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Impact of adult neurogenesis versus preexisting neurons on olfactory perception in complex or changing olfactory environment

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ABBREVIATION LIST **7**

ABSTRACT (ENGLISH) **8**

ABSTRACT (FRANÇAIS) **10**

EXTENDED ABSTRACT (FRANÇAIS) **12**

PART I: INTRODUCTION **16**

1. Olfaction **17**
1.1. Olfactory system organization 17
1.1.1. The olfactory epithelium 17
1.1.1.1. Organization 17
1.1.1.2. Receptors and combinatorial coding 18
1.1.2. The olfactory bulb: the first central relay of olfactory information 19
1.1.2.1. Anatomical organization 19
1.1.2.2. Cell types 20
1.1.2.2.1. Principal/Relay cells: the mitral and tufted cells 20
1.1.2.2.2. Juxtaglomerular neurons 22
1.1.2.2.2.1. Periglomerular cell (PG) 22
1.1.2.2.2.2. Short axon cells 23
1.1.2.2.3. Granule cell (GC) 24
1.1.2.3. Odor coding and computations 25
1.1.2.3.1. Spatial coding 25
1.1.2.3.2. Temporal coding 28
1.1.3. Olfactory bulb connectivity 28
1.1.3.1. Projections from the OB 29
1.1.3.2. Feedback projections to the OB 31
1.1.3.3. Neuromodulatory inputs 31
1.1.4. Conclusion 32

2. Olfactory bulb adult neurogenesis **32**
2.1. Discovery 32
2.2. Differentiation and integration into the preexisting network 33
2.2.1. Periglomerular cells 34
2.2.2. Granule cells 34
2.3. Survival 36

3. Olfactory bulb experience-dependent plasticity **36**
3.1. Environmental changes 37
3.1.1. Sensory deprivation 37

3.1.2. Sensory enrichment 38
3.2. Learning 38
3.2.1. Associative learning 38
3.2.2. Perceptual learning 39

4. Thesis objectives 40

PART II: RESULTS 44

Study 1 - Impact of adult neurogenesis versus preexisting neurons on olfactory perception in complex olfactory environment 45

Study 2 - A competition between transience and persistence of olfactory perceptual memory 96

Study 3 - A model of adult-neurogenesis in the olfactory bulb 126

PART III: GENERAL DISCUSSION 137

1. Behavioral adaptation in changing and complex environment 138

2. Neurogenesis is the central pillar underlying olfactory behavioral adaptation 140

3. Enter preexisting neurons 142

4. Cell types diversity 144

5. Role of adult-born granule cells in pattern separation? 145

Conclusion 147

PART IV: REFERENCES 148

FIGURES LIST 160

PART V: APPENDIX 161

Annexe 1: Forest, J., Midroit, M., Mandairon, N., 2017. La plasticité hors du commun du système olfactif. Pollut. Atmos. 162

Annexe 2: Kermen, F., Midroit, M., Kuczewski, N., Forest, J., Thévenet, M., Sacquet, J., Benetollo, C., Richard, M., Didier, A., Mandairon, N., 2016. Topographical representation of odor hedonics in the olfactory bulb. Nat. Neurosci. 175

Abbreviation list

BrdU = BromodeoxyUridine

BDNF = Brain-Derived Neurotrophic Factor

CldU = Chloro-deoxyUridine

CNS = Central Nervous System

DsRed = DiScosoma Red fluorescent protein

EPL = Exterior Plexiform Layer

eTC = external Tufted Cell

GABA = Gamma-AminoButyric Acid

GAD = Glutamic Acid Decarboxylase

GC = Granule Cell

GFP = Green Fluorescence Protein

IdU = Iodo-deoxyUridine

OB = Olfactory Bulb

OSN = Olfactory Sensory Neuron

PG = PeriGlomerular cell

SA = Short Axon cells

SVZ = Sub-Ventricular Zone

VEGF = Vascular Endothelial Growth Factor

ABSTRACT (English)

Operating in a rich and changing environment requires individuals to continuously permanently discriminate experiences even if they are very similar. The performances of olfactory discrimination can be enhanced through olfactory perceptual learning and a key cerebral structure in this is the olfactory bulb. This structure is the target of a specific form of plasticity that is adult neurogenesis. Neuroblasts coming from the subventricular zone differentiate mostly in granule cells that regulate the activity of the relay cells, the mitral cells. It has previously been shown that these adult-born neurons are required to perform perceptual learning.

The central question of this thesis work is to elucidate both the role and the specificity of adult-born neurons during complex or changing olfactory learning.

We first asked whether increasing the complexity of learning required more neurogenesis than a simpler task. We found that increasing the complexity of learning did not increase the rate of surviving cells but increased the percentage of adult-born neurons responding to the learned odorants as well as their structural plasticity. Interestingly, while a simple learning task seems to only require adult-born neurons a more complex one requires a larger neural network with both preexisting and adult-born neurons.

In addition of being complex, the olfactory environment is also changing. In a second study we studied how the memory of an olfactory information is altered by the acquisition of a new one and what is the role of adult neurogenesis in this process. More specifically, we looked at the role of adult-born neurons in encoding individual memories acquired along successive time windows. We found that adult-born neurons are the definite substrate for olfactory memories and that once allocated to a specific memory they cannot be used to underlie another one.

Lastly, an approach relying on computational neurosciences aimed at outlining a computational framework explaining the role of adult-born granule cells in early olfactory transformations and how sharpened sensory representations emerge from decorrelation.

To conclude, olfactory perception is changing according to environmental modifications and these perceptual adaptations following environmental changes are underlain by an important plasticity of the olfactory bulb circuitry supported in a large part to adult neurogenesis.

ABSTRACT (Français)

Se comporter dans un environnement riche et complexe requiert d'un individu qu'il soit capable de discriminer continuellement de multiples expériences même si celles-ci sont très similaires. Ces performances de discrimination peuvent être améliorées via l'apprentissage perceptif et une structure cérébrale clé : le bulbe olfactif. Cette structure est cible d'une forme de plasticité particulière qui est la neurogenèse adulte. C'est là que des neuroblastes venant de la zone sous-ventriculaire se différencient majoritairement en cellules granulaires qui viennent réguler l'activité des cellules relais, les cellules mitrales. Il a été montré que ces nouveaux neurones sont requis pour un apprentissage perceptif.

La question centrale de cette thèse est d'élucider le rôle et la spécificité des nouveaux neurones lors d'apprentissages olfactifs complexe et changeant.

Nous avons d'abord étudié l'effet d'un apprentissage perceptif complexe sur la neurogenèse adulte. Nous avons montré qu'augmenter la complexité de l'apprentissage n'entraine pas une augmentation de la survie des nouvelles cellules mais accroît le pourcentage de nouveaux neurones qui répondent aux odeurs apprises ainsi que leur plasticité. De façon intéressante, alors qu'un apprentissage simple semble requérir essentiellement les nouveaux neurones, un apprentissage complexe demande le recrutement d'un réseau de neurones plus large contenant à la fois des nouveaux neurones et des neurones préexistants.

En plus d'être complexe, l'environnement olfactif est aussi changeant. Dans une seconde étude nous avons étudié comment la mémoire olfactive est altérée par un nouvel apprentissage et le rôle de la neurogenèse adulte dans ce processus. Plus particulièrement nous nous sommes intéressés au rôle des nouveaux neurones dans l'encodage de mémoires individuelles acquises lors de fenêtres temporelles successives. Nous avons trouvé que les nouveaux neurones sont bien le support physique de la mémoire olfactive et qu'une fois alloués à une mémoire ils ne peuvent pas en sous-tendre une autre.

Finalement, le recours aux neurosciences computationnelles a eu pour but de définir le rôle des nouveaux neurones granulaires dans le premier niveau de transformation de l'information et comment le raffinement des représentations sensorielles émerge par décorrelation.

Pour conclure, la perception olfactive est changeante en fonction des modifications environnementales et l'adaptation de la perception à ces variations de l'environnement est sous tendue par une plasticité du circuit du bulbe olfactif dont le support principal est la neurogenèse adulte.

EXTENDED ABSTRACT (français)

L'olfaction est un sens primordial dans de nombreux comportements comme par exemple la recherche de nourriture, l'évaluation des dangers, l'évitement des prédateurs ou encore les interactions sociales. Pour effectuer ces tâches de façon efficace, le système olfactif doit être capable de discriminer des stimuli parfois extrêmement proches. Les performances de discrimination peuvent être améliorées grâce à l'apprentissage perceptif qui est défini comme une amélioration des capacités de discrimination entre deux odorants très similaires d'un point de vue perceptif après l'exposition passive et répétée à ces mêmes odorants. Une structure cérébrale clé dans cet apprentissage est le bulbe olfactif (BO) qui est le premier relai central de l'information olfactive. Cette structure est la cible d'une forme particulière de plasticité, la neurogenèse adulte. Des cellules souches résidant le long des ventricules latéraux se divisent et donnent naissance à des neuroblastes qui migrent vers l'avant du cerveau, le long du flux rostral migratoire, pour atteindre le BO où ils se différencient majoritairement en cellules granulaires, une population d'interneurones inhibiteurs qui régulent l'activité des cellules relais (i.e. les cellules mitrales). Il a été démontré auparavant que la présence de ces nouveaux neurones est requise pour qu'un apprentissage perceptif puisse avoir lieu.

La question centrale de ce travail de thèse est d'élucider à la fois le rôle et la spécificité des nouveaux neurones dans des apprentissages olfactifs complexe et changeant en utilisant le modèle de la souris.

Premièrement, l'environnement olfactif est complexe. Ainsi les facultés de discrimination doivent être ajustées de façon fine pour guider le comportement de l'animal. Dans ce contexte, nous avons dans un premier temps étudié l'effet de l'apprentissage perceptif sur la neurogenèse adulte dans des conditions plus écologiques que dans les études précédentes, c'est-à-dire mettant en jeu plus d'odorants à discriminer en même temps (de une à six paires). Nous avons tout d'abord observé que les souris étaient capables d'apprendre à discriminer jusqu'à au moins six paires d'odorants en même temps. Nous avons confirmé que la survie des

nouveaux neurones (révélée par un marquage BrdU qui est un marqueur de la synthèse ADN) est augmentée après un apprentissage perceptif simple (une paire d'odeur à apprendre discriminer). Nous avons ensuite recherché si un apprentissage plus complexe (plusieurs paires d'odeurs à apprendre à discriminer) était sous-tendu par la survie d'un plus grand nombre de nouveaux neurones. De façon intéressante, augmenter la complexité de l'apprentissage n'augmente pas le nombre de nouveaux neurones qui survivent mais augmente le nombre de nouveaux neurones qui répondent aux odorants appris (indicé par le marquage du gène précoce Zif268 dans les nouveaux neurones). De plus, nous avons révélé des modifications de la morphologie fine des nouveaux neurones induites par l'apprentissage simple et complexe grâce à l'injection, dans la zone sous ventriculaire de souris adulte, de vecteurs viraux nous permettant de faire exprimer la protéine fluorescente GFP dans les nouveaux neurones. Cette plasticité structurale des nouveaux neurones suggère une modulation de l'activité des cellules relais du bulbe (les cellules mitrales) et par conséquent du message de sortie du bulbe olfactif vers les centres supérieurs. Pour déterminer si ces modifications morphologiques sont spécifiques des nouveaux neurones, nous avons également analysé la morphologie fine des neurones préexistants (nés pendant l'ontogenèse du bulbe olfactif) grâce à l'injection à P0 dans le ventricule latéral d'un virus permettant l'expression d'une autre protéine fluorescente (DsRed) sur les mêmes animaux. Nous révélons un effet de l'apprentissage sur la plasticité structurale des neurones préexistants uniquement dans les cas d'apprentissages les plus complexes. Ces résultats mettent en avant les propriétés uniques des nouveaux neurones dans les adaptations plastiques du bulbe olfactif et l'élargissement du réseau sollicité avec la contribution des neurones préexistants dans les apprentissages plus complexes.

Nous avons ensuite disséqué la contribution fonctionnelle des nouveaux neurones et des préexistants dans l'apprentissage simple versus complexe en utilisant une approche optogénétique. Ainsi, un virus codant pour une protéine halorhodopsine (un canal sensible à la lumière et perméable aux anions chlore) a été inséré dans les neurones préexistants ou les nouveaux neurones permettant l'inhibition sélective de l'activité neuronale de ces 2 populations grâce à la lumière. En lien avec les données morphologiques, nous avons trouvé que l'inhibition des nouveaux neurones altère la discrimination après apprentissage simple et complexe alors

que l'inhibition des neurones préexistants a seulement empêché la discrimination dans un protocole d'apprentissage complexe. Cette première étude a confirmé l'importance des nouveaux neurones pour l'apprentissage perceptif olfactif simple et complexe mais a aussi démontré que lorsque l'apprentissage devient plus complexe, un réseau neural plus large est recruté nécessitant à la fois les nouveaux neurones et les neurones préexistants pour répondre à la demande comportementale.

Deuxièmement, en plus d'être complexe, l'environnement olfactif est changeant. Dans une deuxième étude nous nous sommes demandé comment l'information olfactive déjà stockée est altérée par l'acquisition d'une nouvelle mémoire et quel est le rôle de la neurogenèse adulte dans ce processus. Plus précisément, nous avons effectué des apprentissages perceptifs successifs en variant le délai entre les apprentissages. Nous avons trouvé qu'en absence d'interférence, les nouveaux neurones sauvés par l'apprentissage sont présents dans le BO aussi longtemps que la mémoire de discrimination persiste. De façon intéressante, une deuxième session d'apprentissage survenant à un délai court après la première et utilisant une nouvelle paire d'odorants, conduit à la discrimination des 2 nouvelles odeurs (avec une augmentation de neurogenèse associée) ainsi qu'à l'oubli de la première paire apprise. Cet oubli peut être contrecarré par le maintien dans l'environnement de la première paire d'odeurs pendant la deuxième session d'apprentissage. Lorsque le délai entre les apprentissages successifs est plus long, les mémoires des 2 apprentissages sont maintenus et 2 vagues distinctes de survie des nouveaux neurones sont observées grâce à l'incorporation séquentielle d'analogues de la thymidine (IdU et CldU).

Pour un délai qui permet le maintien de deux apprentissages successifs, afin de déterminer si les mêmes nouveaux neurones peuvent être impliqués dans les deux apprentissages, nous avons inhibé l'activité des nouveaux neurones sauvés par le premier apprentissage en utilisant une approche optogénétique. Cette manipulation a pour effet d'altérer la discrimination de la première paire apprise mais pas de la deuxième ce qui suggère que les nouveaux neurones, dans ce contexte, sont les substrats de la mémoire olfactive et qu'une fois recrutés pour sous tendre un apprentissage, ils ne peuvent pas en sous-tendre un autre.

Finalement, nous nous sommes tournés vers les neurosciences computationnelles pour mieux comprendre, interpréter et former des théories à propos des mécanismes cérébraux de l'apprentissage perceptif. Cette approche a pour but de définir un cadre computationnel expliquant le rôle des cellules granulaires nouvellement formées lors des premières transformations de l'information olfactive au niveau bulbaire. Cela permettra d'aider à comprendre le processus de discrimination, c'est-à-dire comment des représentations neurales de stimuli très similaires sont séparées, i.e décorrélées. Nous essayons actuellement d'intégrer la neurogenèse adulte dans un modèle déjà construit du bulbe olfactif, en collaboration avec le laboratoire de Christiane Linster à l'université de Cornell. Ce modèle computationnel, en nous permettant de manipuler la dynamique des nouveaux neurones, nous montrera comment leur intégration dans le bulbe olfactif peut transformer le message sortant. Nous pourrons alors dériver des règles simples gouvernant la plasticité, induite par l'apprentissage, des nouveaux neurones et des neurones préexistants. Cela nous permettra aussi d'émettre des prédictions et de formuler de nouvelles hypothèses à tester *in vivo*. Idéalement, ce modèle devra être suffisamment simple pour permettre une généralisation vers diffèrent type d'apprentissage (i.e. apprentissage associatif). Ces travaux sont en cours.

Pour conclure, la perception olfactive est constamment modulée par les modifications de l'environnement et cette plasticité perceptive est sous-tendue par une importante plasticité du circuit neuronal bulbaire principalement due à la neurogenèse adulte.

PART I: Introduction

1. Olfaction

Olfaction is a key player of behavioral adaptation in animals as well as in humans (McGann, 2017) and has been shown to be heavily implicated in multiple behaviors such as food seeking (Julliard et al., 2017; Wenner et al., 1969), danger evaluation and predator avoidance (Conover, 2007; Laska et al., 2005), conspecific and social interactions (Gheusi et al., 1994; Hughes et al., 2010; Lin et al., 2005). As such, an olfactory system which keeps the animal's behavior in tune with its environment is essential for survival and adapted responses.

1.1. Olfactory system organization

Olfactory perception starts at the periphery, in the nasal cavity, when the inspiration allows for the entry of volatile molecules and their interaction with specialized olfactory sensory neurons (OSN) at the level of the olfactory epithelium. These OSN transduce the information from their chemical form to an electrical message, which is then sent to the olfactory bulb. The olfactory bulb is the first central nervous system (CNS) relay of olfactory information and thereafter the information is sent to higher cortical and subcortical processing areas (Shepherd, 1972).

In this first part, we are going to review the organization of the olfactory system and how information is processed, putting forward the level of the olfactory bulb, which is our main interest in the work presented further below.

1.1.1. The olfactory epithelium

1.1.1.1. Organization

The olfactory epithelium, which lines the posterior part of the nasal cavity, is a pseudostratified epithelium made of OSN, supporting cells and basal cells (Buck, 1996; Yu and Wu, 2017) (Figure 1A and 1B). OSNs are bipolar cells exhibiting on one side a dendritic process terminated by specialized cilia providing extensive surface and allowing for the sampling of the olfactory mucosa and, on the other side, an axon connecting to the relay cells of the olfactory bulb (OB). Olfactory receptors (OR) are expressed on the surface of the cilia of OSN and sample the exterior medium in order to bind olfactory-related molecules present in the mucus. They transduce volatile chemical molecule into neural code (Buck, 1996). One particularity of OSN is

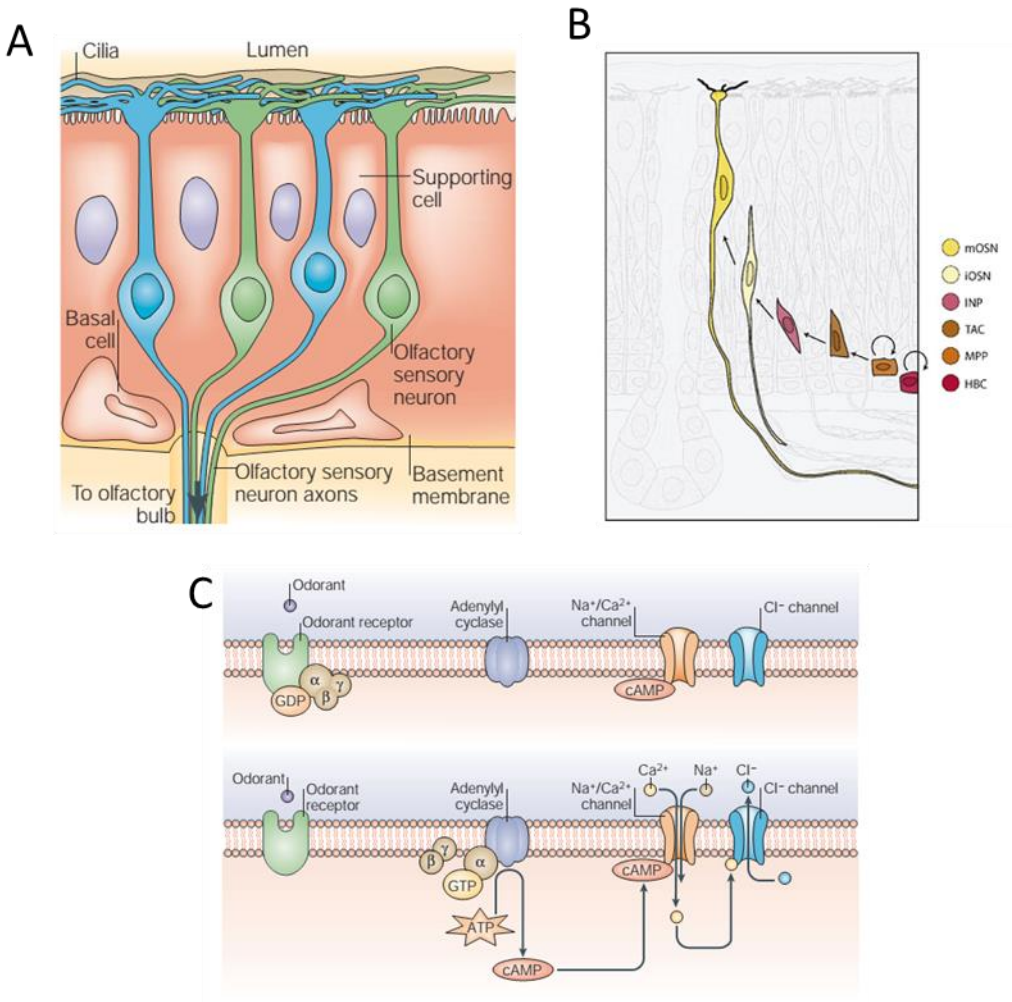

Figure 1 : Olfactory epithelium organization.
A. The olfactory epithelium is made of three types of cells : olfactory sensory neurons (OSN), supporting cells and basal cells. **B.** Basal cells are a type of stem cell with the ability to give rise to new OSN. C. Transduction mechanisms happening in OSN when an odorant binds to a receptor. (Adapted from Mombaerts, 2005, 2006; Yu and Wu, 2017).

that they are short lived, with a lifespan between 30 to 90 days, thus requiring continuous renewal by new sensory cells that are derived from stem cells which are the basal cells.

1.1.1.2. Receptors and combinatorial coding

ORs are seven transmembrane domains G protein-coupled receptor proteins (GPCR) coded by a multigene family (Buck and Axel, 1991)(Figure 1C). There are ~400 genes in humans and more than 1000 genes regrouped into 228 families in the mouse, coding a wide number of receptors making them the largest gene superfamily in vertebrates (Adipietro et al., 2012; Buck, 1996; Nei

et al., 2008; Parmentier et al., 1992; Zhang and Firestein, 2002). ORs are found all across the vertebrate and invertebrate phyla (Nei et al., 2008).

The transduction of the chemical to electrical information is allowed by the conformational change of the GPCR leading to an elevation of cAMP level, opening a cyclic nucleotide cation channel and causing cell depolarization (Figure 1C)(Buck, 1996; Levy et al., 1991).

Only one type of OR is expressed at the surface of each OSN. Like most GPCRs, ORs have a relatively broad receptive field, binding full and partial agonists. The ORs present various ligands affinities and efficacies and they show different levels of activation based on ligand's concentration. An olfactory molecule usually shows differential affinity with several distinct receptors. Thus, considering these two facts, one odorant will activate a combination of OSN generating a spatial pattern of activation that will be odorant specific, a process called combinatorial code (Friedrich and Korsching, 1997). This results in the creation of an "odor map" or "odor image" (Shepherd, 2005; Xu et al., 2000).

This information is then transmitted via the axons of the OSNs crossing the cribiform plate to the principal cells of the olfactory bulb (OB), the mitral and tufted cells (Figure 2A)(Shepherd, 1972).

1.1.2. The olfactory bulb: the first central relay of olfactory information

1.1.2.1. Anatomical organization

The OB is a paleocrotex organized in 6 layers. We can distinguish, from the periphery to the center: the olfactory nerve layer, the glomerular layer, the exterior plexiform layer (EPL), the mitral cell layer, the interior plexiform layer, the granule cell layer (Figure 2B). Historically, each layer was defined on the histological characteristics of the most abundant population of neurons using Golgi staining analysis (Cajal, 1995; Pinching and Powell, 1971; Price and Powell, 1970a, 1970b). Briefly, the information from the OSNs is transmitted to mitral and tufted cells via specialized structures, the glomeruli, localized in the glomerular layer. The activity of mitral and tufted cells, the principal cells, is modulated by several populations of interneurons, among them the granule (GC) and periglomerular (PG) interneurons, residing in the glomerular and

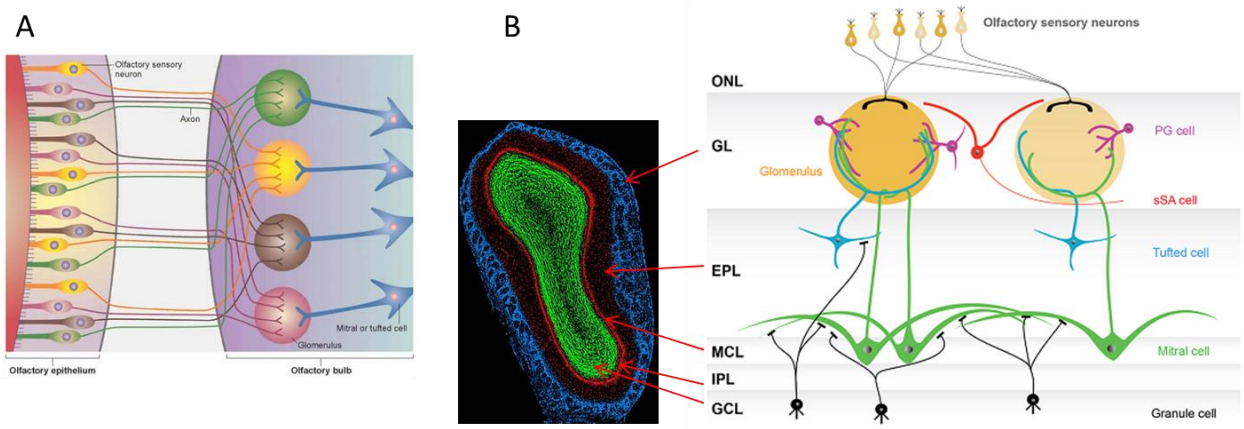

Figure 2: Olfactory bulb structural and functional organization.
A. All OSN from the olfactory epithelium expressing the same receptor project onto the same mitral or tufted cell, creating discrete anatomorfunctional structure that are the glomeruli. **B.** Olfactory bulb organization. ONL: Olfactory Neurons Layer, GL: Glomerular Layer, EPL: External Plexiform Layer, MCL: Mitral Cell Layer, IPL: Internal Plexiform Layer, GCL: Granular Cell Layer, PG cell: PeriGlomerular cell, sSA: superficial Short-Axon cell. (Adapted from Mombaerts, 2006; Nagayama et al., 2014).

granule cell layers respectively (Figure 2B). We are beginning to take into account the great diversity of neuronal types into each layers based on other parameters (electrophysiology, immunochemistry reactivity …)(Kosaka and Kosaka, 2016; Nagayama et al., 2014). In the following parts of this chapter, we are going to develop broad principles as well as particularities and divergences of each layer and cell type, to try to form a comprehensive analysis of the computation realized by the OB.

1.1.2.2. Cell types

The different cell types that we are going to describe with more details are the relay cells of the OB (the mitral and tufted cells), the periglomerular neurons including the diversity of neuronal types surrounding the glomerulus and the granule cells which are the deepest and most numerous neuronal population of the OB.

1.1.2.2.1. Principal/Relay cells: the mitral and tufted cells

As said above, the information is transmitted from the OSNs to the mitral and tufted cells at the level of discrete anatomically and functionally defined structures called glomeruli. Each glomerulus receives projections from OSNs expressing the same OR thus determining each glomerulus receptive field (Mombaerts et al., 1996; Vassar et al., 1994). The level of

convergence is about 1000:1 (OSNs on Mitral/Tufted cell) and allows for better signal to noise ratio (Laurent et al., 2001). Mitral and tufted cells are often grouped in the same category of relay cells of the OB. As such and because many studies did not make the distinction, the specific role of each type of cell is still largely unanswered (see below). The relay cells have been described as the largest cells of the OB with a soma laying in the narrow mitral cell layer. Their primary and secondary dendrites extend into the EPL. The primary dendrite extends radially toward the glomerular layer and interact with incoming OSN axon terminals, forming an extensive and dense arborization extending in the whole glomerulus. The secondary dendrites extend laterally in the mitral cell layer and/or the EPL (for up to 200 µm) and bear synapses with dendrites of interneurons whose cell bodies are located deeper in the OB, the granule cells. Thus, these secondary dendrites back propagate mitral cell activity in the EPL. However, this signal is decrementing with distance (Margrie et al., 2001). Notably, these synapses are reciprocal synapses, i.e. a "two way street of communication" (Price and Powell, 1970a): granule cells are activated by mitral/tufted cells which in turn inhibit them.

Furthermore, populations of neurons like mitral, tufted and juxtaglomerular cells extend their dendrites in usually one and only one glomerulus, generating a columnar architecture in the OB which extends deep into the granule cell layer making them part of that architecture (Willhite et al., 2006). Also, mitral cells connecting to the same glomeruli exhibits similar receptive fields (Chen et al., 2009). Each glomerulus and its associated cells form a functional unit.

Different types of mitral cells and tufted cells exist (Figure 3A and 3B). Mitral cells can be divided into type I and type II based on morphological differences. Type I mitral cells are the majority of the mitral cells and are the one extending long secondary dendrites in the deep EPL. The type II mitral cells extend their secondary dendrite in the intermediate EPL. Even if these morphological variations exist, they have usually been considered as a single population (Mori, 2014).

In the same line, tufted cells can be categorized as external, middle or internal. External tufted cells (eTC) lie just below or within the glomerular layer, middle tufted cells in the two-third part of the EPL and internal tufted cells are localized just superficial to the mitral cell layer (Pinching

and Powell, 1971). These different types of tufted cells also show differences in their morphology: eTC usually don't show secondary dendrites opposed to middle and internal tufted cells demonstrating secondary arborization similar to that seen in mitral cells. In addition, the primary dendritic arborization of eTC is generally simpler and less extended than that of the other types. Because of these divergences, eTC were historically considered as juxtaglomerular cells (JG) as they are localized in the surrounding of the glomerulus (Pinching and Powell, 1971). eTC have the ability to drive sensory activation of mitral cells (Nagayama et al., 2014).

The specific role of mitral versus tufted cells in olfaction is still mostly unanswered. They share lots morphological and biophysical properties. However they differ by the location of their cell body and the extension and location of their secondary dendrites with tufted cells being mostly located in the outer EPL and mitral cells in the deep EPL (Mori et al., 1983; Nagayama et al., 2004). They also exhibit functional differences. For example, tufted cells are more activated by lower odorant concentration than mitral cells (Geramita et al., 2016; Nagayama et al., 2004) and more generally have different odor-evoked responses due to differential PG inhibition (Geramita and Urban, 2017). Finally, they project differently onto secondary olfactory structures suggesting functional differences (Haberly and Price, 1977). This strongly points to the idea that mitral cells and tufted cells form parallel pathways in the OB and probably have different roles in olfactory information processing.

1.1.2.2.2. Juxtaglomerular neurons

Classically, juxtaglomerular neurons is a generic term that encompass PG, superficial short-axon cell (sSA) and eTC(Pinching and Powell, 1971). In the following, we are going to develop the first two classes (PG and sSA) as eTC have already been discussed above.

1.1.2.2.2.1. Periglomerular cell (PG)

As their name indicates, PG are localized around the glomeruli. They have a small cell body and extend their dendritic arborization to one and occasionally two glomeruli and they sometimes have an axon. They have a very variable morphology (Kosaka and Kosaka, 2011; Pinching and Powell, 1971). Their morphologic variability is the reflection of a heterogeneous population

constituted of sub-populations differing also chemically and functionally. Immunochemistry revealed expression and co-expression of GABA (either via GAD65 or GAD67)(~50-55%), dopamine (~13%) and calcium binding protein like calretinin (~28%), neurocalcin (~10%), calbindin (~10%) and paravalbumin (~0.4%)(Parrish-Aungst et al., 2007). These PG cells can be also categorized on their connectivity: type I PG cells receive direct inputs from OSNs while type II PG cells don't receive direct inputs from OSNs (Figure 3A)(Kosaka et al., 1998; S et al., 2014). These subpopulations have thus different role in OB computation (Linster and Cleland, 2009).

1.1.2.2.2.2. Short axon cells

Short axon cells are a relatively old discovery of the OB organization but have been largely ignored for several decades probably due to their relatively small number as well as the fact that they are scattered throughout the OB and difficult to investigate. They are GABAergic interneurons. One type of short-axon cells exhibit dendrites extending in the glomeruli periphery but never entering it, and contrary to PG cells don't show reciprocal synapses (Pinching and Powell, 1971). Based on their localization in the OB, short-axon cells can also be classified as superficial (sSA) or deep (dSA) and based on the type of cell they interact with (tufted cells or mitral cells) they can be further subclassified (Figure 3A and 3B)(Kosaka and Kosaka, 2011). They have also been shown, at least in one sub-population, to receive major top-down cholinergic input (Burton et al., 2017). Overall short-axon cells have the ability to regulate mitral cells, tufted cells and even PG cells' activity and thus to be involved in the temporal patterning of the OB activity.

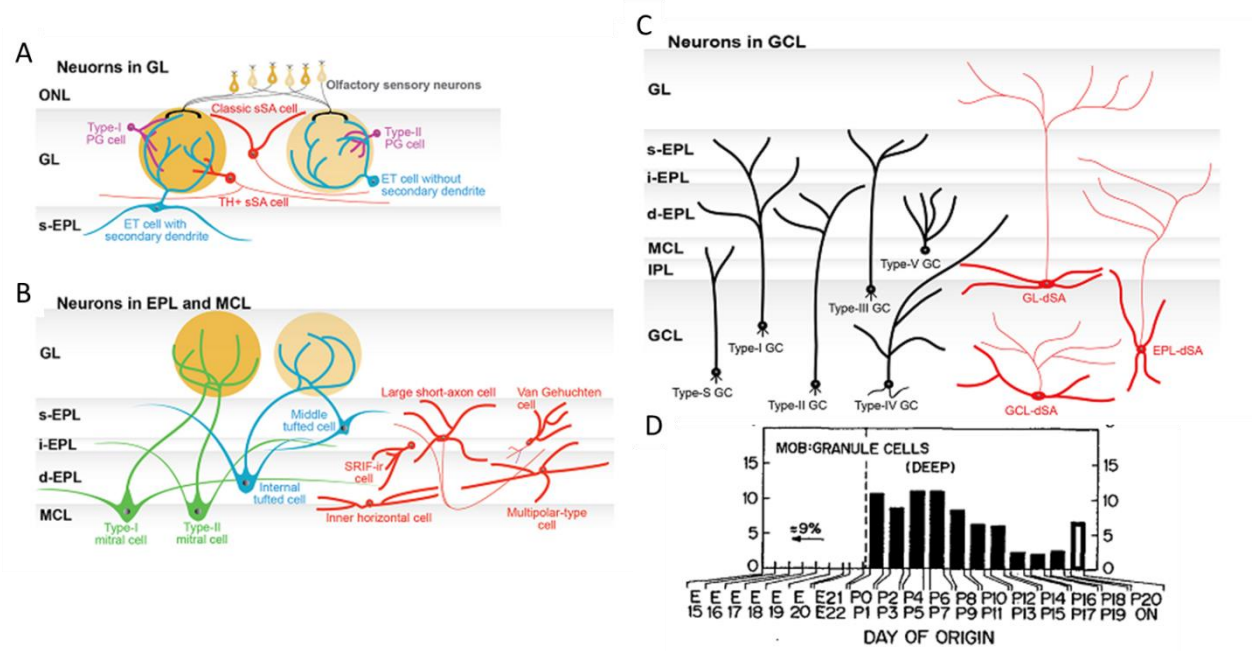

Figure 3: Olfactory bulb cell diversity.
A. Neuronal diversity of the glomerular layer. **B.** Neuronal diversity of the external plexiform and mitral layers. **C.** Neuronal diversity in the granular cell layer. **D.** Granule cells ontogenesis. (Adapted from Nagayama et al., 2014; Bayer et al. 1983).

1.1.2.2.3. Granule cell (GC)

GC are the most abundant population of GABAergic interneurons in the OB. They have a small soma size and have been classified as abnormal for a long time as they are axonless neurons but show both an apical and basal dendrites. Their apical dendrites extend to the EPL and form reciprocal dendrodendritic synapses with mitral and tufted cells while the basal ones extend deeper into the granule cell layer. These dendrites are covered by spines. Dendrites can branch either proximally or distally to the soma. Due to their connections and localization in olfactory circuitry, they are also thought to regulate oscillatory activity (Price and Powell, 1970b; Shepherd et al., 2007). Like other cell types, granule cells can be subdivided in different categories based on morphological and molecular criteria. Deep granule cells that have dendritic arbor connecting to mitral cells lateral dendrites in the deep EPL whereas superficial granule cells connect in the superficial EPL to tufted cells. This could be some evidence of a segregation of mitral and tufted microcircuits (Figure 3C)

During ontogenesis of the OB, GC are generated in the first two post-natal weeks (Altman, 1966; Bayer, 1983)(Figure 3D). Hence, it is possible to label them in the first few postnatal days using lentivirus injections or electroporation targeting the ventricular niche (Figueres-Oñate and López-Mascaraque, 2016).

1.1.2.3. Odor coding and computations

From a computational point of view, early computation in sensory systems are faced with two main problems : global magnitude of activation (i.e. gain control including normalization) and decorrelation (Cleland, 2010; Lepousez et al., 2013). Gain control is the ability of a system to stay between certain bounds even when the environment changes drastically (you can still say that a rose smells like a rose no matter the concentration, i.e concentration invariance). Decorrelation is the ability to discriminate between close stimuli (two odorants that smell much alike) by increasing the difference between their neural representations.

1.1.2.3.1. Spatial coding

The glomerular activation pattern or glomerular map resulting from the ORs activation and bulbar processing lead to an "odor image"(Figure 4A)(Farahbod et al., 2006; Johnson et al., 2004). Interestingly, perceptually similar odorants tends to activate overlapping glomeruli, (Cleland et al., 2002; Johnson and Leon, 2007). The chemotopical organization of the olfactory system is relatively poorly understood compared to the tonotopy or the retinotopy respectively found in the auditory or visual systems (Cleland, 2010). Indeed, retinotopy computations for example are based upon the wavelength of the received light (for the human eye from 400 to 700nm) and spatial proximity of neurons with similar receptive fields allowing for lateral inhibition. For olfaction on the contrary, there is a virtually infinite possibility of odorant molecule configuration and furthermore, fine scale chemotopy studies showed that two spatially closed glomeruli and their associated functional columns don't have similar receptive field. Hence the spatial proximity of glomeruli doesn't predict their respective receptive fields. How is the OB organized then? The high number and molecular diversity of odorants (forming a high dimensional space) make it impossible, from a theoretical point of view, to have fine scale functional chemotopy in olfaction (Cleland et al., 2007; Cleland, 2010;

Cleland and Sethupathy, 2006). Indeed a high dimensionality similarity space (in the case of olfaction about 2^{1000} possible combinations of OSNs activation)(Aimone, 2016) cannot be continuously mapped onto the two-dimensional surface that is the OB. Instead "it will yield patchy, discontinuous maps in which proximity cannot be relied upon to reflect similarities in receptive fields" (Cleland, 2010).

In the OB, there are two main microcircuits responsible for computation: at the OB input level, the glomerular microcircuit and at the OB output level, mitral-granule cells microcircuit (Figure 4B) (Cavarretta et al., 2016; Cleland, 2010). More specifically, the glomerular level seems to have a role in stimulus normalization. The normalization phenomenon allows facilitation of odor recognition independently of odor concentration (Cleland et al., 2007). The mechanism underlying odor concentration normalization are based on the fact that an increased OSNs activity will not increase mitral cells firing at the level of the population, which has been observed *in vivo* and *in vitro*. At the level of individual mitral cells, some differential changes in activity are observed and are consistent with a type of normalization and decorrelation computation named 'mexican-hat' decorrelation (Figure 4C)(Cleland, 2010). It is a way to decorrelate information via specific cell-activity change without mean population activity change. We can also observe normalization via intraglomerular presynaptic inhibition of OSN terminal in the OB (McGann et al., 2005). Normalization needs to be applied across OB columns and this process is thought to be mediated by a network of superficial SA cells and eTC. These cells are activated by sensory inputs and in turn deliver a message carrying a globally averaged level of stimulation of the OB to a specific type of PG cell, the PGe type, which then inhibits mitral cells belonging to the same glomerulus. PGe cells deliver an inhibition to mitral/tufted cells that varies according to the global stimulation level of OB input (Cleland et al., 2007; Cleland, 2010).

Odor concentration normalization is also a requirement for local non-topographical mechanisms of decorrelation which is the type of computations that seem to underlie odor-chemical-similarities-based decorrelation (Cleland and Sethupathy, 2006). In essence, it is a type of intraglomerular inhibition allowing for each glomeruli to construct its own on-center inhibitory-surround decorrelation (Figure 4D)(Cleland and Linster, 2012). It is based on the principle that a

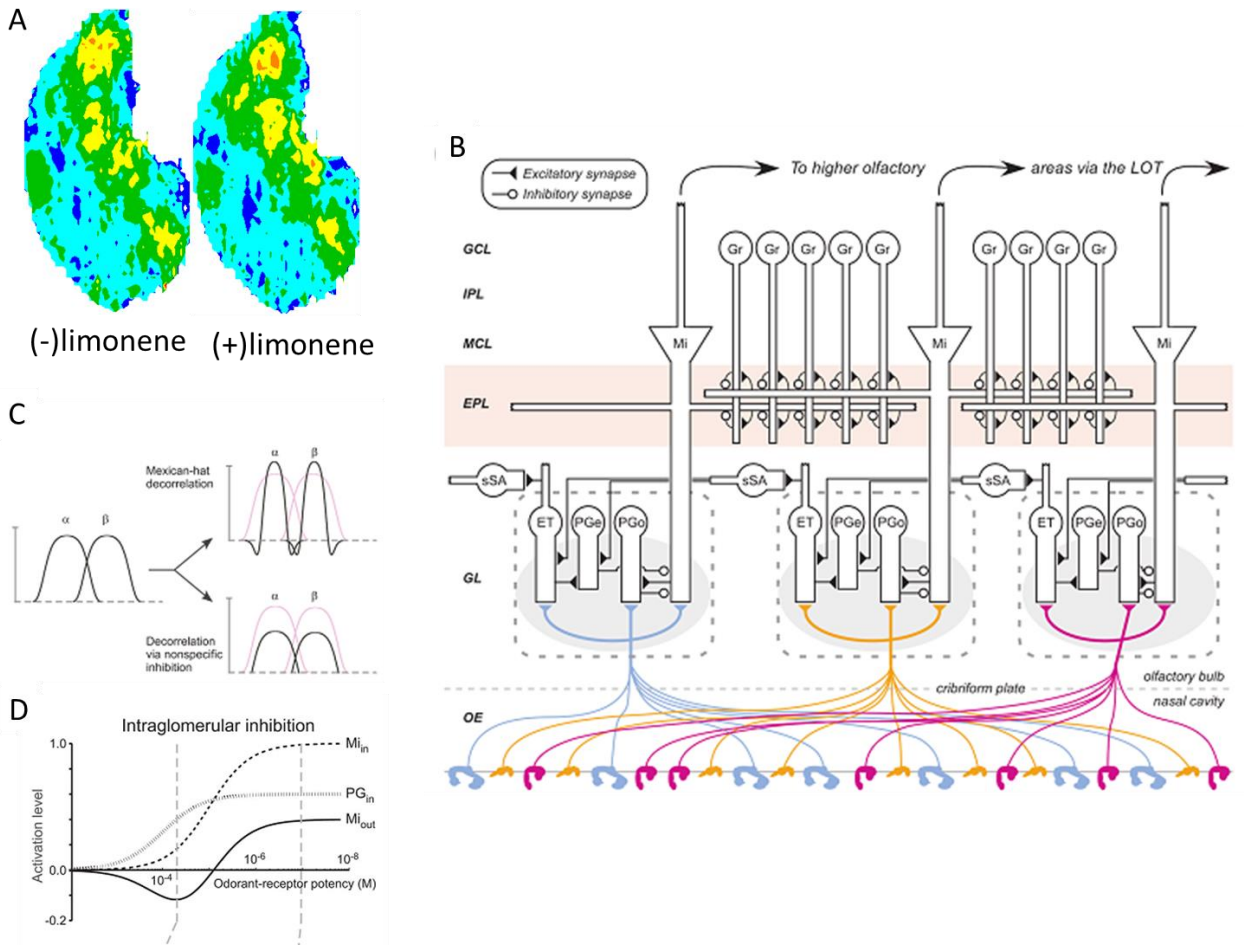

Figure 4: Olfactory bulb projections on olfactory cortices
A. Glomerular activity pattern evoked in response to (-)limonene or (+)limonene. The two pattern are highly similar in basal conditions. **B.** Olfactory bulb glomerular and mitral-granule cells microcircuits. **C.** Decorrelation computations: 'mexican-hat' versus non-specific inhibition. **D.** Intraglomerular inhibition based on on-center inhibitory-surround inhibition computation. (Adapted from Mainland et al. 2014; Cleland, 2010; Linster and Cleland, 2012).

particular group of PG cells (PGo) receiving direct excitatory OSNs stimulation would in turn inhibit the mitral cell of its own glomerulus (Gire and Schoppa, 2009). This PG cell would be more sensitive to OSNs stimulation than the mitral cell but would have a capped activation level that is lower than mitral cell's.

Computations realized at the level of the mitral-granule microcircuit are based on lateral inhibition that also include decorrelation (Arevian et al., 2008). Lateral inhibition circuits are known to perform contrast-enhancement computations. In the OB, enhancement of contrast results from the inhibition of co-activated glomeruli proportionally to their activation response profile and not their spatial closeness. It means that contrast enhancement is realized thanks to

a functional lateral inhibition as opposed to the morphological lateral inhibition which is usually observed (in the retina for example) (Linster et al., 2005). The organization of this microcircuit with properties of distance-independent connections and the ability of learning-dependent plasticity (Moreno et al., 2009, see after) reveal a system able to process high-dimensional odorants representations. However, due to these same properties, this microcircuit has no access to the glomerular microcircuit space and thus cannot perform decorrelation based on odorant structural similarities. One hypothesis is that these decorrelation are performed based on the glomerular microcircuit representation space which is re-transformed based on higher order information like experience, context and learning (Cleland, 2010).

1.1.2.3.2. Temporal coding

The OB high dimensional representation is coded in a complex spatiotemporal code. Indeed individual mitral and tufted cells show odor specific modulation of their firing rate (increase or decrease) (Laurent, 2002) and odor-evoked oscillatory activity is also observed (Laurent et al., 2001). Mitral and tufted cells spiking activity is very precise and mediate synchronized oscillations in specific combinations of output neurons. Long-range synchronization may allow for decorrelation and representation of high-order features (Lepousez et al., 2013; Linster and Cleland, 2010). Synchronized activity of mitral and tufted cells contribute to signal integration, at the level of secondary olfactory areas, in the form of a combination detector (Lepousez et al., 2013; Mori et al., 1999). Indeed, temporal coincidence will increase the probability of cortical neurons firing due to the temporal summation property of neurons. This synchronization process of mitral and tufted cells is thought to be mediated by the lateral inhibition from GC dendrodendritic synapses as well as centrifugal innervation and cortical feedback.

As argued previously (Laurent, 2002) spatial and temporal coding are two sides of the same coin and whose purpose is to solve gain control and decorrelation computations.

1.1.3. Olfactory bulb connectivity

The mitral and tufted cells of the OB project to different cortical and subcortical regions for higher order processing. Among these regions we can find the piriform cortex, the anterior

olfactory nucleus, the olfactory tubercle, the lateral enthorinal cortex and the amygdala (Linster and Cleland, 2003; Mainland et al., 2014; Wilson and Mainen, 2006) (Figure 5). Moreover, these downstream regions send feedback projections to the OB. In addition to these projections, the OB receives projections from neuromodulatory systems such as noradrenergic, cholinergic and serotoninergic fibers coming from the locus coeruleus, the diagonal band of Broca in the basal forebrain and the raphe nucleus respectively (Fletcher and Chen, 2010; Linster and Cleland, 2003). One particularity of olfaction compared to other sensory systems is that bulbar projection directly connect to secondary olfactory areas without thalamic relay (Shepherd, 2005). Below, we will very briefly OB projections and neuromodulatory inputs in term of connectivity as well as established cognitive function.

1.1.3.1. Projections from the OB

The piriform cortex is a three-layer paleocortex to which mitral and tufted cells project, in its layer I, via the lateral olfactory tract (Haberly and Price, 1977). It is subdivided into the anterior piriform cortex and posterior piriform cortex. This subdivision emerges from structural differences in cytoarchitecture and axonal projections that are thought to underlie functional differences (Shepherd, 2004). In contrast to the OB, the spatial activity maps representing the odorants are not observed in the piriform cortex (Stettler and Axel, 2009). However, the odor representation is retrieved as an ensemble of activated neurons across the piriform cortex (Kay, 2011). Piriform cortex neurons have been shown to perform pattern completion (Barnes et al., 2008) allowing for stable representation of perceptual information. Pattern completion is the ability to match a degraded sensory input to a memory template if both are sufficiently close. The piriform cortex has been shown to support discrimination learning where neuron's activity is being decorrelated between odors as the animal learns to discriminate at the behavioral level (Chapuis and Wilson, 2011, 2013; Li et al., 2008). The piriform cortex has also been shown to take part in many other fonctions, among them encoding odor value, identity and perform associations with aversive stimuli (Courtiol and Wilson, 2017; Wilson and Sullivan, 2011). It is a central node of olfactory integration, having reciprocal connections with the orbitofrontal cortex, the lateral entorhinal cortex, the olfactory tubercle, the amygdala and the mediodorsal

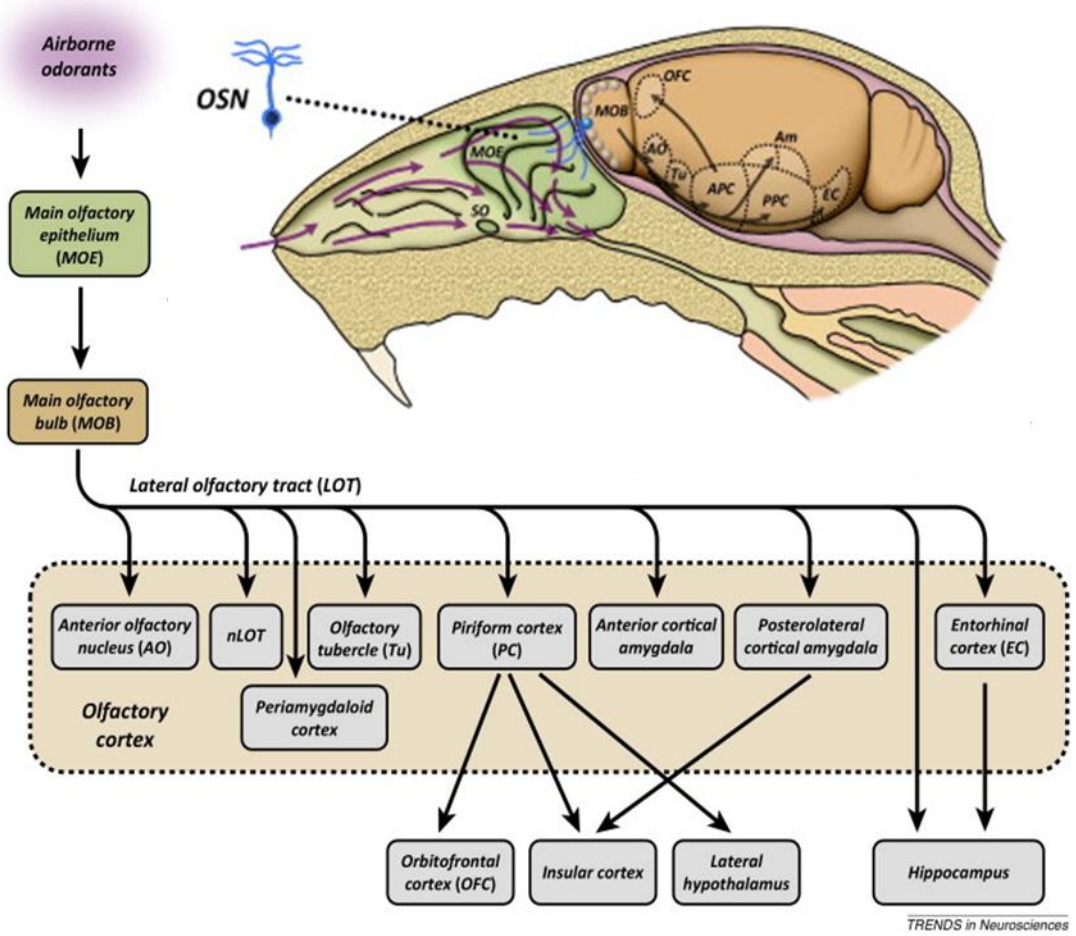

Figure 5: Olfactory bulb projections on olfactory cortices
The olfactory bulb project onto secondary olfactory structures like the anterior olfactory nucleus, the olfactory tubercle, the piriform cortex, the amygdala and the entohinal cortex. Then these secondary structures project onto tertiary areas. (Adapted from Mainland et al. 2014).

thalamus nucleus (Shepherd, 2004). It also has neuromodulatory inputs from cholinergic and monoaminergic brain areas (Linster and Hasselmo, 2001; Wilson et al., 2004).

The **accessory olfactory nucleus** (AON) is located in the posterior continuity to the posterior piriform cortex and receives excitatory inputs both from the ipsilateral OB in a direct pathway and from the contralateral OB via the anterior commissure (Schoenfeld and Macrides, 1984). It might have a role in the "stereo" sensation of an odor by contrasting inputs from each nostril (Kikuta et al., 2010). At the same time it could also have a role in bilateral access to olfactory memory (Imai and Sakano, 2008).

The **olfactory tubercle** is part of the ventral striatum and has a role in motivated behavior and evaluating reward information (Gadziola and Wesson, 2016; Xiong and Wesson, 2016). It has a role in learned odor hedonic processing and state-dependent odor processing as well as arousal (Wesson and Wilson, 2011). Recent evidence from our team put forth its central role in evaluating innate odor hedonics (Midroit et al. 2017, unpublished). It is also a node of cross-modal integration between olfactory and auditory stimuli (Wesson and Wilson, 2010).

The OB also connects directly to the **entorhinal cortex** which is part of the parahippocampal regions which in turn connect to the hippocampus via the performant path (Haberly and Price, 1978; Kosel et al., 1981; Witter et al., 2017). It is thought to be a nodal point in cortico-hippocampal interactions, playing a role in object information processing, memory, attention and motivation (Witter et al., 2017).

Thereafter, secondary olfactory cerebral areas project to tertiary ones like the orbitofrontal cortex (OFC) thought to have a role in conscious perception of odorants (Mori et al., 2013), the thalamus, the prefrontal cortex, and the hippocampus.

1.1.3.2. Feedback projections to the OB

As all primary sensory areas, the OB receives many feedback projections from higher order olfactory-related areas. The piriform cortex for example has a huge number of fibers projecting back onto mitral, glomerular and granule cells of the OB (Luskin and Price, 1983; Shepherd, 2004). This create corticobulbar loops that could be functionally important in regulating plasticity in the OB (Nissant et al., 2009; Oswald and Urban, 2012) in the sense that high order processing could be reverberated thought earlier processing stages.

1.1.3.3. Neuromodulatory inputs

The OB also receives numerous centrifugal neuromodulatory inputs from distant brain structures; among them the diagonal band of Broca (cholinergic and GABAergic), the Locus Coeruleus (noradrenergic) and the basal forebrain (serotoninergic)(Fletcher and Chen, 2010). Neuromodulation is defined as a process serving to modulate network activity (and thus performed computations) as a function of task demand or behavioral state (Linster and Cleland,

2016). The neuromodulatory projections onto olfactory areas have been largely studied (Castillo et al., 1999; Chapuis and Wilson, 2013; Devore and Linster, 2012; Guerin et al., 2008; Mandairon et al., 2006a; Moreno et al., 2012a) and multiple computational models have been use to understand their importance in olfactory processing as well as their interaction with each other (de Almeida et al., 2013, 2016; Li et al., 2015; Linster and Cleland, 2002). Very briefly, neuromodulatory inputs have the ability to influence contrast enhancement, decorrelation and signal to noise ratio computations as well as short-term non associative memory, cortical learning, associative memory and proactive interference (Linster and Cleland, 2016).

1.1.4. Conclusion

The odor code can be modulated to respond to a changing environment and new learning at all the levels of olfactory processing. In the rest of this work, we will focus and limit ourselves to the plasticity occurring in the OB involving changes both in spatial and temporal codes and due in part to the amazing phenomenon of adult neurogenesis.

2. Olfactory bulb adult neurogenesis

2.1. Discovery

For a long time, the accepted view was that neurons were generated only during ontogenesis (Cajal, 1913). However, it is now widely accepted that adult neurogenesis occurs in the adult mammalian brain and more specifically in two main regions: the hippocampus and the OB. Indeed, in the 1960s, Altman and colleagues gathered evidence using ^{3}H thymidine, a DNA analog, that new cells with neuronal characteristics could be found in the hippocampus and the OB (Altman, 1969; Altman and Das, 1965). These results made very little impact on the scientific community at that time. We had to wait twenty years for rediscovery of adult neurogenesis in the singing bird (Goldman and Nottebohm, 1983), in which it is responsible for seasonal singing. They used another DNA marker, more easily handled, the Bromodeoxyuridine (BrdU). Subsequent evidences then appeared such as findings of massive cell migration toward the rodent's OB (Lois and Alvarez-Buylla, 1994) as well as the first isolation of adult neural stem cells

(Reynolds and Weiss, 1992) and the disclosure of adult neurogenesis in the other species among them shrew (Gould et al., 1997), monkey (Gould et al., 1999) and human (Eriksson et al., 1998)(non-exhaustive list).

Thereafter, we are going to limit ourselves to bulbar neurogenesis, only mentioning when needed hippocampal neurogenesis.

Bulbar adult neurogenesis starts in the subventricular zone (SVZ), along the lateral ventricle of the mammalian brain where thousands of neural stem cells (NSCs) are present (Alvarez-Buylla and García-Verdugo, 2002; Lim and Alvarez-Buylla, 2016; Ponti et al., 2013).

Broadly speaking, in the SVZ, NCS are proliferating radial glia-like cells (type B cells) which give rise to transient amplifying cells (type C cells), in turn generating neuroblasts (type A cells) (Figure 6A) (Doetsch et al., 1999; Ming and Song, 2005, 2011). These neuroblasts form a chain in the rostral migratory stream and migrate tangentially toward the OB (Lois et al., 1996). Numerous internal and external factors can contribute to regulate adult neurogenesis at all levels of proliferation and migration (Lledo et al., 2006). For example, hormonal status (thyroid, prolactine), growth factors (VEGF, BDNF), neurotransmitters (serotonin, dopamine) are subject to modulation by environmental changes and in turn have the ability to enhance or decrease adult neurogenesis (Lim and Alvarez-Buylla, 2016; Lledo et al., 2006).

Increasing evidence demonstrates that the spatial position of NSCs in the SVZ will determine their fate, i.e. granule or periglomerular interneuron. NSCs in the dorsal adult SVZ will give rise to superficial GC, calretinin-positive cells and periglomerular tyrosine hydroxylase-positive cells. In contrast, lateral and ventral regions generate mostly deep GC and PG calbindin-positive cells (Fiorelli et al., 2015). On the top of the spatial dimension, the temporal one is also important in the fate of the new born cells. Indeed, some cell types are generated abundantly during the post-natal neurogenesis while other are preferentially generated during adulthood: calbindin-positive PG cells versus calretinin- and tyrosine hydroxylase-positive neurons for example (Batista-Brito et al., 2008; Marchis et al., 2007).

2.2. Differentiation and integration into the preexisting network

Neuroblasts arriving in the OB will stop migrating rostrally to start migrating tangentially in order to reach their final destination in the network. They migrate in the periglomerular and granule cell layers where they differentiate into periglomerular (3%) and granule interneurons (97%) respectively (Figure 6B) (Lledo et al., 2006; Malvaut et al., 2015; Winner et al., 2002).

2.2.1. Periglomerular cells

Overall less is known about the temporal and morphological maturation of adult-born PG compared to adult-born GC. What we do know is that the population of adult-born neurons differentiating into PG interneurons show electrophysiological properties typical of their respective subtype (Belluzzi et al., 2003). The PG population is very diverse and heterogeneous and the same goes for adult-born which mature into the different types already characterized (Belluzzi et al., 2003; Lazarini et al., 2014; Pignatelli and Belluzzi, 2017). However as previously mentioned some types are preferentially generated over others : calbindin-positive PG cells versus calretinin- and tyrosine hydroxylase- expressing neurons (Batista-Brito et al., 2008; Marchis et al., 2007).

2.2.2. Granule cells

Future granule cells follow a stereotypical neuronal development both at the structural and functional levels.

At the structural level, they follow a development in 5 stages (Petreanu and Alvarez-Buylla, 2002) (Figure 6C). At stage 1, young neuroblasts tangentially migrate in the rostral migratory stream. At stage 2, they reach the OB, begin to migrate radially and start extending their first dendrite. At stage 3, they keep extend their main dendrite which stays unbranched. At stage 4, the main dendrite gets branched but is still spineless which then lead to stage 5, a mature granule cell, which developed a heavy spine density.

At the functional level they “listen before they can speak” (Kelsch et al., 2010). Indeed adult-born GC, in contrast with their preexisting counterparts, first receive inhibitory inputs during stage 3 (Carleton et al., 2003). They receive excitatory inputs during stage 4 (Carleton et al., 2003) and at first only on the proximal dendrite (Kelsch et al., 2008). They finally acquire their

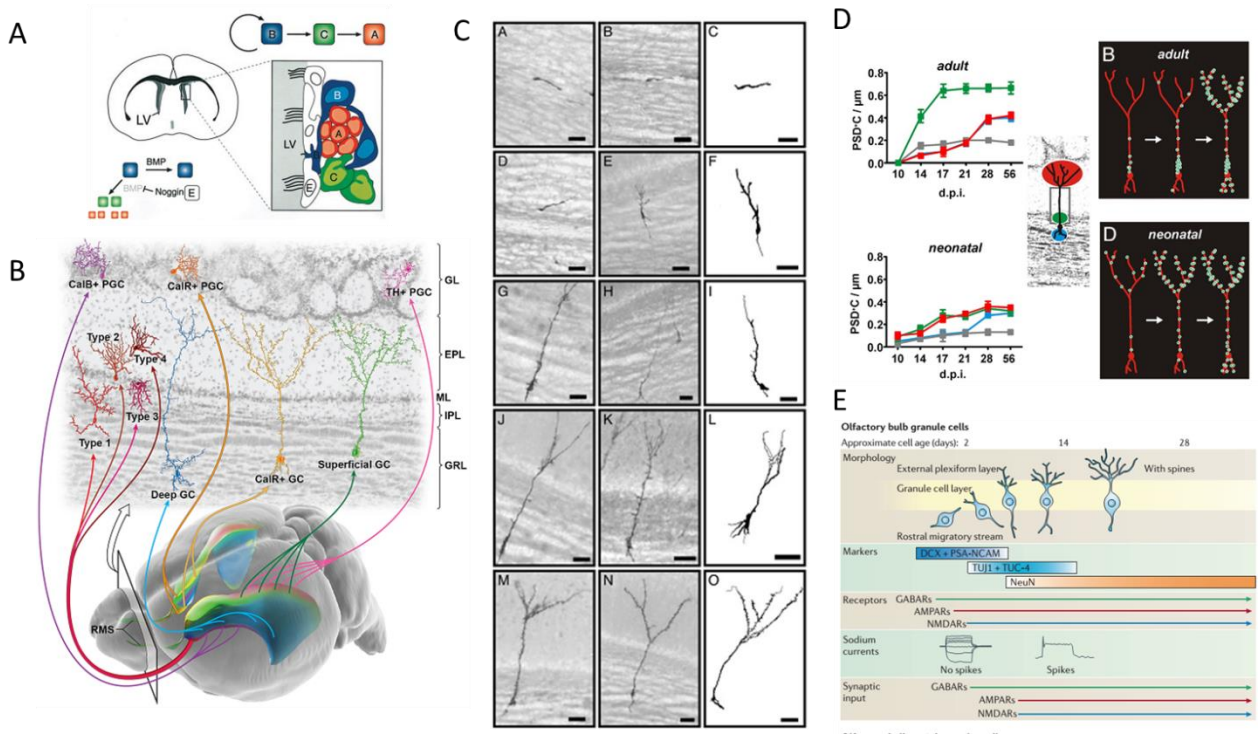

Figure 6: Adult-born interneurons
A. Cells in the SVZ. There are three types of cells: A,B and C. **B.** Stem cells give rise to many different daughter neurons that become either periglomerular or granular neurons. **C.** Stereotypical morphological maturation of granule interneurons. **D.** Morphological development of impinging neurons onto granule cells. **E.** Functional maturation of adult-born granule cells. (Adapted from Alvarez-Buylla and Garcia-Verdugo, 2002; Pretreanu and Alvarez-Buylla, 2002, Lim and Alvarez-Buylla, 2014; Kelsch et al., 2008).

full input/output connectivity and the ability to fire action potential during stage 5 (Carleton et al., 2003; Kelsch et al., 2008) (Figure 6D and 6E).

Even if their stereotyped development is finished by 4 weeks of age, adult-born neurons remain plastic for several additional weeks, notably at the level of the spines (and contrary to dendrites), with a high rate of dendritic spines turnover (Mizrahi, 2007). They also have a transient ability to perform long-term potentiation from cortical inputs (Nissant et al., 2009)

We can also see differences in the spatial distribution of in adult-born neurons inside of the granule cell layer (Imayoshi et al., 2008; Mandairon et al., 2006a). Indeed adult-born neurons will preferentially target the deep granule cell layer, with time replacing the majority of the cells present there, and only replacing about half of the neuronal population in the superficial layer of the granule layer. This could hint at functional differences between superficial versus deep

granule cell layer but this hypothesis is still being tested. It is worth to note though that this is not an addition of new neurons in the OB but a renewal of neurons as the OB volume stays constant.

Due to the amount of GC being generated and integrated every day in the OB of mice (between 10 000 to 30 000 per day depending on the method used), it stands to reason that they can have a great impact on information processing. Even if number is not the only parameter to take into account, one factor reinforcing the importance of these granule cells is that they modulate processing of mitral cells which are the first relay of olfactory information. Hence, like changing the current of a river is better done at the source, modifying olfactory processing in the OB can potentially have a huge impact on downstream received information.

2.3. Survival

Only about 50% of adult-born neurons arriving in the survive and integrate into the preexisting network as fully mature neurons (Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). Furthermore these neurons, during their month of maturation, i.e. their critical (Yamaguchi and Mori, 2005), are highly sensitive to environmental changes which can influence their survival rate. Adult-born neurons survival is thus a potential element of plasticity. In the next section of this work, we are going to develop the role of adult-born neurons as an element of structural and functional plasticity in the OB network.

3. Olfactory bulb experience-dependent plasticity

Plasticity in the brain comes in many forms. One of them is the strengthening or weakening of synaptic connections between cells through long-term potentiation or long-term depression (Hebb, 2005; Kandel, 2001). Neurons also have the ability to change their structure, i.e. structural plasticity, in their ability to create, eliminate and stabilize dendrites and dendritic spines (Holtmaat and Svoboda, 2009). These have been extensively studied from invertebrate to vertebrate species since the 1950s.

In adult neurogenesis-targeted structures like the hippocampus and the OB however, an added level of plasticity is conferred by the constant addition of newly generated neurons which have the ability to (re)shape how the neural network processes information. This adult-born neurons' plasticity comes in many forms: their number or more precisely the number of surviving adult-born neurons in the network, their responsiveness, their position in the network i.e. which neurons they inhibit, and like any other neurons in the central nervous system, their morphology and connectivity.

These parameters are especially important in experience-dependent plasticity which comes from environmental changes (deprivation or enrichment) as well as some specific learning tasks (associative or perceptual learning).

3.1. Environmental changes

Manipulating the environment to understand experience-dependent plasticity is a widely used as a way to observe neuronal changes in response to precise and controlled environmental modifications. Sensory modifications like sensory deprivation or enrichment both demonstrated that activity is a major regulator of adult-born neurons survival and morphology.

3.1.1. Sensory deprivation

Olfactory deprivation consists in preventing activity in the OB and can be obtained using different means like nasal occlusion with plugs (Coppola, 2012; Mandairon et al., 2006) or using genetically modified animals lacking signal transduction proteins in the OSN (Petreanu and Alvarez-Buylla, 2002).

Olfactory deprivation via naris occlusion decreases adult-born granule cell survival rate between 15 and 45 days after occlusion in both glomerular cell layer and granule cell layer, but with a different temporal pattern (Corotto et al., 1994; Mandairon et al., 2006). Worth noting, more cell death was observed in the deep than the superficial granule cell layer (which is probably linked with that fact that more adult-born neurons are migrating to the deep than to the superficial granule cell layer). Genetically engineered anosmic mice showed the same decreased number of adult-born granule cell (Petreanu and Alvarez-Buylla, 2002).

3.1.2. Sensory enrichment

The reverse environmental change to deprivation is enrichment and is a well-known experimental paradigm enhancing neural plasticity and cognitive performances (van Praag et al., 2000). Olfactory enrichment specifically improved the survival of adult-born neurons in the OB (Rochefort et al., 2002). This improvement in survival affects both PG cells and GC but once again with a difference in the temporal pattern (Bovetti et al., 2009; Rochefort et al., 2002) and is associated with a longer olfactory memory (Bovetti et al., 2009; Rey et al., 2012; Rochefort et al., 2002). This has also been seen when conditional inducible activation of *erk5*, a map kinase selectively expressed in adult neurogenic regions (Wang et al., 2015).

3.2. Learning

Specific learning tasks also involve adult-born neurogenesis. Using them allows for dissection of the neural mechanisms associated with behavioral changes. What's more, the new tools recently developed such as optogenetics and chemogenetics allow the determination of causality between specific neuronal population activity and changes in behaviors. This is the basis for a better understanding of the neural correlates of learning.

3.2.1. Associative learning

The role of adult-born neurons in associative learning has been subject to numerous studies and debate in the literature due to conflicting results. On the one side, the reduction of adult-born neurons by irradiation (Lazarini et al., 2009) or an anti-mitotic drug (Sultan et al., 2010) showed no effect on associative learning performances but an effect on memory while in two other studies using . genetically modified mice suppressing adult neurogenesis (Imayoshi et al., 2008) or once again an anti-mitotic agent (Breton-Provencher et al., 2009) revealed no effect on learning or long-term memory. This seemingly opposition in the results provided by these experiments was hypothesized to be due to the difference in the type of associative learning task used. Indeed, the first two used an operant task while the other two a non-operant one. A direct comparison between the two types of tasks (Mandairon et al., 2011) confirmed this and showed that an operant conditioning task increases the number of adult-born neurons in the

OB whereas a non-operant conditioning did not affect neurogenesis and may thus not depend on adult-born neurons.

However more than just being modulated by learning (Alonso et al., 2006; Sultan et al., 2010, 2011a), adult-born neurons actively participate and can be the substrate of olfactory memory. Indeed, on the one hand, going through a behavioral extinction after an associative learning tasks prematurely suppresses adult-born neurons saved by learning (Sultan et al., 2011b). On the other hand, selective optogenetic activation of adult-born neurons accelerated discrimination learning in a go/no-go task and improved memory (Alonso et al., 2012).

Other studies showed that adult-born neurons survival can depend on the delay between neuron birth and learning as well as the timeline of learning itself. Olfactory training can, not only increase, but also decrease or have no effect on adult-born survival depending on the age of adult-born neurons at the time of learning. This highlights the importance of the critical period of adult-born cells in olfactory learning (Mouret et al., 2008). Also, while adult-born neurons survival is increased after a spaced associative learning (Kermen et al., 2010; Sultan et al., 2010) the same training when massed does not modify neurogenesis and a decrease of memory retention is observed (Kermen et al., 2010).

Interestingly, the increase in adult-born neurons' survival after learning is not occurring homogeneously in the OB. Precise quantifications revealed a regionalization in the survival of adult-born neurons (Alonso et al., 2006; Mouret et al., 2008; Sultan et al., 2011a) and the associated neuronal death is necessary for optimal performances (Mouret et al., 2009).

3.2.2. Perceptual learning

Perceptual learning is a passive/implicit form of learning defined as the significant improvement in discrimination abilities induced by previous experiences (Gilbert et al., 2001) reflecting an ongoing process by which animals learn to discriminate common and potentially relevant stimuli within their immediate environment. In the case of olfaction, animals can learn to discriminate between perceptually similar odorants in a relative odor-specific manner thanks to olfactory enrichment (Mandairon et al., 2006a; Moreno et al., 2009). This is a simple learning that

doesn't implicate association to a reward allowing assessment of the discrimination learning process only. Perceptual learning, as associative learning, increases adult-born granule cell survival in the OB (Moreno et al., 2009). In contrast to associative learning, the suppression of adult-born neurons in the OB prevent perceptual learning (Moreno et al., 2009) making them necessary for the task. Furthermore, perceptual learning process is also dependent on noradrenaline transmission that imping on adult-born granule cell survival (Moreno et al., 2012b; Vinera et al., 2015).

Perceptual learning modulates GC inhibition on mitral cells that could underlain odorant pattern separation. Indeed, it has now been showed that, perceptual learning changes odor representation in the OB (Chu et al., 2016; Yamada et al., 2017). A recent paper by the same team also demonstrated that the pattern separation induced by olfactory perceptual learning doesn't happen at the level of the OSN. Indeed using two-photon calcium imaging they found that OSN inputs to the bulb are stable during discrimination learning. Hence, neuronal change underlying discrimination occurs later in olfactory processing (Chu et al., 2017) which contrast with a previous study showing changes in OSN temporal patterns (Kass et al., 2016).

Finally, computational studies showed how adult-born neurons could participate in information's orthogonalization (Cecchi et al., 2001) and how their help in decorrelation could map onto biological knowledge of the OB computation (Chow et al., 2012).

4. Thesis objectives

As mentioned above, olfaction is involved in multiple behaviors from food intake to social interactions. In order to perform these tasks accurately, the olfactory system has to perform fine discrimination between very close stimuli. As we have seen, these discrimination performances can be enhanced through perceptual learning and a key cerebral structure of this learning is the OB due to the requirement of adult neurogenesis (Chu et al., 2016, 2017; Nathalie Mandairon et al., 2006c; Moreno et al., 2009; Vinera et al., 2015).

The central question of this thesis work is the role and the specificity of adult-born neurons in olfactory discrimination learning in complex and changing contexts. To answer this question, we used the perceptual learning paradigm with different variations.

The olfactory environment is complex in the sense that it is made of numerous odors, thus needing fine-tuning of discrimination abilities to guide the animal behavior. In that frame, we first studied the effect of perceptual learning on adult neurogenesis in conditions involving from one to six pairs of perceptually similar odorants. The first question that came up was **1-are mice able to learn to discriminate from one to six odor pairs at a time?** We observed that perceptual learning occurs in all conditions and thus that mice are able to learn to discriminate from one to six pairs of odorants. The second question was **2-do more complex learning save more adult-born neurons?** We confirmed that adult-born neuron survival (evidenced by BrdU labeling) is increased by simple perceptual learning (one odor pair) but interestingly, increasing the complexity of learning does not increase the rate of surviving cell. So, **3-do more complex learning recruit more adult-born neurons to process the learned odorants?** We saw that the percentage of adult-born neuron responding to the learned odorants (shown by immediate early gene Zif268 activity labelling in adult-born neurons) is correlated to the complexity of learning. Modification in number or responsiveness is thus one possible substrate for plasticity. **4-do adult-born neurons exhibit modifications of morphology (and connectivity) associated with learning?** We revealed modifications of adult-born neurons fine morphology induced by both simple and complex learning thanks to the expression of GFP fluorescent proteins expressed in adult-born neurons. This functional and structural plasticity suggests a modulation of the activity of the bulbar relay cells (mitral cells) and thus of the bulbar output towards superior olfactory centers. Next, **5-are these morphological changes specific to adult-born neurons?** To answer that question we labeled granule cells born during the ontogenesis of the (i.e. preexisting neurons) with DsRed fluorescent proteins. We found modifications of preexisting neuron morphology, after complex but not simple learning. These results put forth the unique properties of adult-born neurons in the OB plastic adaptations and the contribution of a larger network involving preexisting neurons in complex learning. Since adult-born neuron plasticity is modulated by both simple and complex learning and that preexisting neuron

plasticity is modulated only by complex learning, we then wanted to **6-dissect the relative functional contribution of each neural population to simple versus complex learning**. To do so we used an optogenetic approach in order to inhibit each neural population during the discrimination test after simple and complex learning. We found that the inhibition of adult-born neurons prevented discrimination in all cases (simple and complex learning) while the inhibition of preexisting neurons only prevented discrimination in complex protocol which is in line with the morphological study. This first study demonstrated the importance of adult-born neurons for simple and complex olfactory learning. It also showed that when learning become more complex, a larger neural network is involved requiring preexisting neurons to answer the behavioral demand.

In addition of being complex, the olfactory environment is changing. In a second study we investigated **7-how the memory of an olfactory information already stored is altered by the acquisition of a new one** and **8-what is the role of adult neurogenesis in this process**. More precisely, we performed successive perceptual learning while varying the delay between learning. We found that in the absence of interference, adult-born neurons saved by learning are present as long as the learned discrimination is present. Interestingly, a second learning of a new pair of odorants occurring shortly after the first one leads to the encoding of the discrimination of this second pair of odorants (with an associated increase in neurogenesis) and the forgetting of the first one. This forgetting process can be counterbalanced by maintaining the first odorant pair in the environment during the second learning session. When the delay between successive learning is longer, both memories are maintained and two distinct waves of adult-born neuron survival can be observed thanks to sequential integration of thymidine analogs (Idu and Cldu).

Finally, maintaining exposure to odors of the first learning during the second learning showed us that it is possible to maintain both memories and each population of adult born cells. In order to go further and causally relate these observations, we inhibited adult born neuron activity using an optogenetic approach. In this last learning paradigm (first learning maintained during the second one), inhibiting, during discrimination testing, the adult-born neuronal population saved by the first learning did not alter the second pair's discrimination. This second study highlighted

the role of adult-born neurons in being the substrate of olfactory memories and that once allocated to a specific memory engram they cannot be used to underlie another one.

Lastly, we turned ourselves toward computational neurosciences to better understand, interpret and frame theories about brain mechanisms on perceptive learning. This approach is aimed at **9-outlining a computational framework explaining the role of adult-born versus preexisting granule cells in early olfactory transformation of the information and how sharpened sensory representations emerge from decorrelation of responses to similar stimuli**. We are currently trying to integrate adult-neurogenesis into an already built model of the OB, in collaboration with the Linster lab at Cornell University. This computational model, by allowing us to manipulate adult-born neurons dynamics, will show us how their integration in the OB can (re)shape the outgoing message. We will then derive simple rules governing learning induced-plasticity of adult-born and preexisting neurons. This will also allow us to make prediction and formulate new hypothesis to be tested in vivo. Ideally, the model needs to be simple enough to be able to generalize to different learning (i.e. associative learning). This work is in progress.

PART II: Results

Study 1 - Impact of adult neurogenesis versus preexisting neurons on olfactory perception in complex olfactory environment

Impact of adult neurogenesis versus preexisting neurons on olfactory perception in complex olfactory environment

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Abstract

Olfactory performances can be enhanced through perceptual learning, defined as an improvement in the discrimination of perceptually close odorants after passive repeated exposure to them. It has been demonstrated that simple olfactory perceptual learning (two odorants learned to be discriminated) increases adult-born neurons survival in the olfactory bulb and that their presence in the olfactory bulb is required for learning to occur. However the olfactory environment is usually more complex needing fine tuning of discrimination abilities to guide the animal behavior. Does more complex learning require more adult-born neurons? We investigated neural mechanisms underlying perceptual learning involving up to six pairs of perceptually similar odorants at the same time. We first observed that perceptual learning occurred in all conditions. We confirmed that adult-born neuron survival is increased by simple perceptual learning but interestingly, increasing the complexity of learning did not increase the rate of surviving cells but increased the percentage of adult-born neurons responding to the learned odorants. In addition, we revealed modifications of adult-born neurons' fine morphology by both simple and complex learning suggesting a modulation of the activity of the bulbar relay cells. To determine whether these morphological changes are specific to adult-born neurons, we analyzed the morphology of granule cells born during the ontogenesis of the olfactory bulb, i.e. preexisting neurons. The gathered data demonstrated far more limited changes in preexisting neurons, revealing only modification of their morphology during complex but not simple learning. These results put forth unique properties of adult-born neurons in the olfactory bulb plastic adaptations. We then deciphered the relative contribution of preexisting versus adult-born neurons to simple versus complex learning using an optogenetic approach and found that the inhibition of adult born neurons prevented discrimination learning in all conditions (simple and complex learning) while

the inhibition of preexisting neurons only prevented discrimination learning in a complex protocol. This study demonstrated that when learning becomes more complex, a larger neural network is involved requiring adult-born and preexisting neurons to answer the behavioral demand.

Introduction

The olfactory bulb (OB) is one of the two main regions of the brain, with the dentate gyrus of the hippocampus, continuously supplied with new neurons during adulthood through a process called adult neurogenesis (Altman, 1969; Lois and Alvarez-Buylla, 1994; Ming and Song, 2005). Stem cells residing in the subventricular zone of the lateral ventricle, divide into neuroblasts that migrate along the rostral migratory stream to reach the OB and differentiate into periglomerular (3%) and granule cells (97%). These inhibitory interneurons shape the output message of the OB carried out by the mitral cells of the OB (Winner et al., 2002; Malvaut and Saghatelyan, 2016). These adult-born neurons play central role in tuning the animal's discrimination abilities and thus its behavior to its environment. More particularly, it has been demonstrated that adult-born neurons have an important role in a specific learning called perceptual learning (Mandairon et al., 2006b; Moreno et al., 2009). Perceptual learning is defined as the significant improvement in discrimination abilities induced by previous passive experiences (Gilbert et al., 2001), reflecting an ongoing process by which animals learn to discriminate common and potentially relevant stimuli within their immediate environment. In the context of olfaction, animals can learn to discriminate between chemically similar odorants in a relative odor-specific manner thanks to olfactory enrichment (Mandairon et al., 2006c; Moreno et al., 2009) . This learning not only increases adult-born neurons' survival but requires their presence in the OB (Moreno et al., 2009). However, first the olfactory system needs to cope with a complex olfactory environment to be able to guide the animal's behavior correctly. What part are adult-born neurons playing in perceptual learning when the system is challenged by a complex olfactory environment? In other words, does complex learning require more adult-born neurons or enhanced structural plasticity? Second, in order to understand the specificity of adult-born neurons' role, it is crucial to compare

their functional properties and structural plasticity to those of preexisting neurons, i.e. neurons born during the ontogenesis of the OB.

Newly integrated adult-born cells show specific electrophysiological properties compared to preexisting neurons: an increase excitability and transient LTP (Carleton et al., 2002; Nissant et al., 2009; Valley et al., 2013) as well as a transient higher responsiveness to odorant stimulation (Livneh et al., 2014). Moreover, adult-born neurons can become specifically more selective to odorants after enrichment, hence acquire functional features distinct from those of preexisting neurons (Magavi et al., 2005; Livneh et al., 2014). So far however, the specificity of adult-born compared to preexisting neurons in a learning context remains unclear. Third, to go beyond correlative evidence for the implication of adult-born versus preexisiting neurons in learned discrimination, experimental designs need to probe their respective contributions in behaving animals.

To answer these questions, we used a perceptual learning paradigm in complex configurations, with mice having to learn to discriminate several pairs of odorants at the time. We assessed the effect of perceptual learning with different levels of complexity on adult-born cell survival, responsiveness to the learned odorants and structural plasticity. In addition, we analyzed the uniqueness of adult-born neurons plasticity by comparing it to the one of preexisting neurons born during ontogenesis. Finally, to determine the functional role of each population on discrimination performances after learning, we measured the effect of selective inactivation, via optogenetics, of the populations of pre-existing or adult-born neurons on learned discrimination.

Materials and Methods

Animals

64 male C57BL/6J mice born and raised in our laboratory were used in this study. Mice were housed in a controlled environment under a 12h light/dark cycle with food and water *ad libitum*. All behavioral training was conducted in the afternoon (12am – 7pm) on mice aged 2-3 months. All efforts were made to minimize the number of animals used and their suffering during the experimental procedure in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and protocols were approved by the French Ethical Committee (DR2013-48 (vM)).

Experimental design.

Five experimental groups of mice were performed based on the number of odor pairs used for the enrichment (from 0 to 6 odor pairs; Figure 1). We assessed on these different groups of animals, the discrimination performances (using a habitution/dishabituation test), the adult-born cell survival (using Bromodeoxyuridine (BrdU) injections) and the neural plasticity using DsRed lentivirus injection at P0 for pre-existing neurons' labelling and GFP lentivirus injection at P60 for adult-born neurons' labelling followed by *ad hoc* post mortem fine morphology analysis. All mice were sacrificed 85 days after birth (Figure 1).

Behavioral experiments

Enrichment. Odor enrichment consisted in exposures to different pairs of odorants per day (1 to 6), with one odorant pair at a time, for 1 hour each during 10 consecutive days. For each pair, the two odorants 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 at least 1 hour. The different experimental groups were as follow: Group 1 was enriched with (+)limonene and (-)limonene ; Group 2 with (+)limonene and (-)limonene, decanal and dodecanone ; Group 3 with (+)limonene and (-)limonene, decanal and dodecanone, acetic acid and propionic acid; Group 4 with (+)limonene and (-)limonene, decanal and dodecanone, acetic acid and propionic acid, butanol and pentanol, propylacetate and butylacetate, (+)terminen-4-ol and (–)terpinen-4-ol (Figure 1 and 2A). Control mice were subjected to the same protocol and under the same conditions except that tea balls contained mineral oil.

Olfactory habituation/dishabituation test. We used a habituation/dishabituation test to assess olfactory discrimination (Moreno et al., 2009). Briefly, this task assessed whether 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 discriminated from the first, it would elicit an increased investigation response from the mouse. Each odorant presentation lasted 50 sec and was separated by 5 min. More specifically, we tested the discrimination between the two odorants of the pair(s) used for the enrichment. All experiments took place in a clean cage similar to the home cage. Odorants were presented by placing 60µL of the diluted odor (1Pa) (Figure 2A) onto a filter paper (Whatman #1) which was put inside a tea ball hanging from the side of the cage. A behavioral 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 time of each odor was recorded and a difference between Ohab4 and Otest indicated discrimination.

Statistical Analyses. All data were analyzed using R and Python. Preliminary tests including Shapiro-Wilk for normality checking and Mauchly's test for sphericity were performed on the

data resulting in the use of non-parametric tests. The data for each odor pair was analyzed by Friedmann tests on the habituation (Ohab1-4) to determine if the time of investigation during Ohab4 is significantly lower than during Ohab1 (habituation). Then to assess discrimination unilateral Wilcoxon tests were performed between Ohab4 and Otest. Only mice that had an investigation time of Ohab1>0s were included in the analysis (9 trials were excluded among 388 Ohab1 total trials). The level of significance was set to 0.05.

Adult-born neuron counts in the OB

BrdU injection. Mice were injected with BrdU (Sigma) (50 mg/kg in saline, three times at 2h intervals, i.p.), 9 days before the beginning of the enrichment period (27 days before sacrifice) in order to have a cohort of labeled adult-born cells in the OB at the beginning of the enrichment period (Figure 1).

Sacrifice. To investigate immediate-early-gene expression in response to the learned odorants, enriched mice were exposed to 100 µl of pure learned odorants (simultaneously for all odorants used during the enrichment period; i.e. 1, 2, 3 or 6 pairs) in tea balls during 1h. Control non-enriched mice were also exposed to 100µl of pure learned odorants (i.e. 0, 1, 2, 3 or 6 pairs) thus become the respective control of each enriched group of mice. Then, 1h after the end of odor stimulation, mice were deeply anesthetized (pentobarbital, 0.25 ml/30 g) and killed by intracardiac perfusion of 40-50 ml of fixative (4% paraformaldehyde in phosphate buffer, pH 7.4). Their brains were removed, postfixed, cryoprotected in sucrose (20%), frozen rapidly, and then stored at -20°C before sectioning with a cryostat (Reichert-Jung, NuBlock, Germany). Brains were sectioned at 14 and 40µm (3*14µm followed by 2*40µm sectioning) for cell counts and morphological analysis respectively.

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 (Sigma).

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 (Molecular Probes).

Cell analysis. All cell counts were conducted blind with regard to the mouse status. BrdU data was collected with the help of a mapping software (Mercator Pro, Explora Nova), coupled with a Zeiss microscope. BrdU-positive cells were counted in the entire granule cell layer of the OB on about 9 sections (14µm thick, 216 µm intervals) of 5-8 mice/group. The number of positive cells was divided by the surface of the granule cell layer to yield the total densities of labeled cells (labeled profiles/μm^2). Densities were compared between experimental groups using a one-way ANOVA followed by Tukey *post-hoc*.

Triple labelled sections were examined using pseudo-confocal scanning microscopy equipped with an Apotome (Zeiss). BrdU-positive cells were examined for colabeling with NeuN and Zif268 (30 cells/animal, n=3-9 animals/group). After normality checking using Shapiro-Wilk

tests, the data was analyzed by two way ANOVA (group and odorant stimulation before sacrifice as factors) followed by regressions analysis in order to demonstrate the enrichment effect as complexity increases or *post-hoc t*-tests to compare the enriched groups with their respective controls (not enriched but stimulated in the same way 1 hour before sacrifice, see above).

Neuronal morphology analysis

***Preexisting neuron labeling*.** At P0, mice were anesthetized on ice and injected in the lateral ventricle with 1 µl of Lenti-PKG-DsRed lentiviruses (6.11 x 10^8 UI/ml, provided through Addgene #12252 by the Trono Lab). The injection was done using a glass-micropipette linked to a Hamilton syringe connected to a programmable syringe controller (infusion rate: 0.2 µL/s).

Adult-born neuron labeling. Prior to surgery, 2 month old mice were anaesthetized with an intraperitoneal cocktail injection of 50 mg/kg ketamine and 7.5 mg/kg xylazine and secured in a stereotaxic instrument (Narishige Scientific Instruments, Tokyo, Japan). 100 nl of Lenti-PGK-GFP lentiviruses (2 x 10^9 UI/ml, provided through Addgene #12252 by the Trono Lab) were injected bilaterally into the subventricular zone (with respect to the bregma: AP, +1 mm; ML, ± 1; DV, −2.3 mm) at a rate of 150nL/min. Mice received antalgic ketoprofen (2 mg/kg) at the end of surgery and once every day until they regained the lost weight. Mice were allowed 8 days to recover from the surgery 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. Triple labeling was performed by using chicken anti-GFP (1:1000, Anaspec), mouse anti-DsRed (1:200, Santa Cruz Biotechnology) and 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 or Alexa 546 for Zif268 revelation were used.

Morphological data analysis. For each neuron, we first determined whether it expressed Zif268 (responsive neuron) thanks to images taken at both 40x and 63x objectives (lateral and *z*-axis resolutions were respectively 160 and 280 nm at 40x and 100 and 200 nm at 63x). Then, spine density on apical and basal dendrites were assessed on images taken at 100x objective using measurement tool (lateral and *z*-axis resolutions were 60 and 200 nm respectively). For the analysis of dendritic arborizations, images were taken with 20x objective (lateral and *z*-axis resolutions were 320 and 400 nm) and 40x objectives. The length of all the distal dendritic segments of the apical domain, the proximal dendritic segment of the apical domain or the basal domain were measured based on previous papers (Kelsch et al., 2008).

Neurons reconstruction was performed using Vias (http://research.mssm.edu/cnic/tools-vias.html) and NeuronStudio software (Rodriguez et al., 2003; Wearne et al., 2005; Scotto-Lomassese et al., 2011)(http://research.mssm.edu/cnic/tools-ns.html). This software allows three dimensional (3D) reconstructions of dendrites and spines from confocal z-series stacks on a spatial scale. Dendritic length was measured semi-automatically and spine lengths were manually assessed with the help of the 3D reconstruction. All morphological analyses were conducted blind with regard to the mouse's group as well as the neuron activation (Zif268-positive or -negative). Images were acquired using Zeiss pseudo-confocal system. Numbers of neurons per group, of neurons belonging to Zif268 sub-categories as well as animal per group are presented in Supplementary Table 1 and 2.

Statistical analysis. Morphological data (spine density and dendritic length) were compared using two-way ANOVA with groups and Zif268 expression as factors followed by *post-hoc* t-

tests using Holm-Bonferroni correction (Aickin and Gensler, 1996) comparing groups within Zif268 activity as well as t-tests comparing Zif268 activity within groups.

Optogenetic in freely behaving mice.

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

Surgery. Mice were injected with pLenti-hSyn-eNpHR3.0-EYFP lentivirus (9.22 x 10^6 IU/ml) (Kermen et al., 2016) at P0 or P60 similarly as above for GFP or DsRed injections (paragraph # 5 of Material and Methods). At P60 all mice were implanted with bilateral optic fibers (200 nm core diameter, 0.22 N.A., Doric Lenses) in the OB, with the following coordinates respective to bregma: antero-posterior +4.6mm, medio-lateral ±0.75mm, dorso-ventral -2mm. Mice were allowed a recovery period of 8 days as described above.

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

Control of light-triggered inhibition. At P106 days (29 days after the end of the enrichment, and just after the end of the post-test), mice were sacrificed in the same way as previously described, with the exception that mice were selected to be either odorants- and light-stimulated while the others were only stimulated with the odorants without light. When present, the light stimulation

followed a pattern mimicking the average light stimulation pattern during the Otest session (0.5s light ON, 2s light OFF, mimicking the averaged light stimulation received during the test trial, repeated over 1h). After brain sectioning (see above), EYFP and Zif268 double immunohistochemistry was performed as described previously (Kermen et al., 2016). GFP-positive Zif268-positive cells were counted in the OB on 1 section covering 14µm just under the injection site to allow assessment of the inhibition.

Results

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

We assessed here whether mice are able to discriminate from 1 to 6 pairs of odorants using a habituation/dishabituation test. Five experimental groups were constituted: non-enriched (NE), enriched with 1 odor pair (Group 1), enriched with 2 odor pairs (Group 2), enriched with 3 odor pairs (Group 3) and enriched with 6 odor pairs (Group 4) (Figure 1). Significant habituation for all groups was observed as evidenced by the reduction in investigation time across trials (Friedman tests, Table 1; Supplementary Figure 1).

Regarding discrimination, all enriched groups showed changes in their ability to discriminate the odorants used for the enrichment. Animals enriched with (+)limonene and (–)limonene (Group 1) are now able to discriminate between these two enantiomers (Figure 2Ai and Table 1). Animals enriched with a slightly more complex environment containing (+)limonene and (-)limonene plus decanal and dodecanone (Group 2) demonstrated the ability to discriminate between the 2 odorants of each pair (Figure 2Aii, Table 1). Going further, animals enriched with the two previous pairs plus a third one, acetic acid and propionic acid, also showed discrimination between the 2 odorants of the three pairs (Figure 2Aiii, Table 1). Finally the most complex

enrichment (6 pairs) allowed mice to discriminate between the odorants of all 6 pairs (Figure 2Aiv, Table 1). Importantly, the control group showed no improvement in discrimination abilities between any of the tested odorant pairs (Figure 2Av, Table 1).

These results showed that increasing the number of odorants used for enrichment enhances the number of discriminated odorants and that mice are able to learn to discriminate at least until 6 odor pairs at a time.

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

The question addressed here is whether increasing the complexity of perceptual learning will increase the number of surviving adult-born cells. 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. We assessed adult-born cells in the granule cell layer since the number of adult-born glomerular interneurons was previously found not to be modulated by perceptual learning (Moreno et al., 2009). BrdU-positive cell density was assessed in the five experimental groups (control NE, Group 1, Group 2, Group 3 and Group 4). We found a significant group effect (one-way-ANOVA: $F_{(4,25)}= 4.77$; $p=0.005$; Figure 3). More specifically, BrdU-positive cell density increased in all enriched groups compared to the non-enriched one (Tukey *post hoc* test - Group1 versus NE: t=3.27, p=0.023; Group2 versus NE: t=3.70, p=0.0086; Group3 versus NE: t=3.06, p=0.038; Group4 versus NE: t=3.55, p=0.01). However, no significant difference between enriched groups were observed (all comparisons: p>0.9) indicating that the density of surviving adult-born cells is not dependent on the complexity of the task.

Increasing the complexity of perceptual learning enhanced the functional recruitment of adult-born cell neurons.

Since we found no increase in the density of adult-born cell surviving when the enrichment became more complex, we hypothesized that a higher proportion of the surviving adult-born neurons would be recruited to process the learned odorants as the task becomes more complex. To test this hypothesis, we thus assessed the percentage of adult-born granule cells expressing Zif268 in response to the learned odorants as an index of cellular activation. To this aim, we exposed the animals on the day of sacrifice to their respective learned odorants ((+)limonene/(-)limonene for Group 1; (+)limonene/(-)limonene plus decanal/dodecanone for Group 2, (+)limonene/(-)limonene, decanal/dodecanone, acetic acid/propionic acid for Group 3; (+)limonene/(-)limonene, decanal/dodecanone, acetic acid/propionic acid, propyl-acetate/butyl-acetate, butanol/pentanol, (+)terpinen-2-ol/(-)terpinen-2-ol for Group 6). In addition, we performed corresponding control groups that were stimulated with the same odorants on the day of sacrifice but were not beforehand enriched with them.

First, we observed that the responsiveness of adult-born neurons to olfactory stimulation was increased in enriched when compared to non-enriched animals (two way ANOVA on BrdU/Zif268/NeuN-positive cells: enrichment factor: $F_{(4,35)}=10.70$, $p=0.000009$; number of stimulating odors factor: $F_{(4,35)}=0.56$, $p=0.70$; no interaction) (Figure 4). Indeed, all enriched groups displayed more BrdU-positive cells expressing Zif268 than their respective non enriched controls indicating that more adult-born neurons responded to the odorants after learning (unilateral t-tests ; Group 1 versus its respective control: $t=-2.05$, $p=0.034$; Group 2 versus its respective control: $t=-2.18$, $p=0.033$; Group 3 versus its respective control: $t=-3.20$, $p=0.005$; Group 4 versus its respective control: $t=-5.30$, $p=0.0009$; Figure 4).

In addition, the more complex the enrichment is, the more BrdU/Zif268-positive cells are retrieved in enriched groups (enriched groups: linear regression: adjusted-R^2=0.35, p=0.003 and control groups: linear regression: adjusted-R^2=-0.20, p=0.81). Since all control non-enriched groups stimulated with a growing number of odorants from group 1 to group 4 present the same percentage of double labeled cells, the positive regression observed between the proportion of activated cell and complexity cannot be ascribed to to the number of odorants used for stimulation the day of sacrifice but rather comes from the number of odorants used for learning.

We then tested whether this result could simply be due to a different duration of odor enrichment between groups (1 hour per day for group 1 versus 6 hours per day for group 4). To test this, we enriched a new group of mice (n=10) with (+)limonene and (-)limonene but this time 6* 1hour per day during 10 days (as for group 4). The percentage of Brdu/Zif268-positive cell is not different from that in group 1 (supplementary Figure 2; unilateral t-test: t=-1.00, p=0.82) suggesting that the diversity of the odorants presented during the enrichment and not the duration of the presentation impacts adult-born neuron recruitment.

Finally, to ensure that the increased in BrdU/Zif268 co-labelling resulted from increased odor responsiveness and not from a change in the basal level of BrdU/Zif268 co-expression in the enriched groups, we performed an additional control group (n=5), in which we assessed BrdU/ Zif268 co-labelling in basal condition (without odor stimulation) in animals enriched with six odorant pairs and compared to co-labelling in non-enriched animals. We did not find any difference between these two experimental groups (unilateral t-test: t=0.32, p=0.38; supplementary Figure 3) suggesting that the observed change in BrdU-positive/Zif268-positive level is due to neurons that respond specifically to the learned odorants and not to an overall increase in adult-born granule cell activity in enriched OB.

We observe no effect of enrichment on the rate of neuronal differentiation of adult-born cells as evidenced by the absence of modulation of the percentage of BrdU/NeuN-positive cells (two way ANOVA: enrichment effect $F_{(4,35)}=1.32$; $p=0.48$; stimulation effect: $F_{(4,35)}=0.66$, $p=0.62$; Supplementary Figure 4).

All together, these results indicate that even if a plateau is reached in the number of adult-born neurons saved by learning, the pool of surviving granule cells is increasingly functionally recruited in the processing of the learned odorants as the complexity of learning increases.

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

To determine whether perceptual learning has an effect on adult-born neuron morphology, we have injected a lentivirus expressing GFP at P60 in the sub-ventricular zone of the mice. We have analyzed dendritic arborization in the apical (the site of interactions with mitral/tufted (M/T) cells) and basal (the site of input of centrifugal fibers) domains and at a finer level, the spine density (Figure 5) (Kelsch et al., 2010). In addition, 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 (Zif268-positive or Zif268-negative neurons) as an index of activation.

Learning affected neither the primary apical dendrite length (two way ANOVA ; group effect: $F_{(4,189)}=1.44$, $p=0.22$; Zif268 effect: $F_{(1,189)}=0.81$, $p=0.37$; interaction: $F_{(4,189)}=0.61$, $p=0.66$; Figure 5A), the apical arborization length (two way ANOVA ; group effect: $F_{(4,188)}=2.16$, $p=0.076$; Zif268 effect: $F_{(1,188)}=0.67$, $p=0.41$; interaction: $F_{(4,188)}=2.36$, $p=0.055$; Figure 5B) nor the basal arborization length (two way ANOVA ; group effect: $F_{(4,181)}=0.17$, $p=0.95$; Zif268 effect: $F_{(1,181)}=0.0001$, $p=0.99$; interaction: $F_{(4,181)}=0.14$, $p=0.97$; Figure 5C). However, we

found that learning increased the spine density in the apical distal (two way ANOVA ; group effect: $F_{(4,197)}$=9.24, p=0.0000007 ; Zif268 effect: $F_{(1,197)}$=8.66, p=0.0036 : interaction: $F_{(4,197)}$=2.29, p=0.061; Figure 5D); apical proximal (two way ANOVA ; group effect: $F_{(4,150)}$=3.73, p=0.0064; Zif268 effect: $F_{(1,150)}$=4.2, p=0.042: interaction: $F_{(4,150)}$=0.55, p=0.69; Figure 5E) and basal domains (two way ANOVA ; group effect: $F_{(4,214)}$=5.44, p=0.00035; Zif268 effect: $F_{(1,214)}$=8.7, p=0.0035: interaction: $F_{(4,214)}$=1.40, p=0.23; Figure 5F).

A closer analysis of the apical domain revealed that the increase of spine density is observed for Zif268-positive neurons in Group1 and Group 2 (*post-hoc* t-test with Holm correction ; Zif268+ neurons: Group 1 versus NE p=0.029, Group 2 versus NE p=0.028 ; Zif268- neurons: Group1 versus NE p=0.12, Group 2 versus NE p=0.15 and intra-group t-test: NE Zif268+ versus Zif268- p=0.3, Group1 Zif268+ versus Zif268- p=0.0082, Group 2 Zif268+ versus Zif268- p=0.014) while in Group 3 and Group 4, the increased spine density is observed for both Zif268-positive and -negative neurons (*post-hoc* t-test with holm correction ; Zif268+ neurons: Group 3 versus NE p=0.031, Group 4 versus NE p=0.029 ; Zif268- neurons: Group 3 versus NE p=0.00004, Group 4 versus NE p=0.00003 ; and intra-group t-test: Group 3 Zif268+ versus Zif268- p=0.64, Group 4 Zif268+ versus Zif268- p=0.58; Figure 5D).

The same phenomenon is observed at the basal domain, with a higher level of spine density in Zif268-positive neurons for Group 1 and 2 (*post-hoc* t-test with holm correction ; Zif268+ neurons: Group 1 versus NE p=0.026, Group 2 versus NE p=0.032 ; Zif268- neurons: Group 1 versus NE p=0.65, Group 2 versus NE p=0.65 and intra-group t-test: NE Zif268+ versus Zif268- p=0.92, Group 1 Zif268+ versus Zif268- p=0.072, Group 2 Zif268+ versus Zif268- p=0.018) while for Group 3 and 4 this is observed for both Zif268-positive and -negative neurons (*post-hoc* t-test with holm correction ; Zif268+ neurons: Group 3 versus NE p=0.039, Group 4 versus NE p=0.001; Zif268- neurons: Group 3 versus NE p=0.01, Group 4 versus NE p=0.009; and intra-

group t-test: Group 3 Zif268+ versus Zif268- p=0.45, Group 4 Zif268+ versus Zif268- p=0.43; Figure 5F).

At the apical proximal domain, we observed an increased spine density only in Group 3 and 4's Zif268-positive neurons compared to NE neurons (*post-hoc* t-test with holm correction ; Zif268+ neurons: Group 1 versus NE p=1.0, Group 2 versus NE p=0.44, Group 3 versus NE p=0.03, Group 4 versus NE p=0.048; Zif268- neurons: Group 1 versus NE p=1.0, Group 2 versus NE p=1.0, Group 3 versus NE p=0.26, Group 4 versus NE p=1.0; and intra-group t-test: NE Group Zif268+ versus Zif268- p=0.88, Group 1 Zif268+ vers Zif268- p=0.18, Group 2 Zif268+ versus Zif268- p=0.44, Group 3 Zif268+ versus Zif268- p=0.22, Group 4 Zif268+ versus Zif268- p=0.38; Figure 5E).

These results indicate that when the behavioral demand was relatively low (enrichment with one and two odor pairs), only Zif268-positive neurons showed increased spine density in the apical distal and basal domains while when the behavioral demand was increased (enrichment with 3 and 4 odor pairs), both Zif268-positive and -negative neurons showed changes in their fine morphology. Thus as the behavioral demand increases, so do the morphological changes, albeit not in a linear manner, not uniformly across the different dendritic domains and in a less specific manner with regards to learned odor processing neurons.

Perceptual learning induces limited morphological changes in preexisting neurons

To investigate the morphology of neurons born in the OB during ontogenesis, a lentivirus expressing DsRed had been injected at P0 in the ventricle of the same animals in which we analyzed adult-born neurons. We found no morphological difference between the different groups regarding the primary apical dendrite length (two way ANOVA ; group effect:

$F_{(4,194)}$=1.75 , p=0.14 ; Zif268 effect: $F_{(1,194)}$=0.0 , p=0.1 ; interaction: $F_{(4,194)}$=0.81, p=0.52; Figure 6A), the apical arborization length (two way ANOVA ; group effect: $F_{(4,193)}$=3.26 , p=0.012 ; Zif268 effect: $F_{(1,193)}$=4.9, p=0.028 ; interaction: $F_{(4,193)}$=1.04, p=0.39 ; but all relevant *post-hoc* comparisons revealed p>0.5; Figure 6B), the basal arborization length (two way ANOVA ; group effect: $F_{(4,157)}$=0.5, p=0.74 ; Zif268 effect: $F_{(1,157)}$=0.0022, p=0.96 ; interaction: $F_{(4,157)}$=0.58, p=0.68) (Figure 6C), the proximal spine density (two way ANOVA ; group effect: $F_{(4,116)}$=3.55, p=0.0091 ; Zif268 effect: $F_{(1,116)}$=3.87, p=0.052; interaction: $F_{(4,116)}$=0.32, p=0.86 ; but all relevant *post-hoc* comparisons revealed p>0.5; Figure 6E) nor the basal spine density (two way ANOVA ; group effect: $F_{(4,107)}$=1.17, p=0.33; Zif268 effect: $F_{(1,107)}$=0.27, p=0.61 ; interaction: $F_{(4,107)}$=1.92, p=0.11; Figure 6F). However, an increase of spine density of the apical distal domain was observed in Group 3 and Group 4 compared to non-enriched animals (two way ANOVA; group effect: $F_{(4,183)}$=4.56, p=0.0016; Zif268 effect: $F_{(1,183)}$=0.038, p=0.84; interaction: $F_{(1,183)}$=0.57, p=0.68 and one way ANOVA on group factor followed by Tukey *post-hoc* test: Group 3 versus NE p=0.015 ; Group 4 versus NE p=0.024; Figure 6D).

All together, these results indicate that the morphology of adult-born neurons is modified by a simple perceptual learning while the morphology of preexisting neurons is modified only when the learning gets complex. Based on these data, we hypothesized that simple perceptual learning requires adult-born neurons plasticity while complex perceptual learning requires modifications of both adult-born and preexisting neurons. To test this, we performed an optogenetic experiment to silence the different populations of neurons (adult-born versus preexisting) during the post learning test in animals submitted to the simplest versus the most complex task.

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

Here, we hypothesized that inhibiting adult-born neurons after simple and complex perceptual learning would alter mice learned discrimination while inhibiting preexisting neurons would only affect the expression of learned discrimination following the complex task.

To test this hypothesis, two new groups of mice (n=11 and n=14) were formed. The first group was infused with halorhodospin lentivirus in the subventricular zone at P60 to transduce and then silence adult-born neurons and the second group of mice was infused with the same virus in the ventricle at P0 to transduce and then silence preexisting neurons (Figure 7). Each group was further divided between mice performing simple or complex perceptual learning. As previously, mice discrimination was tested after learning using habituation/dishabituation tests. Optogenetic inactivation of transduced neurons was achieved in freely behaving mice during the test trial of the habituation/dishabituation paradigm by automatically triggering a light stimulation each time the animal approached the odorized source. All mice were also tested in control condition in which the light remained OFF during the entire test trial. All mice showed habituation (Figure 8, Table 2).

Regarding the discrimination, we found that inhibiting adult-born neurons abolishes the improvement of discrimination induced by simple and complex learning (Figure 8; Table 3). More specifically, animals enriched with (+)limonene/(-)limonene are able to discriminate these two odorants in the light OFF condition but not when light-triggered inactivation of adult-born neurons occurs (Figure 8B; Table 3). The same phenomenon is observed for complex leaning: animals are able to discriminate the 6 odor pairs learned in the light-OFF condition but this is no

longer observed with the light-ON(Figure 8D; Table 3). We assessed the level of viral transduction by first analyzing the density of GFP-positive cells in the OB under the optical fibers and found the same level of transduced neurons in the groups submitted to simple and complex learning (t-test: p=0.098; Supplementary Figure 6A). Second, the effectiveness of light-mediated adult-born granule cell inhibition was assessed by counting Zif268-positive cells among GFP-positive cells in the same region. As expected, a decrease of the percentage of GFP/Zif268-positive cell was observed in the light-ON condition compared to light OFF revealing the efficiency of the inhibition of transfected neurons by light (t-test: complex learning p=0.043 ; Supplementary Figure 6B).

Light-triggered inactivation of preexisting neurons did not alter discrimination of the learned odorants after simple perceptual learning (Figure 8A, Table 3). However, inhibiting preexisting neurons after complex learning mostly impaired discrimination abilities (Figure 8C, Table 3). The level of viral transduction was also analyzed in preexisting neurons. The density of GFP-positive cell in the OB was similar between simple and complex learning (t-test p=0.13; Supplementary Figure 6C) and a decrease of the percentage of GFP/Zif268-positive cell was observed in light-ON condition compared to light OFF revealing, in that case, the efficiency of the inhibition of preexisting neurons by light (t-tests: simple learning p=0.048, complex learning: p=0.015, Supplementary Figure 6D).

Notably, for the inhibition of both preexisting and adult-born neurons, whether after simple or complex learning, the discrimination of dissimilar odorants was not altered (Supplementary Figure 5, supplementary table 3).

Discussion

Perceptual learning contributes to the representation of the sensory environment which guides the animal's behavior. An essential component of the plasticity of this representation in the olfactory system is the adult neurogenesis, consisting in a renewal of interneurons within a preexisting neuronal network. Perceptual learning not only increases adult-born neuron survival but requires them (Moreno et al., 2009). In this study, we did not find a higher level of surviving adult-born cells when enhancing learning complexity, suggesting that a plateau of adult-born cell survival is reached after simple perceptual learning. Furthermore, among the surviving adult-born neurons after perceptual learning, only a portion of them responds to the learned odorants (Moreno et al., 2009; Mandairon et al., 2011; Sultan et al., 2011). Our finding show a positive linear relationship between the proportion of adult-born neurons expressing Zif268 in response to olfactory stimulation and the number of discriminated pairs of odorants. Interestingly, the learning-dependent survival increase reported here is close to the rate of adult-born neurons saved by different olfactory learning paradigms (associative, perceptual; (Alonso et al., 2006: +45% ; Mouret et al., 2008: +31% ; Moreno et al., 2009: +40% ; Kermen et al., 2010 +50% ; Sultan et al., 2010 +55% ; Mandairon et al., 2011 +40% ; this study: +42%) suggesting that survival increase might not be specific to a task but simply a correlate of environmental modifications. Thus learning type or environmental change complexity is not coded by adult-born neurons survival rate. The increased pool of neurons saved is done in a very non-specific manner. However our results strongly suggest that the information about the complexity could be carried by functionally recruiting more adult-born neurons from the pool of available ones.

Beyond the number of surviving cells or the number of adult-born neurons activated, another parameter of plasticity shown to be involved in the improvement of discrimination is the

structural plasticity of adult-born neurons (Daroles et al., 2015). Cell activity is a major regulator of neuronal morphology (Lu, 2003; Kelsch et al., 2010). In line with this, we first found that simple and complex learning increased the density of dendritic spines at the apical domains as well as at the apical proximal and basal domains of adult-born granule cells. Optogenetically inhibiting these neurons prevented discrimination after simple learning (in accordance with Moreno et al., 2009) and also after complex learning. We also report that the morphology of preexisting cells is not modified by simple olfactory learning but an increase of spine density can be observed at the apical domain after complex olfactory learning. Importantly, these modifications seem to correlate what we saw when inhibiting preexisting neurons which only prevented discrimination of the learned odorants after complex but not simple learning. Knowing that there is a direct causal link between morphological modification and learning (Daroles et al., 2015) we can hypothesize that when morphological modifications occur, they are underlying the discrimination changes observed at the behavioral level. There are several ways these morphological modifications could underlie learning. First, apical dendrites are the site of reciprocal dendrodendritic synapses between granule and mitral cells (Price and Powell, 1970; Shepherd, 1972), thus a higher number of spine could provide the elevation of the inhibitory drive on mitral cells seen elsewhere (Moreno et al., 2009) and explain why the absence of these same cell or simply the absence of their activity prevents discrimination (Moreno et al., 2009, this study). Second, the apical proximal and basal domain both receive cortico-bulbar projections from the olfactory cortices (Nissant et al., 2009; Lepousez et al., 2014) as well as neuromodulatory inputs such as noradrenergic, cholinergic and serotoninergic fibers (Fletcher and Chen, 2010). Because the noradrenergic system acts on adult-born cells to allow olfactory perceptual learning (Moreno et al., 2012) and gate dendrodendritic inhibition onto mitral cells (Balu et al., 2007) while not being exclusive of other influences such as cholinergic (Ravel et al.,

1994; Linster and Cleland, 2002, 2016; Mandairon et al., 2006a; de Almeida et al., 2013), this increase in basal inputs could modulate granule cell activity.

Hence, as complexity increases, spine density modifications (both apical and basal) probably lead to a remapping of the synaptic weights which by having a synergic effect with the linear increased involvement of adult-born neurons would underlie behavioral discrimination that gets more and more complex. This could be mediated by a refinement of the representations of each odorant of each pair, refinement done by an increased inhibition onto mitral cells serving as a mean of decorrelation.

The modifications of both basal and apical proximal spine densities observed here are similar to a previous study showing that olfactory associative learning could increase the excitatory drive from the olfactory cortex onto adult-born granule cells (Lepousez et al., 2014). In contrast though, we did observe changes in spine density at the apical distal domain. One possible explanation for this difference lies on the nature of the task (associative learning versus a perceptual learning). Indeed it has been shown that operant or non-operant learning tasks have distinct neurogenic correlates (Mandairon et al., 2011) as well as induce distinct changes in odor representation (Chu et al., 2016).

It has been proposed that adult-born neuron could confer to the OB network, two distinct but complementary forms of circuit plasticity: an early form based on individual neuron survival and a later form based on the fine tuning of synaptic strength (Livneh et al., 2014), The present experiment shows that preexisting neurons could also be recruited to add a third level of plasticity into the network, more particularly during complex learning when adjustments of the network supported by adult-born neurons are not sufficient to answer to the environmental demand.

Interestingly, for complex learning, in addition to Zif268-positive neurons, Zif268–negative neurons unexpectedly demonstrated changes in dendritic spine density. This is true for adult-born and preexisting neurons. Activity being a major regulator of structural morphology (Kelsch et al., 2009; Je et al., 2011) in part via neurotrophic factor release (Lu, 2003), that might induce non-specific morphological changes in the surrounding neurons.

In conclusion, as learning becomes more complex, an overall larger neural network is involved requiring both more and more functionally implicated adult-born neurons presenting morphological adaptations and at one point the recruitment of preexisting neurons showing morphological modifications in order to answer the behavioral demand. Hence this shows at the same time some limitations of adult-born neurons plastic abilities as well as their essential pillar role in bulbar adaptation. Both neuronal populations are highly complementary and together confer the OB high plasticity and stability to answer any behavioral demands no matter it complexity.

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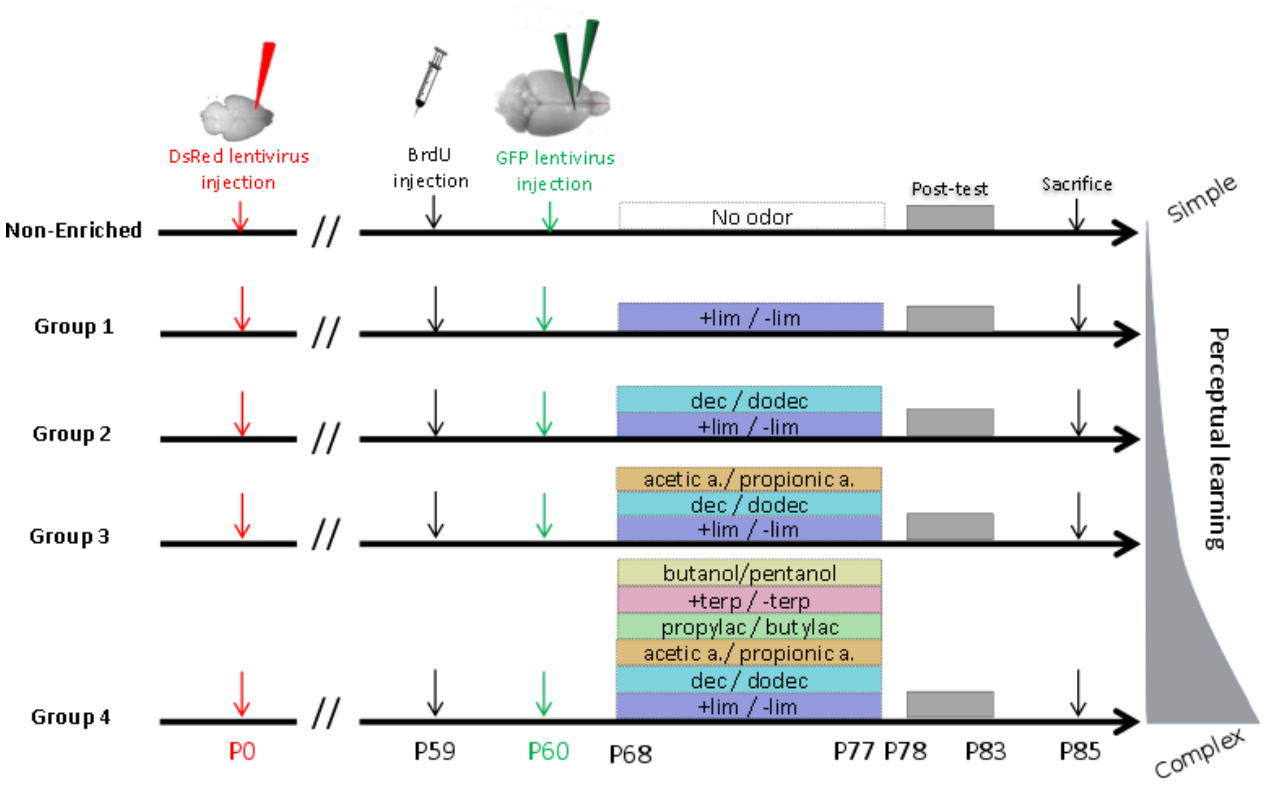
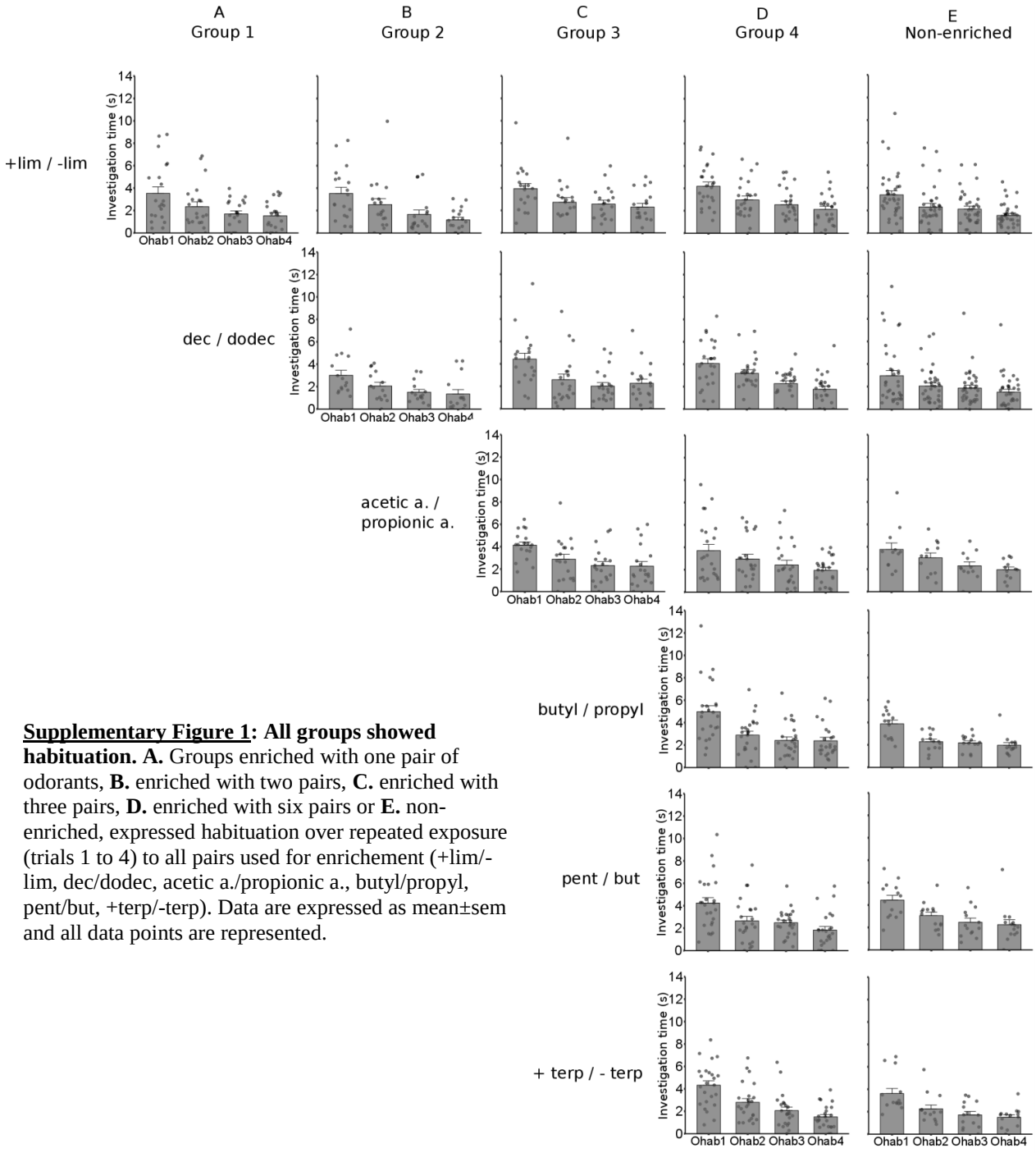


Figure 1: Experimental design.
In mice, preexisting neurons (born during ontogenesis) were labelled by injecting DsRed lentivirus in the lateral ventricle at post-natal day 0 (P0) and adult-born neurons were labelled by injecting BrdU (i.p.) at P59 and GFP lentivirus in the subventricular zone at P60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



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

Group	Odorant	n	Habituation			Discrimination		
			Friedman		Difference	Wilcoxon (1 tail, paired)		Difference
Non Enriched	+lim / -lim	35	X=30,58; df=3	P=0,000001	***	V=260,5	p=0,36	ns
	dec / dodec	35	X=18,22; df=3	P=0,0004	***	V=293	P=0,47	ns
	acetic a. / propionic a.	12	X=12,4; df=3	P=0,0061	***	V=26	P=0,17	ns
	butyl / propyl	14	X=16,6; df=3	P=0,00086	***	V=64	P=0,77	ns
	pent / but	14	X=16,46; df=3	P=0,000909	***	V=50	P=0,45	ns
	+terp / -terp	14	X=22,17; df=3	P=0,00006	***	V=42,5	P=0,28	ns
Group 1	+lim / -lim	21	X=16,72; df=3	P=0,0008	***	V=57	P=0,022	*
Group 2	+lim / -lim	18	X=24,39; df=3	P=0,000021	***	V=18	P=0,0052	**
	dec / dodec	15	X=19,88; df=3	P=0,00018	***	V=56	P=0,028	*
Group 3	+lim / -lim	19	X=12,03; df=3	P=0.0073	***	V=36	P=0,008	**
	dec / dodec	20	X=23,76; df=3	P=0,000028	***	V=53	P=0,027	*
	acetic a. / propionic a.	19	X=20,05; df=3	P=0,00017	***	V=52	P=0,044	*
Group 4	+lim / -lim	24	X=14,55; df=3	P=0,0022	***	V=80	P=0,023	*
	dec / dodec	24	X=20,95; df=3	P=0,0001	***	V=39	P=0,0024	**
	acetic a. / propionic a.	23	X=12,03; df=3	P=0,0073	***	V=65	P=0,024	*
	butyl / propyl	24	X=26,063; df=3	P=0,0000093	***	V=65,5	0,0082	**
	pent / but	24	X=19,75; df=3	P=0,00019	***	V=55	P=0,0061	**
	+terp / -terp	24	X=28,21; df=3	P=0,0000033	***	V=45,5	P=0,0045	**

Table 1: Statistics on habituation and discrimination performances.

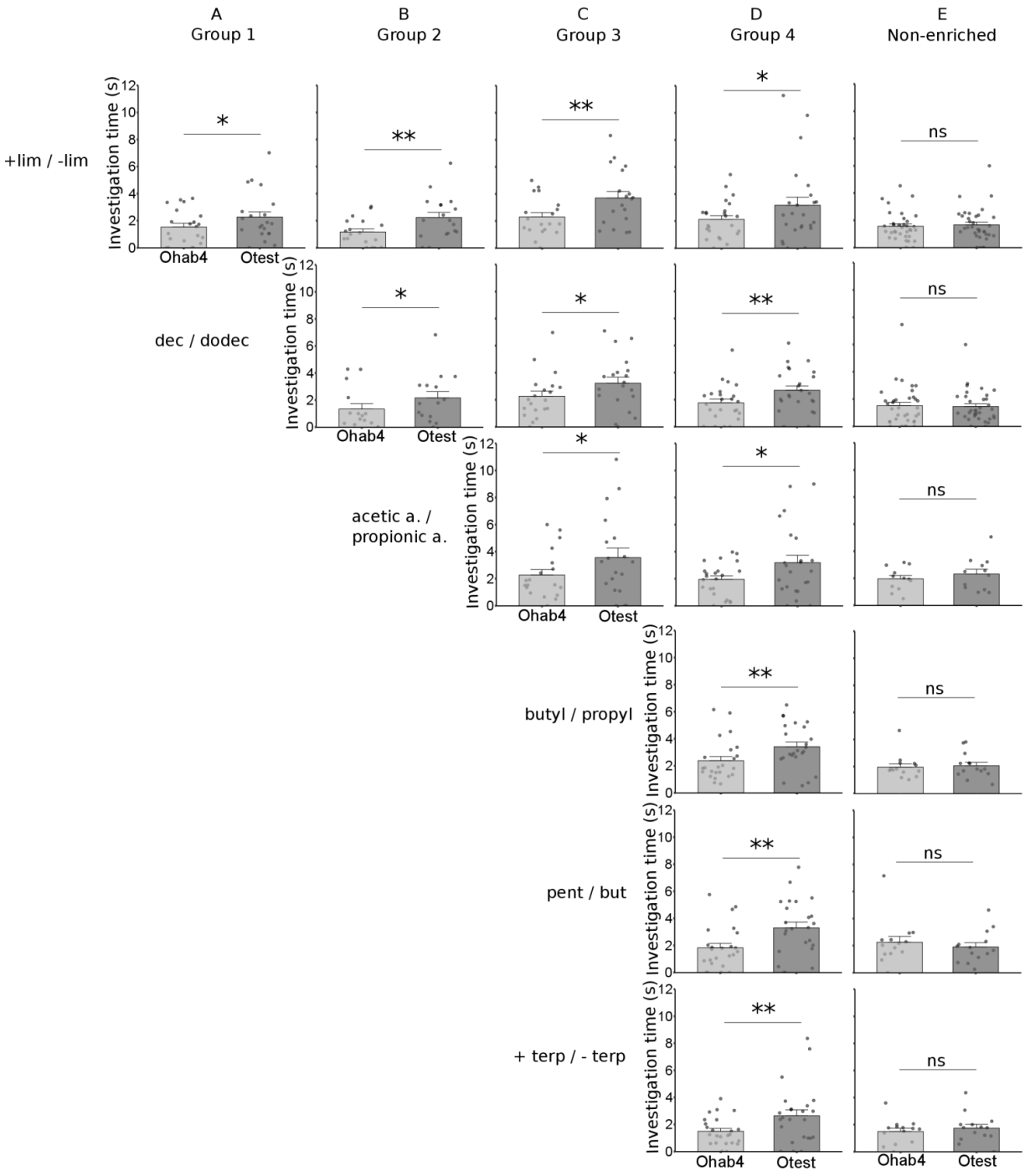


Figure 2: Increasing the complexity of perceptual learning leads to the discrimination of more odor pairs. Behavioral results. Only discrimination trials (trial 4, Ohab4 and trial 5, Otest) are represented. **A.** After **e**nrichment with +lim and –lim (group 1), the investigation time of test trial is superior to hab4 trial indicating discrimination between these 2 odorants. **B.** A **s**imilar result is observed after enrichment with +lim/-lim and dec/dodec (group 2) indicating discrimination between the 2 odorants of the 2 pairs. **C.** Enrichment with +lim/-lim, dec/dodec and ac/prop allows discrimination between the 2 odorants of the 3 pairs. **D.** Enrichment with six pairs of odorants, i.e. +lim/-lim, dec/dodec, ac/prop, propyl/butyl, pent/but, +terp/-terp, allows discrimination between the 2 odorants in all six pairs. **E.** In control non-enriched animals (NE group), no difference is observed between OHab4 and Otest, for any of the six odor pairs (+lim/-lim ; dec/dodec ; ac/prop ; propyl/butyl ; pent/but ; +terp/-terp) indicating no discrimination. *p<0.05. **p<0.01. Data are expressed as mean±sem and all data points are represented.

Odorant	Dilution	Abbreviation
(+)limonene	0.204%	+lim
(-)limonene	0.204%	-lim
decanal	1.776%	dec
2-dodecanone	124.98%	dodec
acetic acid	0.007%	acetic a.
propionic acid	0.0408%	propionic a.
propyl-acetate	0.0062%	propyl
butyl-acetate	0.0202%	butyl
pentanol	0.074%	penta
butanol	0.021%	buta
(+)terpinen-2-ol	6.631%	+terp
(-)terpinene-2-ol	6.631%	-terp

Table 2: Odorants table. All odorants were diluted in mineral oil in order to reach the vapor pressure of 1Pa. All odorants abbreviations are described here for future references.

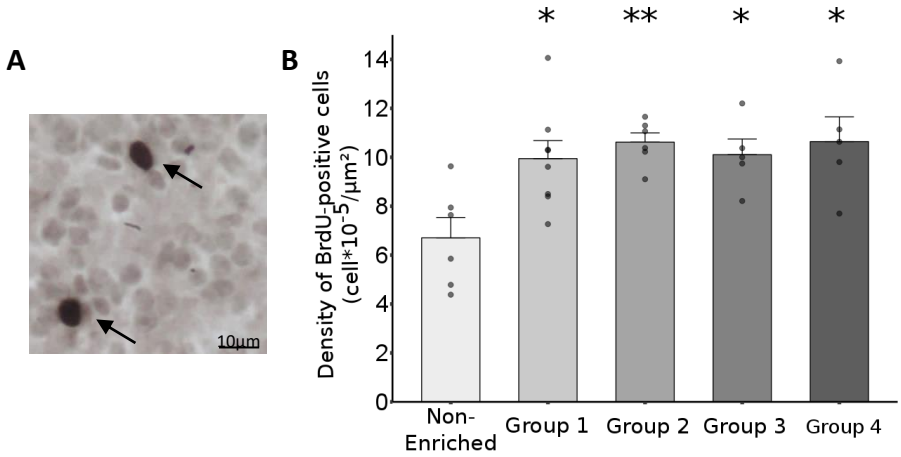
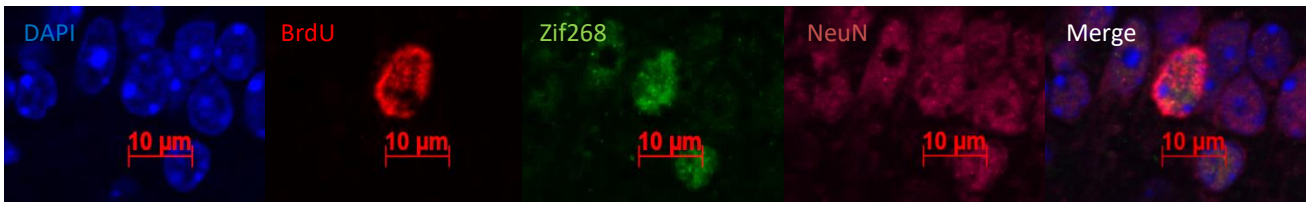
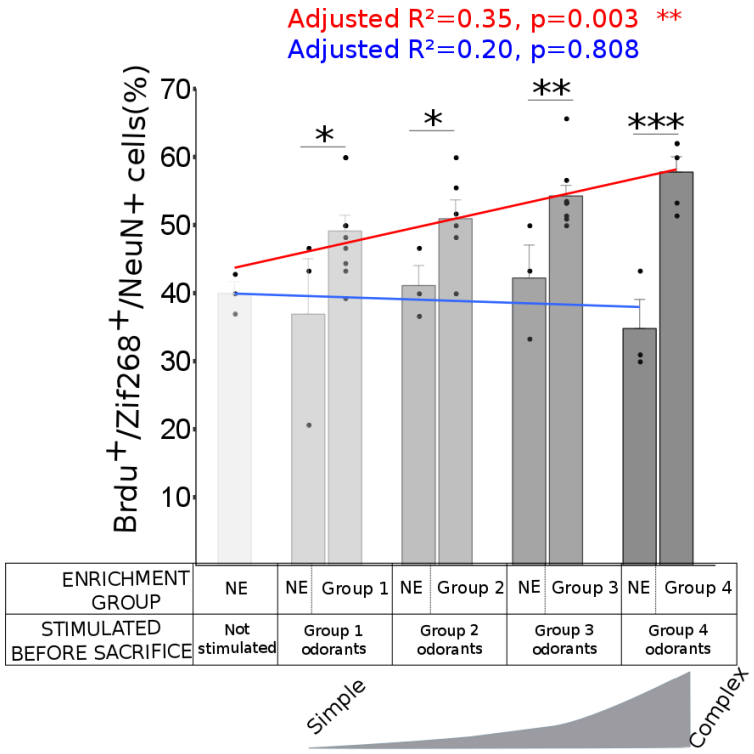


Figure 3: Perceptual learning increased adult-born cell density independently of the enrichment complexity.
A. Example of BrdU-positive immunolabelled cells (arrows). Bar = 10µm. **B.** Enrichment with one odor pair (group 1, n=8), two odor pairs (group 2, n=6), three pairs (group 3, n=5) or six pairs (group 4, n= 5) significantly increased BrdU-positive cell density compared to non enriched group (Non-enriched, n=6). No difference in BrdU-positive cell density was observed between the 4 enriched groups. *$p<0.05$. **$p<0.01$ for comparison with the non-enriched group. Data are expressed as mean±sem and all data points are represented.

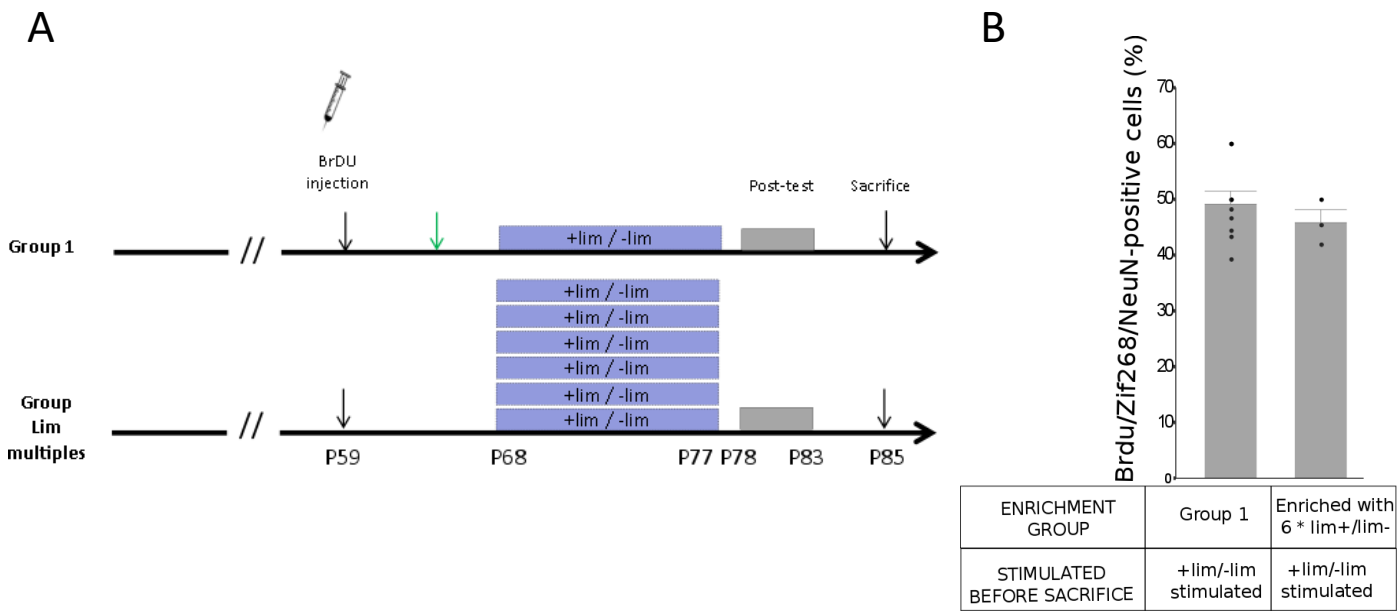
A



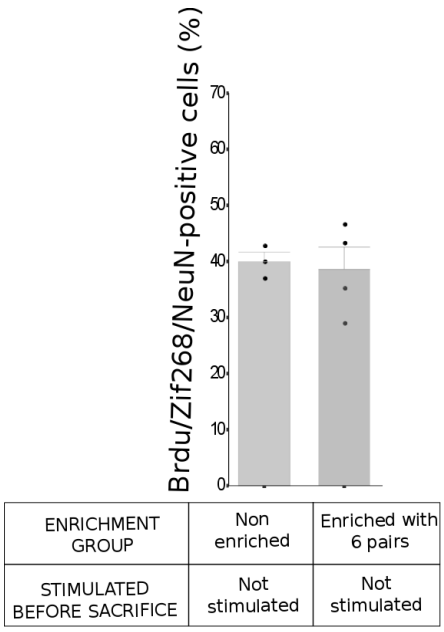
B



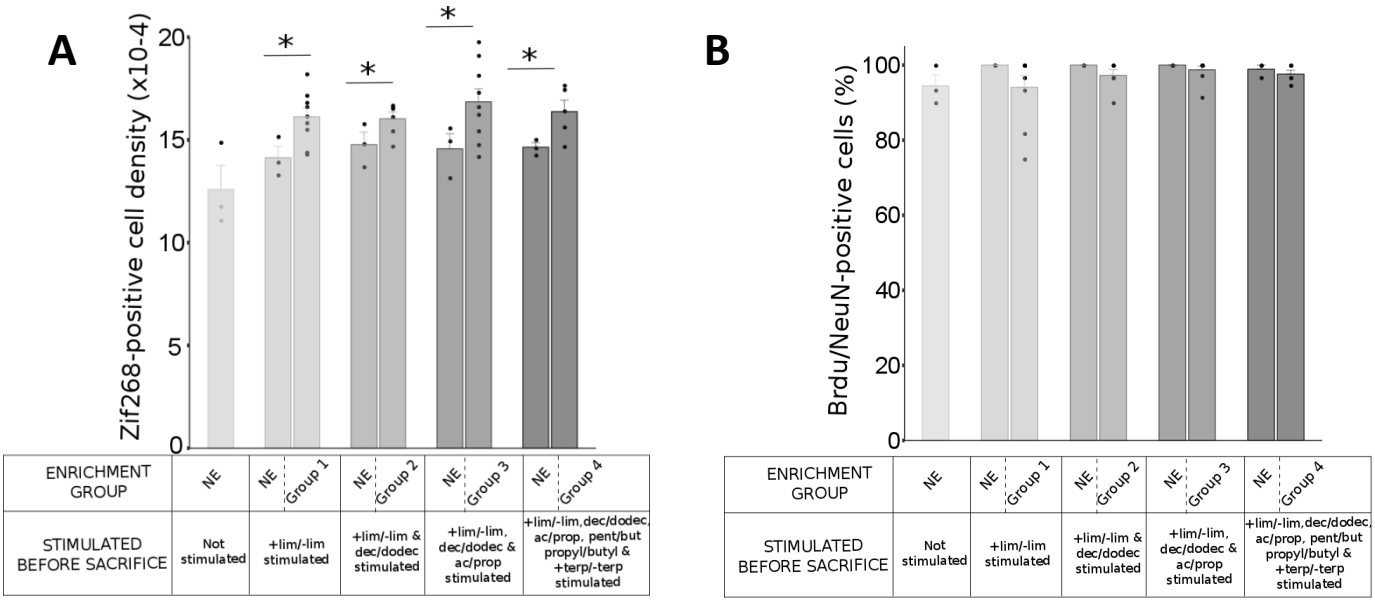
<u>Figure 4</u>: Increasing the complexity of perceptual learning increased the functional recruitment of adult-born neurons.
A. Example of a BrdU/Zif268/NeuN-positive neuron (arrows). **B.** Enrichment with one (n=9), two (n=6), three (n=9) or six (n=5) pairs of odorants increased the percentage of adult-born cells responding to the learned odorants compared to their respective control non-enriched group in response to the same odorants (n=3 for all NE groups). Linear regression analysis indicates that the percentage of BrdU/NeuN/Zif268-positive cells is increasing with enrichment complexity (red line) while this is not the case for control groups (blue line). *p<0.05. Data are expressed as mean±sem and all data points are represented.



<u>Supplementary Figure 2</u>: Multiple enrichments with the same pair of odorants has a similar impact on adult-born neurons' functional recruitment compared to simple enrichment (group1). A. Protocol **B.**The percentage of BrdU/Zif268/NeuN is similar between a group enriched with +lim/-lim once a day during 10 days and a group enriched six times a day during 10 days with +lim/-lim. Data are expressed as mean±sem and all data points are represented.



<u>Supplementary Figure 3</u>: Enrichment does not increase basal BrdU/Zif268 co-labeling. In the absence of odor stimulation, the percentage of BrdU/Zif268/NeuN is similar between non enriched animals and animals enriched with 6 pairs of odorants. Data are expressed as mean±sem and all data points are represented.



<u>Supplementary Figure 4</u>: Enrichment, regardless of complexity does not alter adult-born neuron fate (BrdU/NeuN-positive cells) and increases the density of activated (Zif268-positive) neurons.

A. Enrichment with one pair (group 1), two pairs (group 2), three pairs (group 3) or six pairs (group 4) of odorants does not change the percentage of BrdU/NeuN positive cells. **B.** The density of Zif268-positive cells is superior in enriched group compared to their respective controls. Zif268-positive cell density is similar between all enriched groups suggesting that Zif268 expression is independent of complexity. Data are expressed as mean±sem and all data points are represented.

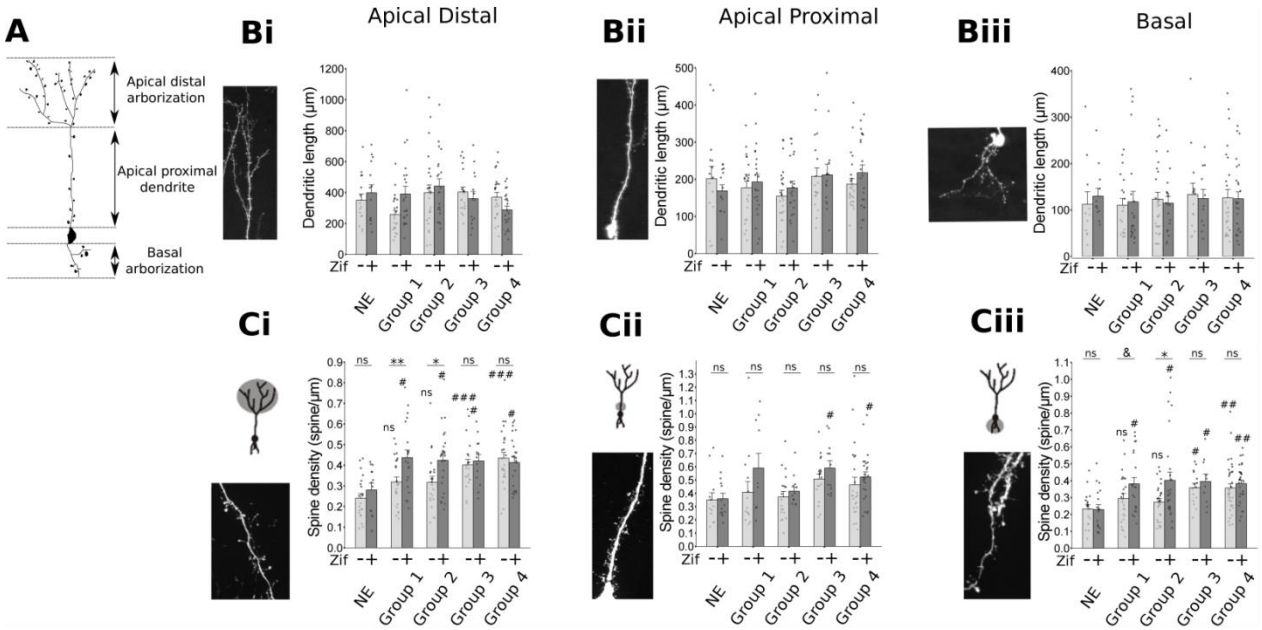


Figure 5: Increasing the complexity of perceptual learning increases structural plasticity of adult-born neurons A. Schematic representation of a granule cell and its dendritic domains**. B.** No effect of perceptual learning was observed on the length of apical dendritic arborization (Bi), of the apical proximal dendrite (Bii) and of the basal dendritic arborization (Biii). **C. S**pine densities after perceptual learning at the apical distal (Ci), apical proximal (Cii) and basal domains (Ciii). For apical distal and basal domains, in Groups 1 and 2 only Zif268-positive neurons showed increased spine density whereas in Groups 3 and 4 both Zif268-positive and Zif268-negative neurons showed increased spine density. Regarding the apical proximal domain, increase in spine density is observed only in groups 3 and 4 and in Zif268-positive neurons. &: p=0.07, *: p<0.05, **: p<0.001 for intra group comparisons. #: p<0.05, ##: p<0.001, ###: p<0.001 for comparison with the NE group. Data are expressed as mean±sem and all data points are represented.

	Apical distal spine density		Apical proximal spine density		Basal spine density	
Group	Zif268-negative	Zif268-positive	Zif268-negative	Zif268-positive	Zif268-negative	Zif268-positive
Non-Enriched	21	13	12	14	18	18
Group 1	24	20	14	10	24	22
Group 2	22	25	18	13	27	27
Group 3	20	13	18	12	15	10
Group 4	25	24	24	25	34	29

	Apical length		Basal_length	
Group	Zif268-negative	Zif268-positive	Zif268-negative	Zif268-positive
Non-Enriched	15	14	11	12
Group 1	23	23	19	23
Group 2	24	21	30	19
Group 3	19	14	14	11
Group 4	24	22	26	26

<u>Supplementary Table 1</u>: Number of adult-born neurons analyzed for each group, each Zif268 condition and each neuronal subdivision.

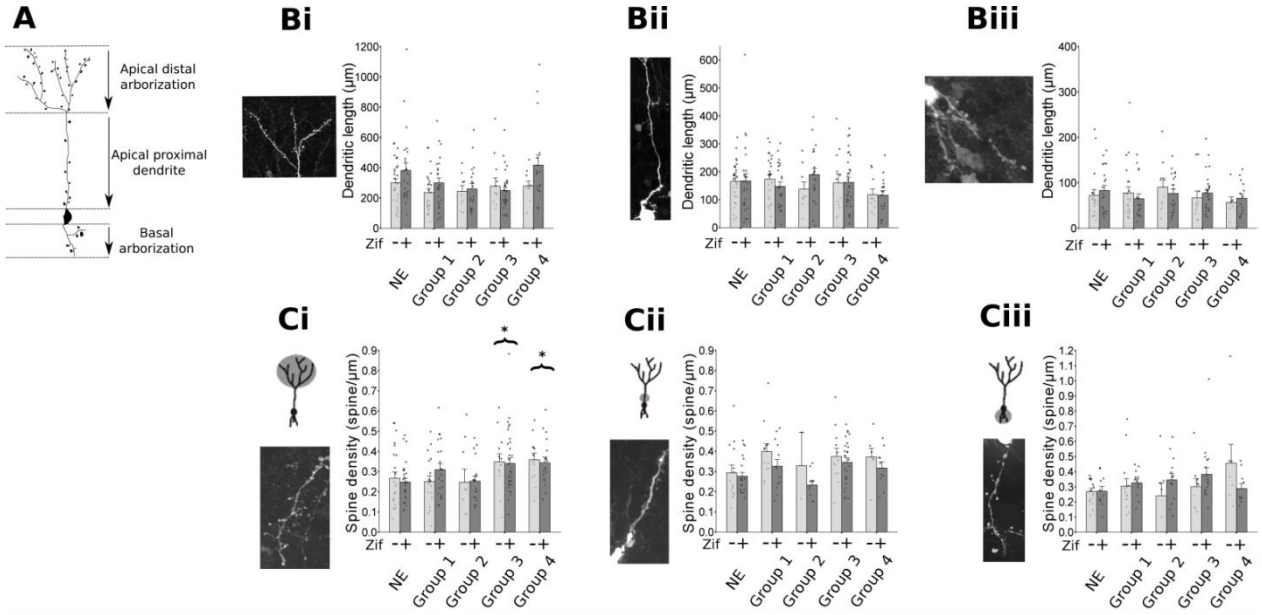
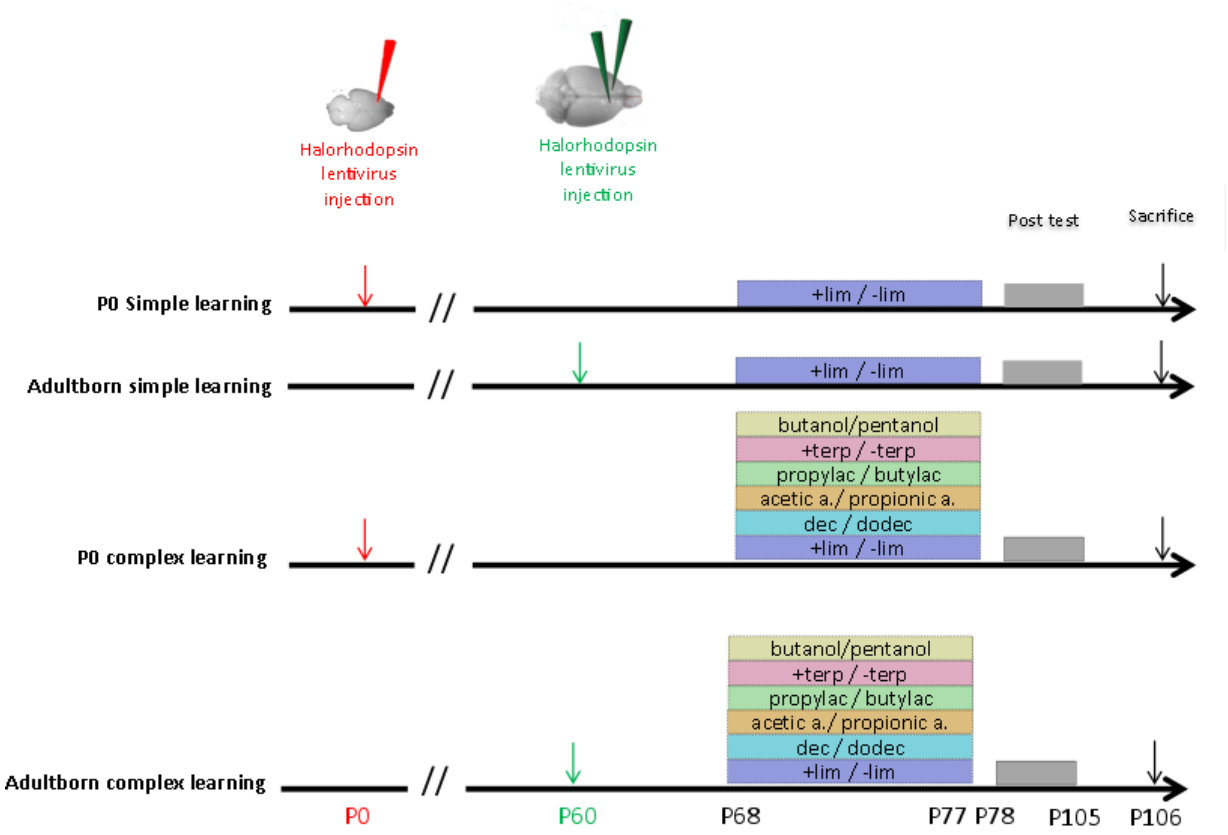


Figure 6: Perceptual learning induces limited morphological changes in preexisting neurons
A. No significant difference was observed between groups regarding apical dendritic arborization length , **B**. primary dendrite length, **C**. basal dendrite length or **E.** basal spine density. **D.** However, an increase in spine density in the apical domain was observed in Zif268-positive and negative neurons of enriched groups 3 and 4 compared to controls. *: $p<0.05$ for comparison with NE group. Data are expressed as mean±sem and all data points are represented.

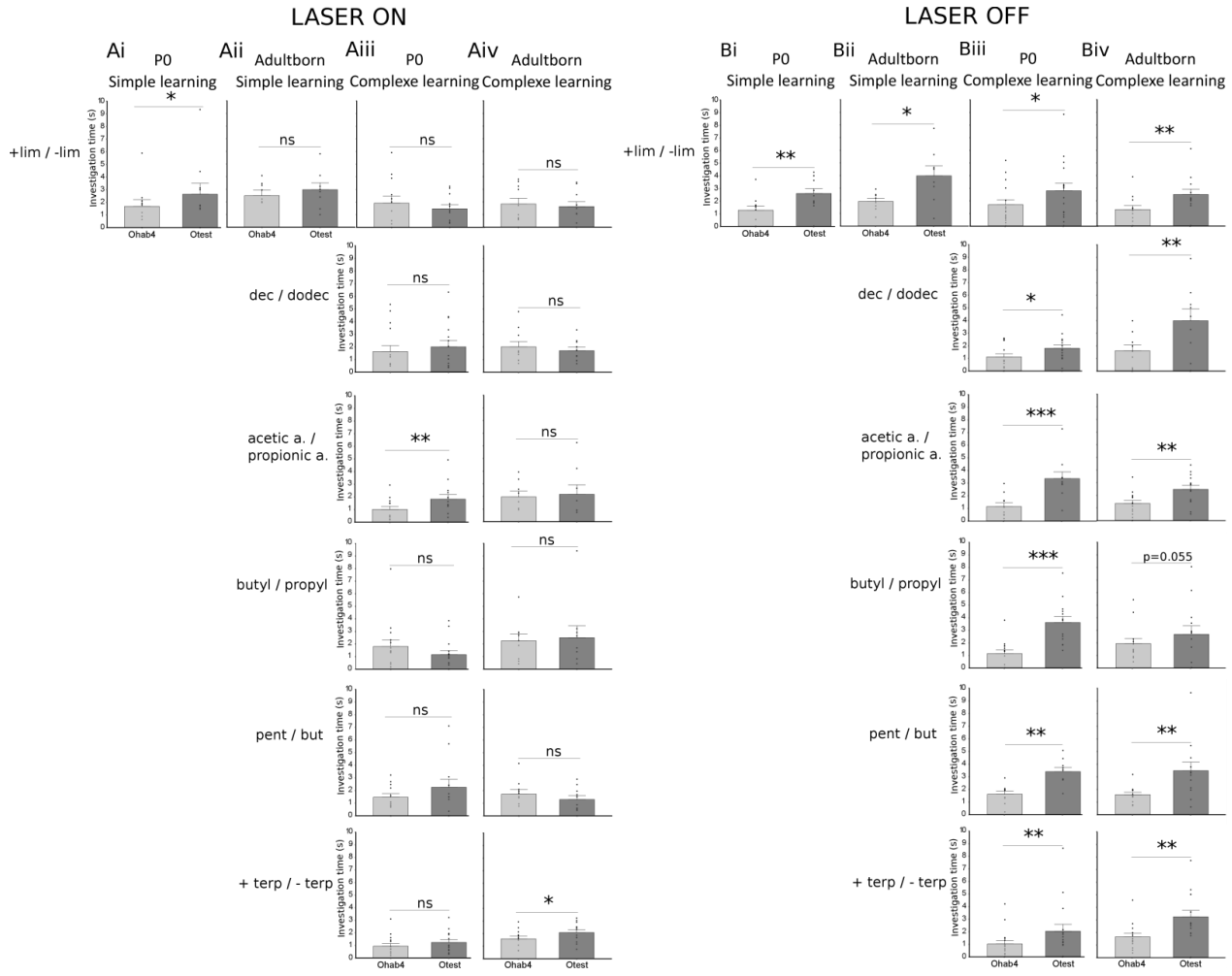
	Apical distal spine density		Apical proximal spine density		Basal spine density	
Group	Zif268-negative	Zif268-positive	Zif268-negative	Zif268-positive	Zif268-negative	Zif268-positive
Non-Enriched	23	27	14	21	15	11
Group 1	18	21	12	13	14	12
Group 2	9	18	2	9	7	14
Group 3	13	34	12	26	9	18
Group 4	12	18	7	10	7	10

	Apical length		Basal_length	
Group	Zif268-negative	Zif268-positive	Zif268-negative	Zif268-positive
Non-Enriched	29	25	18	20
Group 1	26	25	22	18
Group 2	9	20	9	21
Group 3	12	31	11	27
Group 4	10	17	7	14

<u>Supplementary Table 2</u>: Number of preexisting granule neurons analyzed for each group, each Zif268 condition and each neuronal subdivision.

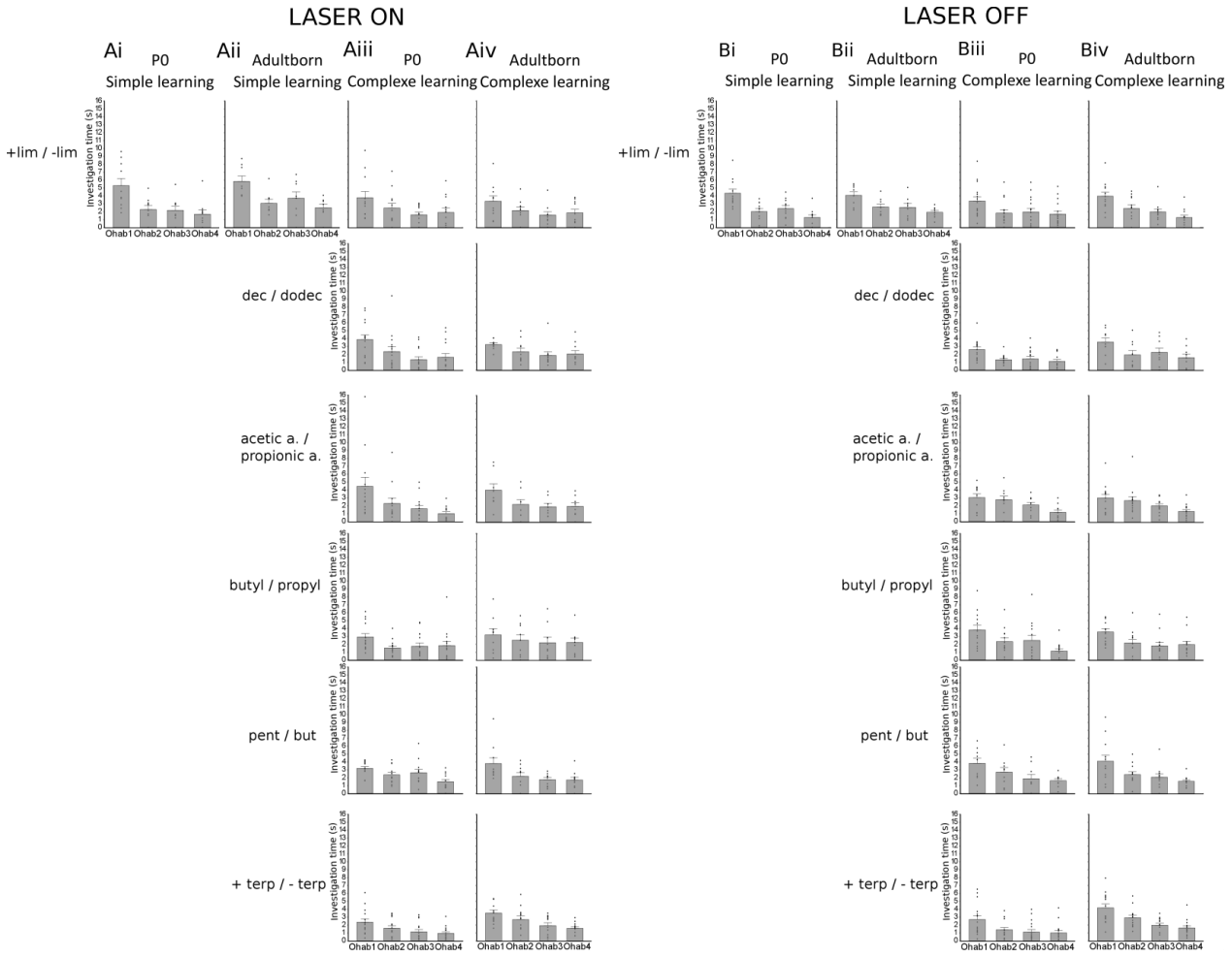


<u>Figure 7</u>: Design of the optogenetic experiment.
Halorhodospin lentivirus was injected in P0 mice for labelling neurons born during ontogenesis (preexisting neurons) and at P60 for labelling adult-born neurons. All mice were bilaterally implanted with optical fibers in the OB. Two groups of mice were trained in a simple perceptual learning (one injected at P0 and one at P60) and two groups of mice were trained in a complex perceptual learning (one injected at P0 and one at P60). After enrichment, mice were tested on their ability to discriminate the learned odor pairs using an olfactory habituation/cross habituation test. Light-stimulation was performed in freely moving mice during test trial when mice approached the odor source. Mice were sacrificed 106 days after their birth.



<u>Figure 8</u>: Optogenetically inhibiting preexisting or adult-born neurons reveals their functionally distinct involvement in simple and complex perceptual learning.

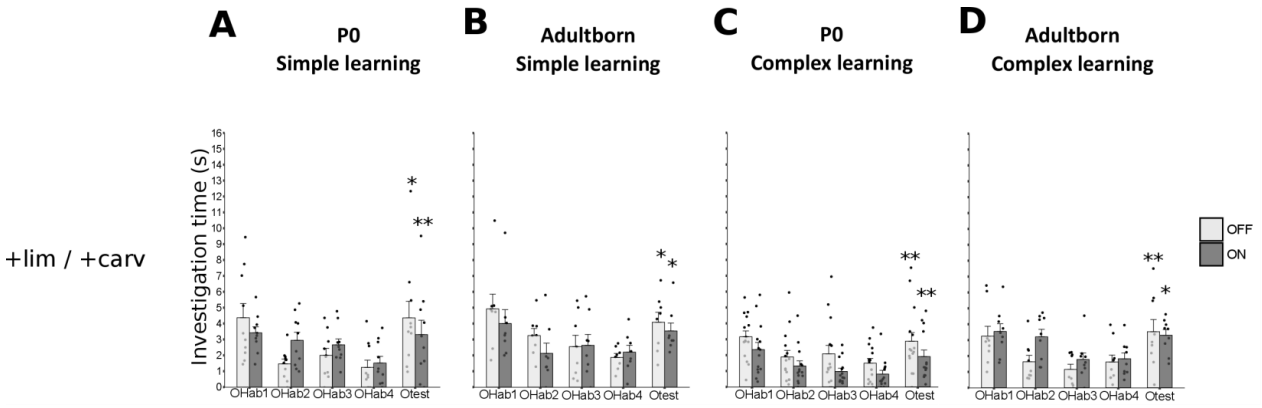
Optogenetic inhibition was performed during the test trial (Otest) when mouse approaches the odor source. After a simple perceptual learning task , **Ai and Bi**. inhibiting preexisting neurons does not prevent discrimination whereas **Aii and Bii.** inhibiting adult-born neurons does. After complex perceptual learning, inhibiting either **Aiii and Biii.** preexisting neurons or **Aiv and Biv** adult-born neurons results in the blockade of the discrimination of all odorant pairs but one (acetic a./propionic a.). Data are expressed as mean±sem and all data points are represented.



Supplementary Figure 5: Groups habituation.
Almost all groups presented an habituation behavior over repeated odorant exposure in the laser ON condition (except for +terp/-terp) and OFF condition (except for dec/dodec). Data are expressed as mean±sem and all data points are represented.

Group	Odorant	Laser	n	Habituation			Discrimination		
				Friedman		Difference	Wilcoxon (1 tail, paired)		Difference
P0, simple enrichment	+lim / -lim	ON	10	X=16,32; df=3	P=0,001	***	V=8	P=0,048	*
		OFF	11	X=24,30; df=3	P=0,00002	***	V=1	P=0,004	**
Adult-born, simple enrichment	+lim / -lim	ON	8	X=13,94; df=3	P=0,003	**	V=11	P=0,19	ns
		OFF	8	X=12,6; df=3	P=0,005	**	V=3	P=0,019	*
P0, complex enrichment	+lim / -lim	ON	12	X=16,15; df=3	P=0,001	***	V=41	P=0,78	ns
		OFF	16	X=17,04; df=3	P=0,0007	***	V=25	P=0,012	*
	dec / dodec	ON	15	X=20,31; df=3	P=0,00015	***	V=29	P=0,13	ns
		OFF	15	X=23,29; df=3	P=0,00004	***	V=22,5	P=0,018	*
	acetic a. / propionic a.	ON	13	X=24,05; df=3	P=0,00002	***	V=5	P=0,0012	**
		OFF	10	X=15,12; df=3	P=0,0017	**	V=0	P=0,00098	***
	butyl / propyl	ON	15	X=15,24; df=3	P=0,0016	**	V=68	P=0,84	ns
		OFF	13	X=20,54; df=3	P=0,00013	***	V=0	P=0,00012	***
	pent / but	ON	12	X=14,6; df=3	P=0,0022	**	V=21	P=0,15	ns
		OFF	9	X=12,33; df=3	P=0,0063	**	V=1	P=0,0039	**
	terp+ / terp-	ON	15	X=17,05; df=3	P=0,00069	***	V=34	P=0,076	ns
		OFF	16	X=23,82; df=3	P=0,00007	***	V=5	P=0,0016	**
Adult-born, complex enrichment	+lim / -lim	ON	10	X=13,08; df=3	P=0,0045	**	V=30	P=0,62	ns
		OFF	12	X=20,24; df=3	P=0,00015	***	V=3	P=0,0044	**
	dec / dodec	ON	10	X=9,55; df=3	P=0,023	*	V=35	P=0,78	ns
		OFF	9	X=7,18; df=3	P=0,066	ns	V=0	P=0,0071	**
	acetic a. / propionic a.	ON	8	X=10,81; df=3	P=0,013	*	V=16	P=0,42	ns
		OFF	14	X=14,31; df=3	P=0,0025	**	V=13	P=0,0072	**
	butyl / propyl	ON	9	X=8,2; df=3	P=0,042	*	V=31	P=0,85	ns
		OFF	13	X=9,47; df=3	P=0,024	*	V=22	P=0,055	ns
	pent / but	ON	10	X=15,24; df=3	P=0,0016	**	V=38	P=0,86	ns
		OFF	12	X=14,4; df=3	P=0,0024	**	V=4	P=0,0017	**
	terp+ / terp-	ON	12	X=5,87; df=3	P=0,12	ns	V=15	P=0,033	*
		OFF	14	X=20,01; df=3	P=0,00017	***	V=8	P=0,0015	**

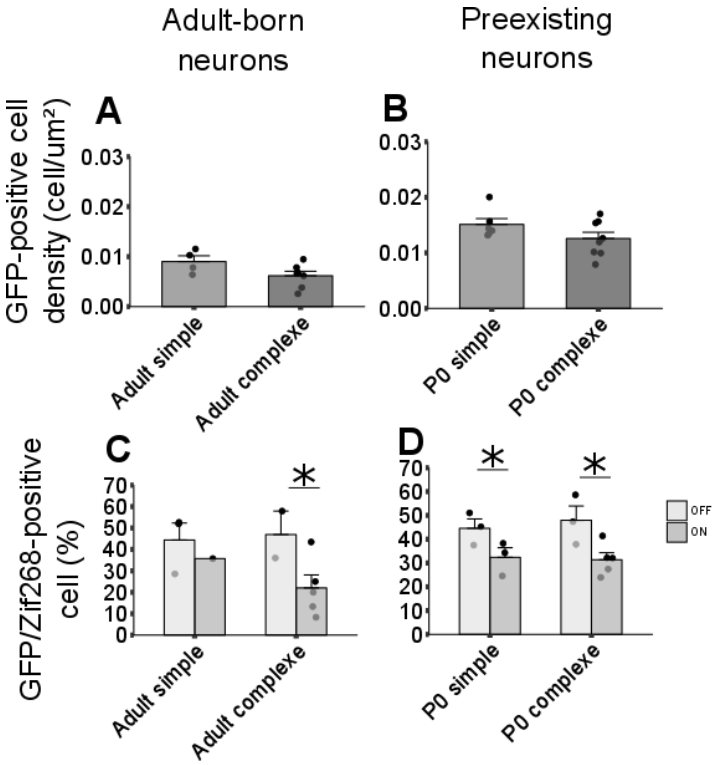
Table 3: Statistics on habituation and discrimination performances during the optogenetic experiment



<u>Supplementary Figure 5</u>: Optogenetically inhibiting preexisting or adult-born neurons did not alter the discrimination of perceptually dissimilar odorants ((+)limonene/(+)carvone).
Optogenetic inhibition was performed during the test trial (Otest) when mouse approaches the odor source. All groups expressed habituation over repeated odorant exposure (trials 1 to 4). In all cases, the light-triggered inhibition of preexisting neurons after simple **(A)** or complex **(C)** learning or adult-born neurons after simple **(B)** or complex **(D)** learning did not impair the discrimination of (+)limonene from (+) carvone.
*p<0.05, **p<0.01. Data are expressed as mean±sem and all data points are represented.

Group	Odorant	Laser	n	Habituation			Discrimination		
				Friedman		Difference	Wilcoxon (1 tail, paired)		Difference
P0, simple enrichment	carv+ / lim+	ON	10	X=15; df=3	P=0,0018	**	V=4	P=0,0068	**
		OFF	10	X=20,25; df=3	P=0,00015	***	V=6	P=0,014	*
Adult-born, simple enrichment	carv+ / lim+	ON	8	X=8,55; df=3	P=0,036	*	V=4	P=0,027	*
		OFF	8	X=10,95; df=3	P=0,012	*	V=2	P=0,012	*
P0, complex enrichment	carv+ / lim+	ON	14	X=15,99; df=3	P=0,0011	**	V=8	P=0,0015	**
		OFF	15	X=21,96; df=3	P=0,000066	***	V=19	P=0,009	**
Adult-born, complex enrichment	carv+ / lim+	ON	9	X=13,93; df=3	P=0,003	**	V=5	P=0,02	*
		OFF	9	X=15; df=3	P=0,0018	**	V=1	P=0,004	**

<u>Supplementary Table 3</u>: Statistics on habituation and discrimination performances of dissimilar odorants ((+)limonene / (+)carvone) during the optogenetic experiment



<u>Supplementary Figure 6</u>: Assessment of viral transfection and light induced inhibition in the olfactory bulb.

The left panel shows the density of cells expressing halorhodpsine (GFP-positive cells) in the OB when labelling adult-born **(A)** or preexisting neurons **(C)**. The right panel shows the percentage of neurons being both GFP-positive and Zif268-positive. Shining light at the OB level before sacrifice reduces Zif268 expression in the transfected cell populations both for adult-born **(B)** and preexisting neurons **(D).** *p<0.05. Data are expressed as mean±sem and all data points are represented.

Study 2 - A competition between transience and persistence of olfactory perceptual memory

A competition between new and already stored memories

OR

Adult-born neurons underlie competition between transience and persistence of olfactory perceptual memory

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ABSTRACT

It is now widely accepted that adult-born neurons in the olfactory bulb play a central role in learning and memory processes. However, the dynamics of memory retention as well as the potential interactions between successive learning and the contributions of adult-born neurons in underlying different memory events are yet to be unraveled. Here, we investigated the role of adult-born neurons in encoding individual memories acquired along successive time windows. For that purpose, we performed successive olfactory perceptual learning while varying the delay between learning. Perceptual learning consists in improvement in discrimination between two similar odorants following enrichment to these odorants. We found that, in the absence of any interference, adult-born neurons saved by perceptual learning are present in the olfactory bulb as long as the learned discrimination ability persists. In contrast, a second learning session, using a new pair of perceptually close odorants and occurring at a short delay after the first one, allowed discrimination of this second pair of odorants but lead to the loss of the earliest formed memory and of the associated increased in adult-born neurons survival. This loss could be counteracted by maintaining the enrichment with the first odor pair during the second learning. For longer delays between successive learning, memory of the first learning is spared and two distinct waves of adult-born neurons survival are evidenced by sequential incorporation of different thymidine analogues (CldU and IdU). Finally, we showed light-inhibition of adult-born neurons saved during the first learning induced loss of the learned discrimination of the first pair of odorants used for enrichment, without affecting that of the second one.

These findings suggest that these adult-born neurons are the definite substrate for olfactory memories and that once allocated to a specific memory they cannot be used to underlie another one.

INTRODUCTION

Adult neurogenesis the OB follows a stereotypical course with as a first step, proliferation of neuronal progenitor in the subventricular zone of the lateral ventricles giving rise to neuroblasts. These immature neurons migrate along the rostral migratory stream to reach the OB (Altman, 1969; Lois and Alvarez-Buylla, 1994; Ming and Song, 2005a, 2011). Once in the OB, they differentiate into granule and periglomerular interneurons (Winner et al., 2002; Malvaut et al., 2015) and establish connections with preexisting cells, integrating functionally in the preexisting circuit. A large proportion of adult-born neurons die within weeks after differentiation in the OB (Petreanu and Alvarez-Buylla, 2002) but their survival can be fostered by sensory activity during a specific time window (Yamaguchi and Mori, 2005). These new neurons, present in the OB during their critical period, can provide potential substrates for new learning. In the context of olfactory associative learning, adult-born neurons have been shown to constitute a critical component of long term memory (Sultan et al., 2010, 2011). In perceptual learning, an implicit form of learning (Gilbert et al., 2001), defined in the olfactory system as an improvement in discrimination between two perceptually similar odorants following passive exposure to them (Mandairon et al., 2006c, b; Mandairon et al., 2006a; Mandairon et al., 2008a; Mandairon and Linster, 2009; Moreno et al., 2009), adult-born neurons are required and their responsiveness is specifically increased in response to the learned odorants (Moreno et al., 2009).

However animals are usually exposed to a changing olfactory environment requiring constant adjustments of odor perception and memory in order for the olfactory system to efficiently guide the animal's behavior. Furthermore, at any time in the olfactory bulb are integrating adult-born neurons of graded ages and maturation levels. How does the acquisition of new memories impact the information already stored in the network and their associated adult-born neurons? Here, using successive perceptual learnings, we show that **1-** adult-born neurons saved by learning are present in the OB as long as the task is remembered, **2-** for a short delay between two learning tasks, the new memory can overwrite the previous one and alter survival of previously recruited adult-born neurons unless 3- an activation of the network with the learned odor pair is maintained. Finally, using sequential labelling of adult-born neurons with IdU and CldU and optogenetics we show 4- that a set of young adult-born neurons, aged between 8 and 18 days are

saved by enrichment period in successive learning and , that two different populations of adult-born neurons underline successive learning.

RESULTS

Adult-born neurons presence correlates with mnesic performances.

Previous studies have reported increased survival of adult-born granule cells after perceptual learning (Moreno et al., 2009). To better understand the potential role of these neurons in long-term bulbar memory, we first asked whether adult-born neurons saved by learning are present in the OB as long as the information is memorized. Thus, different groups of mice were submitted to perceptual learning consisting in a 10-day enrichment period (using (+)limonene and (-)limonene) and learned discrimination between the two odorants was analyzed at different time points post-enrichment and correlated with the density of adult-born neurons tagged before learning and still present in the OB at the time points considered.

More specifically, we tested the performance of discrimination between (+)limonene and (-) limonene at different delays post enrichment (T1, T2, T3, T4 and T5) using a habituation/dishabituation test (Figure 1A). At all time-points analyzed, mice exhibited a habituation behavior (Table 1, Supplementary Figure 1). We observed a significant effect of the post-learning delay on discrimination (trial (hab4 vs test): $F_{(1,84)}$=23.99; p=0.000005, group (T1,T2,T3,T4,T5): $F_{(5,84)}$=3.13; p=0.01, interaction $F_{(5,84)}$=1.18; p=0.32). Indeed, enriched animals were able to discriminate the two enantiomers of limonene ($p<0.05$ between Hab4 and Test) from T1 to T3 but not at longer delays (T4 and T5) ($p>0.05$ between Hab4 and Test ; Table 1, Figure 1Bi). In accordance with our previous works (Mandairon et al., 2006b; Moreno et al., 2009; Moreno et al., 2012), decanal/dodecanone was never discriminated after enrichment with (+)limonene/(-)limonene (trial: $F_{(1,90)}$=0.05; p=0.83, group: $F_{(5,90)}$= 1.18; p=0.32, interaction $F_{(5,90)}$=2.05; p=0.08)($p>0.05$ between Hab4 and Otest, Table 1; Figure 1Bii) indicative of the restricted effect of learning to the enriched odorants. As expected, control non-enriched (NE) animals did not discriminate any of the two odor pairs (T1: $p>0.05$ between Hab4 and Test; Table 1; Figure 1Bi and 1Bii).

In the behaviorally characterized animals, we analyzed the neurogenic parameters. To assess adult-born neuron survival, BrdU was injected 8 days before the enrichment period to label a

cohort of adult-born neurons present in the OB at the beginning of the enrichment period (Moreno et al., 2009, 2012) and BrdU-positive cells were counted in the granule cell layer at the different post learning delays (T1 to T5). We did not assess neurogenesis in the glomerular cell layer of the OB since we previously found no modulation of the rate of neurogenesis in this layer after perceptual learning (Moreno et al., 2009). Learning affected the density of BrdU positive cells ($F_{(2,19)}$=4.08, p=0.034 ; Figure 1Ci). In details, we observed higher density of BrdU-positive cells in groups able to discriminate the two odorants used for enrichment (T1, T2 and T3) compared to groups unable to do so (NE, T4 and T5; Figure 1Ci). The density of BrdU-positive cells was highly correlated to the index of discrimination (Pearson correlation: R=0.88; p=0.021)(Figure 1Cii).

This experiment showed us that perceptual memory natural decay occurred between T3 and T4 and this learning-induced increased number of adult-born neurons was present in the OB as long as the mnesic performances persisted and disappeared when the task was forgotten.

Successive learning delay has a critical impact on stored memory.

4, 14, 24 or 34 days (T2' to T5') after a first enrichment with (+)limonene/(-)limonene, mice were enriched with a new odor pair, decanal/dodecanone, (Figure 2A). As previously, all groups exhibited a habituation behavior (Table 2, Supplementary Figure 2). Mice were tested for discrimination of the two pairs of odorants used during enrichment and we observed significant discrimination differences between groups ((+)limonene/(-)limonene: trial: $F_{(1,94)}$=19.99; p=0.00002, group (T1,T2',T3',T4',T5'): $F_{(4,94)}$=4.63; p=0.002, interaction: $F_{(4,94)}$=2.1; p=0.09 ; decanal/dodecanone: trial: $F_{(1,108)}$=45.08; p=0.0000000009, group: $F_{(4,108)}$=2.19; p=0.08, interaction: $F_{(4,108)}$=5.49; p=0.0005). When the second enrichment was performed 4 days after the first one (T2'), animals were only able to discriminate the second odor pair (Table 2, Figure 2Bi and 2Bii) suggesting that the acquisition of the second learning erased the memory of the first one. However, when the delay between the 2 enrichments increased (14 days, T3'), the animals remembered both learning and were thus able to discriminate the two odor pairs (Table2, Figure 2Bi and 2Bii) suggesting that at this delay the new memory formation did not impact the memory already stored. At higher delays between enrichments (24 and 34 days, respectively T4' and T5'), (+)limonene and (-)limonene were no longer discriminated while decanal and dodecanone were,

which is in accordance with the first experiment and the duration of the memory retention (Table 2, Figure 2Bi and 2Bii).

As in the first experiment, BrdU was injected 8 days before (+)limonene/(-)limonene enrichment. We found an increase in BrdU-positive cell density in animals discriminating the (+)limonene/(-)limonene (T1, T3') compared to the other ones (T2', T4', T5') (group effect. $F_{(4,26)}$=16.1, p=0.000001; Figure 2C). *Post hoc* Tukey corrected t-tests were then performed and showed that when the second enrichment is performed 4 days after the first one (T2'), the density of labeled adult-born cell was significantly decreased compared to T1 (p=0.025) and this is accompanied by the impairment of (+)limonene/(-)limonene discrimination suggesting that at this delay new memory formation altered adult-born neuron survival and already stored information. However, when the second enrichment is performed 14 days after the first one (T3'), both pairs of odorants were discriminated and the density of adult-born cell remained high (T1 versus T3': p=0.2). Finally, when the second enrichment was even more delayed (T4' or T5'), the density of BrdU significantly decreased (T3' versus T4': t=p<0.001 ; T5' versus T3': p<0.001) to a level comparable to T2' (T4' versus T2': p=0.59 ; T5' versus T2': p=0.99) which is in accordance with the observed impairment of (+)limonene/(-)limonene discrimination and the first experiment (Figure 2C).

In summary, the delay between the two enrichments is critical to the upkeep of the memory already stored and its associated adult-born neurons. These data further suggest that the fate of a fraction of adult-born neurons upon learning goes through a fragile, reversible surviving state for one week upon a new learning challenge.

Maintaining the environement prevents cell death, maintains memory and the new learning recruits new adult-born neurons.

With a short delay between the two learning (T2'), we found that the new memory formation altered information already stored and induced apoptosis of adult-born neurons retrieved after the first learning. Since cell survival is known to be input-dependent, we tested whether reactivating the network responding to the odorants used for the enrichment can avoid memory to be overwritten. For that purpose, we maintained the exposure to the first pair of odorants during enrichment with the second pair of odorants. (Figure 3A).

In all groups, mice exhibited a habituation behavior (Table 3, Supplementary Figure 3). Regarding discrimination, we observed significant effects of trial and groups ((+)limonene/ (-)limonene: trial: $F_{(1,164)}$=16.88; p=0.00006, group: $F_{(3,164)}$=12.55; p=0.0000002, interaction: $F_{(3,164)}$=2.34; p=0.08; decanal/dodecanone: trial: $F_{(1,164)}$=31.33; p=0.00000009, group: $F_{(3,164)}$=3.14; p=0.026, interaction: $F_{(3,164)}$=2.7; p=0.047). As the first experiment of this study (Figure 1A), we observed that enrichment with (+)limonene/(-)limonene improved the discrimination between these two odorants when tested at T2' (group 1, see Table 3, Figure 3Bi and 3Bii). Also, once again when a second enrichment with decanal/dodecanone was performed 4 days after the first one, the memory of (+)limonene/(-)limonene discrimination was erased to the benefit of the discrimination between decanal/dodecanone (group 2, Table 3, Figure 3Bi and 3Bii). Interestingly, if we maintained the enrichment with (+)limonene/(-)limonene during the second enrichment period (group 3), the animals were able to discriminate both odor pairs (Table 3, Figure 3Bi and 3Bii) indicating that network reactivation can prevent memory forgetting. Finally, when the retention of the task was tested at T3', mice were, as previously, able to discriminate both odorant pairs (group 4, Table 3, Figure 3Bi and 3Bii).

To further address the neurogenic mechanisms underlying sequential learning, we asked whether distinct populations of adult-born neurons could be saved along different learning time windows. We know that adult-born neurons are saved by learning during their critical period so in our experimental context, we tagged two adult-born neuron populations differing in age, to understand the dynamic of learning-dependent survival of adult-born neurons. More precisely, we tagged with CldU a first pool of adult-born cells aged of 8 days at the beginning of the first enrichment (with (+)limonene/(-)limonene) and we tagged with IdU a second pool of adult-born cells aged of 8 days at the beginning of the second enrichment (with decanal/dodecanone) and not present in the OB at the beginning of the first one (Figure 3A, 3Cii and 3Ciii). The densities of CldU- and IdU-positive cells were assessed in the OB at T2' and showed group effect was found in both cases (CldU: $F_{(3,16)}$=19.06, p=0.00002 ; Idu: $F_{(3,24)}$=7.8 p=0.0008). *Post-hoc* analysis revealed that the level of CldU-positive cells was lower in group 2 in which discrimination of (+)limonene/(-)limonene was impaired compared to the other groups (group 2 versus group 1: p=0.056, group 2 versus group 3: p=0.0029, group 2 versus group 4: p<0.001; Figure 3Cii). Interestingly, the density of CldU-positive cells was not significantly higher in group 3 compared to group 1 (p=0.48) suggesting that the second enrichment recruited a new pool of adult-born

neurons. Indeed, in group 3, the level of IdU-positive cells was increased compared to group 1 ($p=0.017$). It is also true for group 2 and 4 compared to group 1 (respectively: $p=0.03$ and $p<0.001$)(Figure 3Ciii).

We learned from this experiment that first, maintaining the first learning during the second one avoided memory erasure and adult-born cells disappearance and second that a new pool of adult-born neurons is aged of 8 days at the beginning of the second enrichment is recruited. The first pool of neurons saved, although still sensitive to environmental changes (as seen when the first learning is not maintained), may not be recruited to underlie the second learning.

In sum, memory is continuously evolving with the changes of the olfactory environment. The fate of a perceptual learning memory is correlated to the presence of successive waves of survival of adult-born neurons aged between 8 and 18 days during the enrichment.

Once adult-born neurons are allocated to a memory they cannot take part in the formation of a new one.

Next, we used optogenetic in freely behaving mice to test whether a population of adult-born neurons involved in a first learning was, first, functionally involved in that pair's discrimination and, two, if it was also involved in a second learning. We used the same learning paradigm as previously (group 3; Figure 3A and 5A) in which mice underwent a first enrichment period with (+)limonene/(-)limonene and 4 days later a second one with decanal/dodecanone as well as (+)limonene/(-)limonene to avoid memory loss. In this setup, 8 days before the first enrichment period, mice were transfected with halorhodopsin or control lentivirus (same lentivirus without the halorhodopsin cassette) in adult-born neurons and implanted with optical fibers in the OB. The blockade of adult-born cell activity was performed by light stimulation during the test trial of the habituation/dishabituation task. Results showed that light-triggered blockade of neurons born 8 days before the first learning altered the discrimination of (+)limonene/(-)limonene (Figure 5Bi, Table 4) but not that of decanal/dodecanone (Figure 5Bii, Table 4). Notably, the discrimination of a dissimilar pair of odorant (+)limonene/(+)carvone was not impaired by light (Figure 5Biii, Table 4) demonstrating that light-triggered inhibition of adult-born neurons had no deleterious effect on easy odor discrimination. We analyzed the level of viral transfection in control and halorhodopsin groups and found no differences of GFP-positive cell density in the OB (t-test

p=0.5, Figure 5C). We also verified the effectiveness of light-mediated inhibition of adult-born granule cells by assessing the expression of Zif268-positive cell in GFP-positive neurons after light stimulation. As expected, the expression of Zif268 is significantly lower in halorhodospin compared to control group (t-test p=0.03, Figure 5D).

Hence, saving adult-born neurons allows maintaining olfactory memory and conversely, silencing adult-born neurons leads to memory erasure demonstrating the central role of adult-born neurons in being the structural basis of perceptual memory. Furthermore, our data indicate that a given cohort of adult-born neurons underlie learned discrimination as long as they are aged between 8 and 18 days during learning.

DISCUSSION

Perceptual olfactory learning increases the survival of adult-born neurons and these adult-born neurons are present in the olfactory bulb as long as the memory is maintained. Moreover adult-born neurons saved by a first learning are prematurely killed by a second successive learning and behaviorally preventing adult-born neurons death avoid memory erasure. These phenomenon have also been observed after associative olfactory learning (Sultan et al., 2010, 2011).

The OB is continuously receiving waves of adult-born neurons, hence contains adult-born neurons of different age and maturation (Carleton et al., 2002). When learning happens successively, which neurons take part in underlying behavioral change and why? When the delay between the two learning is short (4 days), neurons saved during the first one are prematurely killed which does not happens when the delay is longer (14days) or the first odorant pair is maintained in the environment. This means that there is a critical period, i.e. a time window after neuron's generation, when their survival is very strongly influenced by sensory input (Yamaguchi and Mori, 2005), during which life and death decision is made. Adult-born neuron's plasticity is maximal during their initial development and decrease as they mature (Ge et al., 2008; Kelsch et al., 2009; Nissant et al., 2009). Moreover, they are the most susceptible to death between 14-20 days after birth (Yamaguchi and Mori, 2005). Considering this, the short time window of 14 days allows for waves of neurons to take a snapshot of the neural network when arriving in the OB as well as leave them some time to stabilize. If the information in the environment changes in that length of time, the neuronal population previously saved is not relevant because the information they encoded is not. Hence they are purge from the network via apoptosis. This cell death could be mediated via competition-induced apoptosis due to other waves of adult-born neurons (McAvoy et al., 2016).

This critical period has also been observed in another brain structure, the dentate gyrus (DG). Indeed, new neurons are continuously being produced *de novo* mainly in two main regions, the DG and the OB (Altman and Das, 1965; Praag et al., 2002; Ming and Song, 2005b; Gould, 2007; Ge et al., 2008; Ming and Song, 2011). Granule cells of the hippocampus once functionally integrated also provide substrate for learning and memory in many behavioral tasks (Shors et al., 2001; Snyder et al., 2005; Deng et al., 2010; Cameron and Glover, 2015). In a similar manner than the experiments realized here, spatial learning depends on both the survival improvement of

relatively mature neurons and the removal of more immature ones (Döbrössy et al., 2003; Dupret et al., 2007), hence adult-born neurons at different level of maturation make distinct contribution to hippocampal processing. Additionally there is now ample evidence that neurogenesis rate have a direct impact on learning performances, memory and forgetting (Martinez-Canabal et al., 2013; Akers et al., 2014).

More generally, memory are transient and persist as long as the information is useful to guide the animal behavior appropriately (Richards and Frankland, 2017). As for the DG, memory trace in the OB is subjected to the high renewal rate of neurons due to adult neurogenesis. Increasing the turnover or favoring the integration of new neurons occurs to the detriment of older ones and thus older memory. However at the same time not all ancient memories are erased. The critical period window during which adult-born neurons are more sensitive to environmental information seems to be a way to keep the animal's behavior adapted to a complex and always changing environment, allowing for the detection of potentially relevant information while removing those that are not relevant (thus avoiding conflicting information and retrograde interference). Hence, a faster forgetting allows the system to keep only pertinent information in a changing environment where the action/outcome contingency needs frequent updating (Brea et al., 2014) and this mechanism is allowed by adult neurogenesis processes and adult born neurons survival.

Once their survival in insured by the relevance of the information they encode, i.e. after the critical period, the experiment performed here demonstrate that adult-born neurons do not underlie another perceptual learning and that the new information from the environment is encoded by the next wave of adult-born neurons coming in the OB. Adult-born neurons, once part of a memory, will not underlie another.

In conclusion, on the one hand adult-born neurons are central to the persistence of olfactory memories and on the other hand have a central role in regulating memory transience and keeping up-to-date-only information in the bulbar network. They are an essential pillar of adaptive olfactory processing and memory storage in an olfactory changing environment.

Material and methods

Behavior

Experimental Designs

- Experiment 1

Eight days after BrdU injection, mice were enriched one hour daily during 10 days with (+)limonene and (-)limonene. At the end of the enrichment, mice were tested on spontaneous discrimination between (+)limonene and (–)limonene and also between another pair of perceptually similar odorants (decanal and 2-dodecanone). Discrimination abilities were assessed using an olfactory habituation/dishabituation task. Animals were sacrificed 24, 34, 44, 54 and 64 days after BrdU injections (Figure 1A).

- Experiment 2

Eight days after BrdU injection, new mice were enriched one hour daily during 10 days with (+)limonene and (-)limonene. A second 10-days enrichment period with decanal and dodecanone was performed 4, 14, 24, 34 or 44 days after the first. At the end of both enrichments, mice were tested on spontaneous discrimination between the two odorants of the each odor pairs. As for experiment 1, discrimination abilities were assessed using an olfactory habituation/dishabituation task and animals were sacrificed 24, 34, 44, 54 and 64 days post BrdU injections.

- Experiment 3

In the third experiment, we used CldU and IdU, two analogues of BrdU to label two different populations of adult-born cells. CldU was injected 8 days before the first enrichment (always with (+)limonene and (-)limonene) while IdU was injected 8 days before the second enrichment period. The second enrichment period varied among groups: no enrichment (group 1); decanal and dodecanone (group 2 and group 4), (+)limonene and (-)limonene plus decanal and dodecanone (group 3). The delay between the two enrichments was either 4 or 14 days. As previously, discrimination abilities were assessed using habituation/dishabituation task and animals were sacrificed 34 or 44 days post-CldU injection. Group 2 and 4 comprised mice of previous groups T2’ and T3’ and 10 additional mice.

- Experiment 4

Here, using group 3 experimental configuration from the previous experiment, we performed targetted lentiviral-induced halorhodospin channel expression (NpHR3.0) in the subventricular

zone to specifically inhibit the population of adult-born neurons arriving at the beginning of the (+)limonene/(-)limonene enrichment via optogenetics.

Enrichment. For the olfactory enrichment, swabs containing 100 µL of pure odorant were placed in two tea balls hanging from the cover of the standard breeding cages for one hour daily during 10 days. For the multiple enrichment (experience 3, group 3), the two pairs of odorants were presented with an interval of 1 hour.

Olfactory habituation/dishabituation. We assessed the spontaneous discrimination between two pairs of chemically and perceptually similar odorants: (+)limonene/(-)limonene and decanal/dodecanone. However, decanal is an odorant with little overlapping with (+)limonene, meaning that the enrichment with one pair of similar odorants will not induce the discrimination of the other (Mandairon et al., 2006b; Moreno et al., 2009). The odorants were all diluted in mineral oil proportionally to their vapor pressure in order to reach a pressure of 1 Pa (Cleland et al., 2002; Mandairon et al., 2006b). Experiments were performed in cages similar to the standard home cage and odorants were presented by placing 60 µL of odor stimulus onto a filter paper (Whatman) which was then placed in a tea ball hanging from the cover of the cage. Each mouse was tested on the two odor pairs and the odor pairs were tested in a random order. A test session consisted of one 50-s presentation of mineral oil then four 50-s odor presentations of a first odorant (Hab) at 5 min intervals, followed by one 50-s presentation of the second odorant of the pair (Test). Investigation was defined as active sniffing within 1 cm of the tea ball.

Data analysis. Data analysis was performed using Systat statistical software (SSI, Richmond, CA, USA) and R software (CRAN). Only mice that investigated at least 1 sec during the first presentation of the habituation odorant were included in the analysis. Normality was assessed using Kolmogorov-Smirnov test. Global two-way ANOVAs were performed to evaluate changes in discrimination abilities between groups. Then intra groups one-way RM-ANOVAs followed by paired t-tests were performed in order to determine whether mice exhibited habituation (trial effect) and the discrimination abilities by comparing Hab4 and Test. Discrimination was indicated by a significant increase in investigation time during the test trial. Discrimination index

was calculated as previously (Rey et al., 2012) and is the following calculation: [1-(Hab4/Test)]. The criterion for significance was set to p=0.05.

Adult-born cells

Bromodeoxyuridine (BrdU) administration. To determine the fate of adult-born cells in the OB, BrdU (Sigma) was injected intraperitonally 8 days before the enrichment period. Three injections of BrdU at 2 h intervals (50 mg/kg in saline) were performed.

Histology. 24, 34, 44, 54 and 64 days post BrdU injection, five mice taken randomly from each experimental group were deeply anesthetized by injection of pentobarbital (2 g/kg), and killed with an intracardiac perfusion of 50 mL of cold fixative solution (paraformaldehyde 4% diluted in phosphate-buffered saline). Brains were then removed, cryoprotected in sucrose and frozen rapidly before being stored at -20 °C. Olfactory bulbs were then sectioned with a cryostat (Reichert-Jung, NuBlock, Germany) in 14µm slices.

BrdU immunohistochemistry. The protocol has been previously described (Mandairon, et al. , 2006b). Briefly, sections were incubated overnight in a mouse anti-BrdU antibody (1:100, Chemicon, Temecula, CA, USA) at 4°C followed by a biotinylated anti-mouse secondary antibody (1:200, Vector Laboratories, Burlingame, CA, USA) for 2h. The sections were then processed through an avidin-biotin-peroxydase complex (ABC Elite Kit, Vector Laboratories). Following dehydratation in graded ethanols, the sections were defatted in xylene and cover-slipped in DPX (Fluka, Sigma).

BrdU cell assessment. The method used for positive cell counting has been previously described (Mandairon et al., 2006a). Briefly, positive cells were counted on every fifth section (thickness = 14µm, sampling interval = 70µm) in the granule cell layer of the OB using a mapping software (Mercator, Explora Nova, La Rochelle, France) coupled to Zeiss microscope. BrdU-positive cells were manually counted in the granule cell layer. The mean positive cell density of each array was calculated and averaged within each experimental group. Between-groups comparisons of the mean cell density were performed by ANOVA followed by *post-hoc* t-tests with Tukey corrections. The level of significance was set to 0.05.

Optogenetic in freely behaving mice.

Surgery. 150nL of pLenti-hSyn-eNpHR3.0-EYFP lentivirus (9.22 x 10^6 IU/ml) or 300nL of control pLenti-hSyn-EYFP (1.1 x 10^6 IU/ml, expressing only the reporter gene YFP (Kermen et al., 2016) injections were done bilaterally in the subventricular zone, with the following coordinates respective to bregma: antero-posterior +1mm, medio-lateral ± 1mm, dorso-ventral -2.3mm and at a rate of 150nL/min. Just after virus infusions, mice were implanted with bilateral optic fibers (200nm core diameter, 0.22 N.A., Doric Lenses) in the olfactory bulb, with the following coordinates respective to bregma: antero-posterior +4.6mm, medio-lateral ±0.75mm, dorso-ventral -2mm. Mice were injected with a ketoprophen solution (2 mg/kg) after the surgery as well as during the following days and allowed to recover with food and water *ad libidum* .

Behavior. During the habituation/dishabituation mice were stimulated (crystal laser, 561nm, 10-15mW, continuous stimulation) during the test (Test) when they entered a zone of 2.5cm around the tea ball.

Control of light-triggered inhibition. 36 days post-surgery and lentiviral infusion, mice were stimulated with light 1h before sacrifice and for 1h duration with pattern mimicking the average light stimulating pattern during the test trial (0.75s light ON, 5s light OFF during 1h). After brain sectioning (see above), EYFP and Zif268 double immunohistochemistry was performed as described previously (Kermen et al., 2016) on slices under the injection site to allow assessment of the inhibition.

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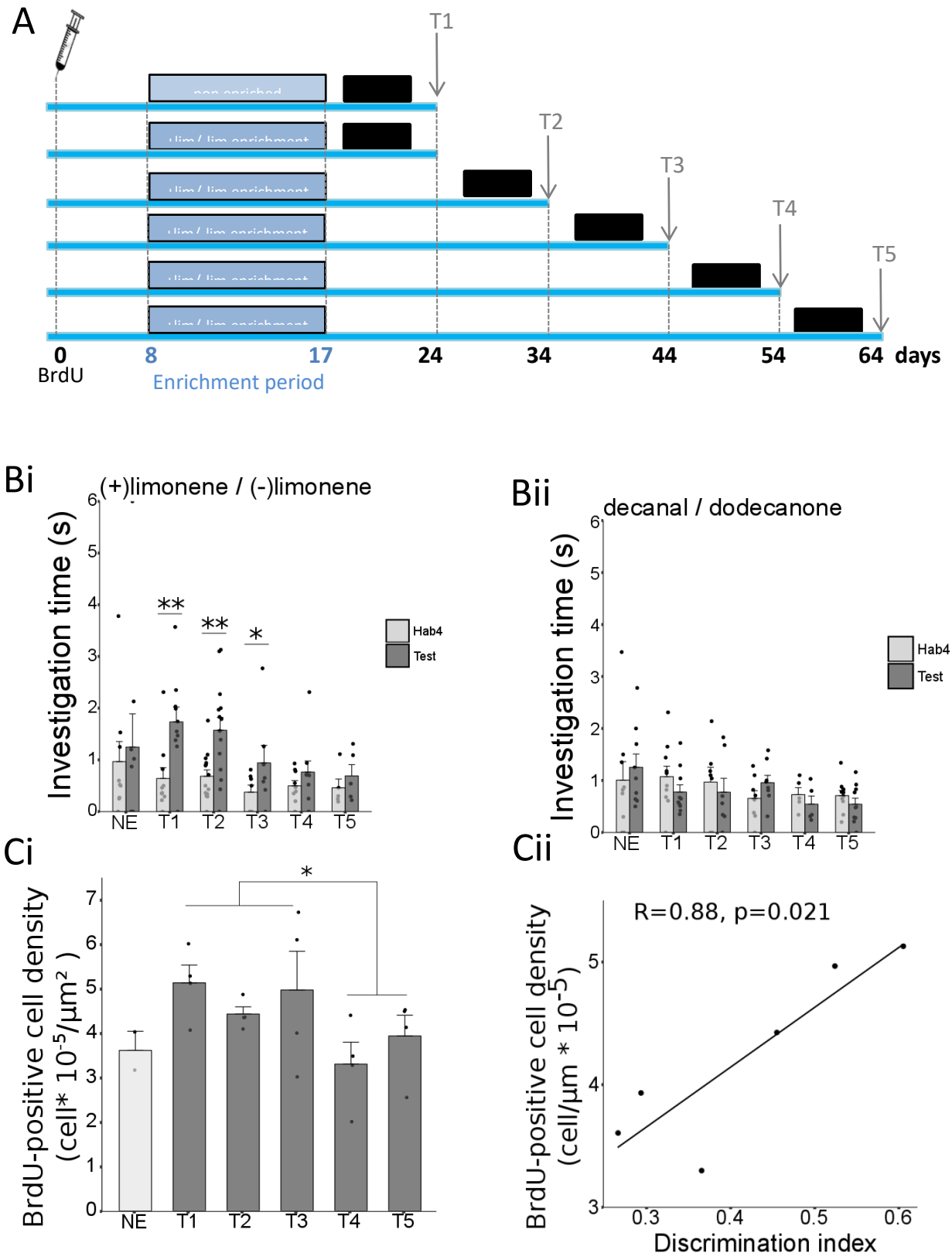
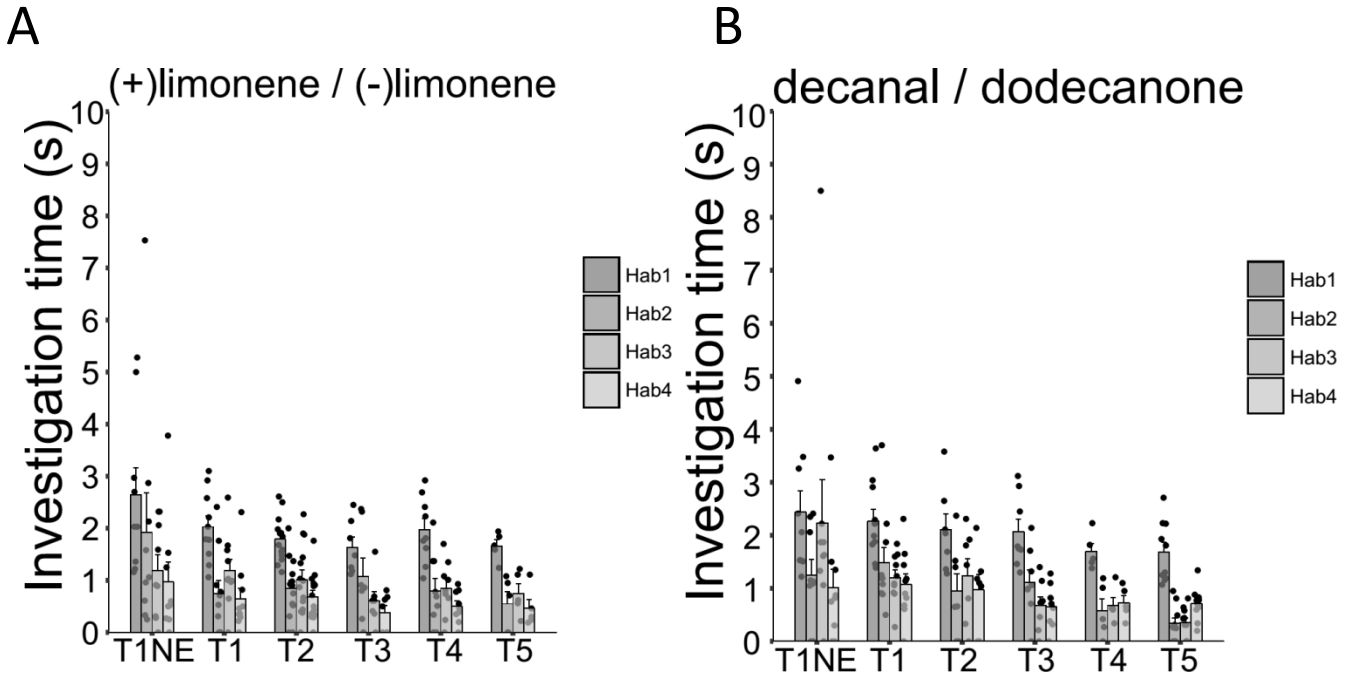


<u>Figure 1</u>: Adult-born neurons saved by learning are present in the OB as long as the mnesic performances persist.
A. Experimental design. **B.** Behavioral results. **Bi**. Discrimination between (+)limonene and (-)limonene is assessed at different delays after the enrichment period. Control non-enriched (NE) do not discriminate the (+)limonene from (-)limonene at T1. After enrichment, the 2 odorants are discriminated at T1, T2, and T3 and no longer at T4 and T5. **Bii.** Decanal and dodecanone are not discriminated at any delay post-learning. **C**. Adult-born neuron density in the OB is higher in groups of enriched animals discriminating +lim from -lim compared to non-enriched animals or to enriched animals that do no longer discriminate. **Cii.** Positive correlation between the discrimination index and adult-born neuron density in the OB. *p<0.05. Data are represented as data points and mean ± sem.

Group	Odorant	Habituation			Discrimination		
		Repeated measure ANOVA		Significant	T-test (1 tail, paired)		Significant
T1NE	+lim/-lim	F(3,24)=3,75	F = 0,024	*	T = -0,74	P=0,24	ns
	dec/dodec	F(3,24)=4,15	P=0,017	*	T=-1,49	P=0,09	ns
T1	+lim/-lim	F(3,29)=11,82	P<0,0001	***	T=-3,3	P=0,005	**
	dec/dodec	F(3,30)=6,89	P=0,001	***	T=1,24	P=0,88	ns
T2	+lim/-lim	F(3,44)=12,74	P<0,0001	***	T=-3,14	P=0,004	**
	dec/dodec	F(3,19)=5,27	P=0,008	***	T=0,69	P=0,74	ns
T3	+lim/-lim	F(3,19)=6,91	P=0,003	**	T=-1,92	P=0,05	*
	dec/dodec	F(3,22)=11,03	P=0,0004	***	T=-1,45	P=0,095	ns
T4	+lim/-lim	F(3,25)=20,21	P<0,0001	***	T=-1,12	P=0,15	ns
	dec/dodec	F(3,13)=21,32	P<0,0001	***	T=0,74	P=0,75	ns
T5	+lim/-lim	F(3,12)=16,88	P=0,0001	***	T=-0,99	P=0,19	ns
	dec/dodec	F(3,28)=31,5	P<0,0001	***	T=1,02	P=0,83	ns

Table 1: Statistical results.



<u>Supplementary Figure 1</u>: Habituation.
A. Habituation (Hab1-Hab4) of the habituation/dishabituation task for the pair (+)limonene / (-)limonene. All groups show habituation with repeated exposure. **B.** Habituation (Hab1-Hab4) of the habituation/dishabituation task for the pair decanal/dodecanone. All groups show habituation with repeated exposure. Data are represented as data points and mean ± sem.

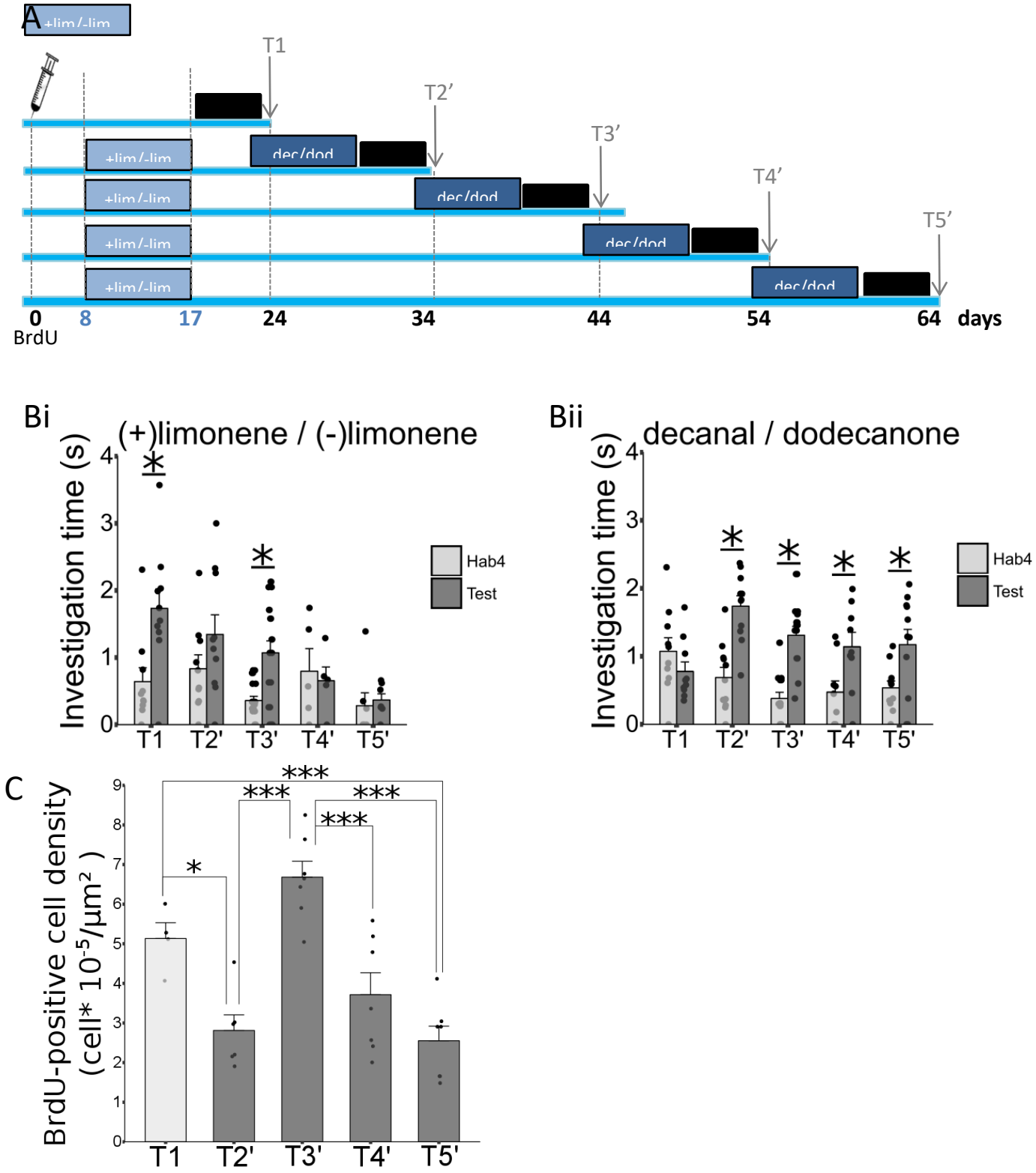
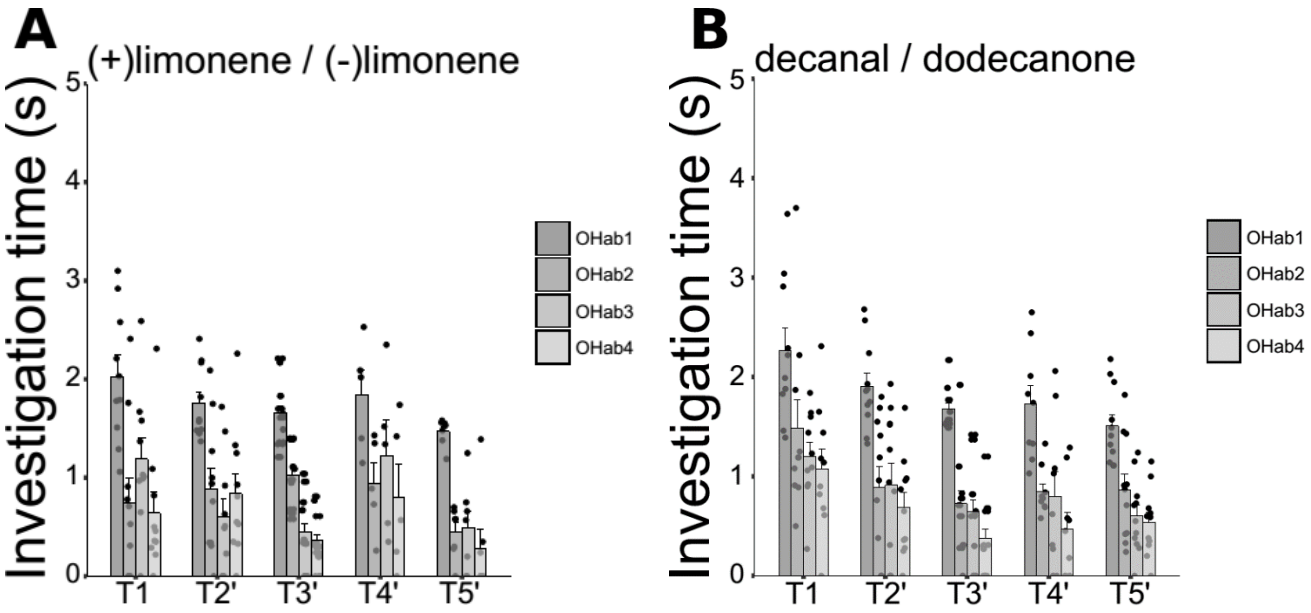


Figure 2: The delay between two sucessive learning is a key determinant for retention of learned dicrimination and adult-born survival

A. Experimental design. The groups differed by the interval between the two enrichment periods: 4, 14, 24 or 34 days separating the two enrichments. **B.** Behavioral results. **Bi.** Discrimination between (+)limonene/(-)limonene. Groups T1 and T3' discriminate. No discrimination is observed for the other groups (T2', T4', T5'). **Bii.** Decanal is discriminated from dodecanone in Group T2' to T5'. **C.** Higher density of adult-born neuron density is observed in groups that are able to discriminate (+)limonene from (-)limonene co. *p<0.05 ; ***p<0.001 Data are represented as data points and mean ± sem.

Group	Odorant	Habituation			Discrimination		
		Repeated measure ANOVA		Significant	T-test (1 tail, paired)		Significant
T1	+lim/-lim	F(3,29)=11.82	P<0,0001	***	T=-3.3, df=9	P=0.005	**
	dec/dodec	F(3,30)=6.89	P=0,001	***	T=1.24, df=9	P=0.88	ns
T2	+lim/-lim	F(3,30)=7.56	P= 0,0004	***	T=-1.38, df=9	P=0.1	ns
	dec/dodec	F(3,33)=9.0	P=0.0002	***	T= -4.52, df=10	P=0.00055	***
T3	+lim/-lim	F(3,70)=76.06	P<0.0001	***	T= -3.55, df=19	P=0.001	***
	dec/dodec	F(3,62)=38.02	P<0.0001	***	T= -6.65, df=17	P=0.000002	***
T4	+lim/-lim	F(3,13)=3.40	P=0.05	*	T=0.39, df=4	P=0.64	ns
	dec/dodec	F(3,27)=10.05	P=0.0001	***	T= -3.71, df=8	P=0.003	**
T5	+lim/-lim	F(3,18)=21.64	P<0.0001	***	T= -0.37, df=6	P=0.36	ns
	dec/dodec	F(3,32)=18.70	P<0.0001	***	T= -3.22, df=10	P=0.0045	**

Table 2: Statistical results.



<u>Supplementary figure 2</u>: Habituation.

A. Habituation (Hab1-Hab4) of the habituation/dishabituation task for (+)limonene/(-)limonene. All groups show habituation with repeated odor exposure. **B.** Habituation (Hab1-Hab4) of the habituation/dishabituation task for decanal/dodecanone. All groups show habituation with repeated exposure. Data are represented as data points and mean ± sem.

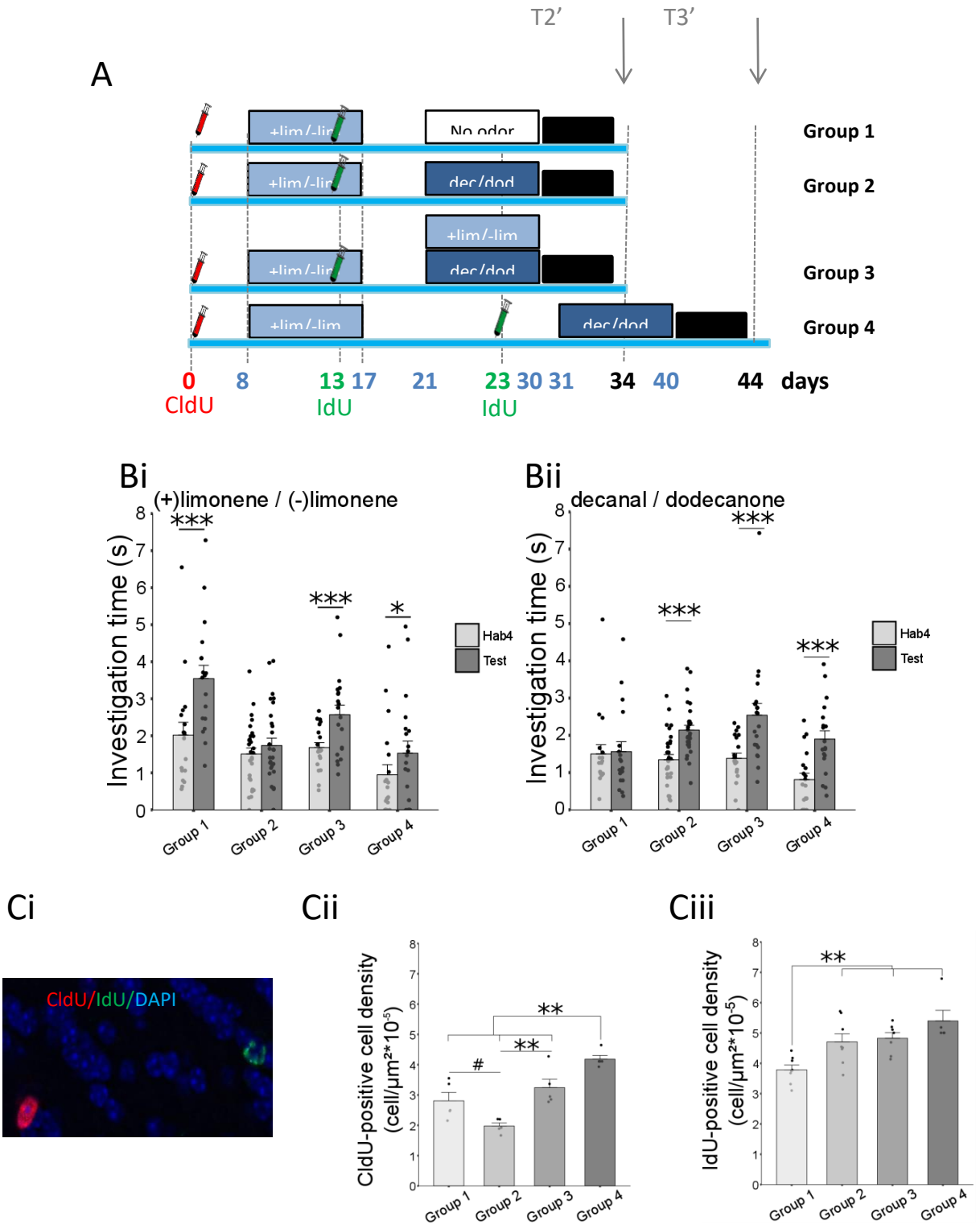
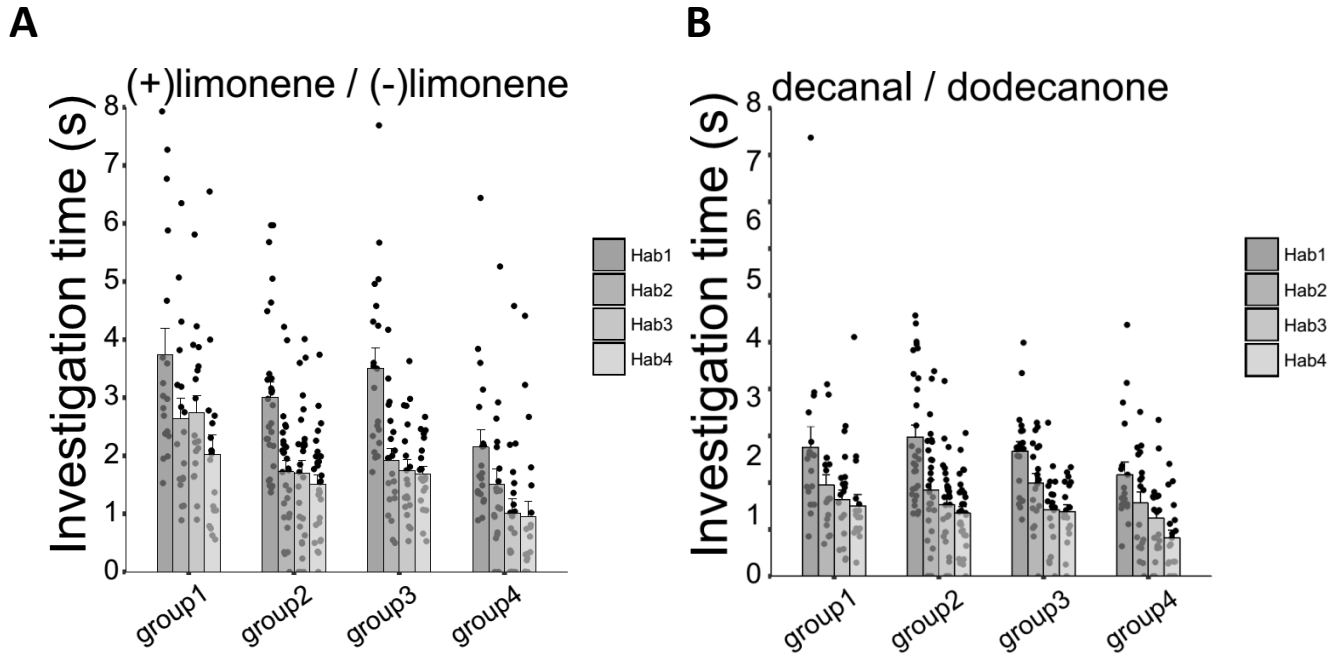


Figure 3: Maintaining the environement prevents cell death, maintains memory and the new learning recruits new adult-born neurons.

A. Experimental design. The groups differed by the interval between the two enrichments and by the odor pairs used for the second enrichment. **B.** Behavioral results. **Bi**. (+)limonene and (-)limonene are discriminated in Group 1 and 3 but not in group 2 and 4. **Bii**. Decanal/dodecanone are discriminated in Group 2, 3 and 4 but not in group 1. **Ci**. Example of Cldu/Idu/Dapi triple labelling. **Cii**. Density of CldU-positive cell is lower in Group 2 compared to group 1, 3 and 4 which correlated with the ability to discriminate (+)limonene from (-)limonene. **Ciii**. Density of IdU-positive cell is higher in group 2, 3 and 4 compared to group 1 which is correlated with the ability to discriminate decanal from dodecanone. #p=0.056;*p<0.05; **p<0.01; ***p<0.001. Data are represented as data points and mean ± sem.

Group	Odorant	Habituation			Discrimination		
		Repeated measure ANOVA		Significant	T-test (1 tail, paired)		Significant
Group 1	+lim/-lim	F(3,60)=7,35	P=0,0004	***	T = -5,43, df=17	P=0,00002	***
	dec/dodec	F(3,60)=5,8	P=0,0015	**	T= -0,43, df=17	P=0,33	ns
Group 2	+lim/-lim	F(3,95)=24,5	p<0.0001	***	T= -1,01, df=27	P=0,15	ns
	dec/dodec	F(3,100)=16,73	P<0.0001	***	T= -5,73, df=28	P=0,000002	***
Group 3	+lim/-lim	F(3,67)=13,83	P<0.0001	***	T= -3,01, df=19	P=0,0036	**
	dec/dodec	F(3,67)=12,78	P<0.0001	***	T= -3,34, df=19	P=0,0017	**
Group 4	+lim/-lim	F(3,65)=7,29	P=0,0001	***	T= -2,27, df=19	P=0,018	**
	dec/dodec	F(3,61)=10,42	P<0.0001	***	T= -4,86, df=18	P=0,00006	***

Table 3: Statistical results.



<u>Supplementary figure 2</u>: Habituation.

A. Habituation (Hab1-Hab4) of the habituation/dishabituation task for the pair (+)limonene/(-)limonene. All groups show habituation with repeated exposure. **B.** Habituation (Hab1-Hab4) of the habituation/dishabituation task for the pair decanal/dodecanone. All groups show habituation with repeated exposure. Data are represented as data points and mean ± sem.

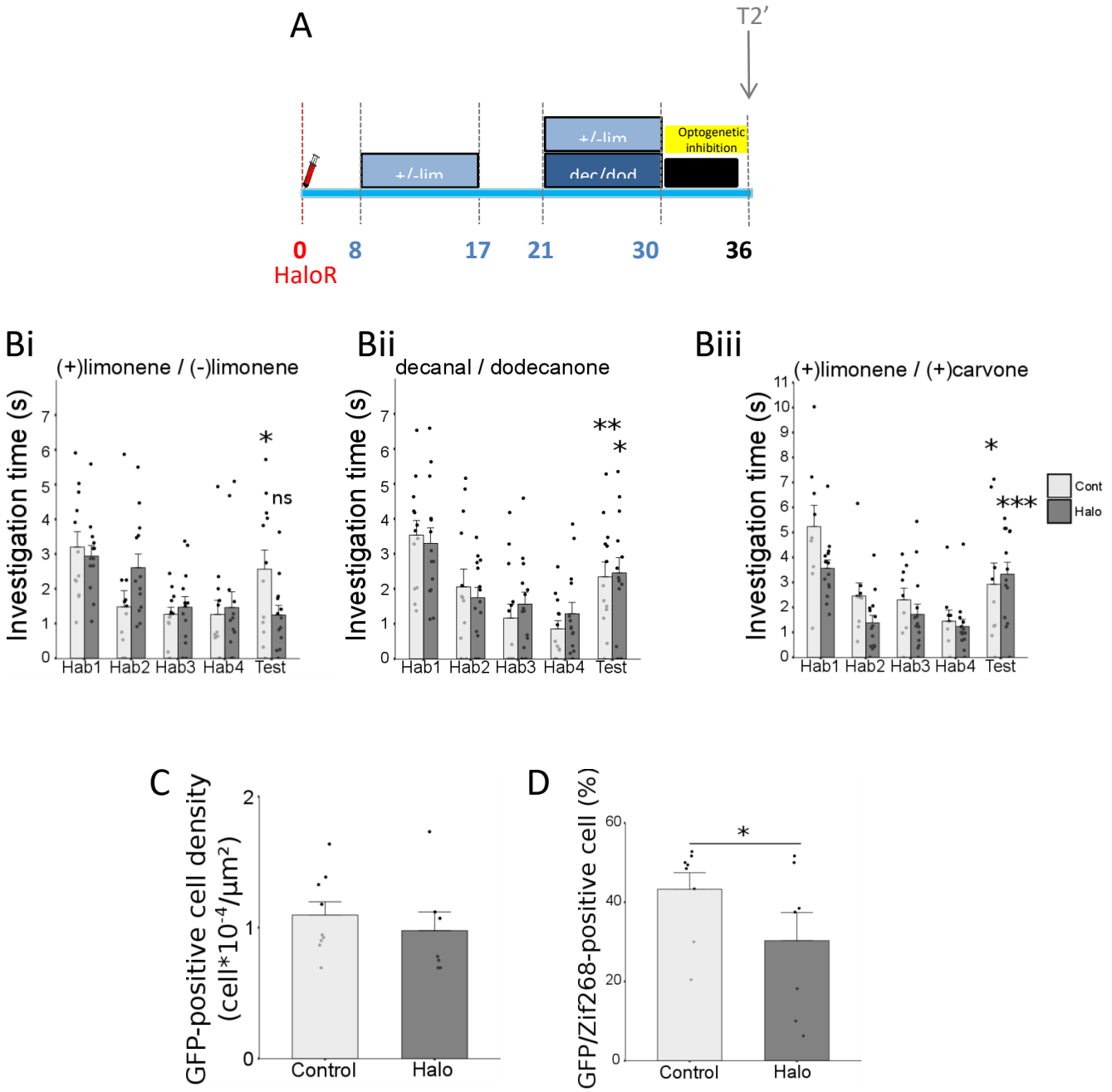


Figure 5: Light-induced inhibition of adult-born neurons induces memory loss.
A. Experimental design. Adult-born neurons aged of 8 days at the begining of the first enrichment were transfected with a lentivirus expressing halorhodopsin (Halo) or with an empty virus (Control). Light stimulation was perfomed during the test trial of the habituation/dishabituation task. **B.** Behavioral results. **Bi**. The learned discrimination of (+)limonene/(-)limonene is abolished by light stimulation in the Halo group, but not in the Control group. **Bii**. Discrimination between decanal/dodecanone is not altered by light stimulation. **Biii**. Discrimination between a dissimilar odorant pair (+)limonene/(+)carvone is not altered by light-triggered inhibition of adult-born neurons. **C**. The density of GFP-positive cells in the OB is similar between Control and Halo animals. **D.** Percentage of BrdU/Zif268-positive cells after light-triggered inhibition is decreased in Halo compared to Control. *p<0,05. **p<0,01. ***p<0,001. Data are represented as data points and mean ± sem.

Group	Odorant	Habituation			Discrimination		
		Repeated measure ANOVA		Signiicant	T-test (1 tail, paired)		Signiicant
Control	+lim/-lim	F(3,53) = 8,65	P= 0,0001	***	T=-1,78, df=15	P=0,048	*
	dec/dodec	F(3,41) = 11,31	p<0,0001	***	T=-3,86, df=12	P=0,001	**
	+limù/+carv	F(3,27) = 9,05	P = 0,0003	**	T=-2,28, df=8	P=0,02	*
Halorhodospin	+lim/-lim	F(3,58) = 8,94	P=0,0001	***	T=-0,12, df=16	P=0,45	ns
	dec/dodec	F(3,46) = 6,96	P=0,0006	**	T=-2,24, df=13	P=0,02	*
	+limù/+carv	F(3,46) = 11,42	P<0,0001	***	T=-4,63, df=13	P=0,0002	***

Table 4: Statistical results.

Study 3 - A model of adult-neurogenesis in the olfactory bulb

A model of adult-neurogenesis in the olfactory bulb

Jeremy Forest, Kathryn Zimmerman, Anne Didier, Nathalie Mandairon*, Christiane Linster*

*equal contribution

Introduction

The olfactory bulb (OB) is the first central relay of olfactory information and performs the first sensory processing operations. As such, and like other structures in similar positions, it has a critical goal in gain control and decorrelation (Cleland, 2010). In the OB, there are two main microcircuits responsible for computation: at the OB input level, the glomerular microcircuit and the OB output level mitral-granule cell microcircuits (Cavarretta et al., 2016; Cleland, 2010). More specifically, the glomerular level seems to have a role in stimulus normalization and decorrelation (Cleland, 2010; Cleland et al., 2012; Cleland and Linster, 2012; Cleland and Sethupathy, 2006; Gire and Schoppa, 2009; Linster and Cleland, 2009; McGann et al., 2005). These normalization processes allow facilitation of odor recognition independently of odor concentration (Cleland et al., 2007). Computations realized at the level of the mitral-granule microcircuit are based on feedback and lateral inhibition and also include decorrelation and discrimination mainly via regulation of spike timing and synchronization (Arevian et al., 2008; Cleland, 2010).

A particularity of the OB however is adult neurogenesis (Altman, 1969; Lois and Alvarez-Buylla, 1994; Ming and Song, 2005). Stem cells residing in the subventricular zone of the lateral ventricle, divide into neuroblasts that migrate along the rostral migratory stream to reach the OB and differentiate into periglomerular (3%) and granule cells (97%). These inhibitory interneurons can reshape the output message of the OB carried out by the mitral cells of the OB (Lledo et al., 2006; Malvaut et al., 2015; Winner et al., 2002).Thus there is a constant interplay between adult-born neurons and preexisting neurons in the OB.

So far however the respective computational role of adult-born versus preexisting granule cells population of neurons in the OB is relatively poorly understood. We know they have different *in vivo* properties (Carleton et al., 2002; Kelsch et al., 2008, 2010; Livneh et al., 2014; Nissant et al., 2009) and seem to not underlie the same behavioral functions (Lemasson et al., 2005; Magavi et al., 2005). However few studies have modeled both of those population in the OB (Aimone, 2016; Cecchi et al., 2001; Chow et al., 2012). What they have shown however is that adult-born neurons are able to maximize the discrimination of odorants and also drive stimulus decorrelation. We want to understand in a computational model, constrained by biological data,

the differential role of adult and preexisting neurons in the computations underlain by the OB and their specific role in perceptual leaning.

This model is still under development. Adult-neurogenesis processes are still being integrated and future development will be discussed.

Materiel & methods

Network architecture. The olfactory network is comprised of four types of cells: olfactory sensory neurons (OSN), Mitral cells (MC), preexisting granule cells (prGC) and adult-born granule cells (abGC). It is based on our previous work (de Almeida et al., 2013). At this time the periglomerular (PG) layer of the network has been removed to simplify computation.

Model neuron equations. All neurons except mitral cells were represented as single compartments. Mitral cells are comprised of two compartments. The evolution of the membrane voltage over time in each compartment is described by a first-order differential equation adopted from previous works (de Almeida et al., 2013; Linster and Cleland, 2002):

$$\tau \frac{dv(t)}{dt} + v(t) = V^{ext}(t) \qquad (1)$$

where τ is the membrane time constant, $V^{ext}(t)$ is the total external input over time and different neurons receive different external inputs. The complete list of parameters is the same as in de Almeida et al., 2013, except when explicitly specified. The input from a specific presynaptic neuron at time *t* is the following:

$$V^{ext}(t) = W_{ij} g_{ij}(t)[E_{N,ij} - v_j(t)] \qquad (2)$$

where W_{ij} is the synaptic strength between neuron *i* and neuron *j*, $g_{it}(t)$ the conductance change in cell *i*, $E_{N,ij}$ is the difference between the Nernst potential of the specific channel type and $v_j(t)$ is the current membrane potential of the postsynaptic neuron.

The communication between neurons in the network occurs either through continuous output or through discrete action potentials (for spiking neurons), both a function of the membrane potential:

$$F_i(V) = \begin{cases} 0 & if\ V \leq\ \theta^{min} \\ \left(\frac{V\ -\ \theta^{min}}{\theta^{max}\ -\ \theta^{min}}\right)^{\beta} & if\ \theta^{max} < V <\ \theta^{min} \\ 1 & if\ V \leq\ \theta^{max} \end{cases} \qquad (3)$$

where $F_i(V)$ represents the continuous output (for OSNs and PGs) or the instantaneous spiking probability (for MC, PrGr, abGr). Θ^{min} and Θ^{max} represent the minimum threshold and the saturation threshold of respectively, the output and probability function. β represents the nonlinearity of $F_i(V)$.

For continuous presynaptic cells $g_i(t) = g^{max} * F_i[v(t)]$; and for spiking presynaptic cells $g_i(t) = g(t - t_i^{fire})$ with the time course of the conductance given by the following equation:

$$g(t) = g^{max}\left(e^{-\frac{t}{\tau 1}} - e^{-\frac{t}{\tau 2}}\right) \qquad (4)$$

where τ_1 and τ_2 are the rising and falling times of the conductance respectively, and $g^{max}$is a constant with no unit representing the maximum conductance of a given channel. After firing the voltage of each spiking neuron is reset to a hyperpolarization potential v^{hyper} and remains inactive for a refractory period τ^{refrac}.

Mitral cells are composed of two distinct compartments: one representing their apical dendrite (apical compartment) and the other representing lateral dendrites and soma (soma compartment). Each of these compartments performs a separate cellular computation by interacting with different cell groups. The two compartments are electrically coupled, and the output computed in the apical compartment $F(V_{apical)}$ is multiplied by the parameter $V^{max}$and directly summed to the membrane potential of the soma compartment. The soma compartment represents the integration site for all mitral cell input and generates action potentials.

Adult-neurogenesis

Adult-born neurons are integrated into the network continuously. Equations governing their membrane potential, input synapses, conductance time course and output are described above.

Knowing that adult-born neurons are more excitable than preexisting neurons however, we set the non-linearity factor β differently in each population (Figure 1) with the firing probability of adult-born neurons being higher than preexisting neurons.

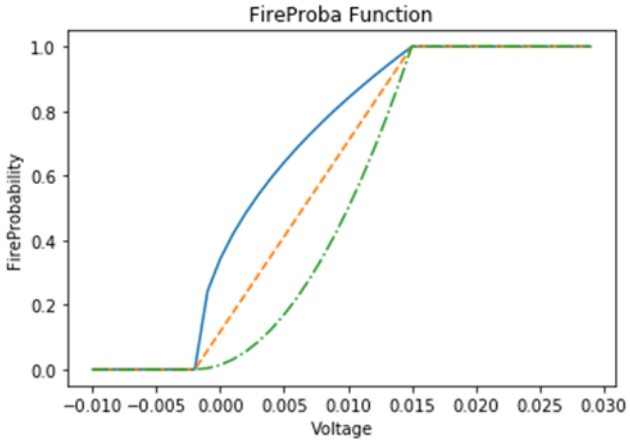


Figure 1: Firing probability of adult-born and preexisting population as a function of cell voltage. The firing probability of adult-born neuron (blue line, $\beta = 0.5$) is higher than the firing probability of preexisting neurons (green line, $\beta = 2$).

Adult-born survival is based on activity-dependent mechanisms developed previously (Cecchi et al., 2001; Chow et al., 2012).

Granule cells (both adult-born and preexisting ones) are removed probabilistically depending on their survival probability which is dependent on a sigmoidal function:

$$p(R_i) = p_{min} + \frac{1}{2}\{tanh(\gamma(R_i - R_0)) + 1\}(p_{max} - p_{\min}) \qquad (5)$$

With R_i defining the resilience of the cell and defined as:

$$R_i = \sum_{\alpha=1}^{N_s} \left[G_i^{(\alpha)} - G_{min} \right]_+ \qquad (6)$$

Briefly, this means that each neuron has a resilience to apoptosis that is dependent on its activity and in turn influences its survival probability. Adult-born neuron addition as well as survival and apoptosis calculations are done every epoch ΔT of 5000ms of neural activity simulation. Simulation was run for $\Delta T = 100$ epoch.

All simulations were implemented within the Python programming language, with a Euler integration method for the differential equations with a time step of 1 ms.

Results, perspectives and discussion

At this date, the model is still in its early stages of development. Accordingly results are limited and I will mostly talk here about model conception and planned perspectives for later implementations.

All computations have so far been done without odor stimulation in order to tune the model in basal conditions. The idea is that once the model is well established according to simple rules, we will be able to build on those and hopefully not having to redefine any new hyperparameters. This would make the model as simple as possible and thus generalizable.

For now, the network is comprised of 20 glomeruli and initialized with 900 preexisting and 100 adult-born granule interneurons. The simulation is run for 100 epoch ΔT each one containing 5000ms of neuronal activity computation. Each cycle begins with the calculation of neuronal survival followed by arrival of new adult-born neurons and establishment of their connexions and ending with cell death and purge of dead cells associated connexions.

Neural activity is higher in adult-born than preexisting neurons in a 5000ms time windows.

We can see that there is a higher activity in adult-born granule than in preexisting granule cells (Figure 2A and 2B). This is to be expected as adult-born neurons have a higher firing probability than preexisting neurons.

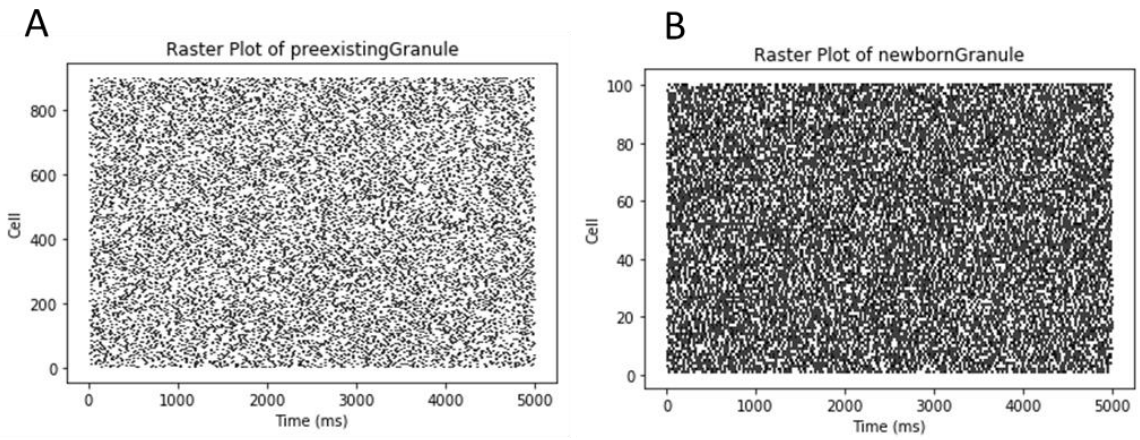


Figure 2: Raster plot of cell discharge during simulation time for preexisting and adult-born granule cells.
A. Preexisting and **B.** adult-born granule cells firing during the last Δ simulation ($\Delta T = 1$). Preexisting granule cells spike a total of 50505 times (average of 56 time per cell) while adult-born granule cells spike 12272 times (average of 123 times per cell).

Adult-born and preexisting neurons population dynamic

We can see first (Figure 3A) that global cell number is decreasing in the OB. Second, preexisting neurons show a slow but steady decrease in number indicating regular apoptosis. Third, adult-born neurons are being generated as their number is increasing. However none of them seem to be dying. Looking at the survival percentage of adult-born neurons along ΔTiteration (Figure 3B), we can see that indeed no adult-born neuron is dying.

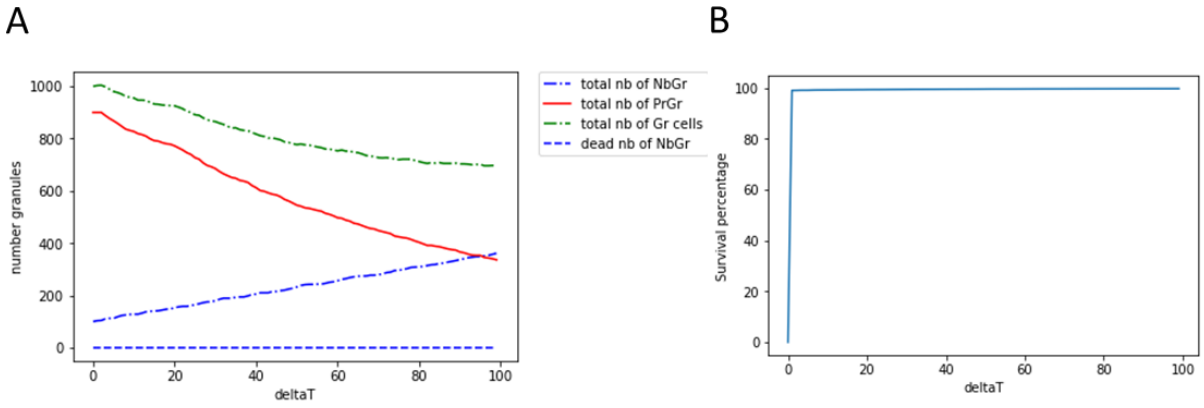


Figure 3: Preexsiting and adult-born granule cell population dynamic
A. Survival and death dynamic of adult-born (NbGr) versus preexisting (PrGr) neurons. Total number of cells in the olfactory bulb is decreasing (green line). Only the preexisting population number of neurons is decreasing, no neuronal death is observe in the adult-born neuron population. **B.** Survival percentage of adult-born neurons born at each ΔT iteration. There is no death of adult-born neurons.
Hyperparameters used for this simulation: $G_{min} = 45, R_0 = 0{,}5, \gamma = 1$.

These results are not in accordance we what can be observed biologically.

Constraining the model

For now, hyperparameters are manually selected and changed. However, that is not efficient and requires manual intervention. The next step is currently being done and consists in rewriting some chunks of the code to be able to perform parallel computation on several CPU cores, speeding up computation. This would be done to allow for the screening of multiples possible combinations of hyperparameters to select the one that better constrain the model according to biological parameters.

These parameters that need to be respected are the following: 1- the OB does not exhibit volume variations due to change in cell number (Petreanu and Alvarez-Buylla, 2002); 2-half of the adult-born neurons should die after a few ΔT iterations (Winner et al., 2002). For now these parameters are not respected (figure 3A and 3B).

In order to do so an hyperparameter search will be implemented using random search algorithm (Bergstra and Bengio, 2012).

Later work will focus on generating olfactory input extracted from the glomerular activation pattern of natural stimuli (Chow et al., 2012; Johnson and Leon, 2007), take into account cell age (already implemented but not yet used) allowing the definition of critical periods (see study 2) and including spine synaptic plasticity which is different in adult-born versus preexisting neurons (see study 1).

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PART III: General discussion

This thesis work was aimed at understanding the role of OB GCs (both adult-born and preexisting) in an environment that is complex and changing.

1. Behavioral adaptation in changing and complex environment

The use of changing and complex environments was done in order to understand the role and contribution of adult-born neurons in environmental conditions that are closer to real life.

In the first study, we observed that mice are able to learn to discriminate multiple pairs of odorants at the same time (from 1 to 6). This result raised the question of the number of similar odorants that the mice are able to learn to discriminate at a same time: is there a limit? We found that when the patterns of activity of the odor pairs are weakly overlapping, which is the case for the first 3 odor pairs used, (+)limonene/(-)limonene, decanal/dodecanone and acetic acid/propionic acid (Figure 7), the enrichment with one of this pair will not lead to the discrimination of the others (Figure 8)(Mandairon et al., 2006c, 2006d; Moreno et al., 2009). However, the activity patterns of these three pairs of odorants cover most of the OB. Adding any odorant pair will evoke activation patterns necessarily overlapping with that of the first three pairs. This overlap will lead the enrichment with one of this pair to improve the discrimination of the enrichment pair of the others (Mandairon et al., 2006c; Moreno et al., 2009). This suggests that enrichments with more than 3 odor pairs will lead to the discrimination of a virtually infinite number of odorants.

In the second study, we observed that when the delay between the 2 learning is long (14 days), mice remember them both while when the delay between the 2 learning is short (4 days), mice remember only the second one. At the same time, maintenance of the first environment while learning the second, allows memory maintenance. At the behavioral level, this suggests that, in the presence of interferences, when the information is stable in the environment and thus pertinent, it is retained. Without any interferences, the information is retained during several weeks even when the environmental information is removed.

This dichotomy between the ability to learn to discriminate a virtually infinite number of odorants and the inability of learned discrimination if the odors are no longer present in the

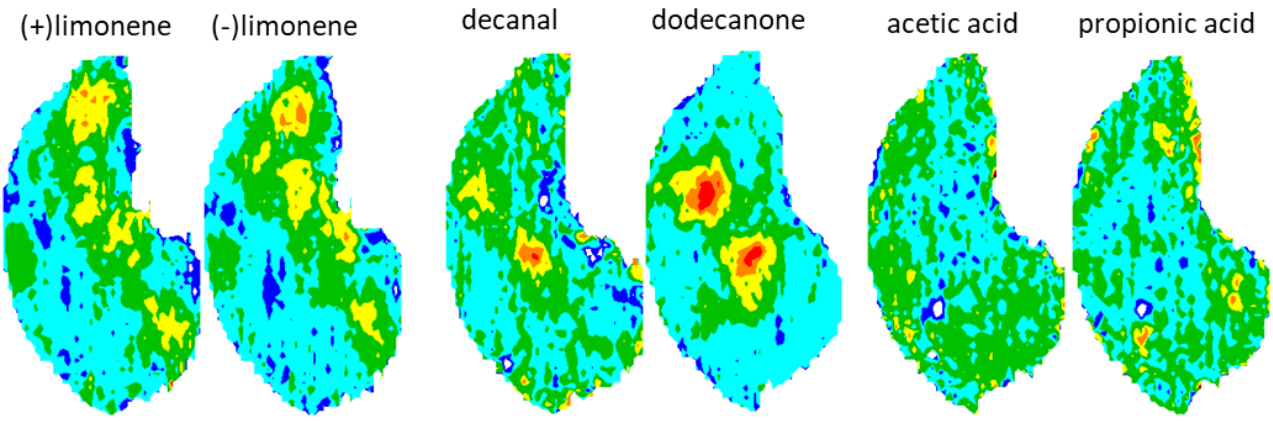

Figure 7: Glomerular activation pattern of (+)limonene, (-)limonene, decanal, dodecanone, acetic acid and propionic acid. Each odor pair ((+)limonene/(-)limonene ; decanal/dodecanone ; acetic acid/propionic acid) evoke similar glomerular activity pattern.

environment and replaced by others, is very interesting. Indeed, one could hypothesized that the information is quickly erased if not pertinent (no longer present in the environment for instance and new discriminatory needs) to avoid filling up the brain capacity storage with non-relevant information potentially damaging to decision-making due to retrograde interferences (Richards and Frankland, 2017) while all important information is encoded and retrieved when needed even a long time after learning. Neural mechanisms underlying this process include neurogenesis and more specifically adult-born neurons within their critical period which allows for memory storage and forgetting depending of the learning time window. Learning ability depends also on the consolidation processes (Kermen et al., 2010). Indeed, in this previous study, the authors analyzed the impact of an associative training which can be either massed or spaced. The authors showed that spaced but not massed learning allows for long-term memory and thus the inter-trial interval is important for memory to last. We revealed in our second study that the improvement in the ability to discriminate between two odorants induced by perceptual learning (which includes consolidation processes over days) is remembered for at least 27 days (Study 2). If we compared this result to the length of memory of odor discrimination after associative olfactory learning, we found relatively similar timing (Sultan et al., 2010). It could be interesting to investigate if complex perceptual learning (Study 1) versus repeated perceptual learning (Study 2) has any influence on the duration of memory maintenance to see if it can be paralleled to associative learning on that parameter.

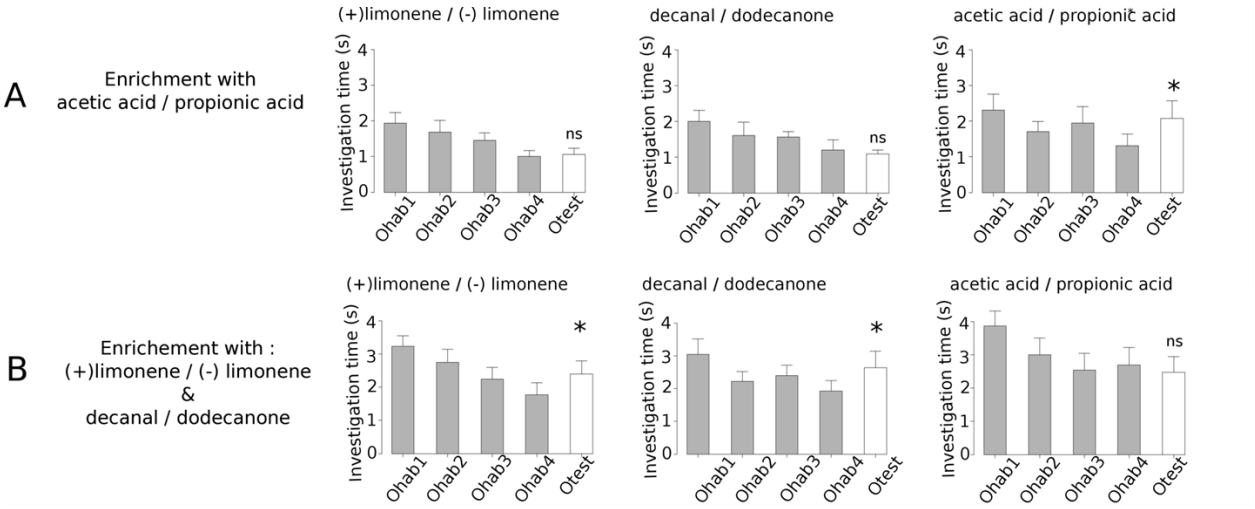

Figure 8: Discrimination tests.
A. Enrichment wit acetic acid and propionic acid. An increased investigation time is seen only when testing discriminatory ability between the learned pair (acetic acid/propionic acid) and not the other two (+limonene/-limonene and decanal/dodecanone). **B.** Enrichment with (+)limonene/(-)limonene and decanal/dodecanone. Animals show increased investigation time to the learned odor pairs but not the third one.

2. Neurogenesis is the central pillar underlying olfactory behavioral adaptation

Perceptual learning requires adult-neurogenesis (Moreno et al., 2009). In the optogenetic experiments that we performed in both studies (Study 1 and Study 2), we blocked, during the discrimination test, the activity of adult-born neurons aged of 8 days at the beginning of the enrichment period and thus being in their integrating period in the OB. This manipulation altered the performances of learned discrimination. These data reinforce and extend the previous finding revealing the requirement for a specific adult-born neurons population during learning and also for memory retrieval of simple and complex perceptual learning.

Adult-born neurons continuously integrate the bulbar circuit. This process coupled with individual neurons survival or death allows for filtering of relevant information. In olfactory learning (associative and perceptual) we see an improvement of adult-born neuron survival (Alonso et al., 2006; Kermen et al., 2010; Mandairon et al., 2011; Moreno et al., 2009; Mouret et al., 2008; Sultan et al., 2010; studies here). Interestingly, the rate of adult-born neuron surviving is broadly similar between olfactory tasks (Moreno et al., 2009; Sultan et al., 2011b; studies

here). Hence, while the rate of adult-born neuron survival does not seem to be specific of any behavioral task, the “where” and “when” of neuronal survival seem to be important parameters.

The second level of plasticity is the functional recruitment of adult-born neurons. As we have observed, the pool of adult-born neurons responding to the learned odorants is enhanced among the surviving neurons in proportion to the complexity of the task. Since learning 2 odor pairs does not double the number of functionally recruited adult-born neurons compared to 1 odor pair, we can make the assumption that each adult-born neuron is participating in the processing of more than one pair of learned odorants. In parallel to that, we observed that successive perceptual learning recruited and then induced cell death of adult-born neurons suggesting that adult-born neurons recruited by perceptual learning can only contribute to one learning. An individual neuron can be recruited to participate to olfactory processing if it is in its critical period but, past that, it either dies or survives to keep only the memory of this information. If during the critical window, learning involves one odor pair, then only that pair will be encoded; if learning involves several pairs, then all of them will be encoded.

We analyzed a third level of plasticity: the structural plasticity of adult-born granule cells. Our data revealed spine but not dendritic plasticity. There is contradictory evidence in the literature on dendrite versus spine plasticity in adult-born neurons as well as the link between the two that remains unclear. Experimental data show that dendritic plasticity can happen at the same time as spine modifications (Daroles et al., 2015) while other demonstrate preferential spine plasticity (Mizrahi, 2007, study 1).

Changes in spine densities are observed in our study at all of the three levels of neuronal subdivision. While another study also observed spines modifications, their localization varied (Lepousez et al., 2014). The explanation for these observed differences might be dependent on the type of learning (associative versus perceptual) which might necessitate, in part, different information processing mechanisms.

We have not considered here other forms of plasticity that have been observed in adult-born neurons. Indeed, adult-born neurons are able to perform, at least temporarily, long-term

potentiation (LTP) upon cortical feedback stimulation (Nissant et al., 2009). It is possible that the amplitude or the duration of the induced LTP vary when spine density is modified. Furthermore, spines not only demonstrate a high turnover (Sailor et al., 2016) but can be classified in different types based on their shapes, which are the reflection of functional attributes like structural stability or synaptic strength (Harris et al., 1992; Hering and Sheng, 2001). So far however, we have not analyzed the variations of spine types. We also haven't looked at a recently demonstrated activity-dependent plasticity mechanisms that is spine relocation (a form of rapid structural plasticity occurring only in adult-born neurons) in the different learning conditions (Breton-Provencher et al., 2016; Hardy and Saghatelyan, 2017) . All these parameters are potential sources for more occurrences of plastic adaptations that would be interesting to study.

Finally, we have not considered glomerular layer modifications because we did not observed an effect of perceptual learning on the number of PG (Moreno et al., 2009). However, that does not mean that their morphology or physiology is not modified (Bovetti et al., 2009). Indeed, at least part of the behavioral adaptation could potentially be supported by glomerular processing modifications.

Adult-born neurons are always required for perceptual learning but not always sufficient depending on the behavioral demand, for example when the environment becomes so complex that their plasticity is no longer sufficient.

3. Enter preexisting neurons

Up to now, the specific contribution of adult-born compared to preexisting neurons to learning has rarely been studied. Several studies focused on the differences between adult-born and older neurons (usually a mixed of preexisting and old adult-born neurons) in basal conditions demonstrating a transient higher responsiveness of adult-born neurons or spine relocation type of plasticity in adult-born but not preexisting (P6) neurons (Livneh et al., 2014; Breton-Provencher et al., 2016). Two studies using broad enrichment (Magavi et al., 2005) or early life learning (Lemasson et al., 2005) analyzed preexisting neurons in term of functional recruitment and experience-dependent plasticity of their response and demonstrated that each population

undergo different modifications. Indeed they respectively showed that a greater proportion of adult-born neurons respond to novel odorants compared to preexisting neurons sand that early olfactory experiences specifically modify the number of GC in pups but doesn't impact adult-neurogenesis. Here we show that when the task gets complex, the plasticity conferred by adult-born neurons is not sufficient to grant the animal enough plasticity; preexisting neurons are then recruited to participate in processing the olfactory information. Indeed, two main points emerge from our data: first, preexisting neurons seem to not be functionally participating to the discrimination of dissimilar odorants or to the discrimination of odorant after simple perceptual learning (as inhibiting their activity via optogenetic does not prevent these behaviors); second they are still able to perform morphological plasticity which could underlie complex perceptual learning (increased apical distal spine density)(as blocking plasticity prevents learning in simple paradigm (Daroles et al., 2015)).

The results obtained here coupled to what is found in the literature about adult-born and preexisting neurons highlight differences in their functions as well as in their intrinsic properties, both able to change with learning but in different ways. Preexisting neurons compared to adult-born neurons seem less plastic, meaning that they seem more likely to be stable over time. Adult-born neurons being a more plastic and changing neuronal population can easily be the substrate of adaptive learning. They would serve an experience-based or circuit-demand form of plasticity, always here, readily available and shaped by environmental demand. In other words, these results could reflect the OB way to reconcile, on one hand the necessity for stability of the encoded information thanks to preexisting neurons and old adult-born neurons and, on the other hand the necessity of having a flexible and very plastic network allowing new information to be acquired and stored rapidly thanks to adult-born neurons.

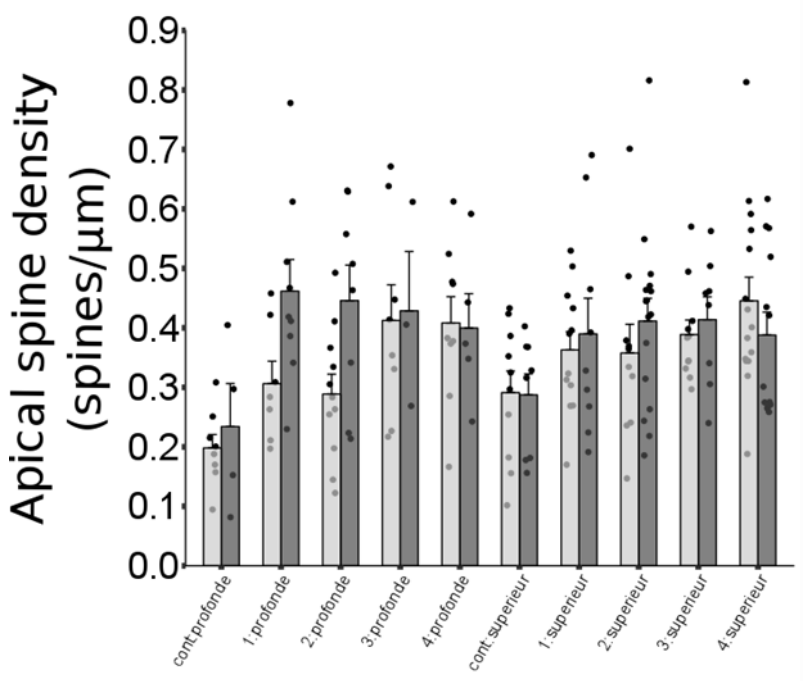

Figure 9: Apical spine density of adult-born neurons from the study 1.
Adult-born neurons previously analyzed in the different conditions (enrichment from 0 to 6 pairs of odorants) have been segregated based on their location in the granule cell layer (deep or superficial).

4. Cell types diversity

As said in the introduction, the cell type diversity of the OB is important and often not taken into account in attemps to understand odor processing and learning mechanisms in the OB. This could be due to several reasons, among which the absence of sufficient knowledge about the cell types diversity and also for simplicity's sake. However many studies have suggested and recently demonstrated functional differences between granule cells in the superficial versus deep granule cell layer which are respectively more associated with tufted and mitral cells (Bourne and Schoppa, 2017; Geramita and Urban, 2017; Geramita et al., 2016; Griff et al., 2008; Haberly and Price, 1977; Imayoshi et al., 2008; Mandairon et al., 2006; Mori et al., 1983; Nagayama et al., 2004). There is now strong evidence that mitral and tufted cells both have segregated microcircuits, probably underlying different computations (Geramita et al., 2016). We also found in the second study some hints about functional differences in the granule cell layers. For example, in our data, some results indicate that there is an increase in apical spine

density of the granule cells residing in both deep and superficial granule cell layer but this increase is specific of Zif268-positive neurons only for the deep granule cells (Figure 9).

5. Role of adult-born granule cells in pattern separation?

More broadly, what can we learn from these experiments on the role of neurogenesis? As stated in the introduction, computational studies based on biological data seem to point granule cells as being perfectly adapted to performed learned pattern discrimination (Cleland, 2010).

Most of the data obtained in the past couple of decade as well as the data obtained here seem to confirm this. Indeed, granule cells thanks to adult-neurogenesis are an extremely dynamic population regulating the spatiotemporal output of the OB which help enhance contrast between stimulus (Mandairon and Linster, 2009; Sahay et al., 2011). Moreover, that function of pattern separation is postulated to be a common mechanisms underlain by adult-born neurons in both the OB and the dentate gyrus of the hippocampus (Aimone et al., 2011; Sahay et al., 2011).

However, discrimination can be attained by two distinct mechanisms (Aimone et al., 2011). One is pattern separation (in the electrophysiological and computational sense) meaning that similar input patterns will see their outputs being orthogonalized and thus becoming more dissimilar. The other possibility however is that the discrimination is attained by adding information on one of the odors, i.e. increasing the odor representation's resolution. If this phenomenon exists, could it be possible to learn discrimination of a pair of similar odorants with exposure to only one odorant of a pair? One study showed that performing perceptual learning with exposure to (+)limonene induced better discrimination of the odor pair (+)limonene/(-)limonene although seemingly less reliably and in a more restricted way than following enrichment with the two odorants of the pair because learning was not transferred to the discrimination of odorants with representations overlapping with the enrichment odors (Figure 10) (Mandairon et al., 2006c).

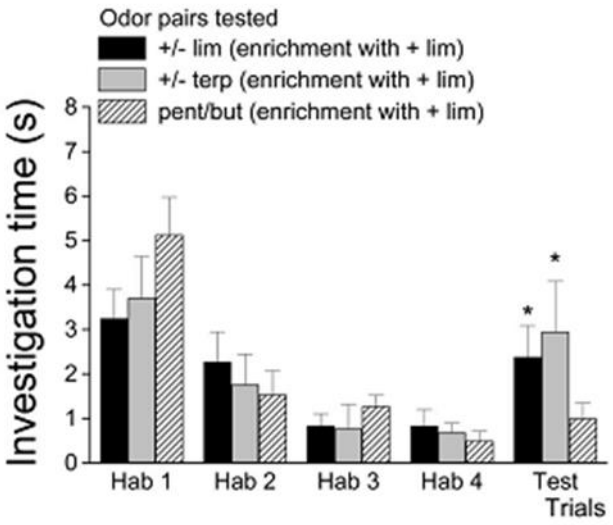

Figure 10: Enrichment with (+)limonene only.
Enrichment with +lim lead to the discrimination of the pair +lim/-lim and +terp/-terp but not pent/but. (Adapted from Mandairon et al. 2006c).

While most of the biological and computational data obtained are consistent and point to granule cells as performing pattern separation for perceptual learning there are also alternative explanation like this increased odor representation's resolution that are worth considering and should not be forgotten.

Conclusion

Overall this thesis work has reinforced the central role of adult-born neurons in behavioral adaptation. By using perceptual learning, it demonstrated that when the task become complex, neurons born during ontogenesis, i.e. preexisting neurons, can be recruited functionally to the processing of the learned odors and are still morphologically dynamic. It also showed not only that individual neurons' critical period is a major regulator of their own death and survival but more importantly that when adult-born neurons, besides underlying perceptual learning, are involved in a specific learning they cannot be recruited to underlie another. This work helps us understand how the olfactory system is able to adapt to environmental changes. More generally it provides new correlative and causative evidence shedding light on for the neural basis of behavior.

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Figures List

Figure 1 : Olfactory epithelium organization 18
Figure 2: Olfactory bulb structural and functional organization. 20
Figure 3: Olfactory bulb cell diversity. 24
Figure 4: Olfactory bulb projections on olfactory cortices 27
Figure 5: Olfactory bulb projections on olfactory cortices 30
Figure 6: Adult-born interneurons 35
Figure 7: Glomerular activation pattern of (+)limonene, (-)limonene, decanal, dodecanone, acetic acid and propionic acid 139
Figure 8: Discrimination tests 140
Figure 9: Apical spine density of adult-born neurons from the study 144
Figure 10: Enrichment with (+)limonene only 146

PART V: Appendix

Annexe 1: Forest, J., Midroit, M., Mandairon, N., 2017. La plasticité hors du commun du système olfactif. Pollut. Atmos.

La plasticité hors du commun du système olfactif

Extraordinary olfactory system plasticity

Jérémy Forest[1], Maëllie Midroit[1], Nathalie Mandairon[1],

Résumé

Le cerveau de mammifère adulte est remarquablement plastique ; en effet, il est capable de se modifier constamment en réponse aux stimulations environnementales changeantes. Ces modifications induites par l'expérience façonnent notre perception de l'environnement.

Dans de nombreuses régions du cerveau, cette plasticité se manifeste par des modifications de la morphologie des neurones ainsi que des changements du nombre et de la position de leurs connexions. Ces modifications anatomiques et fonctionnelles du cerveau permettent les apprentissages et sous-tendent la mémoire. Cependant, dans deux régions cérébrales, cette plasticité devient « extra-ordinaire » puisqu'elle consiste également en l'ajout de nouveaux neurones dans des réseaux complexes de neurones préexistants. Ce phénomène s'appelle la neurogenèse adulte. Les deux régions du cerveau qui sont les cibles de la neurogenèse adulte sont l'hippocampe et le bulbe olfactif, structures importantes pour le traitement de l'information spatiale et olfactive respectivement. Entre ces deux sites de neurogenèse, c'est le bulbe olfactif qui reçoit l'apport le plus important de nouveaux neurones. Ces nouveaux neurones bulbaires sont issus de cellules souches qui vont donner des neuroblastes (neurones immatures) qui migrent jusqu'au bulbe olfactif pour donner des neurones matures et fonctionnels. La neurogenèse adulte, bien que spatialement restreinte dans le cerveau de mammifère, constituerait un processus de plasticité accrue permettant l'apprentissage et la mise en mémoire des informations.

Même si l'olfaction est, parmi nos cinq sens, celui auquel nous attachons souvent le moins d'importance, il est extrêmement présent dans notre quotidien et influence notre qualité de vie. En effet, les odeurs modulent nos humeurs, notre cognition et notre comportement. Ce sens est non seulement essentiel à la prise alimentaire, puisque l'olfaction est un composant majeur de la perception de la flaveur des aliments, mais il intervient également dans l'évitement des dangers comme, par exemple, des incendies, des fuites de gaz, et enfin il joue un rôle dans les interactions sociales. Pour analyser ces milliers d'odeurs que nous percevons tous les jours, nous disposons d'un système olfactif capable de détecter, d'identifier et de discriminer les odeurs. Le traitement sensoriel des odeurs est un processus dynamique tout au long de notre vie, qui est permis par la neurogenèse adulte. Cependant la neurogenèse adulte peut être altérée par le stress ou la pollution, conditions provoquant inévitablement des troubles de la perception des odeurs.

Mots-clés

olfaction, neurogenèse adulte, stress, pollution

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Abstract

The adult mammalian brain is highly plastic. Indeed it is constantly able to remodel itself in response to changing environmental stimulations. These modifications induced by experience shape our environmental perception.

In several brain regions, this plasticity is underlined by changes in the morphology of neurons including modifications of the number or position of their connections. These anatomical and functional modifications allow learning process and memory formation. However in two specific brain regions, this plasticity becomes extraordinary in the sense that new neurons are constantly added in complex preexisting networks. This phenomenon is called adult neurogenesis. The two brain regions where adult neurogenesis occurs are the hippocampus and the olfactory bulb, structures important respectively for spatial and olfactory information processing. Among these two structures, the olfactory bulb receives the most important number of neurons. These new bulbar neurons come from stem cells which divide into neuroblasts (immature neurons) that migrate to the olfactory bulb where they give rise to mature and functioning neurons. Adult neurogenesis, even if spatially restricted in the brain, provides a higher level of plasticity allowing learning and memory.

Even if, among our five senses, olfaction is the one we pay the less attention to, it is extremely present on a daily basis and influences our quality of life. Indeed, odors can have an effect on mood, cognition and behavior.

This sense is not only of primary importance in nutrition behavior because of its importance in flavor perception but it is also essential to avoid dangers like fires or gas leaks. It is also playing a crucial role in social interactions. In order to analyze these millions of odors that we perceive each day, we have an olfactory system capable of detecting, identifying and discriminating odors. The odor sensory processing is a dynamic process occurring during our whole life and permitted by adult neurogenesis. However adult neurogenesis can be altered by stress or pollution, both causing trouble with our odor perception.

Keywords

olfaction, adult neurogenesis, stress, pollution

Introduction

Même si l'olfaction est, parmi nos cinq sens, celui auquel nous attachons souvent le moins d'importance, il est extrêmement présent dans notre quotidien et influence notre qualité de vie. En effet, les odeurs modulent nos humeurs, notre cognition et notre comportement. Ce sens est non seulement essentiel à la prise alimentaire puisque l'olfaction est un composant majeur de la flaveur des aliments (en association avec la gustation et les entrées somatosensorielles) mais il est important également dans l'évitement des dangers tels qu'un incendie, une fuite de gaz ou un prédateur, et enfin il intervient dans les interactions sociales et la reproduction. Les odeurs guident ainsi notre comportement pour répondre aux besoins essentiels à la survie et, au-delà, contribuent fortement aux plaisirs sensoriels et à l'attractivité de l'environnement.

Pour analyser les milliers d'odeurs que nous percevons tous les jours, nous disposons d'un système olfactif capable de les détecter, de les identifier et de les discriminer. Le traitement sensoriel des odeurs est un processus dynamique tout au long de la vie, qui nécessite des apprentissages et met donc en jeu des mécanismes de plasticité cérébrale. Cependant, ce système olfactif très performant peut être perturbé par différents paramètres, comme le stress et la pollution, qui altèrent les réseaux neuronaux sous-jacents.

1. Le bulbe olfactif, première étape de la représentation des odeurs

La perception olfactive se fait schématiquement en trois étapes. Tout d'abord, un odorant, molécule chimique volatile, va atteindre la cavité nasale grâce à la respiration. Cet odorant va alors se lier à des récepteurs olfactifs présents sur les neurones sensoriels de l'épithélium au fond de la cavité nasale. La reconnaissance odorant-récepteur va initier la transformation du message chimique porté par l'odeur en un message électrique. Ensuite, *via* les axones des neurones sensoriels, le message olfactif est acheminé jusqu'au bulbe olfactif, cortex sensoriel et premier relai cérébral de l'information olfactive (figure 1).

Le rôle du bulbe olfactif est d'élaborer une représentation neurale de l'odorant à partir des signaux issus des neurones sensoriels olfactifs de la cavité nasale, mais également de moduler cette représentation en fonction des influences issues d'autres régions du cerveau puisque l'activité du bulbe olfactif est régulée, entre autres, par des voies neuromodulatrices cholinergiques, noradrénergiques et sérotoninergiques. Ces voies neuromodulatrices sont importantes dans les processus d'attention et de mémoire. Ainsi, l'odeur sera encodée dès le bulbe olfactif sous ses aspects perceptifs (identité, intensité…) et mnésiques (contextes, associations diverses…).

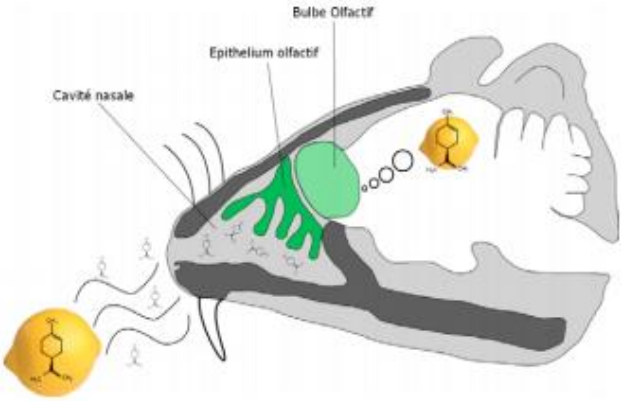

Figure 1. Système olfactif.
Les molécules odorantes entrent dans la cavité nasale où elles se lient avec les récepteurs olfactifs des neurones sensoriels présents au niveau de l'épithélium olfactif. L'information est ensuite transmise au bulbe olfactif puis vers d'autres structures cérébrales, aboutissant ainsi à la perception olfactive.

Olfactory system.

Odorants penetrate into the nasal cavity where they bind the olfactory receptors of the sensory neurons at the level of the olfactory epithelium. The information is then transmitted to the olfactory bulb and to higher brain structures leading to olfactory perception.

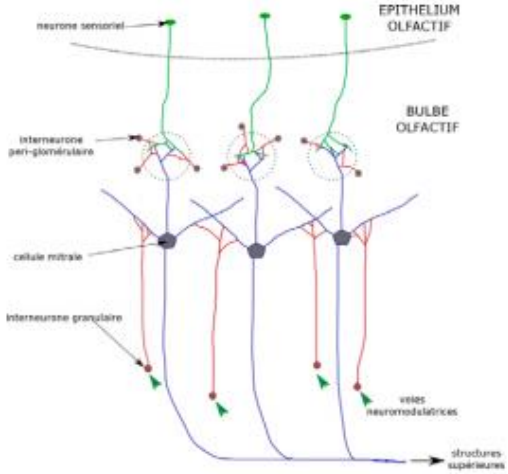

Figure 2. Organisation du bulbe olfactif.
Les neurones sensoriels de l'épithélium olfactif envoient leurs axones vers le bulbe olfactif où ils se connectent aux neurones relais du bulbe (les cellules mitrales) qui, à leur tour, transmettent l'information vers des structures supérieures de traitement. Au niveau du bulbe olfactif, deux populations d'interneurones modulent l'information : les interneurones périglomérulaires et les interneurones granulaires. De plus, les voies neuromodulatrices modulent l'activité des interneurones inhibiteurs.

Olfactory bulb cellular organization.

Olfactory sensory neurons present in the epithelium send their axons to the olfactory bulb where they connect relay neurons (mitral cells) which send the information to higher centers. At the level of the olfactory bulb, two populations of interneurons modulate the information : periglomerular interneurons and granule interneurons. In addition, neuromodulatory fibers modulate inhibitory interneurons activity.

Plus précisément, le bulbe olfactif est constitué de neurones relais (les cellules mitrales), qui reçoivent l'information olfactive grâce à une connexion avec les neurones sensoriels et qui envoient ensuite l'information vers le reste du cerveau. L'information portée par ces cellules mitrales sera sculptée par de petits interneurones inhibiteurs avec qui elles sont connectées (figure 2). Ces petites cellules sont la cible de la neurogenèse adulte.

2. La neurogenèse adulte, une forme originale de plasticité

2. 1. Découverte

La neurogenèse adulte consiste en l'apport permanent de nouveaux neurones dans le circuit neuronal préexistant. En 1906, le neurobiologiste espagnol Santiago Ramon y Cajal (1852-1934) obtient le prix Nobel pour ses travaux sur l'histologie du cerveau. Un de ses postulats affirme que le cerveau adulte est un organe incapable de se régénérer : « *Une fois le développement terminé, la pousse et la régénération axonales et dendritiques s'arrêtent de manière irrévocable. Dans le système nerveux central adulte, le réseau nerveux est fixe, terminé et immuable* ». Ainsi, à la naissance, nous disposons d'un stock de neurones établissant entre eux de nouvelles connexions, mais il n'y aurait pas d'apparition de nouveaux neurones chez l'adulte. Cet énoncé s'érigea en dogme pendant près d'un siècle. Cependant, depuis les années 60, beaucoup de certitudes concernant l'absence de neurogenèse chez l'adulte se sont effondrées. En effet, Joseph Altman, un chercheur américain, découvre dans le cerveau de mammifère des cellules formées à l'âge adulte qui ressemblent à des neurones et qui seront formellement identifiées comme tels grâce à la microscopie électronique par Michael Kaplan quelques années plus tard (Altman, 1969). Après avoir été longtemps ignorée, cette découverte a été confirmée dans les années 90, et il est maintenant bien établi que la neurogenèse persiste tout au long de la vie, principalement dans deux régions du cerveau de mammifère adulte, le gyrus denté de l'hippocampe et le bulbe olfactif (figure 3). Cette neurogenèse a été décrite chez de nombreux mammifères, y compris l'homme (même si la question de la neurogenèse olfactive chez l'homme est discutée par certains auteurs). Entre ces deux sites de neurogenèse, c'est le bulbe olfactif qui reçoit le plus grand nombre de nouveaux neurones.

La neurogenèse adulte, même si spatialement restreinte dans le cerveau de mammifère, permettrait une plasticité accrue dans des structures clés de l'apprentissage et de la mémoire que sont l'hippocampe et le bulbe olfactif. Les études récentes se sont donc attachées à comprendre les interactions entre neurogenèse et apprentissages.

2. 2. Description

2. 2. 1. Mise en évidence de la cellule souche

La cellule souche, cellule capable de donner tous les types cellulaires, a été mise en évidence après mise en culture du tissu neural de mammifère adulte. Les chercheurs ont observé que différentes régions du cerveau, dont la zone sous-ventriculaire bordant les ventricules latéraux, étaient capables de donner des neurosphères, amas de cellules pouvant se différencier en neurones ou en cellules gliales (Reynolds et Weiss, 1992). C'est de cette région que sont issus les neurones formés chez l'adulte du système olfactif.

2. 2. 2. Migration des neurones immatures et intégration dans le réseau neuronal préexistant

La cellule souche adulte présente dans la zone sous-ventriculaire va se différencier en neuroblaste (neurone immature), qui va migrer pendant environ six jours le long d'un trajet très stéréotypé, appelé le flux rostral migratoire, pour rejoindre le centre du bulbe olfactif. Ensuite, ces neuroblastes vont entamer une migration radiale pour rejoindre leur destination finale, la couche granulaire, où ils vont se différencier en interneurones inhibiteurs granulaires (94 %), et la couche glomérulaire où ils donneront des interneurones périglomérulaires (4 %) (Lledo, Valley, 2016). Ainsi, des dizaines de milliers de nouveaux neurones arrivent et s'intègrent chaque jour au niveau du bulbe olfactif. Dans des conditions de base, environ un mois après leur naissance, la moitié de ces neurones meurt par un phénomène d'apoptose, et l'autre moitié va survivre à plus long terme dans le réseau neuronal bulbaire. Cependant cet équilibre mort/survie des nouveaux neurones peut être modulé et jouer un rôle crucial dans de nombreux comportements.

3. Quels rôles pourraient jouer les neurones formés à l'âge adulte dans la fonction olfactive ?

Une question clé associée à la découverte de ce phénomène de neurogenèse concerne le rôle fonctionnel de ces nouveaux neurones. Quelle est leur fonction dans le système olfactif ?

Comme le bulbe olfactif a été impliqué dans de nombreux apprentissages olfactifs et qu'il est le siège d'une neurogenèse importante, l'hypothèse d'un rôle central de la neurogenèse dans les processus d'apprentissage a été proposée. Plusieurs études ont montré que l'activité olfactive simple (être exposé à des odeurs) peut moduler le taux de formation des nouveaux neurones en agissant sur leur capacité à survivre dans le réseau préexistant (Mandairon *et al.*, 2006b, Rochefort *et al.*, 2002). En effet, il existe une fenêtre temporelle (environ un mois après leur naissance) où les nouveaux neurones sont particulièrement sensibles à la présence ou l'absence de stimulation olfactive.

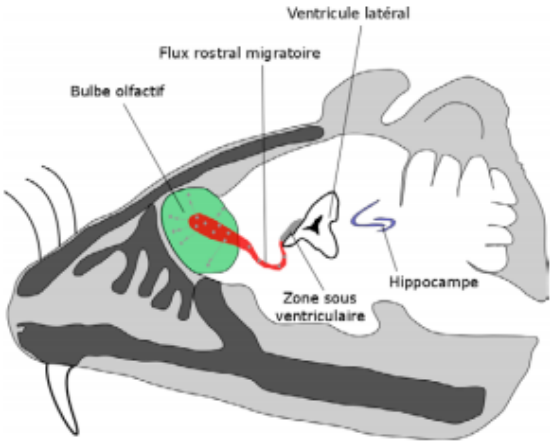

Figure 3. Neurogenèse adulte.
Les nouveaux neurones olfactifs ont pour origine des cellules souches situées dans la zone sous-ventriculaire, le long des ventricules latéraux. Ces cellules souches se différencient en neurones immatures (neuroblastes) qui migrent le long du flux rostral migratoire jusqu'au centre du bulbe olfactif. Ensuite, au sein du bulbe olfactif, ces neurones immatures migrent pour rejoindre leur destination finale et se différencier interneurone granulaire ou périglomérulaire.
Adult neurogenesis.
Olfactory newborn neurons originate from stem cells present in the subventricular zone located along the lateral ventricles. Stem cells differentiate into immature neurons (neuroblasts) that migrate along the rostral migratory stream to reach the olfactory bulb. Within the olfactory bulb, the neuroblasts migrate radially to reach their final destination and differentiate granule or periglomerular interneurons.

Pour comprendre la signification fonctionnelle de la neurogenèse adulte, plusieurs laboratoires ont étudié le rôle de la neurogenèse dans des contextes d'apprentissage et de mémoire. Le paradigme d'apprentissage le plus couramment utilisé est celui de l'apprentissage associatif de discrimination (apprentissage explicite), qui consiste à apprendre à un animal à associer une odeur à une récompense. Au fil des essais d'apprentissage, l'animal parvient à mieux discriminer l'odeur pour trouver sa récompense. Cet apprentissage augmente la neurogenèse (Mandairon *et al.*, 2006a, Sultan *et al.*, 2010). Dans cette lignée, il est maintenant possible, grâce à des vecteurs viraux, de stimuler spécifiquement ces nouveaux neurones, ce qui provoque une amélioration de la discrimination (Alonso *et al.*, 2012). Les nouveaux neurones sélectionnés lors de l'apprentissage deviennent alors le support de la trace mnésique. Ainsi leur suppression pharmarcologique, génétique ou par irradiation, entraîne un déficit de la mémoire (Imayoshi *et al.*, 2008, Lazarini *et al.*, 2009, Sultan *et al.*, 2010). Inversement, un effacement de cette mémoire provoque la suppression prématurée de ces neurones (Sultan *et al.*, 2011).

Parmi les différents types d'apprentissages existe également l'« apprentissage perceptif » qui s'oppose à l'« apprentissage associatif » par son côté implicite, dans le sens où il n'implique pas de conditionnement et pas d'action ciblée de la part de l'animal. L'apprentissage perceptif se traduit par une amélioration de la discrimination entre deux stimuli très similaires, suite à l'exposition répétée à ces mêmes stimuli. Chez les rongeurs, l'apprentissage perceptif olfactif a été mis en évidence par l'exposition répétée à deux odeurs très proches sur le plan perceptuel, donc non discriminées (Mandairon *et al.*, 2006c). Il s'avère que la simple exposition quotidienne à ces odeurs proches pendant une dizaine de jours améliore leur discrimination. Cette amélioration de la perception olfactive est sous-tendue par la neurogenèse, puisque si l'on supprime de façon pharmacologique l'approvisionnement du bulbe olfactif en nouveaux neurones, l'exposition aux odeurs n'améliore plus les performances de discrimination (Moreno *et al.*, 2009). Les mécanismes gouvernant l'intégration et la survie des nouveaux neurones pendant l'apprentissage restent cependant encore mal connus.

Enfin, la neurogenèse adulte semble impliquée dans les apprentissages sociaux et les stratégies de reproduction. Ainsi, les phéromones du mâle dominant sont capables de stimuler la neurogenèse dans le cerveau de souris femelle qui développe une préférence pour l'odeur de ce mâle. Si la neurogenèse est bloquée par

l'injection d'antimitotique, cette préférence sociale n'a plus lieu (Mak *et al.*, 2007). La neurogenèse semble également impliquée lors de la gestation. En effet, ce processus est stimulé par une hormone, la prolactine qui, lorsqu'elle est inhibée, induit une diminution de la neurogenèse (Shingo *et al.*, 2003).

Ainsi, ces différentes études montrent qu'en fonction du nombre de nouveaux neurones présents dans le bulbe olfactif, la perception et la mémoire olfactives peuvent être modulées. Au-delà de leur nombre, la morphologie des nouveaux neurones peut être façonnée par l'apprentissage (Daroles *et al.*, 2016, Lepousez *et al.*, 2014) afin d'améliorer les performances olfactives.

Ainsi, les nouveaux neurones bulbaires, de par une modulation de leur nombre, de leur morphologie ou de leur niveau d'excitabilité (Nissant *et al.*, 2009) changent le message envoyé aux autres régions du cerveau et donc la perception et la mémoire de l'odeur. Il est maintenant de plus en plus évident au sein de la littérature que cette neurogenèse est non seulement essentielle au fonctionnement normal du cerveau mais est en plus extrêmement sensible aux changements de l'environnement, qu'ils soient positifs ou négatifs.

4. Effets du stress et de la pollution

4. 1. Les effets du stress sur la neurogenèse

Nous sommes confrontés quotidiennement à des situations aversives qui émergent de notre environnement : un piéton qui traverse un peu trop précipitamment, un entretien d'embauche, l'odeur de gaz indiquant une fuite, une pollution, auxquelles notre organisme répond par un stress. Le stress est généralement défini comme un état de menace de l'homéostasie, c'est-à-dire de notre équilibre physiologique (Smith et Vale, 2006), auquel nous réagissons en activant une large gamme de réponses comportementales et physiologiques appelée communément la réponse au stress. En situation aversive, le maintien de l'homéostasie nécessite l'activation de nombreux systèmes impliquant le système endocrinien, le système nerveux et le système immunitaire formant la réponse au stress (Chrousos et Gold, 1992, Carrasco et Van De Kar, 2003) ayant pour rôle de maximiser les chances de survie de l'organisme face à une situation menaçante.

Le principal effecteur de la réponse au stress est le système corticotrope ou axe HPA, impliquant l'hypothalamus, l'hypophyse et les glandes surrénales. Son activation, induite par une situation aversive, aboutit à la libération spécifique « d'hormones de stress », appelées glucocorticoïdes et corticostérones, qui agiront entre autres au niveau du cortex préfrontal qui gère les informations et gouverne la prise de décision (comme, par exemple, une fuite), mais également sur l'ensemble de l'organisme grâce à la présence de nombreux récepteurs. Ainsi, en réponse à un challenge de court terme, le stress favorise notre survie en guidant notre comportement et permettant une réponse adaptée de notre organisme. Cependant, lors de situations aversives prolongées et/ou répétées, l'activation de l'axe HPA peut être dérégulée (Miller *et al.*, 2007 ; Mizoguchi *et al.*, 2008), on parle alors de stress chronique. En affectant 13,6 % de la population européenne (95 millions d'individus) et 20 % des personnes âgées, le stress chronique fait partie des troubles les plus fréquents dans nos sociétés selon le National Institue of Mental Health, il est délétère pour l'organisme, notamment pour le système cérébral, et pourrait contribuer au développement de nombreuses pathologies (Mcewen et Stellar, 1993).

Le stress favorise notre survie grâce à la mise en place d'une réponse adaptative extrêmement demandeuse en énergie mais qui s'avère être délétère sur le long terme, lors de stress chronique. Cette réponse représente donc un coût pour certaines régions de l'organisme, mais quel est l'impact sur la neurogenèse ?

Des travaux ont montré que le stress entraîne une profonde perturbation de la neurogenèse adulte, conduisant à une réduction rapide et prolongée des taux de prolifération des neuroblastes et de survie neuronale. Chez le rongeur, un stress chronique établi à l'âge adulte ou un stress précoce après la naissance conduit à un dérèglement de l'axe HPA et à l'élévation de la libération de glucocorticoïdes (Lehmann *et al.*, 2013), qui seraient les médiateurs des altérations de la neurogenèse adulte. En effet, des études ont rapporté une diminution de la prolifération des cellules souches ainsi qu'une diminution de la survie des neurones néoformés dans le bulbe olfactif après apport exogène de glucocorticoïdes (Lau *et al.*, 2007 ; Siopi *et al.*, 2016). Les auteurs rapportent également une perturbation des fonctions olfactives (acuité, discrimination entre deux odeurs et mémoire olfactive). À l'inverse, un traitement avec un antagoniste des récepteurs aux glucocorticoïdes, après l'établissement d'un stress chronique, restaure la neurogenèse à des niveaux contrôles (Oomen *et al.*, 2007). D'autre part, la prolifération des cellules souches est favorisée par la suppression des glucocorticoïdes circulants (Guo *et al.*, 2010). Ces résultats suggèrent donc que la quantité de neurones néoformés est modulée par les glucocorticoïdes qui doivent, pour agir, se lier à leurs récepteurs. Ces récepteurs sont présents en très grande quantité dans le bulbe olfactif (Morimoto *et al.*, 1996) et, de façon intéressante, au niveau des cellules granulaires qui correspondent au type neuronal majoritairement produit lors de la neurogenèse adulte (Winner *et al.*, 2002).

Dans nos sociétés vieillissantes, il est intéressant de noter que le niveau de stress s'accroît avec l'âge (Bryant *et al.*, 2008) ainsi que les taux de glucocorticoïdes circulants (Van Cauter *et al.*, 1996) et pourrait expliquer, au moins en partie, la réduction drastique de la neurogenèse observée (Mobley *et al.*, 2014).

Le stress altère donc la neurogenèse, et nous savons aujourd'hui que cette altération est médiée par l'élévation des taux sanguins de glucocorticoïdes (David *et al.*, 2009 ; Mirescu et Gould, 2006). Toutefois, il n'est pas le seul facteur perturbateur de la neurogenèse.

4. 2. La pollution de l'air

12,6 millions est le nombre de décès causés par la pollution en 2012 selon l'Organisation Mondiale de la Santé (OMS). Il existe différents types de pollution, mais nous traiterons uniquement des effets de la pollution de l'air, qui est à l'origine de plus de la moitié des décès recensés (7 millions).

Selon le code de l'environnement, « *Constitue une pollution atmosphérique [...] l'introduction par l'homme [...] d'agents chimiques, biologiques ou physiques ayant des conséquences préjudiciables de nature à mettre en danger la santé humaine, à nuire aux ressources biologiques et aux écosystèmes, à influer sur les changements climatiques, à détériorer les biens matériels, à provoquer des nuisances olfactives excessives*. ». Il est largement admis que la pollution de l'air impacte notre système nerveux central et pourrait, à force d'exposition, contribuer au développement de pathologies (Genc *et al.*, 2012). Plus particulièrement, l'exposition aux particules en suspension dans l'air a été associée à la perte de l'odorat et à certaines formes de dégénérescence du système nerveux central (Tjalve et Henriksson, 1999). En effet, les individus vivant dans des régions à forte pollution sont susceptibles de présenter un risque accru de développer des maladies telles qu'Alzheimer (Chen *et al.*, 2017), Parkinson ou des scléroses en plaques (Calderon-Garciduenas *et al.*, 2004 ; Calderon-Garciduenas *et al.*, 2013 ; Calderon-Garciduenas et Villarreal-Rios, 2017). Récemment, des travaux ont mis en évidence un effet néfaste de la pollution atmosphérique sur la neurogenèse adulte (Costa *et al.*, 2015). Mais par quels processus ?

4. 2. 1. Neuro-inflammation et stress oxydatif

L'inflammation et le stress oxydatif sont synonymes d'agression cellulaire et représentent les effets les plus importants causés par la pollution de l'air chez l'homme et l'animal (Costa *et al.*, 2015). De par son organisation, le système olfactif est directement exposé et constitue alors une voie d'entrée idéale par laquelle la pollution de l'air pourrait exercer ses méfaits sur l'organisme. En effet, les molécules volatiles présentes dans l'environnement sont constamment en contact avec notre épithélium olfactif, localisé au fond des cavités nasales, qui contient les neurones olfactifs. Ces derniers projettent directement sur le cerveau et plus particulièrement le bulbe olfactif. Ainsi, les particules présentes dans l'air pollué pourraient aisément pénétrer dans la cavité nasale, interagir avec l'épithélium olfactif, accéder au système nerveux central par l'intermédiaire des neurones olfactifs (Calderon-Garciduenas *et al.*, 2003) et y déclencher une réaction inflammatoire et un stress oxydatif. En effet, la présence de particules polluantes a été identifiée dans le bulbe olfactif de rats après inhalation (Oberdorster *et al.*, 2004) et également chez des personnes vivant dans une atmosphère très polluée (Calderon-Garciduenas *et al.*, 2008). Des études montrent une augmentation des niveaux de cytokines pro-inflammatoires et de la peroxydation lipidique, témoins de l'inflammation et du stress oxydatif respectivement, dans le bulbe olfactif des rongeurs exposés à un air pollué (Costa *et al.*, 2015 ; Tin Tin Win *et al.*, 2008), mais également chez l'homme (Calderon-Garciduenas *et al.*, 2004). D'autres soulignent l'action délétère de l'inflammation et du stress oxydatif sur la neurogenèse, qui peut être restaurée par un traitement anti-inflammatoire et antioxydant (Herrera *et al.*, 2003 ; Monje *et al.*, 2003).

4. 2. 2. La pollution de l'air : une source de stress

De par son action durable et néfaste sur notre organisme, la pollution de l'air représente une situation aversive répétée et prolongée et donc une source de stress. En effet, plusieurs études menées chez l'homme et l'animal suggèrent que l'exposition aux polluants atmosphériques augmenterait les taux sanguins d'hormones de stress (Raff *et al.*, 1981 ; Tomei *et al.*, 2003). Les travaux réalisés chez le rat ont montré une activation de l'axe HPA (augmentation de corticostérone) après 8 heures d'exposition à un air concentré en particules ambiantes (Sirivelu *et al.*, 2006). De façon intéressante, cette étude montre une augmentation des taux de noradrénaline dans le bulbe olfactif, qui est un neuromodulateur essentiel à l'activation de l'axe HPA et qui est également connu pour moduler la neurogenèse bulbaire (Bauer *et al.*, 2003). D'autres recherches ont montré que la pollution de l'air altérait les processus cognitifs, induisait un état de stress et une réaction inflammatoire (Fonken *et al.*, 2011), également connue pour activer l'axe HPA (Berkenbosch *et al.*, 1987).

Outre la toxicité induite par les particules polluantes, la pollution de l'air peut être une source de nuisances

olfactives et générer par-là un stress. En faisant partie intégrante du réseau limbique impliqué dans la gestion des émotions, le système olfactif confère aux odeurs une charge émotionnelle importante. Tandis que les odeurs agréables sont largement utilisées en pratique pour réduire le stress et procurer un état de bien-être (aromathérapie, diffuseurs d'odeur...), les odeurs désagréables quant à elles pourraient induire des émotions négatives et générer un stress (Horii *et al.*, 2010). Effectivement, des personnes exposées à des nuisances olfactives se sentent plus stressées (Carlsson *et al.*, 2006).

4. 3. Altérations de la neurogenèse du bulbe olfactif, quelles conséquences ?

La neurogenèse est un mécanisme essentiel à la fonction olfactive. Lors d'un apport exogène chronique de corticostérone, l'apport en nouveaux neurones est réduit de moitié (Siopi *et al.*, 2016), induisant inévitablement des troubles importants de la fonction olfactive. En effet, cette même étude rapporte une altération de l'acuité olfactive, de la détection, de la discrimination et de la mémorisation, qui peuvent être restaurées, en parallèle de la neurogenèse (Hitoshi *et al.*, 2007 ; Oomen *et al.*, 2007), par un traitement pharmacologique réduisant le stress.

Ces altérations peuvent avoir des conséquences dramatiques sur notre organisme en altérant profondément les interactions sociales (perception des parfums, odeurs corporelles), ainsi que notre qualité de vie (Croy *et al.*, 2014), en perturbant les émotions et souvenirs qu'une odeur peut évoquer, mais aussi la reconnaissance et l'appréciation de la nourriture. En conséquence, les déficits olfactifs engendrés par la baisse de neurogenèse induite par le stress et/ou la pollution affecteraient notre santé en provoquant des troubles de la prise alimentaire, des syndromes dépressifs et une incapacité d'adaptation comportementale face à une situation d'urgence (feu, fuite de gaz, par exemple). À l'inverse, un système olfactif défaillant accroît les niveaux de stress (Glinka *et al.*, 2012), suggérant que les déficits olfactifs induits par le stress et/ou la pollution, peuvent en retour conduire à un état de stress chronique. L'état de stress s'aggraverait au fil du temps et, en conséquence, l'altération de la neurogenèse, augmentant à terme les facteurs de risque des troubles psychotiques tels que la schizophrénie (Walker *et al.*, 2008), de la dépression (Van Praag, 2004) et des maladies neurodégénératives (Esch *et al.*, 2002).

Conclusion, traitements et stratégies de prévention

La neurogenèse adulte nous offre de surprenantes capacités de traitement de l'information olfactive, nous permettant ainsi d'évoluer dans un environnement très riche sur le plan sensoriel. Mais cette plasticité « hors du commun » est vulnérable et est aisément perturbée par le stress et la pollution qui nous entourent.

Les niveaux de pollution (OMS, 2016) et de stress (Cohen *et al.*, 2012) étant actuellement en hausse dans nos sociétés, il est important de déployer des stratégies de remédiation pour prévenir, voire compenser ou restaurer, l'altération de la neurogenèse qui en résulte. Des traitements pharmacologiques tels que les antidépresseurs ont révélé des effets bénéfiques sur la neurogenèse. Ils la stimulent chez les rats naïfs (Malberg *et al.*, 2000), la restaurent après l'induction d'un stress chronique (Siopi *et al.*, 2016) et pourrait ainsi limiter voire inverser les dommages causés par le stress. Les anti-inflammatoires et antioxydants ont également fait leurs preuves dans la préservation de la neurogenèse (Herrera *et al.*, 2003 ; Monje *et al.*, 2003).

Outre les approches médicamenteuses, des alternatives plus écologiques existent pour réduire le stress et restaurer la neurogenèse, telles que des épisodes de marche dans la nature (Tyrvainen *et al.*, 2014) et la diffusion d'odeurs. Nous savons aujourd'hui qu'une exposition journalière à une odeur plaisante diminue le stress (Joussain *et al.*, 2014) et que la neurogenèse peut être régulée positivement par des enrichissements olfactifs (Moreno *et al.*, 2009 ; Rey *et al.*, 2012 ; Rochefort *et al.*, 2002). La diffusion répétée d'odeurs plaisantes dans notre environnement pourrait permettre de pallier la réduction de la neurogenèse médiée par le stress et la pollution. En particulier, les odeurs dites « vertes », qui émanent des feuilles des plantes, sont connues pour réduire le stress (Nakashima *et al.*, 2004) et pourraient expliquer la diminution du stress perçu lors d'épisodes de marche dans la nature (Tyrvainen *et al.*, 2014).

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Topographical representation of odor hedonics in the olfactory bulb

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Hedonic value is a dominant aspect of olfactory perception. Using optogenetic manipulation in freely behaving mice paired with immediate early gene mapping, we demonstrate that hedonic information is represented along the antero-posterior axis of the ventral olfactory bulb. Using this representation, we show that the degree of attractiveness of odors can be bidirectionally modulated by local manipulation of the olfactory bulb's neural networks in freely behaving mice.

In all species, including humans, odors strongly impact behavior. Besides odor identity, the hedonic value or pleasantness of an odor is an important variable for smell-triggered behaviors, for example, approaching or avoiding an odor source. The hedonic value or attractiveness of an odorant is thus recognized as the dominant aspect of olfactory perception[1,2]. While experience and culture shape odor hedonics[3,4], spontaneous attraction or avoidance is also observed for unfamiliar odorants[5], suggesting an innate component. This raises the issue of the representation of naive hedonic tone in the brain.

Odor signals are received by odorant receptors expressed by sensory neurons in the olfactory epithelium and can be represented as an odor map in the glomerular layer of the olfactory bulb[6]. Although glomerular maps in the dorsal olfactory bulb have been shown to represent the aversive properties of some odorants[7–9], it remains unclear whether bulbar maps represent innate positive versus negative hedonic tones. In this study, we combine immediate early gene mapping[7] and optogenetics to show that the degree of behavioral attraction to an odorant is represented along the antero-posterior axis of the ventral olfactory bulb, suggesting a functional organization for innate hedonic value.

To determine which odorants trigger innate positive or negative hedonic tone in mice, we tested the olfactory preferences of 50 mice for a set of 16 odorants with a wide range of molecular properties (**Supplementary Table 1** and **Supplementary Fig. 1**), chosen to be unfamiliar to the mice, with no particular biological significance. Odorant investigation time, assessed as an index of attractiveness[7,10], differed significantly across the odorants (permutation test $P = 0.034$; **Fig. 1**).

We selected the five most investigated (camphor, limonene, β-ionone, citronellol and cineole) and the five least investigated (pyridine, thioglycolic acid, 3-hexanol, guaiacol and *p*-cresol) odorants and compare their investigation times to those for 2,3,5-trimethyl-3-thiazoline (TMT) and mouse urine, which are recognized as being, respectively, aversive and attractive to mice (**Supplementary Fig. 2a**). Time spent investigating the five least explored odorants was different from that spent investigating urine, and time spent investigating the five most explored was different from that for TMT, suggesting that the mice found these odorants respectively aversive and attractive. The speed of approach to the odorized hole was closely correlated to investigation time, further strengthening investigation time as a measure of attractiveness (**Supplementary Fig. 2b,c**).

In the olfactory bulb, spatially organized incoming information[6] is transferred to mitral/tufted (M/T) cells, which are the olfactory bulb's relay cells. M/T cell activity is shaped by complex interactions with superficial and deep interneurons, the periglomerular and granule cells, respectively[11]. *Zif268* expression in olfactory bulb interneurons allows mapping of odor-evoked activation in response to odor stimulation[7,12–15]. To reveal the activation patterns that reflect the attractiveness of the odors independently of their identity, we averaged the activation evoked by each of the five most and the five least attractive odorants (**Fig. 2**). The overall densities of Zif268-expressing cells were similar between both groups and layers (Mann-Whitney $P = 0.89$ for glomerular and $P = 0.53$ for granule cell layers; **Fig. 2a,b**). However, differences in the spatial distribution of the odor-responding cells accounted for the innate attraction mice had to the odors. We constructed $Zif268^+$ cell maps by extracting labeled-cell density from 36 sectors in the glomerular and granule cell layers along the antero-posterior axis of the olfactory bulb. These density values were then reported in 2D maps in which each column corresponded to one olfactory bulb section and each bin corresponded to the labeled-cell density in one sector[14,15] (**Fig. 2c**). Compared to pleasant odorants, unpleasant odorants led to a higher density of activated cells in the ventro-posterior glomerular and granule cell layers of the olfactory bulb. In the dorsal olfactory bulb, the response to unpleasant odorants was mostly posterior in the glomerular layer (**Fig. 2d,e**, **Supplementary Figs. 3** and **4**, and **Supplementary Tables 2** and **3**). In contrast, pleasant odorants evoked higher activity in the antero-ventral area of both the glomerular and granule cell layers (**Fig. 2d,e**, **Supplementary Figs. 3** and **4**, and **Supplementary Tables 2** and **3**).

Analysis of overlaps between the odorant-specific maps within each hedonic group yield maps resembling the across-odorant averaged maps, supporting the idea that the latter reflect activation features common to pleasant or unpleasant odorants rather than activation features of individual odorants (**Supplementary Figs. 3d,e** and **5**).

In summary, while the responsiveness of the dorsal glomerular layer to unpleasant odorant has been documented[7], the substantial modulations of Zif268 expression between pleasant and unpleasant

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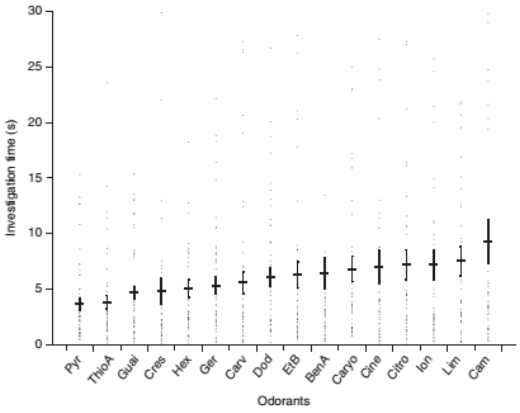

Figure 1 Mice displayed various levels of innate attraction for odorants. Investigation times, automatically measured on a one-hole-board apparatus during 2-min tests, vary for the 16 odorants: pyridine (Pyr), thioglycolic acid (ThioA), guaiacol (Guai), *p*-cresol (Cres), 3-hexanol (Hex), geraniol (Ger), D-carvone (Carv), dodecanal (Dod), ethyl butyrate (EtB), benzyl acetate (BenA), β-caryophyllene (Caryo), cineole (Cine), citronellol (Citro), β-ionone (Ion), limonene (Lim) and camphor (Cam). Odor effect, $F_{(15,735)} = 1.76$, permutation test $P = 0.034$, $n = 50$ mice. Data are represented as mean ± s.e.m. and individual data points. For clearer representation, 20 data points (out of 800) above 30 s are not shown and are distributed as follows: Cres 2; Hex 1; EtB 1; BenA 5; Caryo 2; Cine 1; Citro 1; Ion 2; Lim 2; Cam 3.

odorants were found mostly along the antero-posterior axis of the ventral olfactory bulb.

Because granule cells shape M/T cell activity and therefore the output message read by higher olfactory structures, we hypothesized that changing the output message of the M/T cells by manipulating inhibition should directly affect behavior. We used optogenetic inhibition of localized populations of granule cells with the aim of disinhibiting M/T cells. We achieved this by infusing a lentivirus allowing halorhodopsin-EYFP (enhanced yellow fluorescent protein) expression in neurons (Lenti-hSyn-NpHR-EYFP, NpHR (*Natronomonas* halorhodopsin)) into the postero-ventral or antero-ventral granule cell layer (**Supplementary Fig. 6**). Control animals were infused with an empty lentivirus (Lenti-hSyn-EYFP, control-EYFP). We confirmed that $EYFP^+$ cell density was always higher in the areas targeted by virus infusions compared to non-targeted regions (**Supplementary Fig. 6a**,**b**). In addition, viral diffusion was limited to the granule cell layer as only 0.38% of $EYFP^+$ cells were M/T cells identified by the expression of their specific marker Tbx21 (7,537 M/T cells counted). Light stimulation was effective since it induced a decrease in the number of cells coexpressing EYFP and Zif268 (**Supplementary Figs. 6c** and **7**) and in total $Zif268^+$ cell density (**Supplementary Fig. 6d**). Such differences were not observed in mice infused with the empty virus. Furthermore, light stimulation in olfactory bulb slices inhibited firing in granule cells expressing NpHR but not in those expressing EYFP in the control animals (**Supplementary Fig. 8a**). In addition, using cell-attached recording of M/T cells in olfactory bulb slices, we showed that light-stimulation of granule cells disinhibits M/T cell activity in NpHR infused mice compared to controls (32.88 ± 10.05% of difference between the controls and NpHR, one-sample *t*-test $P = 0.022$). Similarly, postmortem quantification of Zif268 expression in M/T cells showed disinhibition in light-targeted compared to non-light-targeted regions (**Supplementary Fig. 8b**,**c**).

Optogenetic inactivation of transduced neurons was achieved in freely behaving mice during preference testing by automatically triggering a light stimulation each time the animal approached the odorized hole. In the groups with postero-ventral viral infusion, the investigation time for the five unpleasant odorants was increased under posterior light stimulation in the NpHR group compared to the control-EYFP group, showing a shift toward an increased attractiveness of these odorants (**Fig. 3a** and **Supplementary Fig. 9a**). The investigation time of pleasant odorants was unchanged (**Fig. 3c** and **Supplementary Fig. 9c**). Following viral infusion in the antero-ventral olfactory bulb, light-triggered inactivation of this region decreased the investigation time for the five attractive odorants in the NpHR group compared to that of the control-EYFP group, suggesting a shift toward

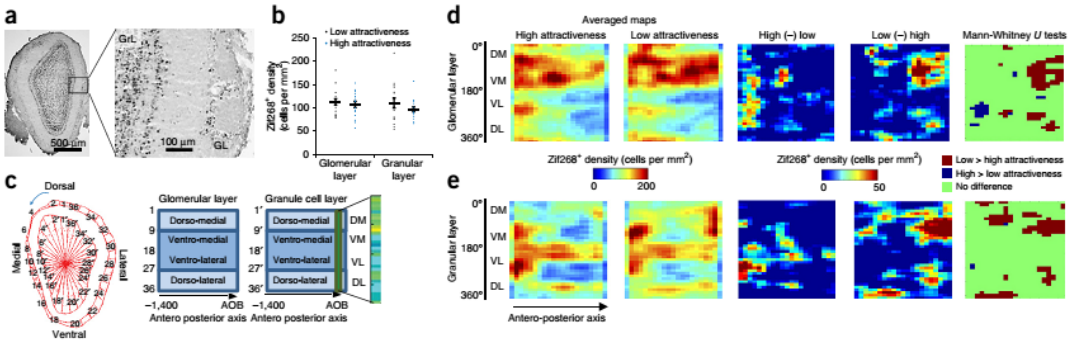

Figure 2 Pleasant and unpleasant odorants evoke different patterns of olfactory bulb activation. (**a**) Coronal sections of the olfactory bulb showing Zif268 labeling (representative of 32 animals) in granular layer (GrL) and glomerular layer (GL). (**b**) $Zif268^+$ cell density is similar in response to unattractive and highly attractive odorants (Mann-Whitney, $P = 0.89$ for glomerular and $P = 0.53$ for granule cell layers; $n = 16$ mice per group) (mean ± s.e.m. and individual data points). (**c**) Cell mapping method. Every $Zif268^+$ cell was counted on each sampled section. Density of labeled profiles was extracted from 36 sectors of 10° of the granule or periglomerular cell layer. For each layer, the cell densities were reported into a matrix in which one column represented all the sectors of one section. (**d**) Left, $Zif268^+$ cell density maps in the glomerular layer in response to high- or low-attractiveness odorants. Middle, subtraction maps. Right, false discovery rate–corrected Mann-Whitney point-to-point comparisons. Significance cutoff was set at $P < 0.05$. (**e**) Same as **d** for the granule cell layer. AOB, accessory olfactory bulb; DM, dorso-medial; VM, ventro-medial; VL, ventro-lateral; DL, dorsolateral.

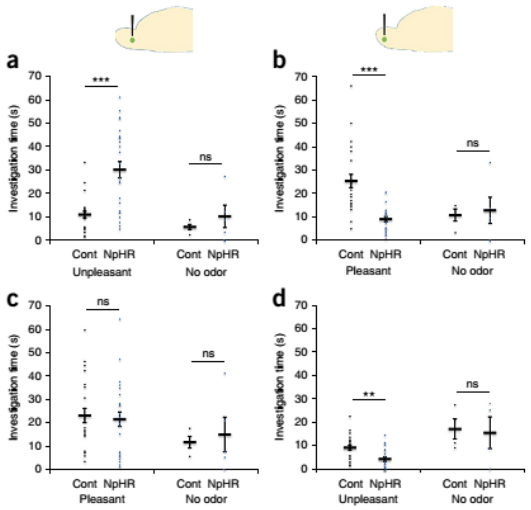

Figure 3 Behavioral effect of optogenetic stimulation. The investigation time of the odorants after postero-ventral (left diagram, **a** and **c**) or antero-ventral (right diagram, **b** and **d**) light stimulation in NpHR-infused (NpHR, blue) and control virus-infused (Cont, black) mice. 5 unpleasant odorants ($n = 5$ mice per odorant), 5 pleasant odorants ($n = 5$ mice per odorant) and one no- (zero-) odorant condition tested. Mann-Whitney group effect; **a**, $P = 0.00008$; **b**, $P = 0.000001$; **c**, $P = 0.75$; **d**, $P = 0.0014$ (mean ± s.e.m. and individual data points); n.s., non-significant.

a reduced attractiveness of the odorants (**Fig. 3b** and **Supplementary Fig. 9b**). Anterior light stimulation further reduced the attractiveness of the unpleasant odorants (**Fig. 3d** and **Supplementary Fig. 9d**). In this optogenetic experiment, the overall increase in investigation time compared to **Figure 1** can be explained by the smaller number of odors experienced by the animals. The effects of light were dependent on sensory stimulation since light stimulation in the absence of the odorant had no behavioral effect.

Inhibition of the anterior olfactory bulb thus produces a reduction in investigation time regardless of odorant attractiveness. This may be due to an increased relative salience of the activity in the posterior olfactory bulb highlighting the role of posterior olfactory bulb activity in encoding odorant unpleasantness. Inhibition of the posterior olfactory bulb failed to further increase the attractiveness of pleasant odorants. This is consistent with the lower level of activity observed in the posterior olfactory bulb in response to pleasant odorants.

In summary, we have demonstrated the presence of a neural trace for positive and negative hedonic tones of odors along the antero-posterior axis of the olfactory bulb. Local optogenetic manipulation of this neural hedonic signature changed the behavioral response to odorants, suggesting that bulbar representations give a reliable indication of the level of odor attractiveness. Even though the degree of attraction to an odor was innate in our mice (i.e., not trained), we were able to modify this by modulating neural activity. Adding to previous experiments showing that aversion to an odor can be abolished by removing specific glomeruli[7], we show here that innate attractiveness is represented along an anatomical axis of the olfactory bulb and that this could be subject to plasticity as it can be modulated by changes in inhibitory activity. Whether this representation of hedonics along the olfactory bulb results from organized projections of olfactory sensory neurons recognizing specific molecular features of pleasant or unpleasant odorants remains unknown but is supported by evidence for a spatial representation of hedonics in the main olfactory epithelium[16]. Finally, it remains to be determined how hedonic-dependent activity in the olfactory bulb is interpreted by higher olfactory structures. M/T cells project to different targets according to their position along the antero-posterior axis, determined by their ontogenetic birth date[17]. Several candidate structures respond to odorant hedonics[18,19], and it would thus be interesting to look for differential projections from anterior and posterior regions of the olfactory bulb to these olfactory brain regions.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

F.K., A.D. and N.M. conceived the experiments; F.K., M.M., N.K., J.F., J.S. and N.M. performed the experiments; C.B. and M.R. engineered the lentiviruses; F.K., M.M., M.T., A.D., M.T., N.K. and N.M. analyzed the data and F.K., M.M., M.R., A.D. and N.M. wrote the article.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animals. 125 adult male C57Bl6/J mice (Charles River Laboratories, L'Arbresle, France) aged 2 months at the beginning of the experiments were used. Experiments were done following procedures in accordance with the European Community Council Directive of 22nd September 2010 (2010/63/UE) and the National Ethics Committee (Agreement DR2013-48 (vM)). Mice were housed in groups of five in standard laboratory cages and were kept on a 12 h light/dark cycle (at a constant temperature of 22 °C) with food and water ad libitum. Behavioral experiments started not earlier than a week after the arrival of the animals and were conducted in the afternoon (2–6 p.m., light phase).

Odorants. 16 monomolecular odorants were selected based on molecular diversity (**Supplementary Fig. 1**). To avoid differences in the concentration of these odorants in the inhaled air due to differences in their volatility, they were diluted in mineral oil so as to achieve an approximate gas-phase partial pressure of 1 Pa (ref. 20; **Supplementary Table 1**). Vapor pressure values were collected from http://www.thegoodscentscompany.com. 2,3,5-Trimethyl-3-thiazoline (TMT, Contech, Canada) and urine from female mice were also used.

Olfactory preference testing. Odor attractiveness was measured using odor investigation time (n = 50 mice). We used a computer-assisted one-hole-board apparatus fitted with sensors to automatically monitor the duration of nose-poking into the central hole[5,10]. A polypropylene swab impregnated with 60 µl of the odorant (1 Pa) was placed at the bottom of the hole, under a grid covered with clean bedding. Every animal was allowed to explore each of the 16 odorants for 2 min. The bedding was replaced after each trial. Total duration of nose-poking into the hole (odor investigation time) was used as a measure of odor preference. Each animal tested one odorant per d. Odorants were randomly presented and animals performed no more than 3 consecutive d of testing. The effect of the odorants on investigation time was analyzed by random permutation test (10 series of 10,000 permutations). This experiment was repeated using 30 additional mice using the 5 most and 5 least explored odorants and investigation times were compared to those for TMT (0.004% in propylene glycol, 60 µl) and urine (20 µl). In this second experiment, we also noted the time elapsed for the mouse's nose to cross the distance of 2 cm around the hole (from which we calculated the speed of approach to the hole) using custom-made video tracking software (n = 15 mice; Volcan) as well as the investigation time of the odorant.

Odorant stimulation, immunohistochemistry and labeled cell mapping. *Odorant selection.* The 5 most attractive odorants, i.e., the most explored (camphor, limonene, β-ionone, citronellol and cineole) and the 5 least attractive, i.e., the least explored (pyridine, thioglycolic acid, 3-hexanol, guaiacol and *p*-cresol), were used for odorant stimulation. The mice were randomly assigned to each odorant.

Odor stimulation and euthanasia. The mice were first placed in individual clean cages for 1 h, with an empty tea ball hanging from the top of the cage. A polypropylene swab impregnated with the odorant (60 µl, 1Pa) was then placed in the tea ball for a further hour. One hour after the end of stimulation, the mice were deeply anaesthetized (pentobarbital, 0.2 ml/30 g) and killed by intracardiac perfusion of 50 ml of fixative (PFA 4%, pH = 7.4). This 1-h delay has previously been shown to enable the expression of Zif268 in response to odorant stimulation[21]. The brains were removed, post-fixed overnight, cryoprotected in sucrose (20%), frozen rapidly and then stored at −20 °C before sectioning at 14 µm with a cryostat.

Immunohistochemistry. The Zif268 immunohistochemistry protocol has been described elsewhere[22]. Briefly, rabbit anti-Zif268 primary antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA, ref: Sc-189) was combined with biotinylated goat anti-rabbit antibody (1:200; Vector Laboratories, Burlingame, CA, USA). Olfactory bulb sections were then processed through an avidin–biotin–peroxidase complex (ABC Elite Kit, Vector Laboratories). Following dehydration in graded ethanols, sections were defatted in xylene and cover-slipped in DPX (Fluka, Sigma).

Zif268⁺cell mapping. For each animal (n = 16 mice for unpleasant odorants (Guai, n = 4; ThioA, n = 3; Pyr, n = 3; Hex, n = 2; Cres, n = 4) and n = 16 for pleasant odorants (Lim, n = 2; Cam, n = 3; Cine, n = 5; Citro, n = 3; Ion, n = 3)), Zif268 immunohistochemistry was performed on adjacent olfactory bulb sections. Immuno-positive cells were counted on 21 sections (14 µm thick, 70 µm intervals) of the right olfactory bulb. All cell counts were conducted blind with regard to the experimental group. Within each section analyzed, Zif268 immuno-positive cells were automatically detected in the granule cell layer and the glomerular layers using mapping software (Mercator, Explora Nova, La Rochelle, France) coupled to a Zeiss microscope.

Two-dimensional olfactory bulb map building. Building an immuno-positive-cell density map has already been described[15]. Briefly, the granule and glomerular cell layers were divided into 36 sectors of 10° each, with a reference axis drawn parallel to the most ventral aspect of the subependymal layer of the olfactory bulb. For each layer, this segmentation yielded 756 bins (36 sectors × 21 sections) for which cell density (number of immuno-positive cells/mm^2) was calculated as shown in **Figure 2c**. Density values for each layer were then merged into a 2D map in which each column represents one section and each bin one sector. The columns were then aligned across animals using the most rostral aspect of the accessory olfactory bulb as an anatomical landmark.

Aligned individual maps were averaged within each group (low- or high-attractiveness odorants). A colored image plot of the data was constructed using Matlab 2015.

To provide an activity map more representative of the olfactory bulb, the bins were rearranged in a profile view scaled to the size of the sections. To do so, homologous sectors of the lateral and medial sides were averaged and the density value thus obtained was reported on a map in which the bins were positioned up for dorsal and down for ventral on a lateral view of the olfactory bulb (**Supplementary Fig. 3**).

Analysis of olfactory bulb maps. *Point-by-point map comparisons.* Comparisons of the distribution of immuno-positive cell density between the groups exposed to low- and high-attractiveness odorants were performed in each layer (granule and glomerular cell layers) using a Mann-Whitney *U* test; each group contained 5 odors × 2–5 animals per odor). More precisely, a sliding area successively centered on each bin and covering 4 adjacent bins (2 × 2 bins around the central bin) was used and statistical significance was set at $P < 0.05$. After FDR correction, bins still showing significant differences between groups were color-coded and an image plot was constructed using Matlab. FDR-corrected Mann-Whitney comparisons were also done in artificial groups composed of 50% of animals exposed to high-attractiveness odorants and to 50% of low-attractiveness odorants. 300 different artificial-group pairs were tested. For eight regions of the olfactory bulb (4 (ventral glomerular, ventral granular, dorsal glomerular, dorsal granular) × 2 (anterior and posterior olfactory bulb)), the surface represented by significant bins was calculated as well as the proportion of total surface it represents. *t*-tests for comparison of proportions were applied to compare anterior and posterior regions (**Supplementary Tables 2** and **3**).

Contrasting the hotspots for high- and low-attractiveness odorants. Here, we searched for bins of activation common to the five odorants of one group but not shared with the five odorants of other group, in other words, bins specifically activated by one group of odorants. To do this we first averaged the maps obtained for each odorant across animals (n = 2 to 5 animals per odor). These maps were then binarized to keep only the 20% of bins with the highest Zif268 density (activation hotspots, value = 1, everywhere else value = 0). An overlap map reflecting the activation hotspots for high-attractiveness odors was then calculated by summing these threshold-based odor maps. In this overlap map, a bin with a value of 0 indicated no strong activation in any of the five high-attractiveness odor maps. Conversely, a bin with a value of 5 indicates that this bin was highly activated in all five odor maps. The same protocol was applied to build the low-attractiveness overlap map.

Finally, subtracting the high-attractiveness overlap map from the low-attractiveness overlap map enabled us to highlight hedonic hotspots.

Code availability. Sliding Mann-Whitney tests, overlaps and artificial group simulation in **Figure 2** and **Supplementary Figure 3** required custom Matlab codes that are provided in **Supplementary Software**.

Optogenetic experiment. *Virus infusion and optical fiber implantation in the olfactory bulb.* Prior to surgery, 55 mice were anaesthetized with an intraperitoneal cocktail injection of 50 mg/kg ketamine and 7.5 mg/kg xylazine and secured in a stereotaxic instrument (Narishige Scientific Instruments, Tokyo,

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Japan). 150 nl of pLenti-hSyn-eNpHR3.0-EYFP lentivirus ($9{,}22 \times 10^6$ IU/ml, expressing halorhodopsin and the yellow fluorescent protein) and 300 nl of control pLenti-hSyn-EYFP lentivirus ($1{,}1 \times 10^6$ IU/ml, expressing only YFP) were injected bilaterally into the posterior olfactory bulb (with respect to the bregma: AP, +4.3 mm; ML, ± 0.75; DV, −2 mm) or anterior olfactory bulb (with respect to the bregma: AP, +5 mm; ML, ± 0.75; DV, −2 mm) at a rate of 150 nl/min. Following virus infusions, a dual optical fiber (200-nm core diameter, 0.22 N.A.; Doric Lenses) was implanted into the olfactory bulb at the same coordinates as the virus infusion.

The pLenti-hSyn-eNpHR 3.0-EYFP was a gift from Karl Deisseroth[23] and obtained through Addgene (plasmid #26775). The control pLenti-hSyn-EYFP lentivirus (empty virus containing only the EYFP insert) was obtained from the pLenti-hSyn-eNpHR 3.0-EYFP plasmid by PCR amplification of the EYFP sequence and insertion into the pLenti-hSyn-eNpHR 3.0-EYFP backbone by recombination (In-fusion cloning kit, Clontech).

Optical stimulation in freely moving animals. Animals injected with the lentivirus into the posterior granule cell layer and implanted with optical fibers 8–10 weeks beforehand performed the 2-min olfactory preference test for the 5 unpleasant odorants (60 µl of pyridine, thioglycolic acid, 3-hexanol, guaiacol and *p*-cresol at 1 Pa; $n = 20$ mice) and the 5 pleasant odorants (60 µl of camphor, limonene, β-ionone, citronellol and cineole at 1 Pa, $n = 20$ mice). One odorant was tested per d. All mice were also tested in a no-odor condition. Bilateral continuous light stimulation (crystal laser, 561 nm, 10–15 mW) was automatically triggered when the mouse's nose came within 1 cm of the hole (VideoTrack, Viewpoint) and stopped automatically when the nose exited the zone. Total duration of nose-poking into the hole (odor investigation time) was used as a measure of odor preference. Animals injected with lentivirus into the anterior granule cell layer were subjected to the same behavioral test. Investigation time between the control and NpHR mice was compared using Mann-Whitney tests. Behavioral experiments were conducted blind with regard to the experimental group (control versus NpHR). Nine trials out of 240 were discarded because unwanted events during testing disturbed the animals.

Control of light-triggered inhibition. A few days after the olfactory preference testing, all mice were light stimulated (5 s of light stimulation every 15 s for 2 min at 10–15 mW, mimicking the light stimulation received during the preference test) one hour before euthanasia. Each mouse olfactory bulb was coronally sectioned (14 µm). EYFP (inhibitory channel expressing cells) and Zif268 double staining was performed on olfactory bulb sections as described previously (incubation with rabbit Zif268 antibody (1:1,000, Santa Cruz, ref: Sc-189), chicken GFP antibody (1:1,000, Anaspec TEBU, ref: 55423) and guinea pig Tbx21 (1:5,000, gift from Y. Yoshihara). The density of EYFP, $Zif268^+$ and the percentage of double-stained cells were counted in the ventral olfactory bulb on 2–4 sections (inter section interval = 70 µm) covering 140–280 µm under the injection site and at a distance from it (about 500 µm anterior or posterior depending on the injection site). As a control, YFP^+ cells were also assessed in the dorsal olfactory bulb. Positive cell densities or percentages of double stained cells were calculated for each animal, averaged within groups and compared using Wilcoxon tests. Assessment of cell density experiments were conducted blind with regard to the experimental group. In some cases, histology was not sufficiently well preserved to efficiently perform cell counts ($n = 5$ mice discarded).

In addition, we did slice recording on granule cells: animals infused in the olfactory bulb with NpHR ($n = 5$) or the control virus ($n = 3$) (with respect to the bregma: AP, +4.3 mm; ML, ± 0.75; DV, −2 mm; $n = 5$) 8 weeks earlier were anaesthetized with an intra-peritoneal injection of ketamine (50 mg/ml) and decapitated. The head was quickly immersed in ice-cold (2–4 °C) carbogenated artificial cerebrospinal fluid (cACSF; composition: 125 mM NaCl, 4 mM KCl, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 7 mM $MgCl_2$ and 5.5 mM glucose; pH = 7.4) oxygenated with 95% O_2/5% CO_2. The osmolarity was adjusted to 320 mOsm with sucrose. Olfactory bulbs were removed and cut in horizontal slices (400 µm thick) using a Leica VT1000s vibratome (Leica Biosystems, France). Slices were incubated in a Gibb's chamber at 30 ± 1 °C using an ACSF solution with a composition similar to the cACSF, except that the $CaCl_2$ and $MgCl_2$ concentrations were 2 mM and 1 mM, respectively.

Slices were transferred to a recording chamber mounted on an upright microscope (Axioskop FS, Zeiss) and were continuously perfused with oxygenated ACSF (4 ml/min) at 30 ± 1 °C. Granule cells expressing the EYFP were visualized using an epifluorescence microscope (Zeiss Axio Scope) with a 40× objective (Zeiss Plan-Apochromat) and the bandpass filters set 38HE (Zeiss, excitation 470/40 nm, emission 525/50 nm). The illumination was produced by a white LED (Dual Port OptoLED, Cairn Research, UK). Measurements were performed with an RK 400 amplifier (BioLogic, France). The data were acquired with a sampling frequency of 20 kHz on a PC-Pentium D computer using a 12-bit A/D-D/A converter (Digidata 1440A, Axon Instruments) and PClamp10 software (Axon Instruments). Patch-clamp configurations were achieved with borosilicate pipettes (o.d.: 1.5 mm; i.d.: 1.17 mm; Clark Electromedical Instruments). The recording pipette was filled with the following intracellular solution (131 mM K-gluconate, 10 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 2 mM ATP-Na_2, 0.3 mM GTP-Na_3 and 10 mM phosphocreatine; pH = 7.3, 290 mOsm). Halorhodopsin was excited at 470–570 nm.

Activated mitral cells were assessed using labeling with Zif268 (1:1,000, Santa Cruz, ref: Sc-189) and Tbx21 (1:5,000, a gift from Y. Yoshihara), a specific marker of mitral cells. Double-labeled cells were counted in the dorsal and the ventral parts of the OB.

In the olfactory bulb slices prepared from the mice as described above (control $n = 3$ mice and $n = 6$ cells, NpHR, $n = 4$ mice and $n = 6$ cells), the spontaneous firing activity of the mitral cells was recorded in a cell-attached configuration. 5-s recordings in the control condition were alternated with 5 s in the presence of LED illumination (between 6 and 10 recording sessions for each condition). The effect of light stimulation was compared between NpHR and the control mice.

Statistics. A Kolmogorov-Smirnov test was used to assess normality of the data. Due to differences in normality across the different data sets, we chose to use Mann-Whitney tests, permutation tests or Wilcoxon tests for paired data (Statistica) in this study. No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications[14,21,24,25]. Data collection and animal assignation to the various experimental groups were randomized.

A **Supplementary Methods Checklist** is available.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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