Olfactory enrichment improves the recognition of individual components in mixtures. Physiol Behav 89:379–384.
Mandairon N, Sultan S, Nouvian M, Sacquet J, Didier A (2011) Involvement of Newborn Neurons in Olfactory Associative Learning? The Operant or Non-operant Component of the Task Makes All the Difference. Journal of Neuroscience 31:12455–12460.
Marrone DF, Ramirez-Amaya V, Barnes CA (2012) Neurons generated in senescence maintain capacity for functional integration. Hippocampus 22:1134–1142.
Ming G, Song H (2011) Adult Neurogenesis in the Mammalian Brain: Significant Answers and Significant Questions. Neuron 70:687–702.
Mirich JM, Williams NC, Berlau DJ, Brunjes PC (2002) Comparative study of aging in the mouse olfactory bulb. Journal of Comparative Neurology 454:361–372.
Montaron M-F, Charrier V, Blin N, Garcia P, Abrous DN (2020) Responsiveness of dentate neurons generated throughout adult life is associated with resilience to cognitive aging. Aging Cell:e13161.
Moreno M, Richard M, Landrein B, Sacquet J, Didier A, Mandairon N (2014) Alteration of olfactory perceptual learning and its cellular basis in aged mice. Neurobiology of Aging 35:680–691.
Moreno MM, Bath K, Kuczewski N, Sacquet J, Didier A, Mandairon N (2012) Action of the Noradrenergic System on Adult-Born Cells Is Required for Olfactory Learning in Mice. Journal of Neuroscience 32:3748–3758.
Moreno MM, Linster C, Escanilla O, Sacquet J, Didier A, Mandairon N (2009) Olfactory perceptual learning requires adult neurogenesis. Proc Natl Acad Sci USA 106:17980–17985.
Nissant A, Bardy C, Katagiri H, Murray K, Lledo P-M (2009) Adult neurogenesis promotes synaptic plasticity in the olfactory bulb. Nat Neurosci 12:728–730.
Panzanelli P, Bardy C, Nissant A, Pallotto M, Sassoe-Pognetto M, Lledo P-M, Fritschy J-M (2009) Early Synapse Formation in Developing Interneurons of the Adult Olfactory Bulb. Journal of Neuroscience 29:15039–15052.
Patel RC, Larson J (2009) Impaired olfactory discrimination learning and decreased olfactory sensitivity in aged C57Bl/6 mice. Neurobiology of Aging 30:829–837.
Price JL, Powell TPS (1970) The Synaptology of the Granule Cells of the Olfactory Bulb. Journal of Cell Science 7:125–155.
Rey NL, Sacquet J, Veyrac A, Jourdan F, Didier A (2012) Behavioral and cellular markers of olfactory aging and their response to enrichment. Neurobiology of Aging 33:626.e9-626.e23.
Richard MB, Taylor SR, Greer CA (2010) Age-induced disruption of selective olfactory bulb synaptic circuits. Proc Natl Acad Sci U S A 107:15613–15618.
Rochefort C, Gheusi G, Vincent J-D, Lledo P-M (2002) Enriched Odor Exposure Increases the Number of Newborn Neurons in the Adult Olfactory Bulb and Improves Odor Memory. J Neurosci 22:2679–2689.
Rodriguez A, Ehlenberger DB, Dickstein DL, Hof PR, Wearne SL (2008) Automated Three-Dimensional Detection and Shape Classification of Dendritic Spines from Fluorescence Microscopy Images Sabatini B, ed. PLoS ONE 3:e1997.
Roman FS, Alescio-Lautier B, Soumireu-Mourat B (1996) Age-related learning and memory deficits in odor-reward association in rats. Neurobiol Aging 17:31–40.
Sailor KA, Valley MT, Wiechert MT, Riecke H, Sun GJ, Adams W, Dennis JC, Sharafi S, Ming G, Song H, Lledo P-M (2016) Persistent Structural Plasticity Optimizes Sensory Information Processing in the Olfactory Bulb. Neuron 91:384–396.
Shepherd GM, Chen WR, Willhite D, Migliore M, Greer CA (2007) The olfactory granule cell: From classical enigma to central role in olfactory processing. Brain Research Reviews 55:373–382.
Sultan S, Mandairon N, Kermen F, Garcia S, Sacquet J, Didier A (2010) Learning-dependent neurogenesis in the olfactory bulb determines long-term olfactory memory. The FASEB Journal 24:2355–2363.
Ueha R, Shichino S, Ueha S, Kondo K, Kikuta S, Nishijima H, Matsushima K, Yamasoba T (2018) Reduction of Proliferating Olfactory Cells and Low Expression of Extracellular Matrix Genes Are Hallmarks of the Aged Olfactory Mucosa. Front Aging Neurosci 10 Available at: https://www.frontiersin.org/articles/10.3389/fnagi.2018.00086/full.
Vinera J, Kermen F, Sacquet J, Didier A, Mandairon N, Richard M (2015) Olfactory perceptual learning requires action of noradrenaline in the olfactory bulb: comparison with olfactory associative learning. Learning & Memory 22:192–196.
Wearne SL, Rodriguez A, Ehlenberger DB, Rocher AB, Henderson SC, Hof PR (2005) New techniques for imaging, digitization and analysis of three-dimensional neural morphology on multiple scales. Neuroscience 136:661–680.
Weiler E, Farbman AI (1997) Proliferation in the Rat Olfactory Epithelium: Age-Dependent Changes. J Neurosci 17:3610–3622.
Whitman MC, Greer CA (2007) Synaptic integration of adult-generated olfactory bulb granule cells: basal

axodendritic centrifugal input precedes apical dendrodendritic local circuits. J Neurosci 27:9951–9961.
Yoder WM, Gaynor LS, Burke SN, Setlow B, Smith DW, Bizon JL (2017) Interaction between age and perceptual similarity in olfactory discrimination learning in F344 rats: relationships with spatial learning. Neurobiol Aging 53:122–137.

FIGURE LEGENDS

Figure 1: Olfactory perceptual learning and retention are progressively impaired with aging.

(A) Experimental design. For each age group (2, 6, 12 and 18 months), two sub-groups of mice (control and learning) were tested for discrimination between (+) Limonene and (–) Limonene, before (pre-test) and after the olfactory enrichment period (short-term test) before sacrifice. Half of the mice performed an additional test before sacrifice (long-term test). Olfactory enrichment was performed during 10 days by daily exposure to both (+) Limonene and (–) Limonene. For the control group, odorants were replaced by odorless mineral oil. All mice received an intra-cerebral injection of a GFP-encoding lentivirus at D0 to label a cohort of adult-born neurons born 8 days before the beginning of learning.

(B) Discrimination was tested by comparing investigation time between OHab4 (4^{th} presentation of the habituation odorant) and OTest (presentation of the test odorant). When investigation time was longer for OTest than OHab4, this indicates that the mouse is able to discriminate between the two odorants; when investigation time in OTest is similar to or shorter than OHab4, this indicates that the mouse is not able to discriminate between the two odorants. Before any olfactory enrichment (pre-tests), mice of all ages were not able to discriminate (+) from (–) Limonene. The control groups of all ages never discriminated (+) from (–) Limonene, whether tested at short- or long-term. In the learning groups, the 2-and 6-month-old mice discriminated the two odorants at both short- and long-term. The 12-month-old group discriminated shortly after the enrichment period but not at the later time point, indicating learning but with long-term retention deficit. The 18-month-old mice never discriminated (+) from (–) Limonene, indicating no learning at all. All results are given as mean ± SEM; dots represent individual mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (n=5-52 mice per group, see Suppl. Table 1 for details).

Figure 2: Structural plasticity induced by olfactory perceptual learning in apical dendrites of adult-born granule cells is impaired by aging.

(A) Representative image of an adult-born granule cell expressing GFP in the OB. Inset shows an apical dendritic segment with dendritic spines. Scale bars = 50µm and 5µm in inset.

(B-C) Apical dendrites of adult-born granule cells show no significant changes in total length (B) or number of branch points (C) according to age (2 *vs* 12 months), post-learning interval (D25 *vs* D60) or learning (control *vs* learning group). (n=20-39 neurons per group, from 4-5

animals).

(D) Apical spine density of adult-born granule cells is increased by learning at D25 in 2-month-old mice and at D60 in 12-month-old mice. (n=15-22 neurons per group, from 4-5 animals).

(E) Apical dendrites of pre-existing granule cells show no changes in spine density with learning in 12-month-old mice (control *vs* learning). (n=10-14 neurons per group, from 3-4 animals).

All results are given as mean ± SEM, dots represent individual neurons. * $p<0.05$, ** $p<0.01$

Figure 3: Structural plasticity induced by olfactory perceptual learning in basal dendrites of adult-born granule cells is impaired by aging.

(A) Representative image of an adult-born granule cell expressing GFP in the OB. Inset shows a basal dendritic segment with dendritic spines. Scale bars = 50µm and 5µm in inset.

(B-C) Basal dendrites of adult-born granule cells show no significant changes in total length (B) or number of dendrites (C) according to the age of the animal (2 *vs* 12 months), post-learning interval (D25 *vs* D60) or learning status (control *vs* learning). (n=16-34 neurons per group, from 4-5 animals).

(D) Basal spine density of adult-born granule cells is higher at D60 in control 12-month-old mice than control 2-month-old mice. Perceptual learning specifically increases basal spine density in the long term after learning in 2-month-old mice: basal density is higher in the learning group at D60 compared to the control group. 12-month-old mice display no learning-induced structural plasticity in adult-born granule cells. (n=15-24 neurons per group, from 4-5 animals).

(E) Basal dendrites of pre-existing granule cells show no significant changes in spine density with learning (control *vs* learning) in 12-month-old mice. (n=8-10 neurons per group, from 3-4 animals).

All results are given as mean ± SEM; dots represent individual neurons. * $p<0.05$

Figure 4: Structural plasticity induced by olfactory perceptual learning is specific to adult-born granule cells responding to the learned odorants and loses this specificity with aging.

(A) ZIF expression distinguishes adult-born granule cells responding to the learned odorants (GFP+/ZIF+, top panel) from non-responding cells (GFP+/ZIF-, bottom). Scale bars = 10µm.

(B) Learning-induced structural plasticity of apical spines is restricted to ZIF+ adult-born granule cells at D25 in 2-month-old mice. In 12-month-old mice, learning-induced structural

plasticity of apical spines is observed only at D60 and affects both ZIF- and ZIF+ adult-born granule cells.

(C) Learning-induced structural plasticity of basal spines is restricted to ZIF+ adult-born granule cells in 2-month-old mice and is visible at both short- and long-term post-learning (D25 and D60). In 12-month-old mice, no learning-induced structural plasticity is observed on basal spines of adult-born granule cells.

All results are given as mean ± SEM; dots represent individual neurons (n=5-13 neurons per group, from 3-5 animals). * $p<0.05$, ** $p<0.01$.

SUPPLEMENTARY FIGURES AND TABLES

Suppl. Figure 1: Habituation to odorants and discrimination of dissimilar odorants are preserved during aging.

In pre-enrichment tests **(A-B)**, short-term tests **(C-D)** and long-term tests **(E-F)**, mice of all ages and all groups (control and learning) displayed habituation to the presented odorant of both odorant pairs (Dodecanone/Carvone and (+) Limonene / (-) Limonene), as shown by a decreasing investigation time between OHab1 and OHab4 ($p<0.05$, see Supp. Table 1 for detailed statistical results). Discrimination was tested by comparing the investigation time between OHab4 and OTest. In pre-enrichment tests, mice of all ages did not discriminate (+) Limonene and (-) Limonene (**A**, $p>0.05$) but discriminated Dodecanone and Carvone (**B**, $p<0.001$). See Fig.1 for complete description of the (+) Limonene / (-) Limonene discrimination results in short-term and long-term tests. All results are given as mean ± SEM, dots represent individual mice. OHab1 *vs* OHab4: # $p<0.05$, ## $p<0.01$, ### $p<0.001$, § $p=0.082$; OHab4 *vs* OTest: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (n=5-52 mice per group, see Suppl. Table 1 for details).

Supp. Figure 2: Olfactory perceptual learning does not induce structural plasticity in pre-existing granule cells of 12-month-old mice.

(A) Experimental design. In order to label a cohort of pre-existing granule cells, a group of 12-month-old mice received an intra-cerebral injection of lentivirus expressing DsRed 3 months before the beginning of the enrichment period. These mice were subdivided in a control and a learning group and tested for discrimination as previously described. Note that these groups of mice were sacrificed after the short-term test.

(B-C) Apical dendrites of pre-existing granule cells show no significant changes in their total length **(B)** and number of branch points **(C)** with learning (control *vs* learning). (n=10-14 neurons per group, from 3-4 animals).

(D-E) Basal dendrites of pre-existing granule cells show no significant changes in their total length **(D)** and number of dendrites **(E)** with learning (control *vs* learning). (n=8-10 neurons per group, from 3-4 animals).

All results are given as mean ± SEM, dots represent individual neurons.

<u>Suppl. Table 1:</u> Full statistical report of the behavioral data (Fig.1 and Suppl.Fig.1).

Pre-tests

		Friedman's ANOVA	Wilcoxon (unilateral, paired)	
Odorant	**Age**	**OHab1-4 + OTest**	**OHab1 vs OHab4**	**OHab4 vs OTest**
Dodec/Carv	**2 months**	n=19, Friedman chi-squared = 37.095, df = 4, p = 1.722e-07 ***	V = 190, p = 1.907e-06 ***	V = 9, p = 6.294e-05 ***
	6 months	n=39, Friedman chi-squared = 52.821, df = 4, p = 9.291e-11 ***	V = 755, p = 1.822e-07 ***	V = 101.5, p = 2.921e-05 ***
	12 months	n=30, Friedman chi-squared = 56.602, df = 4, p = 1.499e-11 ***	V = 436, p = 1.487e-05 ***	V = 30, p = 1.895e-06 ***
	18 months	n=28, Friedman chi-squared = 26.6, df = 4, p = 2.395e-05 ***	V = 377, p = 6.475e-06 ***	V = 40, p = 0.0001076 ***
(+)Lim/(-)Lim	**2 months**	n=20, Friedman chi-squared = 39.709, df = 4, p = 4.973e-08 ***	V = 210, p = 9.537e-07 ***	V = 94, p = 0.3506 n.s.
	6 months	n=36, Friedman chi-squared = 18.175, df = 4, p = 0.00114 ***	V = 480, p = 0.01003 *	V = 366, p = 0.8005 n.s.
	12 months	n=52, Friedman chi-squared = 69.478, df = 4, p = 2.925e-14 ***	V = 1309, p = 8.416e-09 ***	V = 555, p = 0.112 n.s.
	18 months	n=41, Friedman chi-squared = 30.029, df = 4, p = 4.828e-06 ***	V = 736, p = 1.413e-05 ***	V = 326, p = 0.08888 n.s.

Short-term post-tests (only the (+)Lim/(-)Lim odorant pair was tested)

		Friedman's ANOVA	Wilcoxon (unilateral, paired)	
Age	**Learning group**	**OHab1-4 + OTest**	**OHab1 vs OHab4**	**OHab4 vs OTest**
2 months	**Control**	n=10 Friedman chi-squared = 9.28, df = 4, p = 0.05447 n.s.	V = 45, p = 0.04199 *	V = 20, p = 0.2461 n.s.
	Learning	n=10, Friedman chi-squared = 12.965, df = 4, p = 0.01145 *	V = 54, p = 0.001953 **	V = 6, p = 0.01367 *
6 months	**Control**	n=18, Friedman chi-squared = 25.911, df = 4, p = 3.299e-05 ***	V = 149, p = 0.0003268 ***	V = 56, p = 0.1719 n.s.
	Learning	n=18, Friedman chi-squared = 26.622, df = 4, p = 2.37e-05 ***	V = 152, p = 0.001167 **	V = 12.5, p = 0.0007949 ***
12 months	**Control**	n=25, Friedman chi-squared = 22.132, df = 4, p = 0.0001886 ***	V = 300, p = 2.694e-05 ***	V = 148.5, p = 0.3582 n.s.
	Learning	n=25, Friedman chi-squared = 37.86, df = 4, p = 1.198e-07 ***	V = 290, p = 0.0001249 ***	V = 12, p = 4.269e-05 ***
18 months	**Control**	n=18, Friedman chi-squared = 9.1111, df = 4, p = 0.05838 n.s.	V = 145, p = 0.003845 **	V = 56, p = 0.1061 n.s.
	Learning	n=20, Friedman chi-squared = 16.131, df = 4, p = 0.002849 **	V = 149, p = 0.01566 *	V = 89, p = 0.2853 n.s.

Long-term post-tests (only the (+)Lim/(-)Lim odorant pair was tested)

		Friedman's ANOVA	Wilcoxon (unilateral, paired)	
Age	**Learning group**	**OHab1-4 + OTest**	**OHab1 vs OHab4**	**OHab4 vs OTest**
2 months	**Control**	n=5, Friedman chi-squared = 13.6, df = 4, p = 0.008687 **	V = 15, p = 0.03125 *	V = 9, p = 0.6875 n.s.
	Learning	n=5, Friedman chi-squared = 9.28, df = 4, p = 0.05447 n.s.	V = 15, p = 0.03125 *	V = 0, p = 0.03125 *
6 months	**Control**	n=9, Friedman chi-squared = 5.6, df = 4, p = 0.2311 n.s.	V = 35, p = 0.08203 n.s.	V = 23, p = 0.5449 n.s.
	Learning	n=8, Friedman chi-squared = 14.918, df = 4, p = 0.004874 **	V = 36, p = 0.003906 **	V = 1, p = 0.007813 **
12 months	**Control**	n=13, Friedman chi-squared = 12.738, df = 4, p = 0.01263 *	V = 79, p = 0.008545 **	V = 51, p = 0.6576 n.s.
	Learning	n=16, Friedman chi-squared = 18.814, df = 4, p = 0.000855 ***	V = 128, p = 0.0003815 ***	V = 69, p = 0.5301 n.s.
18 months	**Control**	n=10, Friedman chi-squared = 10.513, df = 4, p = 0.03262 *	V = 53, p = 0.00293 **	V = 28, p = 0.5391 n.s.
	Learning	n=10, Friedman chi-squared = 7.36, df = 4, p = 0.118 n.s.	V = 47, p = 0.02441 *	V = 18, p = 0.1875 n.s.

* p<0.05, ** p<0.01, *** p<0.001

Suppl. Table 2: Full statistical report of the morphological data in Fig.2.

Apical dendrites – Adult-born neurons

	Wilcoxon bilateral unpaired tests, corrected for multiple comparisons by FDR (12 selected comparisons)	
Total dendritic length (Fig.2B)	2m.D25.Control vs 12m.D25.Control	p=0.9174545
	2m.D25.Learning vs 12m.D25.Learning	p=0.9160000
	2m.D60.Control vs 12m.D60.Control	p=0.9174545
	2m.D60.Learning vs 12m.D60.Learning	p=0.9160000
	2m.D25.Control vs 2m.D25.Learning	p=0.9174545
	2m.D60.Control vs 2m.D60.Learning	p=0.9174545
	12m.D25.Control vs 12m.D25.Learning	p=0.9174545
	12m.D60.Control vs 12m.D60.Learning	p=0.6864000
	2m.D25.Control vs 2m.D60.Control	p=0.9370000
	2m.D25.Learning vs 2m.D60.Learning	p=0.9174545
	12m.D25.Control vs 12m.D60.Control	p=0.9174545
	12m.D25.Learning vs 12m.D60.Learning	p=0.9174545
n=number of neurons	2m.D25.Control (n=32) ; 2m.D25.Learning (n=39) 2m.D60.Control (n=23) ; 2m.D60.Learning (n=22) 12m.D25.Control (n=21) ; 12m.D25.Learning (n=24) 12m.D60.Control (n=24) ; 12m.D60.Learning (n=20)	

	Wilcoxon bilateral unpaired tests, corrected for multiple comparisons by FDR (12 selected comparisons)	
Number of branch points (Fig.2C)	2m.D25.Control vs 12m.D25.Control	p=0.4920000
	2m.D25.Learning vs 12m.D25.Learning	p=0.4280000
	2m.D60.Control vs 12m.D60.Control	p=0.4920000
	2m.D60.Learning vs 12m.D60.Learning	p=0.4920000
	2m.D25.Control vs 2m.D25.Learning	p=0.4074000
	2m.D60.Control vs 2m.D60.Learning	p=0.4920000
	12m.D25.Control vs 12m.D25.Learning	p=0.4920000
	12m.D60.Control vs 12m.D60.Learning	p=0.4920000
	2m.D25.Control vs 2m.D60.Control	p=0.9350000
	2m.D25.Learning vs 2m.D60.Learning	p=0.1668000
	12m.D25.Control vs 12m.D60.Control	p=0.7974545
	12m.D25.Learning vs 12m.D60.Learning	p=0.7974545
n=number of neurons	2m.D25.Control (n=32) ; 2m.D25.Learning (n=39) 2m.D60.Control (n=23) ; 2m.D60.Learning (n=22) 12m.D25.Control (n=21) ; 12m.D25.Learning (n=24) 12m.D60.Control (n=24) ; 12m.D60.Learning (n=20)	

	3-way ANOVA		Post-hoc bilateral unpaired t-tests, corrected for multiple comparisons by FDR (12 selected comparisons)	
Spine density (Fig.2D)	**Animal age**	$F_{(1,136)}$=3.7090; p=0.0562051	2m.D25.Control vs 12m.D25.Control	p=0.0748800
			2m.D25.Learning vs 12m.D25.Learning	p=0.6229091
	Post-learning interval	$F_{(1,136)}$=0.1530; p=0.6963328	2m.D60.Control vs 12m.D60.Control	p=0.2266667
			2m.D60.Learning vs 12m.D60.Learning	p=0.0050940 **
	Learning	$F_{(1,136)}$=8.7775; p=0.0036010 **	2m.D25.Control vs 2m.D25.Learning	p=0.0250800 *
			2m.D60.Control vs 2m.D60.Learning	p=0.6229091
	Animal age * Post-learning interval * Learning	$F_{(1,136)}$=14.4096; p=0.0002207 ***	12m.D25.Control vs 12m.D25.Learning	p=0.7760000
			12m.D60.Control vs 12m.D60.Learning	p=0.0025800 **
			2m.D25.Control vs 2m.D60.Control	p=0.0910000
			2m.D25.Learning vs 2m.D60.Learning	p=0.2175000
			12m.D25.Control vs 12m.D60.Control	p=0.1937143
			12m.D25.Learning vs 12m.D60.Learning	p=0.0456000 *
n=number of neurons	2m.D25.Control (n=22) ; 2m.D25.Learning (n=19) 2m.D60.Control (n=18) ; 2m.D60.Learning (n=15) 12m.D25.Control (n=18) ; 12m.D25.Learning (n=17) 12m.D60.Control (n=17) ; 12m.D60.Learning (n=18)			

Apical dendrites – Pre-existing neurons

	Wilcoxon bilateral unpaired test	
Spine density (Fig.2E)	12m Control vs Learning	W = 63, p-value = 0.4668
n=number of neurons	12m.Control (n=11); 12m.Learning (n=14)	

Suppl. Table 3: Full statistical report of the morphological data in Fig.3.

Basal dendrites – Adult-born neurons

	Wilcoxon bilateral unpaired tests, corrected for multiple comparisons by FDR (12 selected comparisons)	
Total dendritic length (Fig.3B)	2m.D25.Control vs 12m.D25.Control	p=0.9109091
	2m.D25.Learning vs 12m.D25.Learning	p=0.8331429
	2m.D60.Control vs 12m.D60.Control	p=0.9109091
	2m.D60.Learning vs 12m.D60.Learning	p=0.8331429
	2m.D25.Control vs 2m.D25.Learning	p=0.8331429
	2m.D60.Control vs 2m.D60.Learning	p=0.8331429
	12m.D25.Control vs 12m.D25.Learning	p=0.9109091
	12m.D60.Control vs 12m.D60.Learning	p=0.9260000
	2m.D25.Control vs 2m.D60.Control	p=0.8331429
	2m.D25.Learning vs 2m.D60.Learning	p=0.9109091
	12m.D25.Control vs 12m.D60.Control	p=0.8331429
	12m.D25.Learning vs 12m.D60.Learning	p=0.8331429
n=number of neurons	2m.D25.Control (n=18) ; 2m.D25.Learning (n=34) 2m.D60.Control (n=21) ; 2m.D60.Learning (n=16) 12m.D25.Control (n=18) ; 12m.D25.Learning (n=21) 12m.D60.Control (n=16) ; 12m.D60.Learning (n=16)	

	Wilcoxon bilateral unpaired tests, corrected for multiple comparisons by FDR (12 selected comparisons)	
Number of dendrites (Fig.3C)	2m.D25.Control vs 12m.D25.Control	p=0.5448000
	2m.D25.Learning vs 12m.D25.Learning	p=0.9740000
	2m.D60.Control vs 12m.D60.Control	p=0.5448000
	2m.D60.Learning vs 12m.D60.Learning	p=0.5448000
	2m.D25.Control vs 2m.D25.Learning	p=0.9308571
	2m.D60.Control vs 2m.D60.Learning	p=0.9740000
	12m.D25.Control vs 12m.D25.Learning	p=0.8280000
	12m.D60.Control vs 12m.D60.Learning	p=0.9740000
	2m.D25.Control vs 2m.D60.Control	p=0.9740000
	2m.D25.Learning vs 2m.D60.Learning	p=0.9740000
	12m.D25.Control vs 12m.D60.Control	p=0.5448000
	12m.D25.Learning vs 12m.D60.Learning	p=0.2352000
n=number of neurons	2m.D25.Control (n=18) ; 2m.D25.Learning (n=34) 2m.D60.Control (n=21) ; 2m.D60.Learning (n=16) 12m.D25.Control (n=18) ; 12m.D25.Learning (n=21) 12m.D60.Control (n=16) ; 12m.D60.Learning (n=16)	

	Wilcoxon bilateral unpaired tests, corrected for multiple comparisons by FDR (12 selected comparisons)	
Spine density (Fig.3D)	2m.D25.Control vs 12m.D25.Control	p=0.7525714
	2m.D25.Learning vs 12m.D25.Learning	p=0.8661818
	2m.D60.Control vs 12m.D60.Control	p=0.0190800 *
	2m.D60.Learning vs 12m.D60.Learning	p=0.7525714
	2m.D25.Control vs 2m.D25.Learning	p=0.7525714
	2m.D60.Control vs 2m.D60.Learning	p=0.0190800 *
	12m.D25.Control vs 12m.D25.Learning	p=0.8661818
	12m.D60.Control vs 12m.D60.Learning	p=1.0000000
	2m.D25.Control vs 2m.D60.Control	p=0.7525714
	2m.D25.Learning vs 2m.D60.Learning	p=0.7525714
	12m.D25.Control vs 12m.D60.Control	p=0.8661818
	12m.D25.Learning vs 12m.D60.Learning	p=0.8661818
n=number of neurons	2m.D25.Control (n=16) ; 2m.D25.Learning (n=24) 2m.D60.Control (n=20) ; 2m.D60.Learning (n=17) 12m.D25.Control (n=13) ; 12m.D25.Learning (n=17) 12m.D60.Control (n=18) ; 12m.D60.Learning (n=15)	

Basal dendrites – Pre-existing neurons

	Wilcoxon bilateral unpaired test	
Spine density (Fig.3E)	12m Control vs Learning	W = 45, p-value = 0.4234
n=number of neurons	12m.Control (n=9); 12m.Learning (n=8)	

Suppl. Table 4: Full statistical report of the morphological data in Fig.4.

Apical dendrites - Adult-born neurons

	4-way ANOVA		Post-hoc bilateral unpaired t-tests, corrected for multiple comparisons by FDR (16 selected comparisons)	
Spine density (Fig.4B)	**Animal age**	$F_{(1,128)}$=1.2206; p=0.27131	Learning effect:	
	Post-learning interval	$F_{(1,128)}$=0.0804 ; p=0.77725	2m.D25.Zif- Control *vs* Learning	p=0.91600000
	Learning	$F_{(1,128)}$=5.0582; p=0.02622 *	2m.D25.Zif+ Control *vs* Learning	p=0.00672800 **
	Zif	$F_{(1,128)}$=5.4402; p=0.02124 *	2m.D60.Zif- Control *vs* Learning	p=0.65600000
	interaction Animal age * Learning	$F_{(1,128)}$=6.5911; p=0.01140 *	2m.D60.Zif+ Control *vs* Learning	p=0.96400000
	interaction Animal age * Post-learning interval * Learning	$F_{(1,128)}$= 3.8694 ; p=0.05134	12m.D25.Zif- Control *vs* Learning	p=0.75840000
	interaction Animal age * Learning* Zif	$F_{(1,128)}$= 6.2562 ; p=0.01364 *	12m.D25.Zif+ Control *vs* Learning	p=0.74844444
			12m.D60.Zif- Control *vs* Learning	p=0.00672800 **
			12m.D60.Zif+ Control *vs* Learning	p=0.06848000 #
			Zif effect:	
			2m.J25.Control Zif- *vs* Zif+	p=0.01466667 *
			2m.J25.Learning Zif- *vs* Zif+	p=0.96400000
			2m.J60.Control Zif- *vs* Zif+	p=0.65600000
			2m.J60.Learning Zif- *vs* Zif+	p=0.96400000
			12m.J25.Control Zif- *vs* Zif+	p=0.91600000
			12m.J25.Learning Zif- *vs* Zif+	p=0.65600000
			12m.J60.Control Zif- *vs* Zif+	p=0.96400000
			12m.J60.Learning Zif- *vs* Zif+	p=0.06200000 #
n=number of neurons	2m.D25.Zif+.Control (n=13) ; 2m.D25.Zif+.Learning (n=11) 2m.D25.Zif-.Control (n=9) ; 2m.D25.Zif-.Learning (n=8) 2m.D60.Zif+.Control (n=10) ; 2m.D60.Zif+.Learning (n=5) 2m.D60.Zif-.Control (n=8) ; 2m.D60.Zif-.Learning (n=10) 12m.D25.Zif+.Control (n=7) ; 12m.D25.Zif+.Learning (n=12) 12m.D25.Zif-.Control (n=11) ; 12m.D25.Zif-.Learning (n=5) 12m.D60.Zif+.Control (n=5) ; 12m.D60.Zif+.Learning (n=9) 12m.D60.Zif-.Control (n=12) ; 12m.D60.Zif-.Learning (n=9)			

Note that all interactions that are not listed above were statistically non-significant.

Basal dendrites - Adult-born neurons

	Wilcoxon bilateral unpaired tests, corrected for multiple comparisons by FDR (16 comparisons)	
Spine density (Fig.4C)	Learning effect:	
	2m.D25.Zif- Control *vs* Learning	p=0.69371429
	2m.D25.Zif+ Control *vs* Learning	p=0.03608000 *
	2m.D60.Zif- Control *vs* Learning	p=0.32888889
	2m.D60.Zif+ Control *vs* Learning	p=0.03608000 *
	12m.D25.Zif- Control *vs* Learning	p=0.42909091
	12m.D25.Zif+ Control *vs* Learning	p=0.32888889
	12m.D60.Zif- Control *vs* Learning	p=0.69371429
	12m.D60.Zif+ Control *vs* Learning	p=0.37760000
	Zif effect:	
	2m.J25.Control Zif- *vs* Zif+	p=0.13408000
	2m.J25.Learning Zif- *vs* Zif+	p=0.15146667
	2m.J60.Control Zif- *vs* Zif+	p=0.79573333
	2m.J60.Learning Zif- *vs* Zif+	p=0.32888889
	12m.J25.Control Zif- *vs* Zif+	p=0.09280000 #
	12m.J25.Learning Zif- *vs* Zif+	p=0.81800000
	12m.J60.Control Zif- *vs* Zif+	p=0.06613333 #
	12m.J60.Learning Zif- *vs* Zif+	p=0.48800000
n=number of neurons	2m.D25.Zif+.Control (n=6) ; 2m.D25.Zif+.Learning (n=12) 2m.D25.Zif-.Control (n=10) ; 2m.D25.Zif-.Learning (n=12) 2m.D60.Zif+.Control (n=12) ; 2m.D60.Zif+.Learning (n=9) 2m.D60.Zif-.Control (n=8) ; 2m.D60.Zif-.Learning (n=8) 12m.D25.Zif+.Control (n=8) ; 12m.D25.Zif+.Learning (n=11) 12m.D25.Zif-.Control (n=5) ; 12m.D25.Zif-.Learning (n=6) 12m.D60.Zif+.Control (n=7) ; 12m.D60.Zif+.Learning (n=7) 12m.D60.Zif-.Control (n=11) ; 12m.D60.Zif-.Learning (n=8)	

<u>Suppl. Table 5:</u> Full statistical report of the morphological data in Supp.Fig.2.

Pre-existing neurons

	Student t-test (unpaired, bilateral)	
Apical dendritic length (Supp.Fig.2B)	12m Control vs Learning	t = -0.34014, df = 22, p-value = 0.737
n=number of neurons	12m.Control (n=10); 12m.Learning (n=14)	

	Wilcoxon test (unpaired, bilateral)	
Apical dendritic branch points (Supp.Fig.2C)	12m Control vs Learning	W = 68.5, p-value = 0.9517
n=number of neurons	12m.Control (n=10); 12m.Learning (n=14)	

	Wilcoxon test (unpaired, bilateral)	
Basal dendritic length (Supp.Fig.2D)	12m Control vs Learning	W = 64, p-value = 0.315
n=number of neurons	12m.Control (n=10); 12m.Learning (n=9)	

	Wilcoxon test (unpaired, bilateral)	
Basal dendrite number (Supp.Fig.2E)	12m Control vs Learning	W = 54, p-value = 0.7807
n=number of neurons	12m.Control (n=10); 12m.Learning (n=9)	

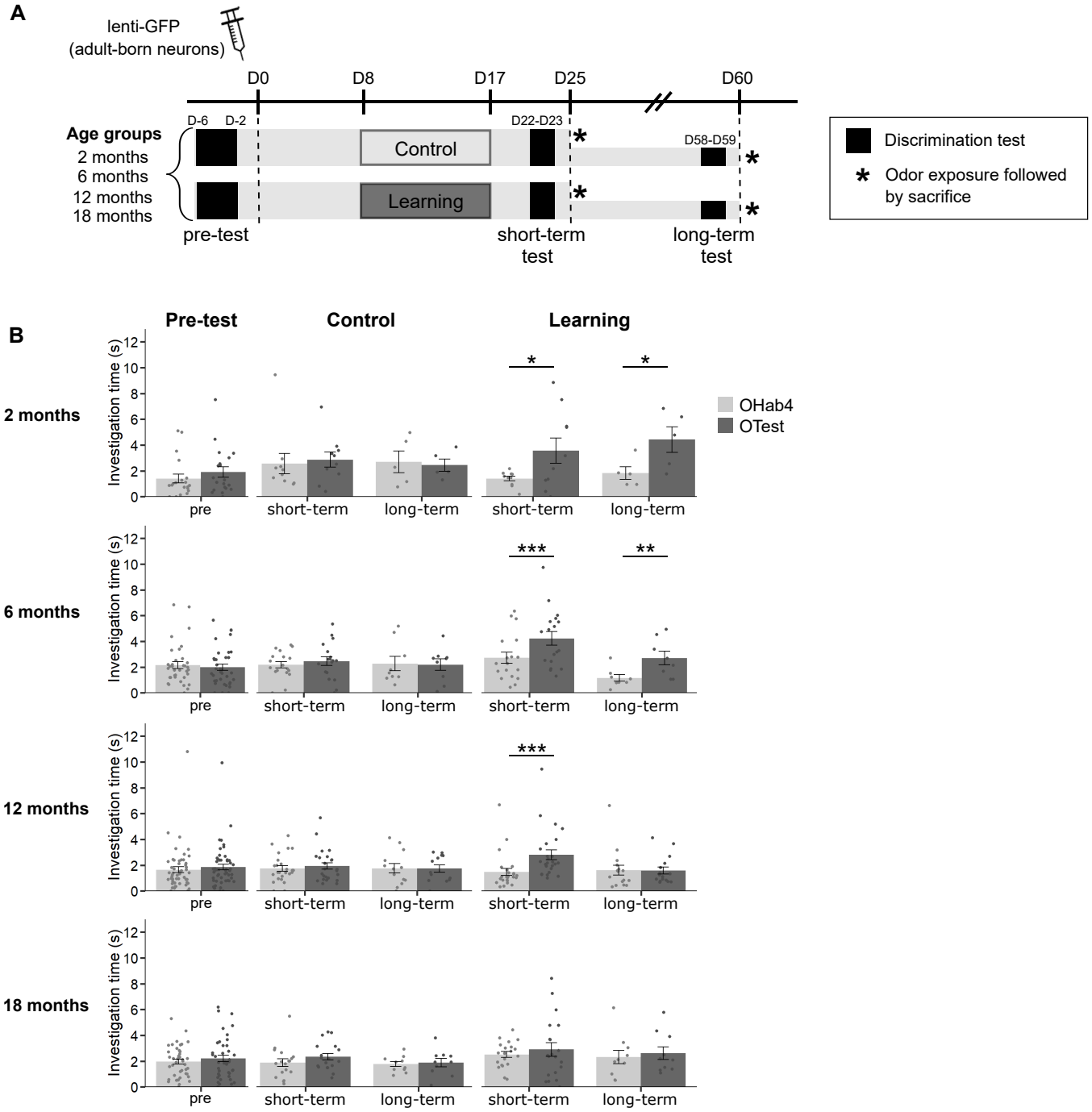


Figure 1: Olfactory perceptual learning and retention are progressively impaired with aging.

(A) Experimental design. For each age group (2, 6, 12 and 18 months), two sub-groups of mice (control and learning) were tested for discrimination between (+) Limonene and (–) Limonene, before (pre-test) and after the olfactory enrichment period (short-term test) before sacrifice. Half of the mice performed an additional test before sacrifice (long-term test). Olfactory enrichment was performed during 10 days by daily exposure to both (+) Limonene and (–) Limonene. For the control group, odorants were replaced by odorless mineral oil. All mice received an intra-cerebral injection of a GFP-encoding lentivirus at D0 to label a cohort of adult-born neurons born 8 days before the beginning of learning.

(B) Discrimination was tested by comparing investigation time between OHab4 (4th presentation of the habituation odorant) and OTest (presentation of the test odorant). When investigation time was longer for OTest than OHab4, this indicates that the mouse is able to discriminate between the two odorants; when investigation time in OTest is similar to or shorter than OHab4, this indicates that the mouse is not able to discriminate between the two odorants. Before any olfactory enrichment (pre-tests), mice of all ages were not able to discriminate (+) from (–) Limonene. The control groups of all ages never discriminated (+) from (–) Limonene, whether tested at short- or long-term. In the learning groups, the 2-and 6-month-old mice discriminated the two odorants at both short- and long-term. The 12-month-old group discriminated shortly after the enrichment period but not at the later time point, indicating learning but with long-term retention deficit. The 18-month-old mice never discriminated (+) from (–) Limonene, indicating no learning at all. All results are given as mean ± SEM; dots represent individual mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (n=5-52 mice per group, see Suppl. Table 1 for details).

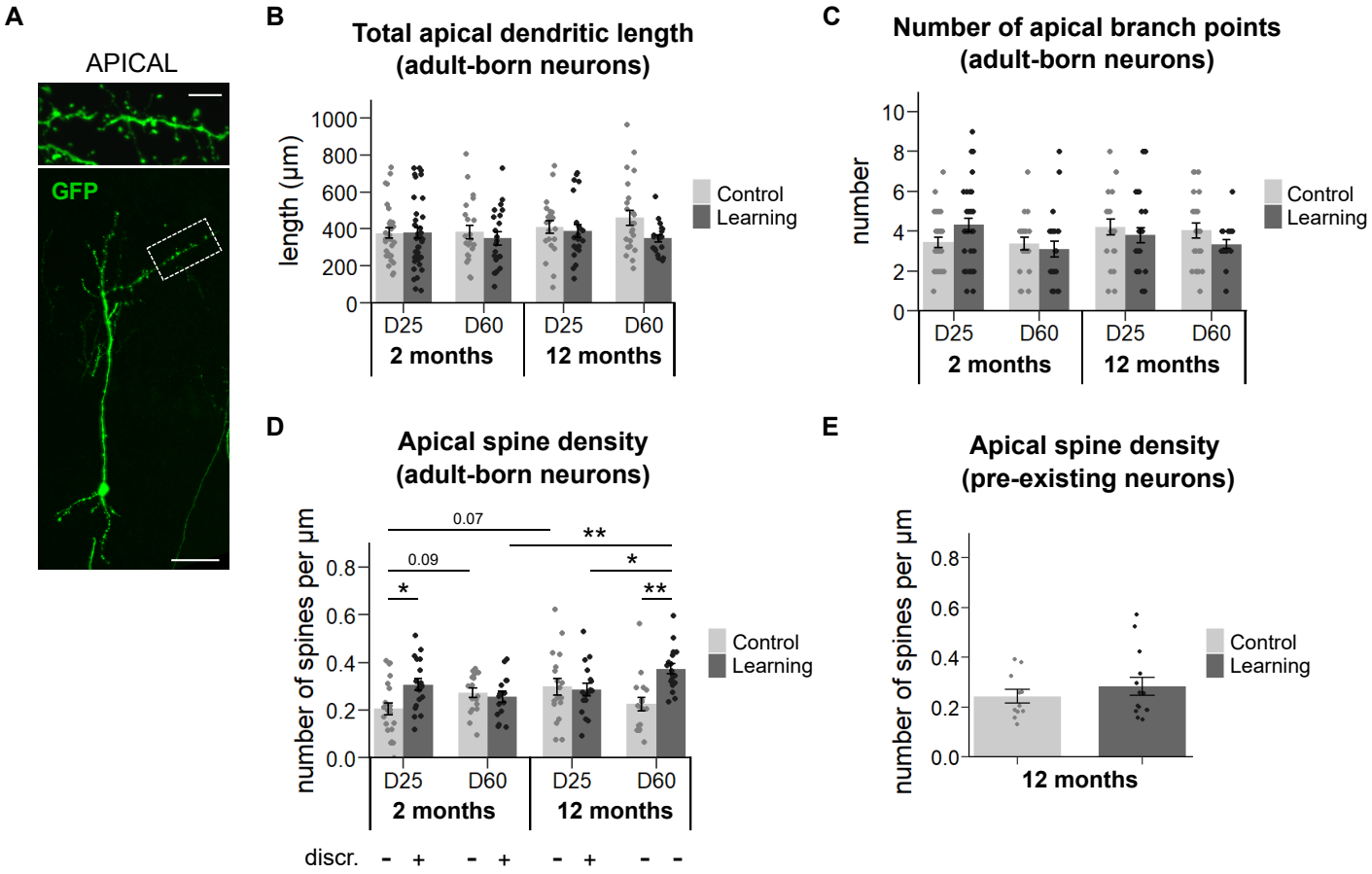


Figure 2: Structural plasticity induced by olfactory perceptual learning in apical dendrites of adult-born granule cells is impaired by aging.

(A) Representative image of an adult-born granule cell expressing GFP in the OB. Inset shows an apical dendritic segment with dendritic spines. Scale bars = 50µm and 5µm in inset.
(B-C) Apical dendrites of adult-born granule cells show no significant changes in total length (B) or number of branch points (C) according to age (2 vs 12 months), post-learning interval (D25 vs D60) or learning (control vs learning group). (n=20-39 neurons per group, from 4-5 animals).
(D) Apical spine density of adult-born granule cells is increased by learning at D25 in 2-month-old mice and at D60 in 12-month-old mice. (n=15-22 neurons per group, from 4-5 animals).
(E) Apical dendrites of pre-existing granule cells show no changes in spine density with learning in 12-month-old mice (control vs learning). (n=10-14 neurons per group, from 3-4 animals).
All results are given as mean ± SEM, dots represent individual neurons. * p<0.05, ** p<0.01

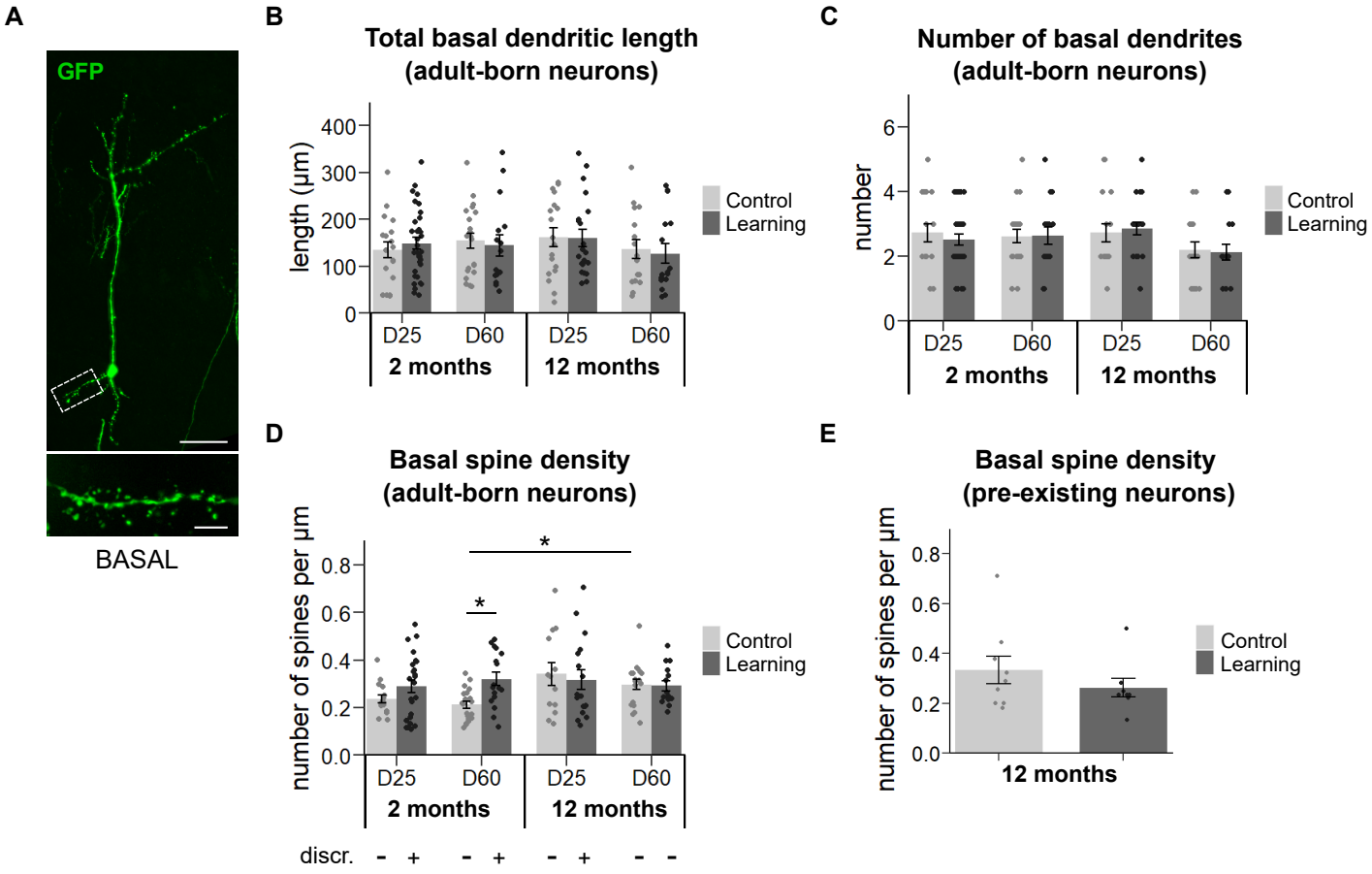


Figure 3: Structural plasticity induced by olfactory perceptual learning in basal dendrites of adult-born granule cells is impaired by aging.

(A) Representative image of an adult-born granule cell expressing GFP in the OB. Inset shows a basal dendritic segment with dendritic spines. Scale bars = 50µm and 5µm in inset. (B-C) Basal dendrites of adult-born granule cells show no significant changes in total length (B) or number of dendrites (C) according to the age of the animal (2 vs 12 months), post-learning interval (D25 vs D60) or learning status (control vs learning). (n=16-34 neurons per group, from 4-5 animals).
(D) Basal spine density of adult-born granule cells is higher at D60 in control 12-month-old mice than control 2-month-old mice. Perceptual learning specifically increases basal spine density in the long term after learning in 2-month-old mice: basal density is higher in the learning group at D60 compared to the control group. 12-month-old mice display no learning-induced structural plasticity in adult-born granule cells. (n=15-24 neurons per group, from 4-5 animals).
(E) Basal dendrites of pre-existing granule cells show no significant changes in spine density with learning (control vs learning) in 12-month-old mice. (n=8-10 neurons per group, from 3-4 animals).
All results are given as mean ± SEM; dots represent individual neurons. * $p<0.05$

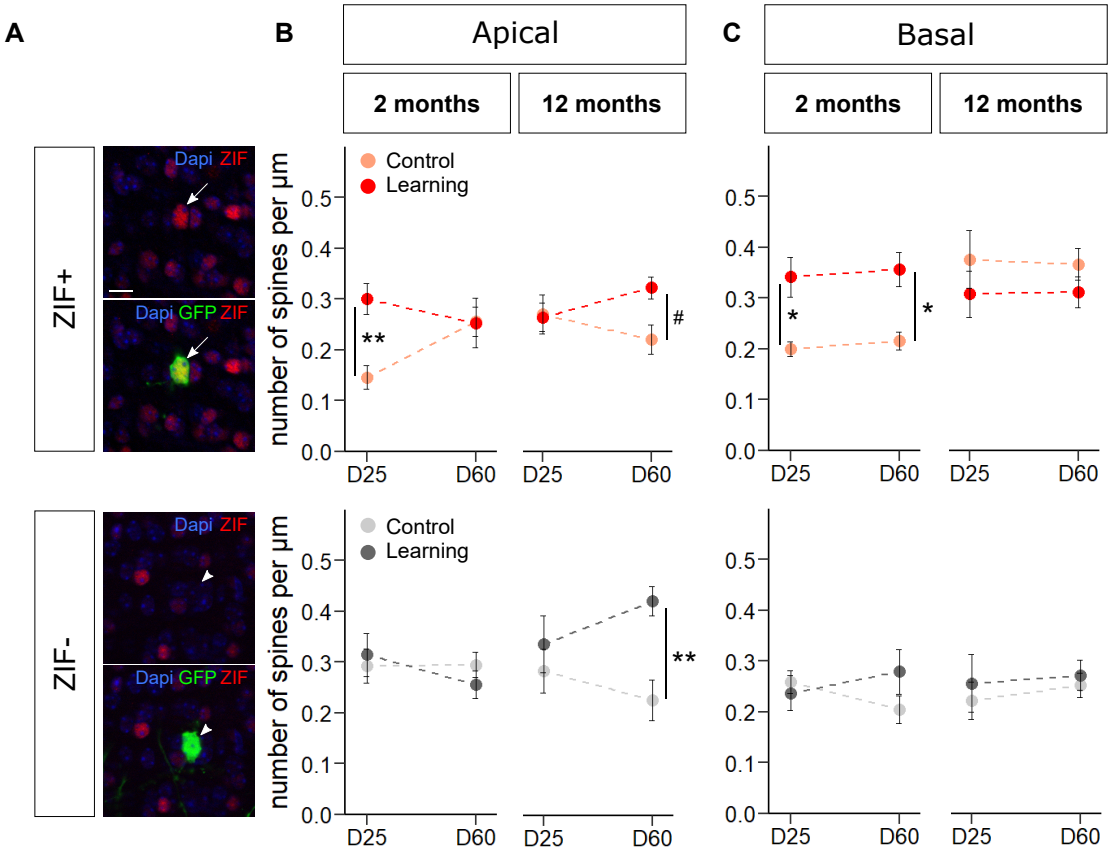
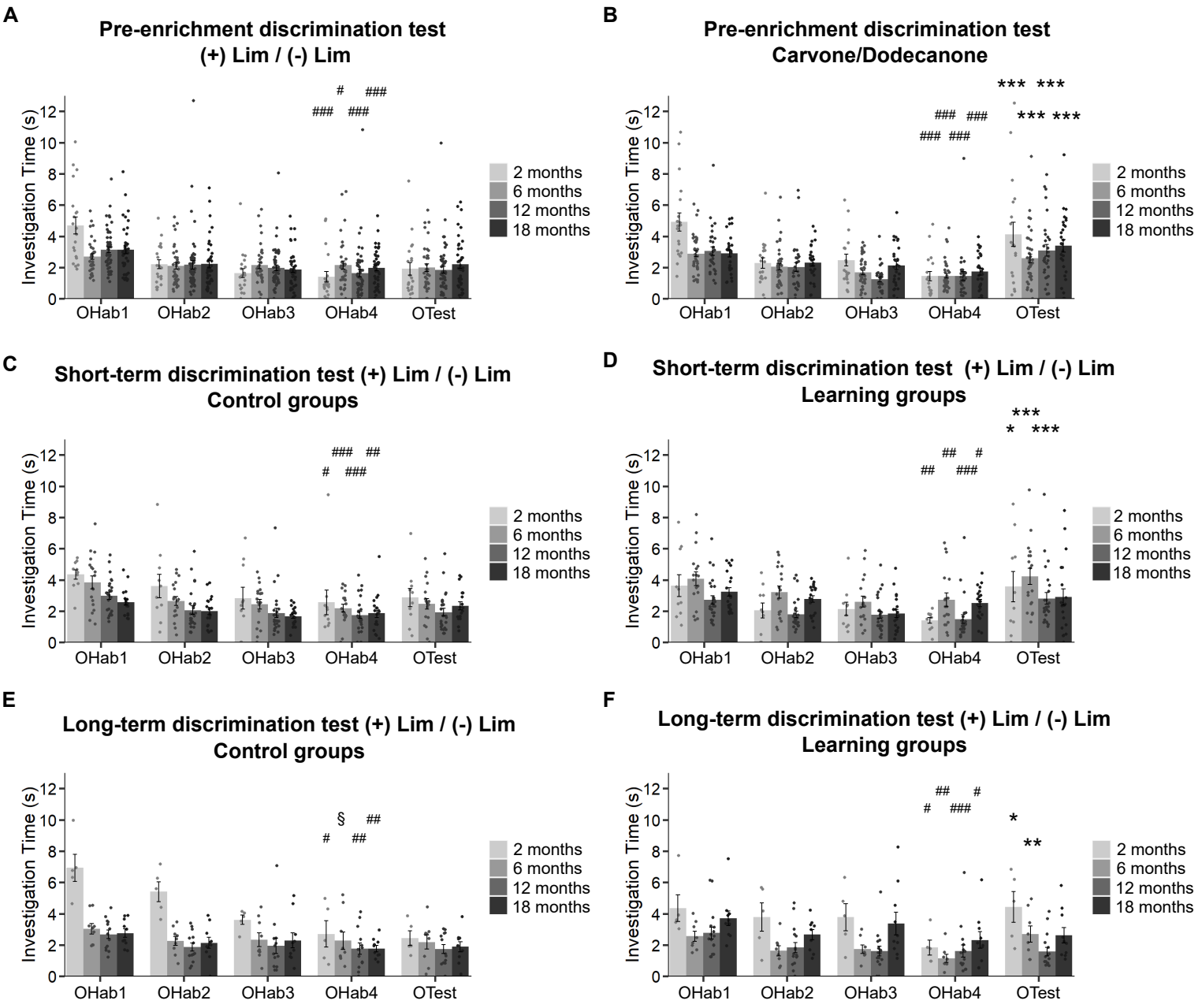


Figure 4: Structural plasticity induced by olfactory perceptual learning is specific to adult-born granule cells responding to the learned odorants and loses this specificity with aging.

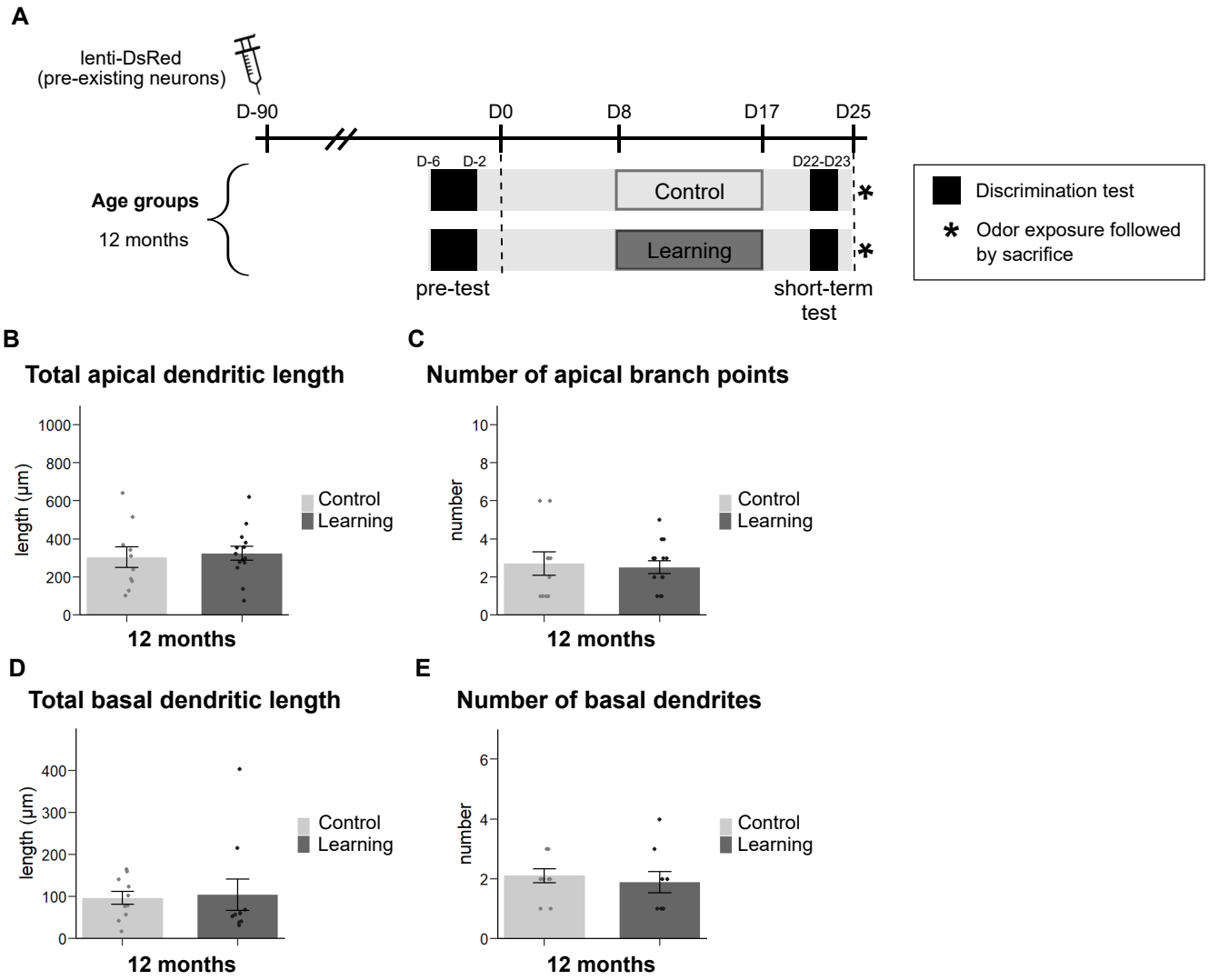
(A) ZIF expression distinguishes adult-born granule cells responding to the learned odorants (GFP+/ ZIF+, top panel) from non-responding cells (GFP+/ZIF-, bottom). Scale bars = 10µm. (B) Learning-induced structural plasticity of apical spines is restricted to ZIF+ adult-born granule cells at D25 in 2-month-old mice. In 12-month-old mice, learning-induced structural plasticity of apical spines is observed only at D60 and affects both ZIF- and ZIF+ adult-born granule cells. (C) Learning-induced structural plasticity of basal spines is restricted to ZIF+ adult-born granule cells in 2-month-old mice and is visible at both short- and long-term post-learning (D25 and D60). In 12-month-old mice, no learning-induced structural plasticity is observed on basal spines of adult-born granule cells.

All results are given as mean ± SEM; dots represent individual neurons (n=5-13 neurons per group, from 3-5 animals). * $p<0.05$, ** $p<0.01$.



Suppl. Figure 1: Habituation to odorants and discrimination of dissimilar odorants are preserved during aging.

In pre-enrichment tests (A-B), short-term tests (C-D) and long-term tests (E-F), mice of all ages and all groups (control and learning) displayed habituation to the presented odorant of both odorant pairs (Dodecanone/Carvone and (+) Limonene / (-) Limonene), as shown by a decreasing investigation time between OHab1 and OHab4 ($p<0.05$, see Supp. Table 1 for detailed statistical results). Discrimination was tested by comparing the investigation time between OHab4 and OTest. In pre-enrichment tests, mice of all ages did not discriminate (+) Limonene and (-) Limonene (A, $p>0.05$) but discriminated Dodecanone and Carvone (B, $p<0.001$). See Fig.1 for complete description of the (+) Limonene / (-) Limonene discrimination results in short-term and long-term tests. All results are given as mean ± SEM, dots represent individual mice. OHab1 vs OHab4: # $p<0.05$, ## $p<0.01$, ### $p<0.001$, § $p=0.082$; OHab4 vs OTest: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (n=5-52 mice per group, see Suppl. Table 1 for details).



Supp. Figure 2: Olfactory perceptual learning does not induce structural plasticity in pre-existing granule cells of 12-month-old mice.

(A) Experimental design. In order to label a cohort of pre-existing granule cells, a group of 12-month-old mice received an intra-cerebral injection of lentivirus expressing DsRed 3 months before the beginning of the enrichment period. These mice were subdivided in a control and a learning group and tested for discrimination as previously described. Note that these groups of mice were sacrificed after the short-term test. (B-C) Apical dendrites of pre-existing granule cells show no significant changes in their total length (B) and number of branch points (C) with learning (control vs learning). (n=10-14 neurons per group, from 3-4 animals). (D-E) Basal dendrites of pre-existing granule cells show no significant changes in their total length (D) and number of dendrites (E) with learning (control vs learning). (n=8-10 neurons per group, from 3-4 animals). All results are given as mean ± SEM, dots represent individual neurons.