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Neuronal plasticity in the olfactory bulb during simple and complex learning

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Résumé:

L'olfaction est un sens primordial chez les animaux : il est critique pour l'identification de la nourriture, l'évitement de prédateurs ou encore la reproduction. Pour parvenir à effectuer ces tâches, l'animal a besoin d'un système olfactif capable de réaliser des discriminations olfactives fines. Ces performances de discrimination peuvent être améliorées grâce à l'apprentissage perceptif qui est défini comme une amélioration de la discrimination de deux odeurs très proches d'un point de vue perceptif, après une exposition répétée à celles-ci. La clé d'un tel apprentissage se trouve au niveau du premier relai cérébral de l'information olfactive, le bulbe olfactif, puisque l'exposition aux odeurs permet de potentialiser leur discrimination grâce à un phénomène de plasticité hors du commun : la neurogenèse adulte.

Dans les conditions naturelles, l'environnement olfactif est complexe et varié nécessitant une flexibilité et un ajustement de la discrimination fine pour guider de façon adaptée le comportement. Dans cette étude, nous avons recherché les corrélats neurogéniques de l'apprentissage perceptif dans des conditions à la fois simple en présentant une seule paire d'odorants lors de l'exposition mais aussi plus complexes et donc plus écologiques en présentant plusieurs paires d'odorants simultanément. Nous avons observé que les animaux étaient capables d'apprendre à discriminer plusieurs paires d'odeur proches et que cette amélioration des performances olfactives était accompagnée d'une augmentation de l'implication des nouveaux neurones dans le traitement des odeurs apprises (mis en évidence par l'expression du gène précoce Zif268) sans augmentation de leur nombre. De plus, nous avons mis en évidence une modification de la morphologie fine des nouveaux neurones (interneurones granulaires) par l'apprentissage grâce à l'injection à l'âge adulte d'un vecteur viral exprimant une protéine fluorescente (GFP). Cette plasticité structurale suggère une modulation de l'activité des neurones relais par les nouveaux neurones et donc du message de sortie du bulbe olfactif vers les centres olfactifs supérieurs. Pour déterminer si cette plasticité structurelle était spécifique des nouveaux neurones, nous avons injecté un vecteur viral exprimant une autre protéine fluorescente (DsRed) lors de l'ontogenèse des cellules granulaires (animal à P1). Des résultats préliminaires révèlent l'absence de plasticité structurale des neurones préexistants lors de l'apprentissage perceptif et mettent en avant les propriétés uniques de plasticité des nouveaux neurones.

Ainsi, l'apprentissage perceptif est sous-tendu principalement par une augmentation de la plasticité structurale des nouveaux neurones ainsi qu'une augmentation de leur implication dans le traitement des odeurs apprises. Une sollicitation accrue du système lors d'apprentissages plus complexes conduit à augmenter ces phénomènes de plasticité.

Introduction

Olfaction is crucial for everyday life both in animals and humans. This sensory system devoted to the detection, discrimination and recognition of odorants from the environment is involved in numerous behaviors, among them social interactions, food choice and preferences, escaping dangers…

1. Olfactory system organization

Odorant molecules from the air are inhaled and interact with olfactory sensory neurons present in the olfactory epithelium. Each of these neurons expresses only one type of receptor (Buck and Axel, 1991) but is able to respond to many odorants (Duchamp-Viret *et al.*, 1999). Thus odorants activate specific combinations of sensory neurons. These sensory neurons' axons go through the cribriform plate to reach the olfactory bulb (OB) (Figure 1A) where they establish synapses with the relay cells (mitral and tufted cells) in specific regions called glomeruli (Figure 1B), establishing an odor-specific activation map. In the OB, the activity of the relay cells is regulated by two types of inhibitory interneurons: the periglomerular and the granule cells residing in the glomerular and the granule cell layers respectively. The periglomerular cells are connected with the mitral/tufted cells at the level of the glomeruli and modulate information entering the OB network. The granule cells are deeper in the OB, connected with the mitral/tufted cells at the level of their secondary lateral dendrites which makes them regulators of the outgoing information (Shepherd, 1972). Because of their number and position in the network, the granule cells have been proposed to have a major role in the regulation of olfactory information (Figure 1B). The olfactory information goes from the OB which is the first relay of the olfactory information in the central nervous system to higher olfactory centers, mainly, the olfactory piriform cortex.

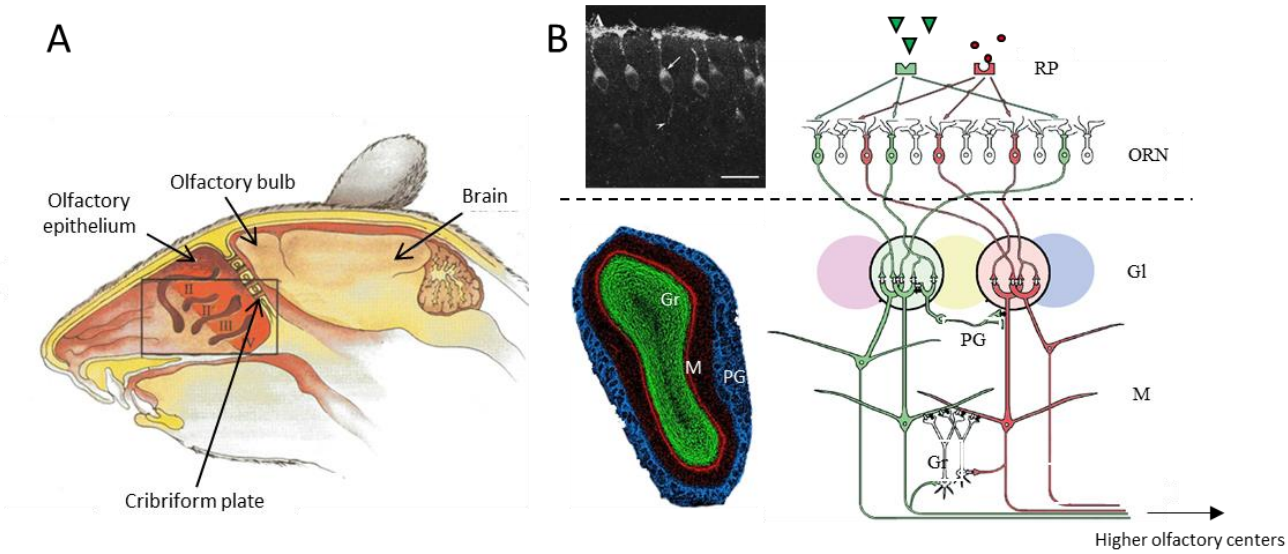

Figure 1: The mouse olfactory system.
A. Odorants present in the air bind olfactory receptor neurons present in the olfactory epithelium. These neurons send their axons to the olfactory bulb. the first central relay of the olfactory system. **B.** The olfactory bulb organization: the olfactory receptor neurons connect the mitral cells that are regulated by periglomerular and granule interneurons. (RP: receptor protein; Gl: glomeruli; PG: periglomerular cell; M: Mitral cells; Gr: granule cells)

2. Perceptual learning

Previous experience can lead to significant improvements in discrimination abilities which is called perceptual learning (Gilbert *et al.*, 2001). This learning reflects an ongoing process by which animals learn to discriminate common and potentially relevant stimuli within their immediate environment.

In human, perceptual learning can lead to acquisition of expertise. For instance, expertise in wine improves its discriminability (Wilson and Stevenson, 2003). In rodents, olfactory enrichment improves olfactory discrimination (Mandairon *et al.*, 2006c). Interestingly, odor enrichment enhances animals' ability to discriminate between chemically similar odorants in a relatively odor-specific manner. Indeed, the discrimination of a pair of similar odorants is improved by enrichment with the same odorants but not with different odorants that activate regions of the OB not overlapping with the regions activated by the discriminated pair. Even if the mechanisms underlying this learning remain unclear, it has been shown that infusions of NMDA into the OB improves odor discrimination in a manner similar to odor enrichment indicating that changes in OB processing contribute at least partially to the perceptual plasticity (Mandairon *et al.*, 2006c). A computational model proposed that activation of OB neurons produces widespread changes in inhibitory processing, which can underlie the observed improvement of odor discrimination (Mandairon *et al.*, 2006c). In line with this model, odor enrichment has been shown to increase inhibition of mitral cells (Buonviso *et al.*, 1998) and to increase the responsiveness of the inhibitory granule cells to odorants, as measured by expression of an immediate early gene (Mandairon *et al.*, 2008).

All these data support that modulation of inhibitory processing in the OB, which is mediated by local inhibitory interneurons, can underlie the observed improvement of odor discrimination during perceptual learning. Interestingly, these bulbar inhibitory interneurons are continuously generated in adulthood thanks to a process called adult neurogenesis.

3. Adult neurogenesis

Contrary to previous beliefs, the adult brain is in fact capable of generating adult-born neurons that can integrate into its complex circuitry throughout life.

The first study, demonstrating that neurogenesis was indeed occurring in full grown adults, used tritiated thymidine which is taken up by dividing cells and revealed by autoradiography. It revealed the presence of adult-born cells in the OB and hippocampus (Altman, 1969). However that work was mostly dismissed up until 20 years later when adult neurogenesis was once again put forth as multi potential neural stem cells were isolated from adult mammals brain (Reynolds and Weiss, 1992) and shown to be able to become neurons (Lois and Alvarez-Buylla, 1993). That is when adult mammalian neurogenesis became widely accepted.

Concerning more specifically adulthood OB neurogenesis, stem cells reside alongside the lateral ventricle, in the subventricular zone. These stem cells divide into neuroblasts which migrate along the rostral migratory stream toward the OB. Once in the OB, neuroblasts then migrate tangentially to reach the glomerular and

granule cell layers to differentiate into periglomerular (20%) and granule interneurons (80%) respectively, which shape the output message of the OB (Figure 2) (Lledo *et al.*, 2006). Migration from the subventricular zone to the OB takes 5 to 10 days.

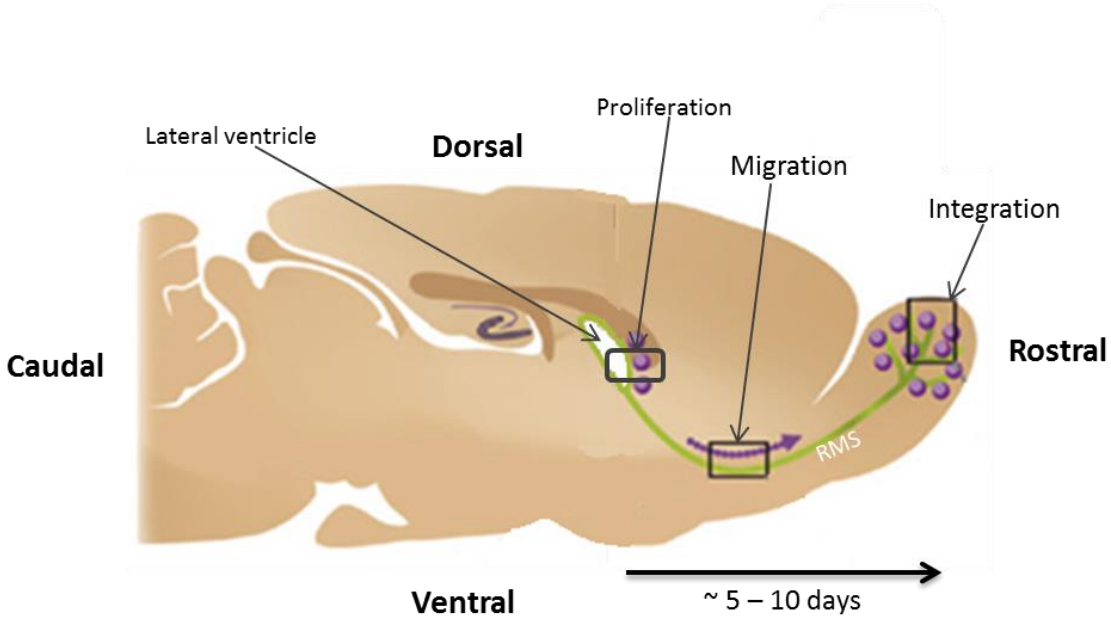

Figure 2: Adult neurogenesis in the olfactory bulb.
Stem cells residing in the subventricular zone of the lateral ventricles divide and differentiate into neuroblasts that migrate toward the OB (in about 8 to 10 days) along the rostral migratory stream (RMS). Once in the OB, they migrate tangentially to reach the granule and periglomerular layers where they differentiate into respectively granule and periglomerular interneurons.

Between 15 and 45 days after generation, approximately 50% of the adult-generated granule cells die. Activity plays an important role in the modulation of their survival rate. For instance, odor exposure increases survival (Rochefort *et al.*, 2002), while olfactory deprivation by naris occlusion decreases survival of adult-born granule cells (Mandairon *et al.*, 2006b). Olfactory associative learning also influences adult-born cells survival (Alonso *et al.*, 2006; Mandairon *et al.*, 2006a). Furthermore, numerous studies have investigated the necessity of adult-born neuron presence in the OB during olfactory associative learning and have yielded conflicting results (Imayoshi *et al.*, 2008; Breton-Provencher *et al.*, 2009; Lazarini *et al.*, 2009; Sultan *et al.*, 2010; Mandairon *et al.*, 2011). However, regarding perceptual learning, these adult-born neurons have been shown to be necessary for the improvement of discrimination to occur after exposure (Moreno *et al.*, 2009). In this study, the authors showed that perceptual learning is accompanied by an elevated survival rate of adult-born granule neurons, preferentially involved in processing the learned odor, within the OB. This suggests that perceptual plasticity relies on long-term changes in odor processing at the level of the OB involving increased survival of adult-born granule interneurons.

4. Study objectives

Adult neurogenesis has been functionally linked to learning. It allows the shaping of the bulbar network to the olfactory environment, playing a crucial adaptive role.

Until now, studies have analyzed behavioral performances and neurogenic correlates during simple olfactory perceptual learning, involving only one pair of odorants. However, in real life, animals are exposed to various and complex olfactory environments and the olfactory system has to adapt to be able to guide the animal's behavior correctly. Thus, in this study, we will use a more realistic context to investigate how the animal adapts its perceptive capacities to a more complex environment and examine the neurogenic mechanisms underlying the plasticity of the olfactory perception in this condition. In other words, we will first investigate whether the pool of surviving or recruited adult-born neurons can be increased by making the learning more complex. To do so, we will use the model of perceptual learning in a more complex configuration, using several pairs of odorants during the enrichment, to study its effect on **1-** olfactory discrimination, **2-** adult-born cell survival, adult-born neuron involvement in processing the learned odorants and adult-born neuron structural plasticity as an index of their integration in the network. Structural plasticity will be assessed through fine characterization of changes in dendritic arborization and dendritic spines. This should help us to evaluate the adaptive potential of adult neurogenesis.

In addition, the uniqueness of the contribution of adult-born neurons to olfactory learning and its underlying plasticity remains essentially unknown. In order to shed light on this issue, we will **3**- compare learning-induced structural plasticity in adult-born neurons to that of preexisting neurons born during ontogenesis. This will tell us whether adult-born neurons show specific plastic properties to support olfactory learning.

Materials and Methods

1- Animals

35 male C57BL/6J mice, aged from post natal day 1 (P1) to 3 months, were used in this study. These mice were born and raised in our laboratory but their mothers came from Charles River laboratory (L'Arbresles, France). Mice were raised in a controlled environment under a 12h light/dark cycle with food and water *ad libidum*. All behavioral training was conducted in the afternoon (1pm – 7pm). All efforts were made to minimize the number of animals used and their suffering during the experimental procedure in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and protocols were approved by the French Ethical Committee (DR2013-48 (vM)).

2- Experimental design.

Here, we analysed the effect of a 10-day period of olfactory enrichment on adult mice's ability to discriminate between odorants using an olfactory habituation/cross habituation test (see below). Three groups of mice were performed depending on the number of odor pairs used for the enrichment, and thus its complexity (Figure 3). Then, to evaluate the effect of these different enrichments on the rate of adult neurogenesis, we injected Bromodeoxyuridine (BrdU) 9 days before the beginning of the enrichment period. On the same mice, we analyzed the morphology of preexisting and adult-born neurons by labelling

preexisting neurons using the injection of DsRed lentivirus at P1 and then, GFP lentivirus at P60 (8 days before enrichment). Mice were sacrificed 26 days after the GFP injection (Figure 3).

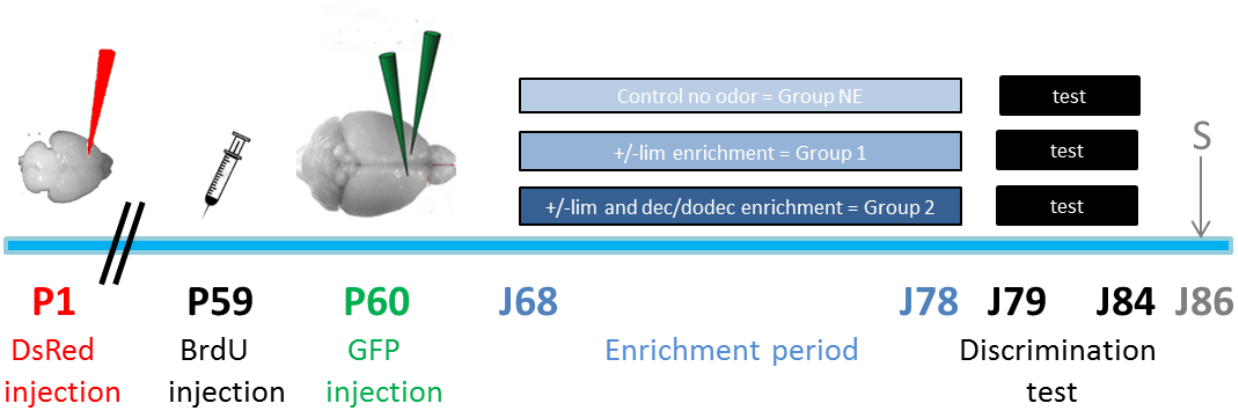

Figure 3: Experimental design.
In mice, preexisting neurons were labelled by injecting DsRed lentivirus in the lateral ventricle at P1 then adult-born neurons were labelled by injecting BrdU (i.p.) at P59 and GFP lentivirus in the subventricular zone at P60. Then, 3 groups of mice were performed depending on the number of odor pairs used for the 10-day enrichment period (non-enriched: Group NE; enriched with one odor pair: Group 1; enriched with two odor pairs: Group 2). At the end of the enrichment, mice were tested on their ability to discriminate these odor pairs using an olfactory habituation/cross habituation test. Mice were sacrificed 86 days after their birth.

3- Behavioral experiments

Enrichment. Odor enrichment consisted of exposure with one odor pair at a time for 1-hr per day for 10 consecutive days. Odorants of the pair were presented simultaneously on two separate swabs containing 100 µl of pure odorant placed in two separate tea balls hanging from the cover of the cage. For multiple enrichments, odor pairs were presented with an interval of 2 hours. 9 mice were enriched with +limonene and –limonene; 7 mice with first +limonene and -limonene followed by decanal and dodecanone. Control mice were housed under the same conditions except that tea balls contained mineral oil (n=12).

Olfactory habituation/cross habituation test. We used a habituation/cross-habituation test to assess olfactory discrimination (see Moreno et al 2009). Briefly, this task assessed the degree to which mice were able to spontaneously discriminate between odorants by habituating them to an odorant (Ohab) and measuring their cross-habituation to a second odorant (Otest). If the second odorant was not discriminated from the first, it would not elicit an increased investigation response by the mouse. Each presentation lasted 50 sec and was separated by 5 min. More specifically, we tested the discrimination between +limonene and - limonene, decanal and dodecanone. All experiments took place in a clean cage similar to the home cage. Mice were allowed a 10 min habituation period before the start of the experiment. Odorants were presented by placing 60µL of the diluted odor (1Pa) onto a filter paper (Whatman #1) which was put inside a tea ball hanging from the side of the cage. A test session consisted of one presentation of mineral oil then four odor presentations of the habituation odor (Ohab1-4) followed by one presentation of the test odor (Otest). Each odor of each pair was used alternatively as habituation or test odor. Investigation times of each odor were recorded and a difference between Ohab4 and Otest indicated discrimination.

Statistical Analyses. All data were analyzed using R statistical software. The data for each odor pair was analyzed by a one-way repeated measure ANOVA on the habituation part (Ohab1-4) to determine if the time of investigation during Ohab4 is significantly lower than during Ohab1 (habituation). Then to assess discrimination a paired-t-test was performed between Ohab4 and Otest. Only mice that had an investigation time of Ohab1>0s were included in the analysis (6 trials were excluded among 112 total trials). The level of significance was set to 0.05.

4- Adult-born neuron counts in the OB

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

Sacrifice and Histology. To investigate immediate-early-gene expression in response to the learned odorants, mice were presented with tea balls containing 100 µl (pure) of learned odorants (depending on the experimental group: no odor, +limonene/- limonene or +/- limonene and decanal/dodecanone) for 1 h. One hour after the end of the odor stimulation, mice were deeply anesthetized (pentobarbital, 0.2 ml/30 g) and killed by intracardiac perfusion of 50 ml of fixative (4% paraformaldehyde in phosphate buffer, pH 7.4). Their brains were removed, postfixed, cryoprotected in sucrose (20%), frozen rapidly, and then stored at -20°C before sectioning with a cryostat (Reichert-Jung, NuBlock, Germany).

BrdU immunohistochemistry. 14µm thick sections were incubated in Target Retrieval Solution (Dako) for 20 min at 98°C. After cooling, they were treated with pepsin (0,43 U/mL in 0,1N HCl, Sigma) for 3 min. Sections were transferred to a blocking solution (5% normal horse serum (Sigma) with 5% BSA and 0,125% Triton X-100), and were then incubated overnight at 4°C with a mouse anti-BrdU antibody (1/100, Chemicon), followed by a biotylated anti-mouse secondary antibody (1/200, Vector Laboratories) for 2h. The sections were then processed through an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). After dehydration in graded ethanols, the sections were defatted in xylene and cover-slipped in DEPEX (Fluka, Sigma).

BrdU-positive cell quantification. All cell counts were conducted blind with regard to the mouse status. Data were collected with the help of mapping software (Mercator Pro, Explora Nova), coupled with a Zeiss microscope. BrdU-positive cells were counted in the granule cell layer of the OB on about 9 sections (14µm thick, 216 µm intervals) of 4-6 mice/group. The number of positive cells was divided by the surface of the region of interest to yield the total densities of labeled cells (labeled profiles/μm^2). Densities were compared between experimental groups using a one-way ANOVA followed by *post-hoc* t-test with holm correction for multiple comparisons.

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

Triple labelling analysis. Once again, all cell counts were conducted blind with regard to the mouse status. Triple labelled slices were examined using pseudo-confocal scanning microscopy equipped with an Apotome (Zeiss). BrdU-positive cells were examined for colabeling with NeuN and Zif268 (a minimum of 30 cells/animal, n=3-6 animals/group). The percentage of double and triple labeled cells was calculated for each group and compared using a one-way ANOVA followed by *post-hoc t*-tests to compare only the enriched groups with their respective controls (not enriched but stimulated in the same way 1-hr before sacrifice, see above).

5- Neurons morphology

Preexisting neuron labeling. At P1, mice were anesthetized in ice and injected in the lateral ventricle with 1 µl of lentiviruses expressing DsRed fluorescent protein. The injection was done using a glass-micropipette linked to a Hamilthon syringe. Injection was performed using programmable syringe controller at a rate of 0.2 µL/s.

Adult-born neuron labeling. At 2 months (8 days before the beginning of the enrichment period, see Figure 3), the same mice were injected with lentiviruses expressing GFP fluorescent protein in the subventricular zone (100 nl per side). To do so, mice were anesthetized with a cocktail injection of 50 mg/kg ketamine and 7,5 mg/kg xylazine (i.p.), secured in a stereotaxic instrument (Narishige Scientific Instruments, Tokyo, Japan) and injected in the subventricular zone at the following coordinated with respect to bregma: anteroposterior +1mm, mediolateral ± 1mm, dorsoventral – 2.3mm. Injection was performed using a Hamilton syringe and a programmable syringe controller at a rate of 150nL/min. Mice received antalgic ketoprofen (2 mg/kg) at the end of surgery and once every day during 2 days. Mice were allowed 8 days to recover from the surgery with food and water *ad libitum.*

DsRed and GFP neurons immunohistochemistry. The functional involvement of preexisting and adult-born neurons was assessed using Zif268 expression coupled with GFP or DsRed labeling on 40µm thick sections. Thus, two different double labeling were performed: a GFP/Zif268 and a DsRed/Zif268 labeling using a mouse anti-DsRed (1:200, Santa Cruz Biotachnology), a chicken anti-GFP (1:1000, Anaspec) and a rabbit anti-Zif268 (1:1000, Santa Cruz Biotechnology). Appropriate secondary antibodies coupled with Alexa 546 for DsRed revelation, Alexa 488 for GFP revelation and Alexa 633 for Zif268 revelation were used.

Morphological data analysis. All morphological analyses were conducted blind with regard to the mouse status. Images were acquired using Zeiss pseudo-confocal system. A total of 10 adult-born (GFP-positive) neurons per animal were examined (n= 2-3 animals for each experimental group).

For preexisting neurons, 5-7 Dsred-positive neurons were analyzed (n=1-2 animals for each experimental group). In order to compare the morphology of odor responsive versus non responsive neurons, among these labeled preexisting and adult-born neurons, half were chosen to be Zif268-positive and the other Zif268-negative. For each neuron, we first analyzed whether it expressed Zif268 thanks to images taken with 40x objective (lateral and *z*-axis resolutions were 160 and 280 nm) (Figure 4A) then the spine density and spine neck length of apical and basal dendrites were manually assessed on images taken with 100x objective using measurement tool (lateral and *z*-axis resolutions were 60 and 200 nm) (Figure 4B).

For the analysis of the dendritic arborization, images were taken with 20x (lateral and *z*-axis resolutions were 320 and 400 nm) and 40x objectives (Figure 4B). It consisted in the addition of the length of all the dendritic segments in the apical or basal domain (Figure4B).

Neurons reconstruction was performed using NeuronStudio software (Wearne *et al.*, 2005; Rodriguez *et al.*, 2008) (Figure 4C). This software allows for three dimensional (3D) reconstructions of dendrites and spines from confocal z-series stacks on a spatial scale. Dendritic length was measured automatically and spine lengths were manually assessed with the help of the 3D reconstruction.

Statistical analysis. Morphological data (spine density, spine length and dendritic length) were compared between groups using an ANOVA followed by *post-hoc* t-test with holm correction for multiple comparisons. When differentiating responding versus non responding neurons to learned odorants (Zif+ versus Zif-), data were analyzed by a two-way ANOVA with groups and Zif268 expression as factors followed when necessary by *post-hoc* one-way ANOVA on either one of the previous factor or/and *post-hoc* t-test with holm correction for multiple comparisons.

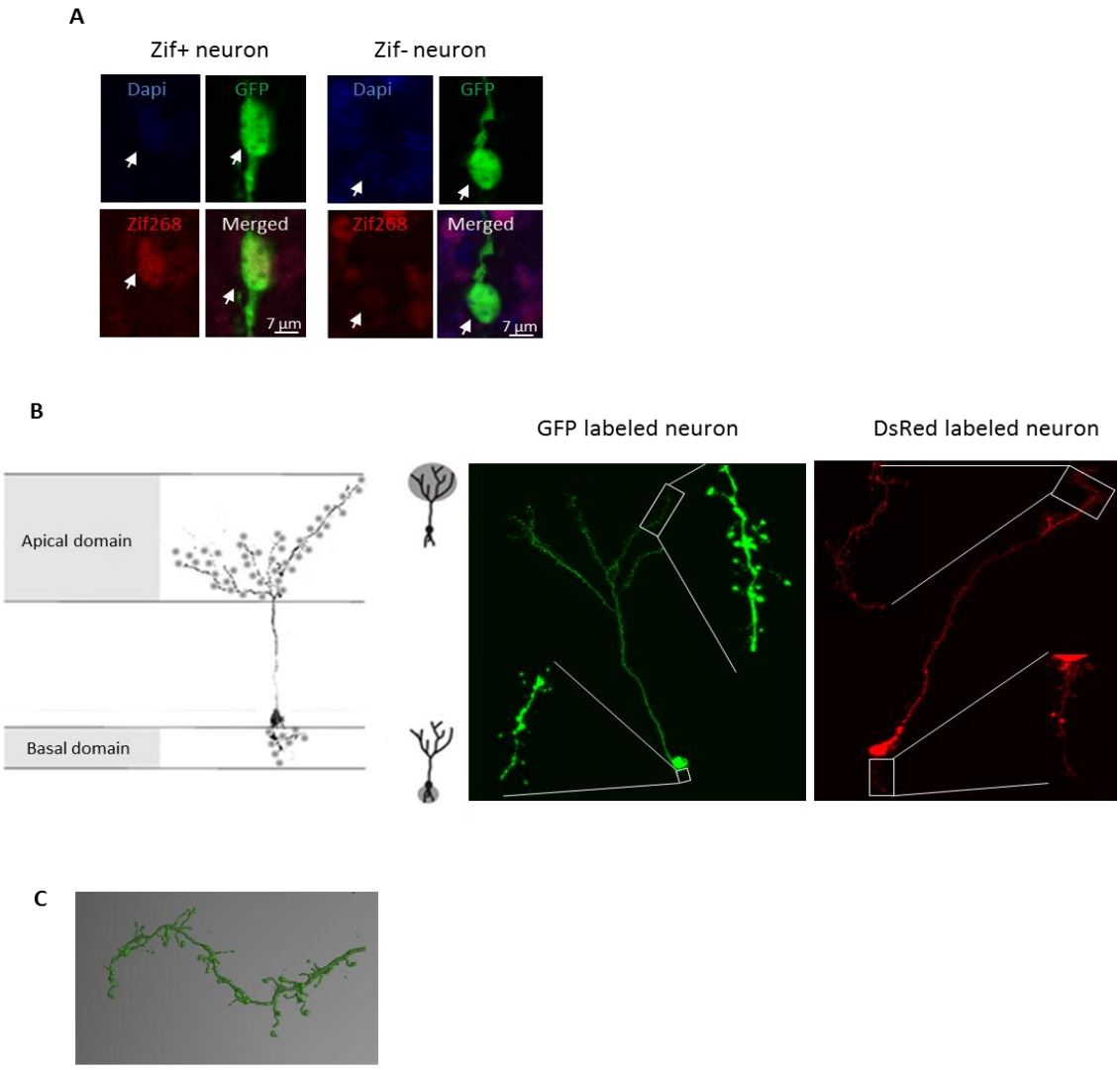

Figure 4: Methods.
A. Example of Zif268-positive (Zif+) and Zif268-negative (Zif-) neuron. **B.** Morphological analysis of neurons: example of preexisting (injected with DsRed at P1) and adult-born (injected with GFP at P60) neurons Analysis of dendritic length, spine density and length on the apical and basal domain (Modified from Kelsh et al. 2009) **C.** Example of 3D modelisation of an apical dendrite.

Internship progress

The first cohort of mice was injected with a virus expressing DsRed at the beginning of December with the help of Isabelle Caille, a researcher from Paris who taught us how to inject virus in the lateral ventricle of P1 mice. In January, I learned to perform stereotaxic injections in adult animals and constituted my first cohort of mice that had the double injection (DsRed and GFP). In parallel, I developed with the help of our lab's engineer (Marc Thevenet) our own system of virus injection in P1 mice. At the beginning of February, I tested olfactory discrimination before enrichment on this first cohort of mice to ensure that odor pairs used for the enrichment are not discriminated (data not shown here). This first cohort of mice was composed of both males and females since they were injected at P1 and thus not yet differentiated. We removed females from all cohorts since all data obtained in the team came from male mice. During that month, I also performed the enrichment and the behavioral post-test and we injected with DsRed two more cohorts of mice. We euthanized the first cohort at the beginning of March, and then sliced the brains and assessed BrdU, BrdU/NeuN/Zif268 cells after immunohistochemistry performed by the lab's assistant engineer of the group (Joelle Sacquet). In parallel, I took pictures for morphological analysis. At the same time, behavioral pre-test of the second and third cohort were done. Injection of GFP virus, enrichment, behavioral post-test and sacrifice were all done between mid-March and the end of April in parallel with the data analysis of the first cohort. In addition, when we designed our behavioral protocol, we constituted three enriched groups (enriched with one, two and three pair of odorants) but it turned out that the enrichment with +limonene and -limonene (one odor pair) was enough to induce discrimination between our third odor pair (+terpinen and -terpinen). Thus, that third group was removed from further analysis due to the high overlap between odorants. May was spent finishing analyzing BrdU, BrdU/NeuN/Zif268 data as well as some morphological data from the second and third cohorts. The present document was written all along the internship. Introduction and materiel and methods parts began to be written at the beginning of the internship. Then results were added as the research progressed. However the most part including the discussion was written during the last two weeks.

Results

<u>Increasing the complexity of perceptual learning leads to the discrimination of more odor pairs</u>

The purpose of this experiment was to assess whether mice, after a 10 day olfactory enrichment period, were able to discriminate between different but perceptually similar odor pairs. In order to test this, we used a habituation/cross habituation test (see material and method section).

To evaluate the effect of more than one odor pair used during enrichment on discrimination ability, we performed three experimental groups: non-enriched, enriched with 1 odor pair (Group 1), enriched with 2 odor pairs (Group 2).

Significant habituation for all groups was observed as evidenced by the reduction in investigation time across trials (trial effect $F_{(3,400)}$= 18.91 ; p<0.0001 and a one-way repeated measure ANOVA for each group, see table 1)(Figure 3) with no group effect ($F_{(2,400)}$= 1.6721 ; p = 0.18) nor odor effect ($F_{(1,400)}$= 0.92 ; p= 0,33).

Regarding discrimination, as expected, the control group showed no improvement in discrimination abilities between +limonene and -limonene nor between decanal and dodecanone as observed by the absence of significant difference between Ohab4 and Otest (Figure 3Bi, Table1). In contrast, both enriched groups showed changes in their ability to discriminate the odorants they were enriched with. Indeed, animals enriched with only +limonene and –limonene (Group 1) are now able to discriminate between these two enantiomers, but are still unable to discriminate between decanal and dodecanone (Figure 3Bii, Table1) while animals enriched with a more complex environment containing +limonene and -limonene plus decanal and dodecanone (Group 2) demonstrated the ability to discriminate between each odor of each pair as demonstrated by a significant difference between OHab4 and OTest (Figure 3Biii, Table1).

These results showed that increasing the number of odorants used for enrichment enhances the number of discriminated odorants.

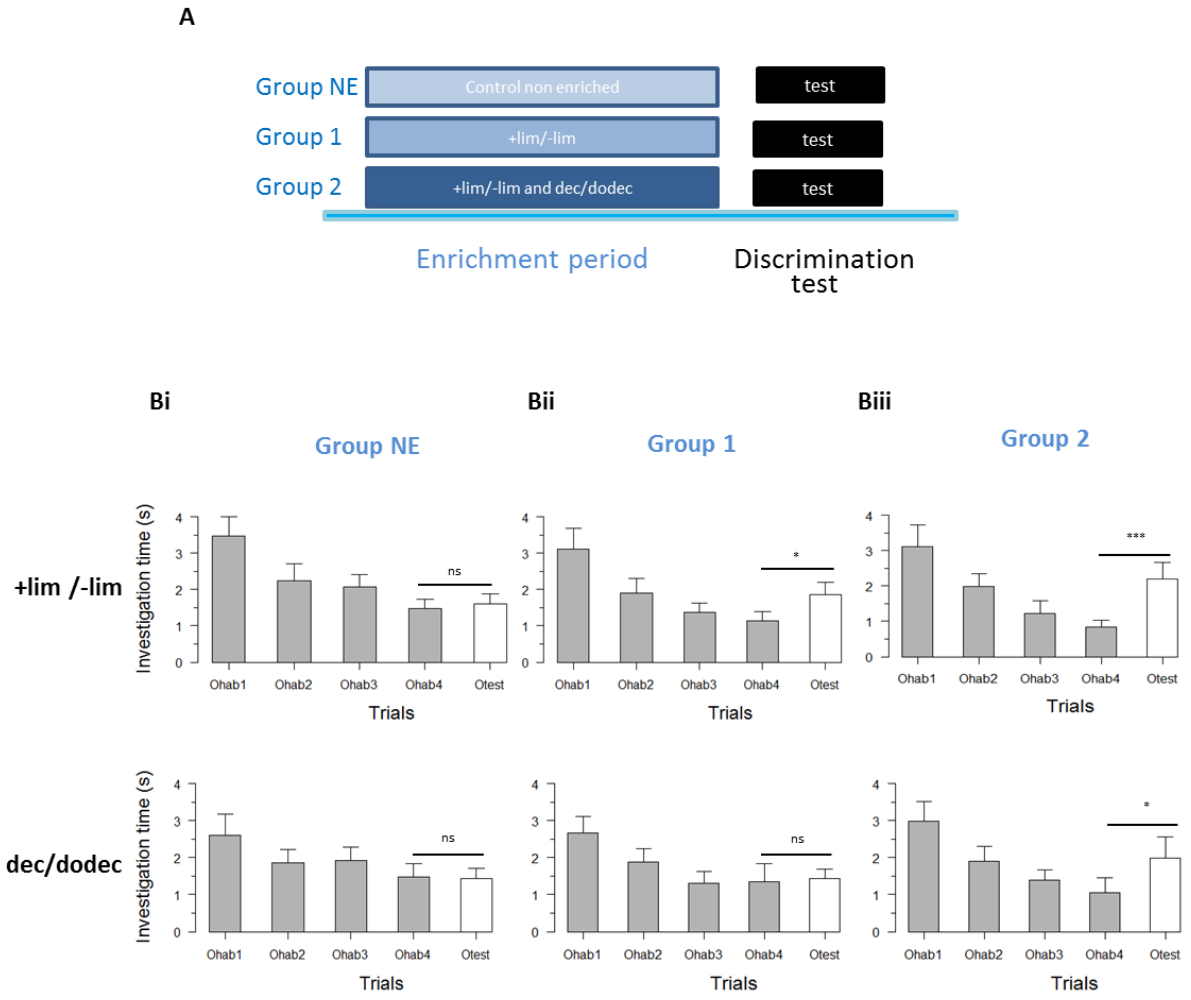

Figure 5: Increasing the complexity of perceptual learning leads to the discrimination of more odor pairs

A. Experimental design. **B.** Behavioral results. **(Bi).** In control non enriched animals, none of the two odorant pairs (+lim/-lim and dec/dodec) is discriminated. **(Bii).** Enrichment with +lim and –lim (group 1) improves the discrimination between these 2 odorants (up) but not the discrimination between dec and dodec (down). **(Biii).** Enrichment with +lim/-lim and dec/dodec (Group 2) improves the discrimination between the 2 odorants in both pairs. *p<0.05. ***p<0.001. Data are expressed as mean±sem. Group NE = Group Non-Enriched.

Groups	Odor pair	One way repeated measure ANOVA Habituation		Paired t test Discrimination
NE group	lim +/-	F (3,66) = 6.595893	p = 5.753726e-04. **yes**	p = 0.32833. **no**
	dec / dodec	F (3,66) = 3.163707	p = 3.018429e-02. **yes**	p = 0.43684. **no**
Group 1 (+lim/-lim enriched)	lim +/-	F (3,48) = 8.647106	p = 1.080394e-04. **yes**	p = 0.03741. **yes**
	dec / dodec	F (3,48) = 3.725818	p = 1.733122e-02. **yes**	p = 0.4524. **no**
Group 2 (+lim/-lim and dec/dodec enriched	lim +/-	F (3,39) = 10.70130	p = 2.878780e-05. **yes**	p = 0.003921. **yes**
	dec / dodec	F (3,33) = 8.665583	p = 0.0002211715. **yes**	p = 0.02272. **yes**

Table 1: Habituation and discrimination performances

Perceptual learning increased adult-born cell density independently of the complexity of enrichment.

The question addressed here is whether the pool of adult-born cell saved by "simple" learning can be enhanced by increasing the complexity of learning. In order to label adult-born cells present in the OB at the beginning of the enrichment, we injected BrdU 9 days before the enrichment period (the migration time of the neuroblasts from the subventricular zone to the OB is about 5 to 10 days). We assessed adult-born cells only in the granule cell layer since adult-born glomerular interneurons were not found to be modulated by perceptual learning (Moreno *et al.*, 2009).

BrdU-positive cell density was assessed in the three experimental groups (control non enriched, Group 1 and Group 2). We found a significant group effect ($F_{(2,13)}$= 8.07 ; p=0.005)(Figure 6B). More specifically, we found an increase in BrdU-positive cell density in Group 1 (p=0.02) and Group 2 (p=0.006) compared to non-enriched animals. However, no significant difference between Group 1 and 2 was observed (p=0.24).

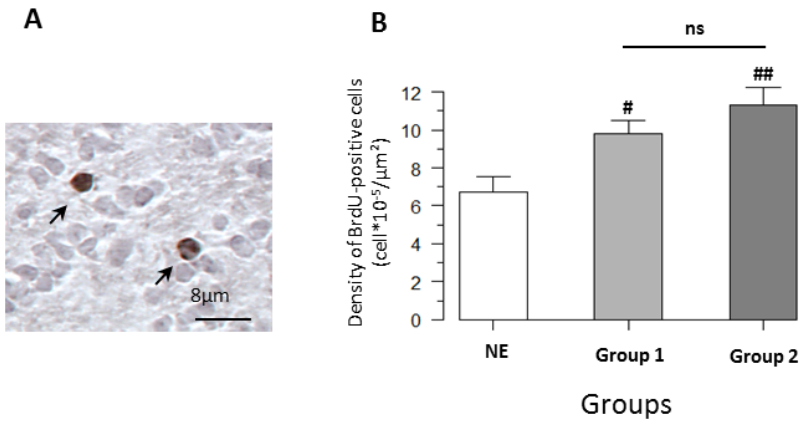

Figure 6: Perceptual learning increased adult-born cell density independently of the enrichment complexity.
A. Example of BrdU immunohistochemistry. **B.** Enrichment with one odorant pair (Group 1) and two odorant pairs (Group 2) both significantly increase BrdU-positive cell density. No difference in BrdU-positive cell density was observed between group 1 and 2. #p<0.05. ##p<0.01 for comparison with NE group. Data are expressed as mean±sem.

Increasing the complexity of perceptual learning enhances the recruitment of adult-born cell neurons

Since we found no increase in the density of adult-born cell surviving when the enrichment was more complex, we hypothesized that to be able to perform the task; a higher portion among the pool of surviving adult-born neurons would be recruited to process the learned odorants. To test it, we further examined the involvement of adult-born cells in perceptual learning by assessing the percentage of adult-born granule cells

expressing Zif268 as an index of cellular activation following odor stimulations. More specifically, to evaluate in each group the involvement of adult-born neurons in processing the learned odorants, we stimulated animals on the day of sacrifice with their respective learned odorants (+limonene/-limonene for Group 1 and +limonene/-limonene plus decanal/dodecanone for Group 2). Furthermore, to compare Zif268 in enriched versus control non-enriched animals, we divided the latter group into 3 subgroups differing in the odor stimulation on the day of sacrifice: no odor; +limonene/-limonene; +limonene/-limonene plus decanal/dodecanone. First, comparison between the 3 control non-enriched groups in which only the odor stimulation on the day of sacrifice varies, indicated no modulation of the percentage of BrdU/Zif268-positive cells ($F_{(2,5)}$= 0.0391 p=0.962). In contrast, significant differences were observed between experimental groups (group effect: $F_{(4,16)}$: p = 0.002). Indeed, Group 1 displayed more BrdU-positive cells expressing Zif268 than its control non enriched group (p= 0.02) indicating that more adult-born neurons responded to the learned odorants. It was also true for Group 2 in comparison to its control group (p=0.002). Interestingly, we found an increase in BrdU/Zif268-positive cells in Group 2 compared to Group 1 (p=0.03) (Figure 7A).
It is important to note that we did not observe any effect of enrichment on the level of neuronal differentiation of adult-born cells as evidenced by the absence of modulation of the percentage of BrdU/NeuN-positive cells (group effect $F_{(4,16)}$= 0.6606 ; p=0.6283) (Figure 7B).

All together, these results indicate that when the task gets more complex, the neural network does not save more adult-born neurons but increases their recruitment in processing the information.

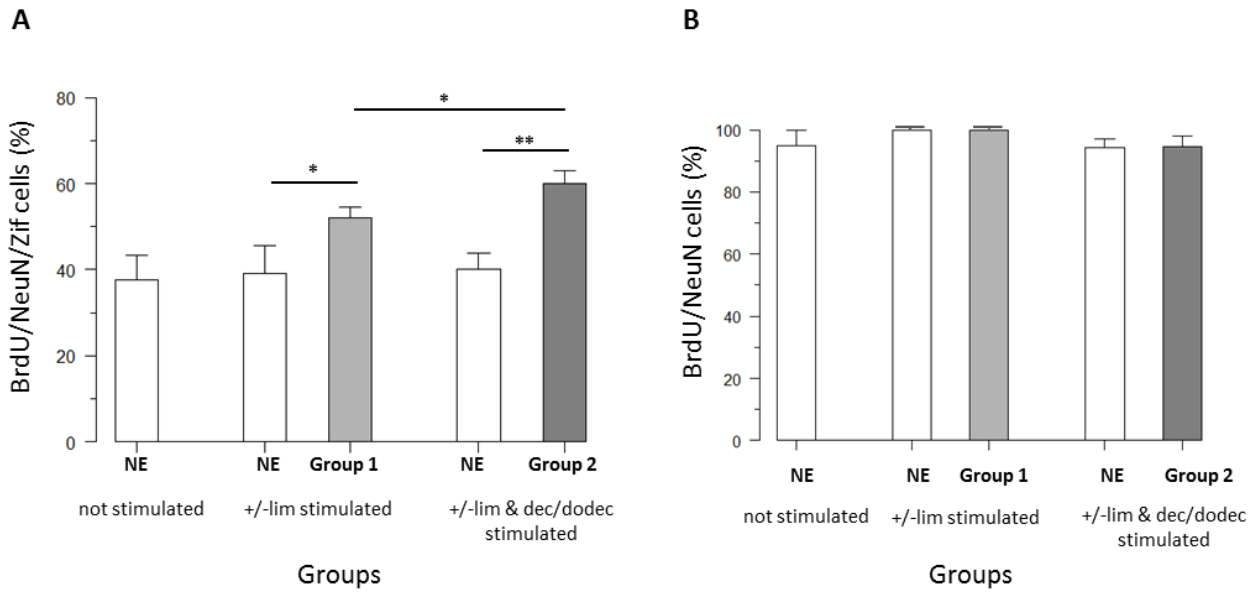

Figure 7: Increasing the complexity of perceptual learning increases the recruitment of adult-born neurons
A. Enrichment with +lim/-lim (Group 1) increases the percentage of adult-born cells responding these learned odorants compared to control non-enriched group in response to the same odorants. Same phenomenon was observed in Group 2. Interestingly, the percentage of BrdU/NeuN/Zif268-positive cells is higher in Group 2 compared to Group 1. **B.** Quantification of BrdU/NeuN double-labeling showed no effect of the enrichment on the neuronal fate of adult-born cells. *p<0.05. **p<0.01. Data are expressed as mean±sem. Group NE = Group Non-Enriched.

Increasing the complexity of perceptual learning induces a higher structural plasticity of adult-born neurons

To determine whether perceptual learning has an effect on adult-born neuron morphology, we first analyzed dendritic arborization in the apical and basal domains and then at a finer level, the spine density and length.

At the apical level, we did not find any effect of learning on the dendritic arborization ($F_{(2,73)}$=0.7281 ; p=0.4863) or on spine length between groups ($F_{(2,54)}$=0.1053 ; p=0.9001)(Figure 8A). However, we found a difference in spine density between groups (group effect: $F_{(2,77)}$= 9.76 ; p=0.0001). A closer analysis revealed an increase in spine density both in Group 1 and 2 compared to non-enriched group (p= 0.006 and p<0.0001 respectively) and also in Group 2 compared to Group 1 (p= 0.033) (Figure 8A). At the basal level, we did not see any effect of learning on the dendritic arborization ($F_{(2,61)}$= 0.7526 p=0.4755) or on the spine length ($F_{(2,656)}$= 0.0182 p=0.982) but we found a higher level of spine density in both Group 1 and 2 compared to control non-enriched (group effect: $F_{(2,54)}$= 5.4783 p= 0.00682; NE group versus Group 1: p= 0.0039; NE group versus Group 2: 0.0081; Group 1 versus Group 2: p= 0.6390).

Next, to test whether adult-born neurons involved in processing the learned odorants displayed higher levels of morphological plasticity than adult-born neurons that do not respond to the learned odorants, we differentiated neurons upon their Zif268 expression (Zif+ or Zif-). This approach yielded interesting results regarding both the apical and basal dendritic spine densities in adult-born neurons. Indeed, at the apical level, we observed an increase in spine density in Zif+ neurons after enrichment compared to controls (group effect, $F_{(2,41)}$= 8.6703 p= 0.0007). More specifically, in Groups 1 and 2, Zif+ neurons showed an increased in apical spine density compared to the non-enriched group (p= 0.0114 and p= 0.0003 respectively) and Group 2 showed an increase in apical spine density compared to Group 1 (p= 0.0475). Finally, no difference was observed between Zif- neurons of all groups ($F_{(2,33)}$= 2.0937; p= 0.1393). These results indicate that this modification of the morphology is specific to neurons responding to the learned odorants.

Regarding spine density at the basal dendritic level, an effect of learning was found (group effect, $F_{(2,51)}$= 5.21 p= 0.008) but no difference between Zif+ and Zif- neurons was retrieved (Zif268 effect: $F_{(1,51)}$= 0.0757 p= 0.784315). We did not observe any differences between Group 1 and 2 (p= 0.614). Thus, neural plasticity occurring at the basal level differs from the one at the apical level since both Zif+ and Zif- neurons showed the same morphological changes. In addition, these changes were not dependent on an increase of the learning complexity.

All together, these results indicate that adult-born neurons displayed a morphological plasticity due to perceptual learning and that this plasticity is enhanced when the behavioral demand is higher.

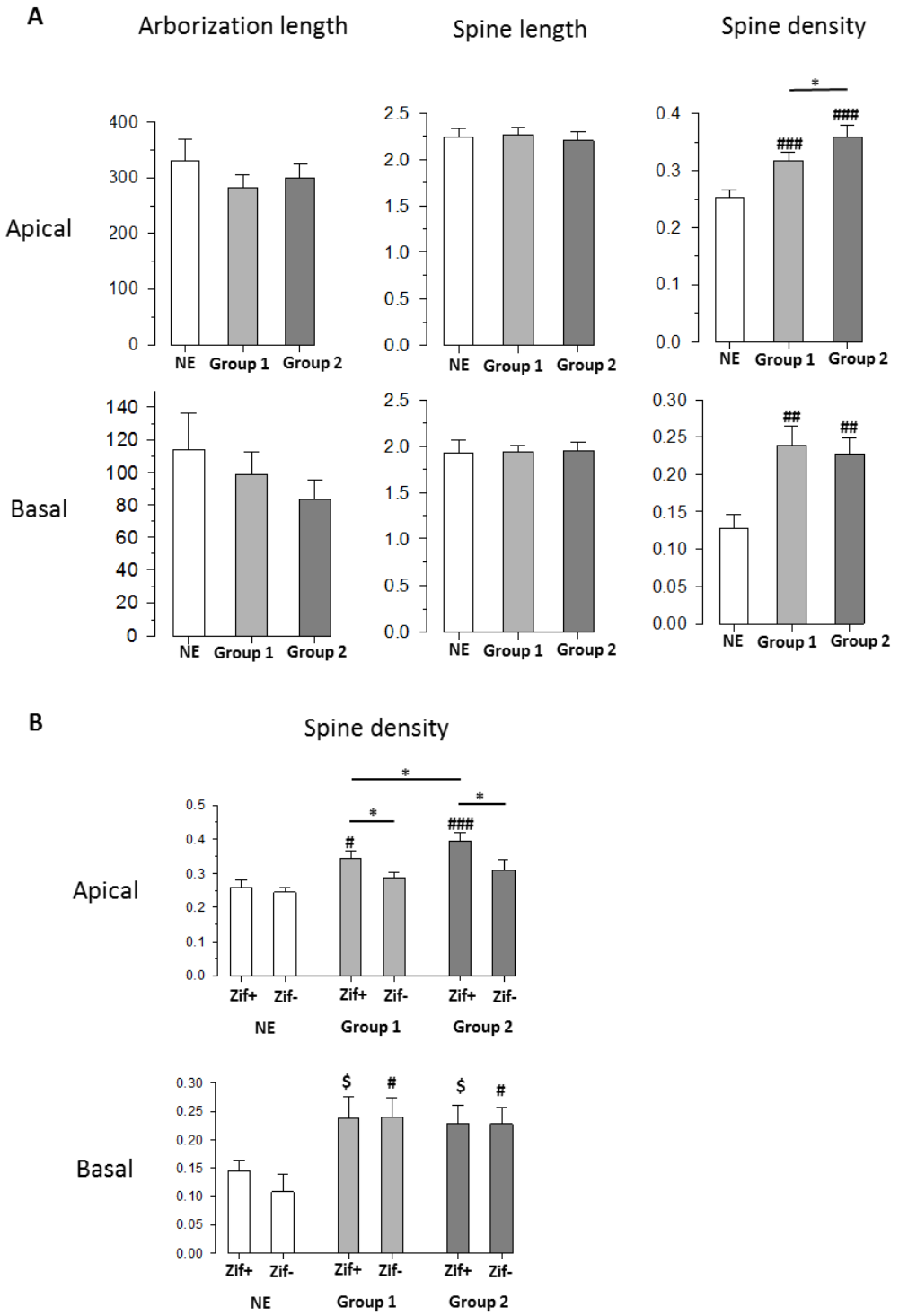

Figure 8: Increasing the complexity of perceptual learning induces a higher structural plasticity of adult-born neurons
A. In apical (up) and basal (down) dendrites, no effect of perceptual learning on dendritic arborization length was found. Group 1 displayed a higher spine density compared to control non-enriched group. Same effect was observed for Group 2. In addition, spine density in the apical dendrites was higher in Group 2 compared to Group 1. No effect of perceptual learning on spine length neither at the apical nor basal level was found **B.** In the apical dendrites, Zif+ neurons presented a higher spine density compared to Zif- neurons. This finding was not retrieved in the basal dendrites. #: $p<0.05$, ##: $p<0.01$, ###: $p<0.001$ for comparison with NE group. *$p<0.05$. $: $p=0.1$. Data are expressed as mean±sem.

Perceptual learning induces no morphological change in preexisting neurons

DsRed injections at P1 allowed us to investigate the morphology of neurons born in the OB during ontogenesis. Interestingly, we found no group effect on apical and basal dendritic arborization length ($F_{(2,34)}$= 0.7835 ; p=0.4649 and $F_{(2,14)}$= 0.8827 ; p=0.4355 respectively) and on spine length ($F_{(2,245)}$= 0.8186 ; p=0.4423 and $F_{(2,186)}$= 0.8863 ; p=0.4139 respectively) or on dendritic spine density ($F_{(2,28)}$= 0.5811 ; p=0.56 and $F_{(2,15)}$= 0.0105 ; p=0.98 respectively).

All together, these preliminary results indicate that preexisting neurons showed no morphological plasticity in response to perceptual learning no matter the task complexity.

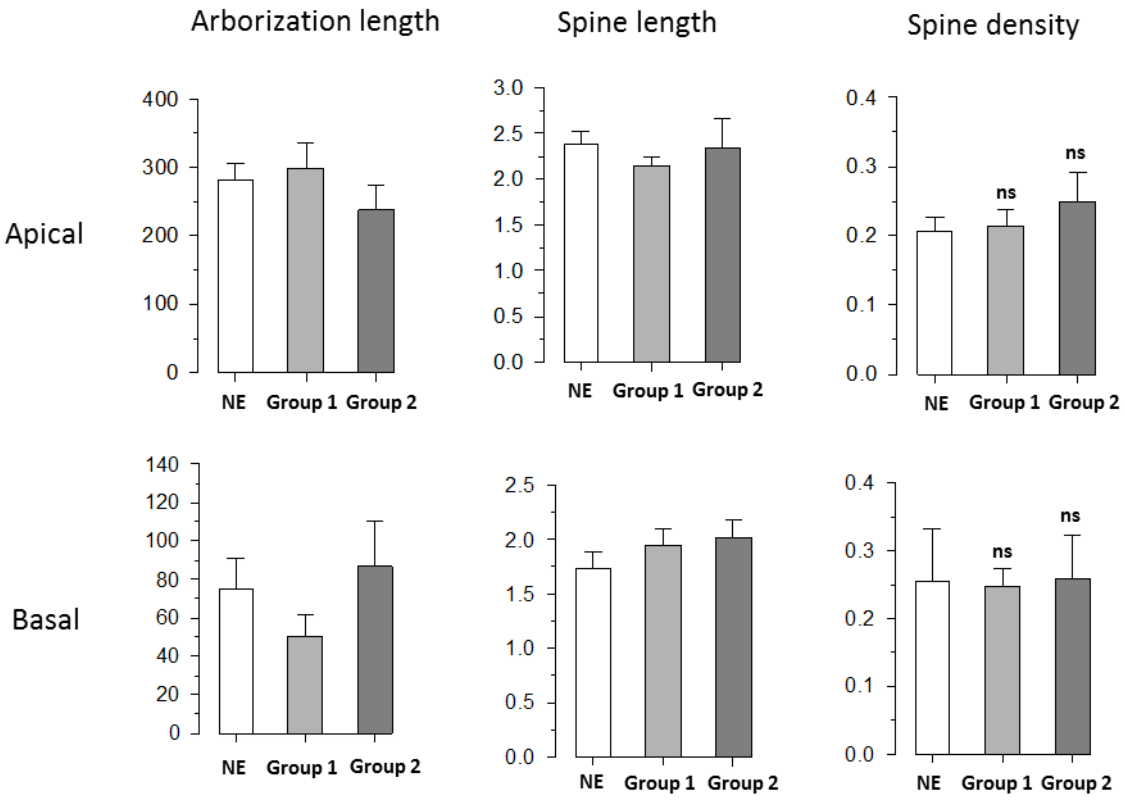

Figure 9: Perceptual learning induces no morphological change in preexisting neurons
No significant difference was observed between groups regarding dendritic arborization length or spine density and spine length at the apical and basal domains. ns: no difference for comparison with NE group. Data are expressed as mean±sem.

Discussion

Discrimination abilities can be modulated by experience through a mechanism called perceptual learning corresponding to an improvement in discrimination between close stimuli following enrichment. This type of learning contributes to the perceptual representation of the environment which guides the animal's behavior.

Adult neurogenesis, a specific form of plasticity within the olfactory bulb, is consisting in a renewal of granule cells within a preexisting neuronal network. Perceptual learning has been shown to be dependent on adult neurogenesis (Moreno *et al.*, 2009). Furthermore these authors showed an increased adult-born neuron survival after a perceptual learning paradigm. In this study, we first tested whether a more complex form of perceptual learning task would increase adult-born cell survival. We did not find a higher level of surviving adult-born cells after a more complex learning (enrichment with 2 odor pairs) compared to the simple one (enrichment with one odor pair), suggesting that a plateau of adult-born cell survival has been reached after simple perceptual learning. When looking closely at studies on neurogenesis using different models of learning (associative or perceptual) (Alonso *et al.*, 2006; Moreno *et al.*, 2009; Kermen *et al.*, 2010; Sultan *et al.*, 2010), the number of adult-born neurons saved by different paradigms is broadly similar, which is in accordance with our finding and suggests that the number of adult-born neurons saved is not sensitive to the type of learning or its complexity even though an even higher level of complexity should be tested (3 or more odor pairs). Most of the adult-born cells saved by learning are neurons since we observed 90% of co-expression with NeuN, a neuronal marker. This ratio stays constant among the experimental groups indicating that learning does not modulate the level of neuronal differentiation which is in accordance with previous studies (Moreno *et al.*, 2009, 2012; Sultan *et al.*, 2010).

Among surviving adult-born neurons after perceptual learning (Moreno *et al.*, 2009), only a portion of them is actively responding to the learned odorants (about 50%). Thus, we hypothesized that the improvement of olfactory discrimination after complex learning could be underlined by an increase of adult-born neurons involved in processing the learned odorants. To test this hypothesis, we quantified the expression of the immediate-early-gene Zif268 in adult-born neurons and found that the magnitude of this functional involvement in response to the learned odorants could predict the performances of olfactory discrimination. Indeed, when the number of odor pairs discriminated increases, the percentage of adult-born neurons expressing Zif268 increases too. This result strongly suggests that when the task gets more complex, the system solicits more adult-born neurons to participate into the network processing the learned odorants. However, this increase is not twice as high in animals enriched with two pairs compared to one pair of odorants, meaning some overlaps between learned odorant networks and thus some adult-born neurons are probably involved in processing several odor pairs.

In this first part of the study, we found that the number of adult-born neurons involved in processing the learned odorants is increased when the number of odor pair discriminated is enhanced. However, beyond the number of cells, we investigated whether adult-born neuron structure is plastic. Since cell activity is a major regulator of adult-born neuron's morphology (Nägerl *et al.*, 2004; Kelsch *et al.*, 2010), we investigated whether adult-born neurons morphology is modulated by learning. Toward this goal, we injected a virus expressing fluorescent protein in the adult that allowed us to examine the fine morphology of the adult-born neurons. Interestingly, we found an increase in spine density in the apical domain after learning. The apical dendrites are the site of reciprocal dendro-dendritic synapses between granule and mitral cells (Shepherd, 1972). More particularly, the increased density of dendritic apical spines occurred only in neurons strongly

responding to the learned odorants and expressing Zif268. Thus, the increased responsiveness accompanied by the increased number of spines in adult-born neurons should lead to an increased level of inhibition on mitral cells and ultimately to refinement of odor representation and better discrimination of highly similar odorants.

Interestingly, adult-born neurons showed an increased dendritic spine density also in their basal domains. The basal dendrites receive glutamatergic inputs from axons collateral of mitral /tufted cells and neuromodulatory inputs such as noradrenergic, cholinergic and serotoninergic fibers coming from the locus coeruleus, diagonal band of Broca in the basal forebrain and raphe nucleus respectively (Fletcher and Chen, 2010). Recently, it has been shown that perceptual learning raised noradrenergic system activity and that noradrenergic fibers contact adult-born neurons in the OB (Moreno *et al.*, 2012) suggesting that an increase of noradrenergic input onto adult-born cells could be involved in this neural plasticity. However we do not know if other neuromodulatory systems projecting into the OB are involved such as the cholinergic system known to modulate olfactory discrimination or memory (Ravel *et al.*, 1994; Mandairon *et al.*, 2006b). Such increase in basal inputs has been proposed to be able to modulate granule cells and gate dendrodendritic inhibition onto mitral cells (Balu *et al.*, 2007) and could thus contribute to increase inhibition onto mitral cells, in addition to the increase spine density on apical dendrites (Figure 10).

However, the bulbar neural network is not only composed of adult-born neurons but also of preexisting neurons arising from ontogenesis. In order to analyze whether the plasticity of adult-born neurons is unique or special, we compared it to that of preexisting neurons. We thus analyzed the morphology of preexisting neurons thanks to the injection of a virus expressing another fluorescent protein in one-day old animals. Preliminary results obtained here showed no morphological modification of preexisting neurons, no matter the complexity of the perceptual learning task. This finding needs to be confirmed by analyzing more neurons and in addition, we question whether a further increase of the complexity of the task could lead to the same outcome.

In order to go further with these results it would be interesting to analyze the different forms of the spines (mushroom versus filopodia, stubby and thin) to differentiate mature and thus active synapses from immature ones (Zhao *et al.*, 2006) in both preexisting and adult-born neurons after learning. Another interesting point would be to differentiate neurons according to their position in the granule cell layer. Indeed, subpopulations of adult-born neurons can be differentiated between either being superficial or deep in the granule cell layer (Imayoshi *et al.*, 2008) suggesting different functional roles of these adult-born neurons based on their localization in the OB granule cell layer.

All together, these results showed that perceptual learning is accompanied by a structural plasticity of adult-born neurons as well as by an increase in adult-born neurons involved in processing the olfactory information. Enhancing the complexity of the task intensifies these phenomena of plasticity resulting, at the behavioral level, in discrimination of a higher number of perceptually similar odorants.

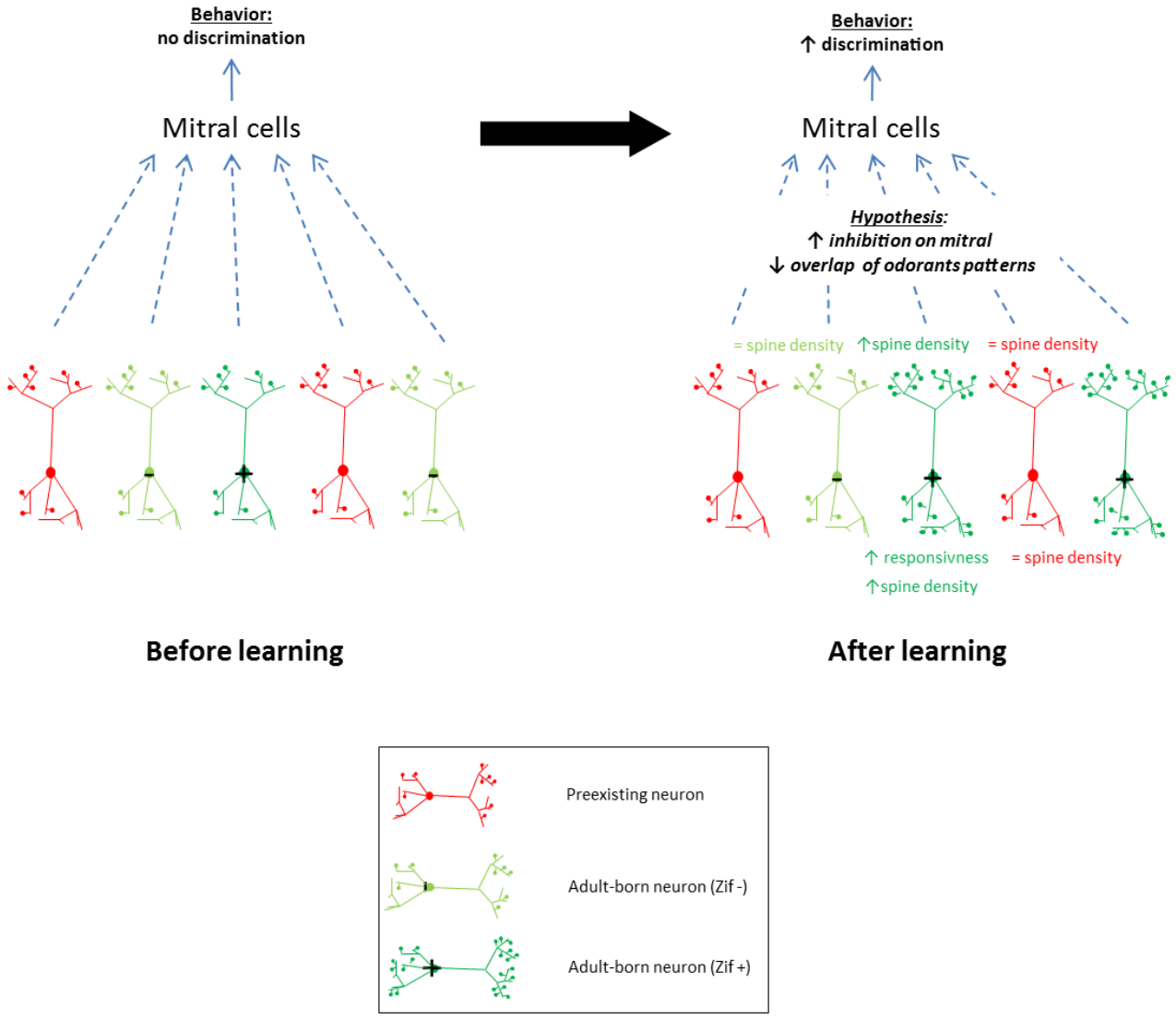

Figure 10: Summary
Perceptual learning induces a modulation of adult-born neurons' morphology (increased spine density) accompanied by an enhancement of adult-born neurons responsiveness to the learned odorants. These modifications could lead to an increased level of inhibition on mitral cells and ultimately to the refinement of odorants representations and the observed improved discrimination of highly similar odorants.

Perspectives

The bulbar network is composed of preexisting and adult-born neurons. Our study suggested a modulation of plasticity only in the adult-born neurons after perceptual learning. However, behavioral data (capacity of discrimination) and neurogenic aspects are only correlative. The use of another technique, such as optogenetic would allow revealing the direct roles of adult-born and preexisting neurons in perceptual learning. More precisely, the injection, at P1 or P60 of lentivirus containing an inhibitory Halorhodopsin (NpHR) channel under specific neuronal promoter (synapsin) would enable to selectively shut down preexisting or adult-born neurons. Indeed, the light stimulation induces hyperpolarizing currents leading to the inhibition of the lentivirus-transfected neurons. We would proceed by direct laser stimulation using

implanted optical fibers in the olfactory bulb during olfactory discrimination test (habituation / cross habituation task). The efficiency of the light stimulation can be assessed by the mapping in the olfactory bulb of Zif268 expression known to be modulated by neuronal activity. The experiment will answer the question of the role of preexisting versus adult-born neurons in perceptual learning. It is a complementary technique to the ones we used since it does not allow assessing the effect of learning on structural plasticity, number of adult-born cell survival or involvement of neurons in the learning task.

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