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Neural basis of perceptual learning: structural plasticity and noradrenergic control

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Bases neurales de l'apprentissage olfactif perceptif : plasticité structurale et contrôle noradrénergique

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Le champ des neurosciences connaît depuis quelques décades un développement très important dans la compréhension des corrélats neuronaux de la perception. Le cerveau adulte répond aux variations de l'environnement et à l'expérience par des modifications fonctionnelles et structurales, regroupées sous le terme générique de plasticité, plasticité qui sous-tend l'apprentissage. Cette plasticité affecte la perception sensorielle, olfactive puisque c'est cette modalité qui va nous intéresser, mais également la perception de stimuli dans d'autres modalités sensorielles.

Contrairement à des convictions longtemps érigées en dogme mais maintenant dépassées sur la nature fixe du cerveau, il est établi désormais que le cerveau adulte est capable de générer tout au long de la vie de nouveaux neurones qui s'intègrent dans la circuiterie cérébrale complexe, en particulier dans le bulbe olfactif et qui pourraient jouer un rôle dans l'apprentissage. Des travaux antérieurs de l'équipe ont démontré que l'acquisition de l'apprentissage perceptif défini comme l'amélioration des performances de discrimination après exposition répétées à une paires d'odorants perceptivement très proches, dépend de la présence des neurones formés chez l'adulte. Par ailleurs, le système noradrénergique innerve massivement le bulbe olfactif et en particulier les neurones cibles principales de la neurogenèse adulte, les interneurons granulaires et son implication dans les apprentissages olfactifs, perceptif en particulier est connu. Le substrat des interactions possibles entre système noradrénergique et neurones formés chez l'adulte sont par contre inconnus.

Les objectifs de la thèse étaient donc (1) d'évaluer le rôle fonctionnel des contacts noradrénergiques mis en place par l'apprentissage en utilisant l'outil et (2) de dévoiler le pattern temporel et spatial de l'innervation des neurones formés chez l'adulte dans le bulbe olfactif et sa modification potentielle par l'apprentissage en comparaison des neurones formés pendant l'ontogenèse. Pour cela nous avons utilisé une approche comportementale combinée à des approches neuro-anatomique et optogénétique.

Pour évaluer le rôle fonctionnel des contacts noradrénergiques après apprentissage, nous avons mis en place la manipulation optogénétique des fibres noradrénergiques dans le bulbe olfactif. Cette approche permet une sélectivité spatiale (manipulation des fibres dans le bulbe olfactif uniquement) temporelle (manipulation au moment du test et quand la souris approche l'odeur) et neurochimique (manipulation des fibres issues du *Locus Coeruleus* uniquement). L'inhibition des fibres noradrénergiques dans ce contexte a bloqué la discrimination apprise, suggérant fortement que les contacts noradrénergiques contribuent à l'expression de l'apprentissage.

Les résultats morphologiques indiquent que l'innervation noradrénergique des neurones formés chez l'adulte s'installe dès le huitième jour après la naissance des neurones. L'apprentissage induit une augmentation massive de ces contacts sur les neurones formés chez l'adulte pointant le système noradrénergique comme un acteur majeur de la plasticité induite par l'apprentissage perceptif. Aucune modification n'est observée sur les neurones préexistants formés pendant l'ontogenèse. En regard de l'augmentation des contacts noradrénergiques, les neurones formés chez l'adulte montrent après apprentissage une augmentation des épines dendritiques qui là encore n'est pas retrouvée sur les neurones formés pendant l'ontogenèse.

Ce travail apporté des données originales mettant en évidence une plasticité structurale du système noradrénergique et la contribution fonctionnelle de ce système à la discrimination apprise. Il a de plus permis de mettre en évidence que cette plasticité a pour cible sélective les neurones formés chez l'adulte qui montrent eux aussi un développement dendritique accru en regard de l'augmentation des contacts noradrénergiques. La comparaison avec l'effet de l'apprentissage sur les neurones formés pendant l'ontogenèse a aussi apporté des données décisives concernant les propriétés spécifiques de nouveaux neurones qui leur confère un rôle unique dans le réseau bulbaire pour sous-tendre l'apprentissage.

SUMMARY

The field of neuroscience has experienced explosive growth over the past decade toward understanding the neural correlates of perception. More specifically, the adult brain responds to environmental experience by significant functional and structural modifications, called "neural plasticity" which underlies learning. A main issue in neuroscience is to understand the cellular basis of perceptive plasticity and subsequent behavioral adaptations.

Contrary to previously held beliefs about its static nature, the adult brain is in fact capable of generating new neurons that can integrate into its complex circuitry. The birth of new neurons constitutively occurs in two specific regions of the adult mammalian brain (OB and hippocampal dentate gyrus). Adult neurogenesis is a sophisticated biological process whose function has remained a mystery to neuroscience researchers but a role in learning and memory has been proposed. Previous works in our group have shown that perceptive olfactory learning depends on adult neurogenesis. In addition, neuromodulatory systems, including noradrenergic and cholinergic systems massively innervate the olfactory bulb and more specifically the inhibitory interneurons targeted by adult neurogenesis and are long-known for their role in learning and memory. The issue of the interactions between the noradrenergic system and adult-born neurons is raised by these data but their substratum is unknown.

One objective of the present work was thus to (1) to assess the functional role of centrifugal contacts using an optogenetic approach and (2) to determine the spatial and temporal pattern of the innervation by noradrenergic inputs of developing adult-born neurons and to investigate its modulation by learning. For that purpose, we used behavioral and neuro-anatomical approaches.

Results indicate that the noradrenergic innervation is selectively increased on adult born neurons following perceptual olfactory learning, pointing the noradrenergic system as a key mechanisms involved in perceptual learning. Interestingly, noradrenergic contacts on neurons born during ontogenesis were not affected by learning, suggesting a very specific part played by adult-born neuron in learning associated plasticity. In the same brains, we have analyzed the structural plasticity induced by learning in adult-born and pre-existing neurons. The major finding is that mirroring the increased number of noradrenergic contacts, learning induced an increase in dendritic spines on adult-born, but not on pre-existing neurons.

To assess the functional role of noradrenergic contacts following perceptual learning, we have inhibited the noradrenergic fibers in a spatially (olfactory bulb only) and temporally (upon odor investigation during the behavioral testing) selective manner by optogenetic. For that purpose, a lentivirus expressing Halorhodopsine, a light-sensitive inhibitory channel, was beforehand infused in the noradrenergic neurons of the Locus Coeruleus projecting to the olfactory bulb, and optic fibers were chronically implanted in the olfactory bulb. Inhibiting noradrenergic fibers during behavioral testing blocked learned discrimination the expression of the learned discrimination, strongly suggesting that they contribute to the expression of the learning.

To examine the function of cholinergic inputs on abGCs (another principle inputs on abGCs) during perceptual learning, cholinergic innervation on abGCs is studied. Preliminary results show that learning decreased the cholinergic innervation on abGCs, which is opposite to the effect of perceptual learning on noradrenergic innervation. These distinctive phenotypes

induced by perceptual learning strongly indicate the different roles of these two main centrifugal inputs on olfactory perceptual learning.

The PhD work provides original evidence for a structural plasticity of the noradrenergic system and its functional contribution to learned discrimination. In addition, it shows that this plastic change of noradrenergic contacts target adult-born neuron selectively and is parallel to the increase in spine density in these adult-born neurons. Comparison with neurons born during ontogenesis that do not show such plasticity brings decisive arguments favoring a unique role of adult neurogenesis in underlying olfactory learning.

Le champ des neurosciences connaît depuis quelques décades un développement très important dans la compréhension des corrélats neuronaux de la perception. Le cerveau adulte répond aux variations de l'environnement et à l'expérience par des modifications fonctionnelles et structurales, regroupées sous le terme générique de plasticité, plasticité qui sous-tend l'apprentissage. Cette plasticité affecte la perception sensorielle, olfactive puisque c'est cette modalité qui va nous intéresser, mais également la perception de stimuli dans d'autres modalités sensorielles. Par exemple, une odeur présente en permanence dans l'environnement finit par ne plus être perçue et sur une autre échelle de temps, l'exposition répétée à une odeur la rend familière, le plus souvent agréable et permet de la discriminer plus facilement d'odeurs proches. Une question centrale en neuroscience est donc de mettre à jour les mécanismes neuronaux de la plasticité perceptive et des adaptations comportementales qui en résulte.

La neurogenèse adulte et l'apprentissage

Contrairement à des convictions longtemps érigées en dogme mais maintenant dépassées sur la nature fixe du cerveau, il est établi désormais que le cerveau adulte est capable de générer tout au long de la vie de nouveaux neurones qui s'intègrent dans la circuiterie cérébrale complexe. La naissance de nouveaux neurones a lieu de manière constitutive dans deux régions cérébrales, le bulbe olfactif et le gyrus denté de l'hippocampe. La neurogenèse adulte est un processus biologique sophistiqué dont la fonction reste un mystère. D'un point de vue théorique, l'incorporation de nouveaux neurones dans le cerveau adulte représente un challenge particulièrement intrigant. Parce que le réseau neuronal est en perpétuel changement en raison de l'addition de nouveaux neurones, le bulbe olfactif et l'hippocampe ne peuvent pas être considérés comme stables dans leur cyto-architecture. Comment cette réorganisation cellulaire contribue à la fonction de l'hippocampe ou du bulbe olfactif et à la cognition n'est pas élucidé. Dans le débat actuel, les données issues des approches computationnelles proposent que les nouveaux neurones agissent comme des modulateurs des neurones principaux pour influencer la séparation de patterns d'activation proches et permettent ainsi de reconnaître comme distincts des motifs d'activation très recouvrant, permettant la discrimination entre deux stimuli olfactifs ou deux contextes proches dans le cas du bulbe olfactif et de l'hippocampe respectivement (Bakker *et al.*, 2008; Barnes *et al.*, 2008; Aimone *et al.*, 2011; Sahay *et al.*, 2011a; Sahay *et al.*, 2011b). Ce raffinement du message neuronal pourrait reposer sur un renforcement de l'inhibition.

L'hippocampe doit encoder des expériences très similaires au sein de représentation séparées pour permettre la mémoire épisodique ou spatiale. Récemment, il a été proposé que les neurones formés chez l'adulte jouent un rôle dans ce processus. En effet, la capacité de souris à distinguer entre deux contextes spatiaux très peu différents est altérée par le blocage de la neurogenèse adulte (Clelland *et al.*, 2009). Cependant, le rôle exact joué par les nouveaux neurones dans les circuits hippocampiques responsables de cette discrimination observée à l'échelle du comportement demeure incompris.

Le bulbe olfactif est impliqué dans la discrimination de centaines de milliers de molécules odorantes pures ou en mélange et cette discrimination requiert souvent de distinguer ou de séparer des motifs

d'activation très proches. En effet, chaque odeur évoque un motif spatial d'activation et la similarité entre ces motifs est inversement corrélé aux capacités de discrimination (Rubin & Katz, 2001; Linster *et al.*, 2002). De plus, les capacités de discrimination peuvent être modulées par l'expérience. L'exposition répétée au mêmes stimuli proches perceptivement, en absence de renforcement comportemental (récompense) améliore la discrimination, un processus de plasticité perceptive appelé apprentissage perceptif (Gilbert *et al.*, 2001; Mandairon *et al.*, 2006a; Mandairon *et al.*, 2006d; e; *Mandairon *et al.*, 2008; Mandairon & Linster, 2009; Moreno *et al.*, 2009). De manière remarquable, les travaux antérieurs de l'équipe ont démontré que l'acquisition de l'apprentissage perceptif dépend de la présence des neurones formés chez l'adulte (Moreno *et al.*, 2009).

Le système noradrénergique, médiateur des effets de l'expérience sur les réseaux neuronaux

Les systèmes neuromodulateurs comme les systèmes noradrénergique et cholinergique innervent massivement le bulbe olfactif et en particulier les neurones cibles de la neurogenèse adulte, les interneurons granulaires. Ils sont depuis longtemps connus pour leur implication dans les processus d'apprentissage en général et olfactif en particulier. Les manipulations pharmacologiques de ces systèmes altèrent l'activité du bulbe olfactif et les performances comportementales dans différentes tâches, chez le jeune et chez l'adulte (Sullivan & Wilson, 1994),(Veyrac *et al.*, 2007; Veyrac *et al.*, 2009), (Doucette *et al.*, 2007; Guerin *et al.*, 2008; Mandairon *et al.*, 2008).

L'apprentissage perceptif olfactif n'a pas lieu si l'action de la noradrénaline est bloquée par des antagonistes des récepteurs alpha et Bêta adrénergiques (Moreno et al 2012) et ces antagonistes empêchent aussi l'augmentation de la neurogenèse adulte observée pendant l'apprentissage et indispensable à sa réalisation. La noradrénaline est donc un bon candidat à la médiation des effets de l'expérience sur la plasticité cérébrale. Cependant, à ce jour comment elle interagit avec les neurones issus de la neurogenèse est méconnu. Plus largement, elle pourrait jouer un rôle clé dans les mécanismes par lesquelles l'expérience et l'activité cognitive s'inscrit de manière durable dans les réseaux neuronaux en maintenant un niveau élevé de plasticité perceptive et neurale. De ce point de vue, ce rôle pourrait correspondre au mécanisme actuellement recherché par lequel l'activité cognitive tout au long de la vie contribue au maintien des capacités cognitives.

Objectifs

Les systèmes neuromodulateurs, noradrénergique principalement mais aussi cholinergique sont des régulateurs puissants de l'apprentissage olfactif et de la neurogenèse adulte dans le bulbe olfactif. (Bauer *et al.*, 2003; Veyrac *et al.*, 2005; Veyrac *et al.*, 2009).

Un objectif de la thèse est de déterminer le pattern temporel et spatial de l'innervation des neurones formés chez l'adulte dans le bulbe olfactif et sa modification potentielle par l'apprentissage. Pour examiner les changements dans la connectivité des neurones avec les fibres noradrénergiques issues du Locus Coeruleus, nous avons eu recours à des méthodes neuroanatomiques. Les neurones formés chez l'adulte ou formés pendant l'ontogenèse (à des fins de comparaison) ont été marqués par des vecteurs lentiviraux exprimant une protéine fluorescente, préalablement injectés dans les zones d'intérêt chez le nouveau-né ou chez l'adulte de façon à pouvoir examiner la morphologie fine des neurones et la plasticité structurale (densité et morphologie des épines dendritiques) induite dans ces neurones par l'apprentissage. En parallèle, nous avons utilisé le marquage par le transporteur de la noradrénaline (NET) pour visualiser les fibres noradrénergiques et les contacts synaptiques qu'elles forment avec les neurones du bulbe olfactif, formés chez l'adulte ou au cours de l'ontogenèse.

Un autre objectif était d'évaluer le rôle fonctionnel des contacts noradrénergiques mis en place par l'apprentissage. Pour atteindre cet objectif, nous avons par la technique d'optogénétique inactivés les terminaisons noradrénergiques dans le bulbe olfactif et évalué l'effet de cette inactivation sur les capacités de discrimination apprises. Après injection d'un lentivirus exprimant l'halorhodopsine, canal ionique photosensible inhibiteur dans le *Locus Coeruleus* contenant les corps cellulaires noradrénergiques projetant sur le bulbe olfactif, nous avons obtenu l'expression de l'halorhodopsine dans les fibres noradrénergiques du bulbe olfactif. Des fibres optiques ont été implantées bilatéralement dans le bulbe olfactif et la stimulation lumineuse destinée à inhiber la libération de noradrénaline dans le bulbe olfactif a été délivrée au cours du test de discrimination olfactive suivant la période d'apprentissage perceptif.

Résultats

Les résultats indiquent que l'innervation des neurones formés chez l'adulte s'installe dès le huitième jour après la naissance des neurones pour le système cholinergique, comme pour le système noradrénergique. L'apprentissage induit une augmentation massive des contacts noradrénergiques sur les neurones formés chez l'adulte qui n'est pas retrouvée pour les fibres cholinergiques, pointant le système noradrénergique comme un acteur majeur de la plasticité induite par l'apprentissage perceptif. Dans la suite du projet, nous nous sommes alors concentrés sur la plasticité et le rôle du système noradrénergique. La caractérisation des épines avec un marqueur des synapses fonctionnelles indique que les contacts induits par l'apprentissage sont fonctionnels dans la même proportion que ceux existant chez les contrôles. De manière très intéressante et originale, nous avons également pu montrer que l'augmentation des contacts noradrénergiques n'avait lieu que sur les neurones néoformés et qu'aucune modification n'était observée sur les neurones formés pendant l'ontogenèse. Ceci est un argument nouveau en faveur d'un rôle très spécifique des néo neurones dans l'apprentissage.

En parallèle, nous avons examiné la morphologie fine des neurones formés chez l'adulte et pré-existants (formés pendant l'ontogenèse). Le résultat majeur est que, en regard de l'augmentation des contacts noradrénergiques, les neurones formés chez l'adulte montrent après apprentissage une augmentation des épines dendritiques qui là encore n'est pas retrouvée sur les neurones formés pendant l'ontogenèse.

En résumé de la première partie de ce travail, nous avons montré une forte réorganisation de l'innervation noradrénergique après apprentissage olfactif, au bénéfice exclusif des neurones formés chez l'adulte.

Pour évaluer le rôle fonctionnel des contacts noradrénergiques après apprentissage, nous avons mis en place la manipulation optogénétique des fibres noradrénergiques dans le bulbe olfactif. Cette approche permet une sélectivité spatiale (manipulation des fibres dans le bulbe olfactif uniquement) temporelle (manipulation au moment du test et quand la souris approche l'odeur) et neurochimique (manipulation des fibres issues du *Locus Coeruleus* uniquement). L'inhibition des fibres noradrénergiques dans ce contexte a bloqué la discrimination apprise, suggérant fortement que les contacts noradrénergiques contribuent à l'expression de l'apprentissage et donc à la discrimination apprise.

Ces données ont été présentées en novembre 2015 au congrès de la Société américaine de neurosciences et ont donné lieu à la sélection de l'abstract pour une présentation orale.

Le travail de thèse a apporté des données totalement originales qui pour la première fois mettent en évidence une plasticité structurale du système noradrénergique et la contribution fonctionnelle de ce système à la discrimination apprise. Il a de plus permis de mettre en évidence que cette plasticité a pour cible les neurones formés chez l'adulte qui montrent eux aussi un développement dendritique accru en regard de l'augmentation des contacts noradrénergiques. La comparaison avec l'effet de l'apprentissage sur les neurones formés pendant l'ontogenèse a aussi apporté des données décisives concernant les propriétés spécifiques de nouveaux neurones qui leur confèrent un rôle unique dans le réseau bulbaire pour sous-tendre l'apprentissage.

La mise en œuvre de projet a nécessité une approche multidisciplinaire incluant l'approche comportementales, la mise en œuvre de l'outil optogénétique et une analyse morphologique poussée.

En permettant de mieux comprendre comment les systèmes neuromodulateurs régulent la neurogenèse et le réseau bulbaire, ce travail est une contribution au champ de la plasticité cérébrale, de la neurogenèse adulte et de l'étude de l'influence des systèmes neuromodulateurs dans ces processus.

1. OLFACTION: FROM ODORANT MOLECULES TO PERCEPTION

1.1 GENERAL ORGANIZATION OF THE PERIPHERAL OLFACTORY SYSTEM

Olfaction is a key sense, enabling diverse vital activities including food seeking, danger detection, socialization etc. Olfactory reception starts from the olfactory sensory neurons within the olfactory epithelium which is lining the nasal cavity (Lledo *et al.*, 2005) (Figure 1). Olfactory sensory neurons detect volatile molecules (odorants) through molecular odorant receptors expressed in the dendrites of these neurons (Buck & Axel, 1991; Allingham *et al.*, 1999; Buck, 2000). Each neuron expresses only one molecular olfactory receptor. Each odorant receptor can interact with a number of odorant molecules with rather moderate affinity (with some exception for very selective receptors) (Kauer, 2002). Thus, individual receptors are remarkably cross-reactive in response to odorants. Therefore, each odorant will activate a specific receptor repertoire according to the diversity, as well as the concentration of odorant compounds (Duchamp-Viret *et al.*, 1999). This combinatorial code allows a specific neural signature for each odorant molecule. Olfactory sensory neurons project their axons to the olfactory bulb (OB) which is the first cortical relay of the olfactory information. More specifically, they connect the projection cells of the OB (mitral/ tufted cells) in specific structures called glomeruli (Moulton, 1974; Costanzo & Morrison, 1989). Olfactory sensory neurons expressing the same odorant receptor send their axons only to few glomeruli in the main OB (Mombaerts *et al.*, 1996). Since a given odorant activates a combination of olfactory sensory neurons, a combination of olfactory glomeruli will be activated by one odorant, leading to an odor map (Sharp *et al.*, 1975; Stewart *et al.*, 1979; Johnson *et al.*, 1998; Johnson *et al.*, 1999). Convergence of sensory neurons onto projection cells in the glomeruli is impressive; nearly 1,000 sensory neurons converge and connect with a single projection cell (Lledo *et al.*, 2005), increasing sensitivity. Within the OB, the activity of these projection cells is highly controlled by inhibitory (mostly GABAergic) interneurons, the periglomerular cells and the granule cells, via reciprocal dendro-dendritic synapses (Rall *et al.*, 1966; Price & Powell, 1970; Pinching & Powell, 1971).

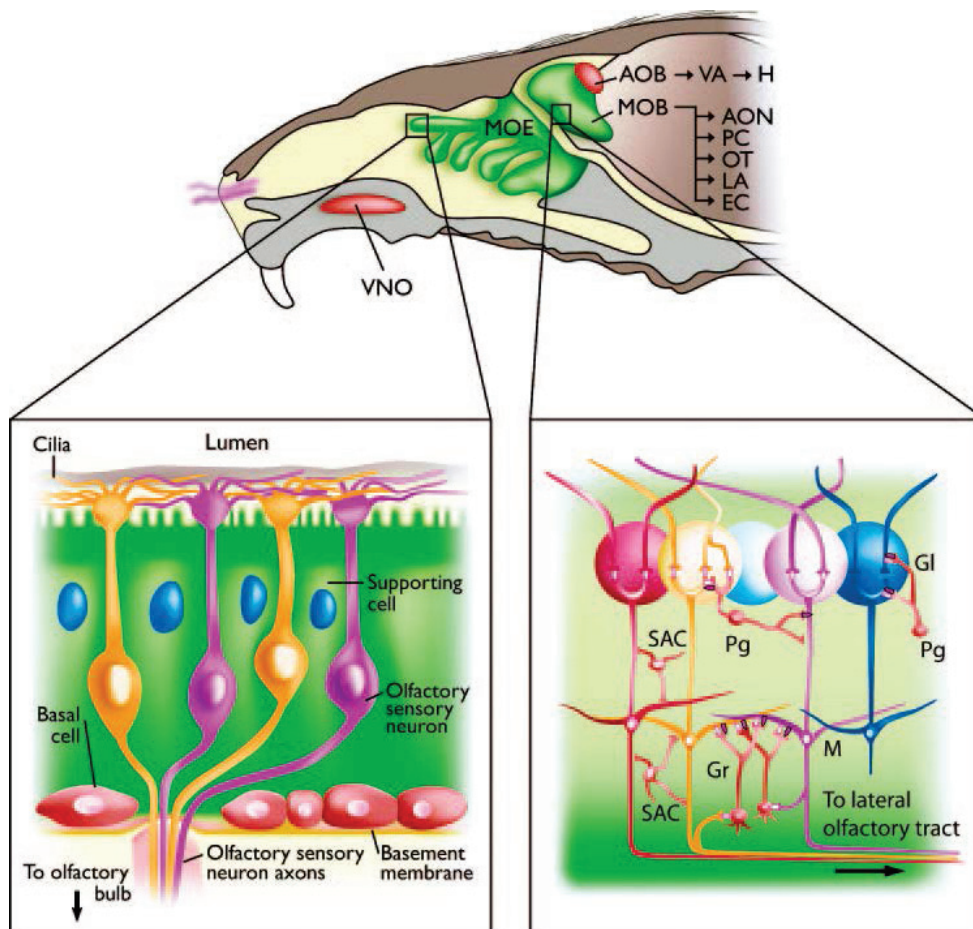


Figure 1. The olfactory system in rodents. Diagram in the top shows a simplified olfactory system in the brain in a sagittal view. The main olfactory epithelium (MOE) and main OB are highlighted in green. Sensory neurons are the principle sensors of odorants. The MOE receive odorants stimulation and relay the olfactory information to the MOB. After computation in the MOB, the olfactory information relays from the MOB to various cortices, including anterior olfactory nucleus (AON), the piriform cortex (PC), the olfactory tubercle (OT), the lateral part of the cortical amygdala (LA) and entorhinal cortex (EC). The diagram in the bottom left shows the structure in the MOE. Three principle cell types exist in the MOE, which are olfactory sensory neurons (OSN), supporting cells and basal cells. The basal cell is neural stem-cell, producing OSN. OSN are the odorant sensors through odorant receptors. The diagram in the bottom right indicates the simplified synaptic organization of the OB. Each OSN expresses one and only one of ~1000 odorant receptor, and each glomeruli (Gl) in the OB only receives the axon from the OSN expressing the same odorants receptor. The approximately 2,000 GL in the rodent OB are the loci where axons of OSN, dendrites of mitral/tufted cells, plus dendrites and axon from periglomerular cells converge. Mitral and tufted cells are main projection neurons in the OB. These projection neurons receive sensory signals from sensory neurons and receive regulation from two types of interneurons, periglomerular cells (Pg) and granule cells (Gr) (both in purple), and short axon cells (SAC) as well. After regulation, mitral/ tufted cells project to olfactory primary cortices (Lledo *et al.*, 2005).

This dendro-dendritic circuitry between projection neurons and interneurons provides a precise and localized inhibition, allowing a spatial and temporal shaping of mitral cell discharges. The olfactory message shaped by interneurons is then sent by projection

neurons to various cortices, including anterior olfactory nucleus, the piriform cortex, the olfactory tubercle, the lateral part of the cortical amygdala and the entorhinal cortex (Lledo *et al.*, 2005). Like many other sensory systems, the olfactory information is finally relayed to the neocortex through thalamus. Moreover, the olfactory tubercle sends axons to the medial dorsal nucleus of the thalamus, which in turn projects to the orbitofrontal cortex, the brain region believed to be related with the conscious perception of smell (Rolls, 2001; Lledo *et al.*, 2005).

1.2 OLFACTORY BULB INTERNEURONS

Periglomerular and granule cells are the two main interneuron populations in the OB. Periglomerular cells are co-activated by sensory inputs and mitral cells. Within the glomeruli, they form dendro-dendritic synapses with mitral cells and exert an inhibitory action on them. Periglomerular cells, more excitable than mitral cells, may inhibit mitral cells as a function of the strength of the sensory input. Inhibition of mitral cells would thus be low for weak inputs and higher for stronger inputs, providing a mean to increase the signal-to-noise ratio (Cleland & Sethupathy, 2006). Periglomerular cells also contribute to more classical lateral inhibition by connecting in an inhibitory way adjacent glomeruli (Wilson & Mainen, 2006). Periglomerular cells present a great neurochemical diversity (Kosaka *et al.*, 1995). Most of them are GABAergic and co-synthesize and release Dopamine. Others are not GABAergic and express enkephaline or calcium binding proteins like calretinin, calbindin (Kosaka *et al.*, 1995) or regulatory protein such as neurogranine (Gribaudo *et al.*, 2009).

Granule cells are GABAergic cells and the most numerous cellular population in the OB. Their cell bodies are lying in the granule cell layer with their dendrites extending throughout the external plexiform layer, where lateral dendrites of mitral cells radiate (Price & Powell, 1970). The action potentials in the lateral dendrites of a mitral cell can spread throughout the length of the dendrites (Xiong & Chen, 2002; Debarbieux *et al.*, 2003), therefore, mitral cell spikes can activate granule cells over long distances (up to 200 μm from the soma). In turn the granule cells can modulate the spread of action potentials in lateral dendrites of mitral

cells, via reciprocal dendro-dendritic synapses (Price & Powell, 1970; Shepherd *et al.*, 2007). This inhibition on lateral dendrites of mitral cells is called lateral inhibition, playing a crucial role in regulating activities of mitral cells

The maps of odorants are extremely complex in a spatial and temporal manner, and plus exhibit overlapping patterns across individual odorant stimuli. The proximity of individual patterns predicts discrimination abilities between odorants, odorants evoking highly overlapping maps not being discriminated (Linster *et al.*, 2002). Thus the mitral/ tufted cells may send ambiguous outputs to the olfactory cortices. Granule cells exert a regulation on the activation of mitral/ tufted cells and thus mediate signal contrast enhancement between odor stimuli (Mori & Yoshihara, 1995; Yokoi *et al.*, 1995; Shepherd *et al.*, 2007).

Furthermore, granule cells undergo neurogenesis throughout life. This special property may provide this cell special and crucial role in olfactory processing. The specific features and possible roles of adult-born granule cells (abGCs) are detailed in the following chapter.

2. ADULT NEUROGENESIS IN THE OLFACTORY BULB

Adult neurogenesis in the mammalian brain mainly occurs in the hippocampus, in the OB (Lledo *et al.*, 2006) and in the accessory olfactory bulb (Peretto *et al.*, 2014). In the following, we will focus on neurogenesis targeting the OB.

The OB receives throughout life thousands of adult-born neurons every day (Altman, 1969; Lois & Alvarez-Buylla, 1994). Neuroblasts are generated in the subventricular zone (SVZ) from stem-cells exhibiting radial glia-like properties (Doetsch *et al.*, 1999; Merkle *et al.*, 2004). They leave the SVZ and migrate towards the OB at an average speed of 30 μm per hour (Lois & Alvarez-Buylla, 1994; Lois *et al.*, 1996). Migration occurs longitudinally towards the OB along an anatomically well-defined route called the rostral migratory stream (RMS) (Altman, 1969; Lois & Alvarez-Buylla, 1994; Lledo *et al.*, 2006). After arriving in the OB, neuroblasts detach from the RMS and migrate radially from the core towards the surface of the OB and integrate distinct OB layers where they differentiate into the two subtypes of inhibitory interneurons, granule cells and periglomerular cells (Lois & Alvarez-Buylla, 1994) (Figure 2). The exact number of interneurons generated in adulthood remains uncertain (Lim & Alvarez-Buylla, 2014) due to technical limitations but it is likely to be in the range of several thousand per day.

The differentiation of individual adult-born interneurons in the OB highly depends on the geographical origin within the ventricle walls of the cells from which it was generated (Merkle *et al.*, 2007; Alvarez-Buylla *et al.*, 2008; Lim & Alvarez-Buylla, 2014; Fiorelli *et al.*, 2015) (Figure 3). Labeling fifteen distinct populations of radial glia in the SVZ of neonatal mice, Merkle and his colleagues found that each region in SVZ gives rise to only a very specific subset of interneuron subtypes (Merkle *et al.*, 2007).

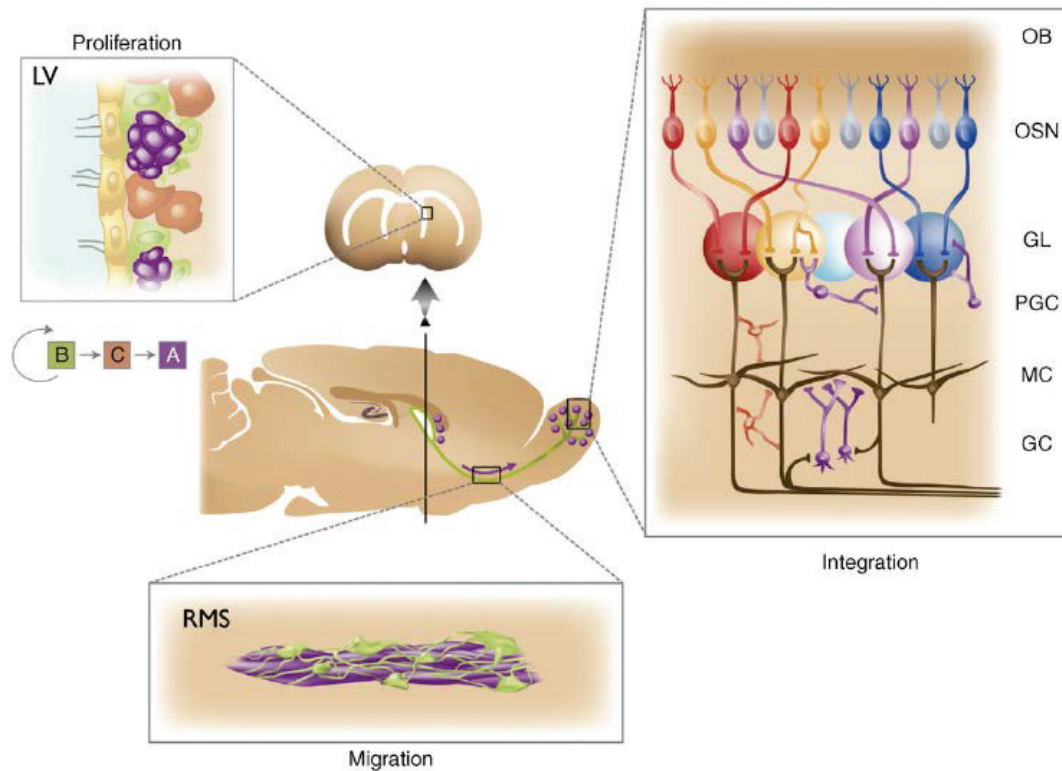


Figure 2. The subventricular zone (SVZ)-OB pathway of neurogenesis. Diagram in the center shows a sagittal view of rodent brain. The arrow (purple) indicates the migrating direction of neuroblasts (purple dots) from the SVZ to the OB. Diagram in the bottom illustrates the tangential migration of neuroblasts (in green) along the rostral migratory stream (RMS) (in purple), which is mainly comprised of radial glia-like cells. Diagram in the up left indicates the location of SVZ, where neural stem cells proliferate and differentiate. There are 3 main types of cells related to neurogenesis in the SVZ, stem cells (in green, type B cells), transit-amplifying cells (in brown, type C cells) and neuroblasts (in purple, type A cells). Panel in the right indicates the simplified structure of the OB showing the position of granule cells (GC) and periglomerular cells (PGC) which are the renewed populations in the OB. Mitral and tufted cells are main projection neurons in the OB. These projection neurons receive sensory signals from sensory neurons and receive regulation from the two types of interneurons PGC and GC (both in purple), and short axon cells (in red) as well (Lledo *et al.*, 2008).

Dorsal regions produce the highest percentage of tyrosine hydroxylase-positive (TH+, dopaminergic) periglomerular cells while calbindin-positive periglomerular cells are generated mainly in ventral regions. Anterior medial wall region generates Calretinin-positive periglomerular cells. Each targeted region gave rise to granule cells but with preference: dorsal regions tend to generate superficial granule cells and ventral regions mostly deep granule cells. Calretinin-positive granule cells were mostly from anterior regions, where many Calretinin-positive periglomerular cells were produced as well (Merkle *et al.*, 2007; Lledo *et al.*, 2008). Studies labeling neural stem cells in SVZ of adult mice showed that the place of birth of neuroblasts governs differentiation in a way similar to neonates (Weinandy *et al.*, 2011).

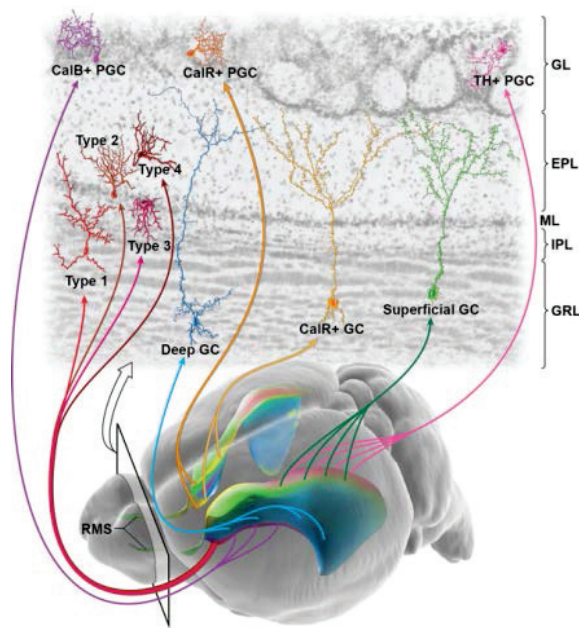


Figure 3. Regional organization of subventricular zone (SVZ) neural stem cells and their differentiation in the OB. Diagram in the bottom shows an oblique view of the adult mouse brain. The colored regions illustrate regional organization of the neurogenic niche. Top diagram: simplified layer structure in the OB, where are located identified subtypes of interneurons. Cells born in discrete sub-regions of SVZ migrate along the rostral migratory stream (RMS) into the OB to differentiate into granule cells (GC) and periglomerular cells (PGC) and subtypes of them. Details of these interneurons are seen in the introduction text. In the diagram four novel subtypes (type 1–4) of interneurons are shown, which are originally born in the most anterior SVZ. Abbreviations: CalB, calbindin; CalR, calretinin; TH, tyrosine hydroxylase; PGC, periglomerular cell; GC, granule cell; GL, glomerular layer; EPL, external plexiform layer; ML, mitral cell layer; IPL, internal plexiform layer; GRL, granular layer (Lim & Alvarez-Buylla, 2014).

Once in the OB, adult-born cells integrate into the existing network as they morphologically and functionally differentiate. During this period, adult-born cells are highly sensitive for their survival and integration to olfactory inputs and learned significance of odors. Although periglomerular cells are sensitive to olfactory inputs during this critical period (Bovetti *et al.*, 2009; Bonzano *et al.*, 2014), we will focus in the following presentation mainly on granule cells whose survival has been shown to be highly affected by learning (Moreno *et al.*, 2009; Sultan *et al.*, 2010).

2.1 MORPHOLOGICAL MATURATION OF ADULT-BORN GRANULE CELLS

The maturation of abGCs can be divided into five stages according to morphological criteria (Petreanu & Alvarez-Buylla, 2002; Carleton *et al.*, 2003) (Figure 4). In stage 1 (days 2–7 post retroviral infection in the SVZ, dpi), cells are only found tangentially migrating from SVZ to the OB along the RMS and its rostral extension in the core of the OB. These cells show a round or elongated soma with a prominent leading process and growth cone, and sometimes a small trailing process. In stage 2 (5–7 dpi), cells detach from the RMS and radially migrate toward the superficial layers of the OB. At this stage, cells present a longer and frequently bifurcated leading process comparing to stage 1 cell, with a longer and smooth trailing process.

AbGCs in stage 3 (9–13 dpi) have their cell bodies settled in a specific location in the granule cell layer and their migration is completed in this stage. Cells in stage 3 exhibit an irregular border of the cell body, which showed a smooth contour in stage 1 and stage 2 cells. A single process of these cells extends towards the external plexiform layer, which is supposed to develop into the growing apical dendrite, but at this stage, initial branching mostly stays within in the granule cell layer. Moreover, neurites on the basal side are found, which are in the process of developing into basal dendrites.

After stage 3, both basal and apical dendrites of abGCs continue to grow and apical dendrites extend through mitral cell layer towards the external plexiform layer. Furthermore, spines are generated and majority of synaptic contacts with local networks are developed (13-22 dpi). Later maturation of abGCs is divided into 2 stages. Stage 4 (11–22 dpi), granule cells exhibit branched apical dendrites in the external plexiform layer which are devoid of or bear only few spines and gemmules (spine-like protrusions from the dendrites). Stage 5 (15–30 dpi), granule cells present mature morphological features, with dense spines on both of basal and apical dendrites.

For all granule cells (born in adulthood or not), the basal dendrites and the primary unbranched apical dendrite (from soma to the first branching) only exhibit axo-dendritic synapses, which are involved in receiving inputs from axon collaterals of mitral/tufted cells and centrifugal inputs. In contrast, the branched apical dendrites contain bidirectional dendro-dendritic synapses where granule cells receive glutamatergic inputs from the lateral dendrites of mitral/tufted cells, and perform GABAergic inhibition back onto mitral/tufted cells (Shepherd *et al.*, 2007). These two dendritic domains are thus involved in distinct functions of granule cells.

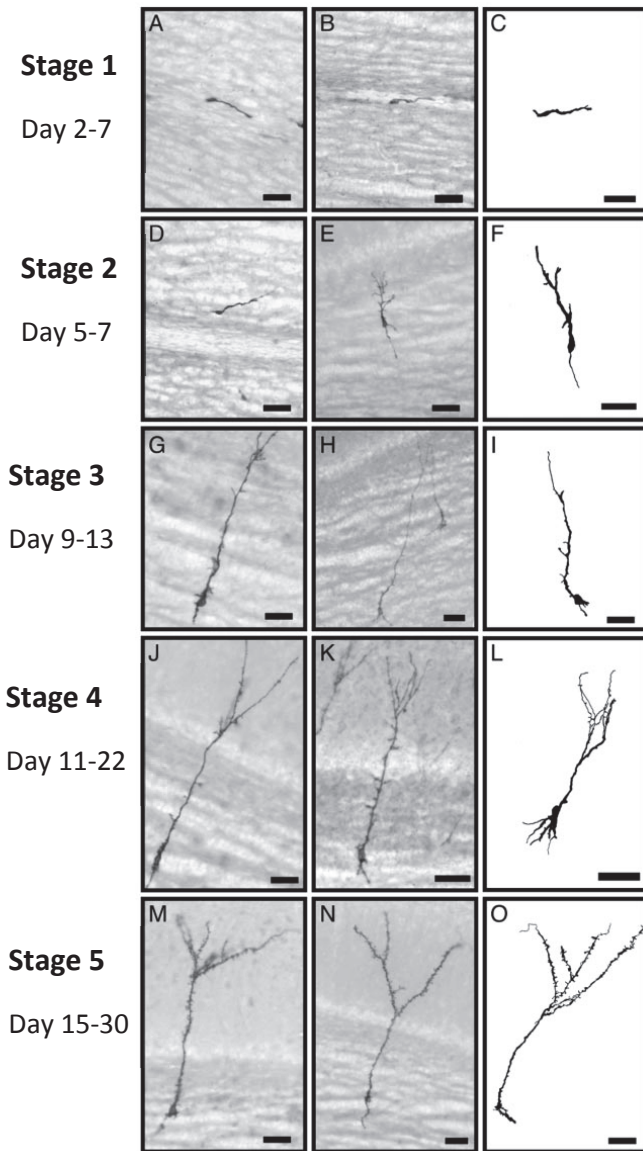


Figure 4. Morphological maturation of abGCs. Diagram shows examples of five stages of abGCs in photomicrographs (left and center columns) and camera lucida drawings (right column). Details of the definition of stages are given in the text. A–C, stage 1; D–F, stage 2; G–I, stage 3; J–L, stage 4; M–O, stage 5. Scale bars, 25 μm . (Petreanu & Alvarez-Buylla, 2002).

Therefore, distinct maturation pattern may occur in these separated domains of abGCs. Indeed, a study using immunohistochemistry of synaptic markers and ultrastructural analysis showed that glutamatergic inputs (positive for PSD-95, a scaffolding protein that localizes to the postsynaptic density of glutamatergic synapses, and to GluR2/3 AMPA receptor subunits) were formed at 10 dpi (stage 3 or stage 4) on the cell body and basal dendrites of abGCs. In contrast, spines on the apical dendrites are found starting 14 dpi whereas reciprocal synapses are not formed until 21 dpi (stage 5) (Whitman & Greer, 2007b). Another study (Kelsch *et al.*, 2008) using PSD-95 clusters confirmed these previous finding (Figure 5). In addition, synaptophysin (synaptic vesicle protein serving as a presynaptic marker) clusters were detected only in the distal dendritic domain from 17 dpi and reached their stable density at 28 dpi (Kelsch *et al.*, 2008), which indicates that the appearance of GABAergic outputs of abGCs followed by several days that of glutamatergic inputs. This sequential

development indicates that abGCs may silently integrate into the pre-existing circuitry since they develop output synapses only after the acquisition of the capability of receiving inputs. In addition, this pattern is different from the one of neurons born during the neonatal period (Figure 5).

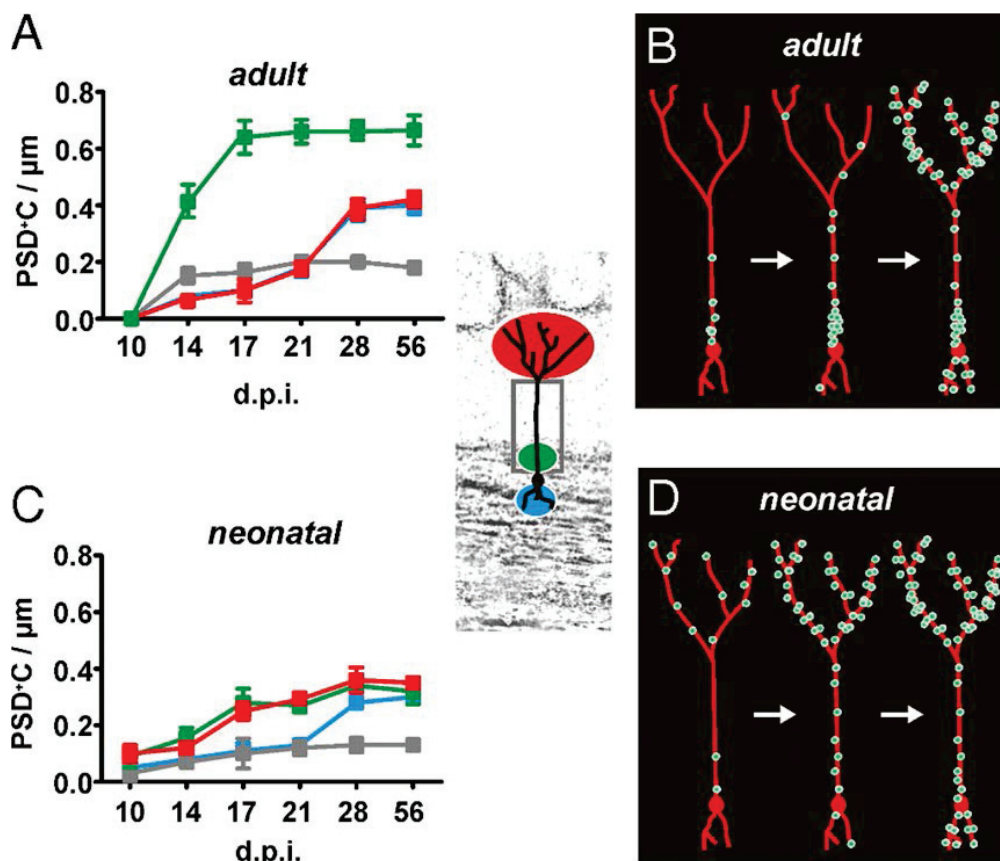


Figure 5. Domain-specific development of PSD+Cs during maturation of adult- and neonatal-generated GCs (with branching in the superficial external plexiform layer). (A) Mean PSD+C density at different stages (d.p.i.) during the maturation of new GCs generated in adult animals. The dendritic domains are indicated in the graph: basal (blue), proximal (green), and distal domain (red line) as well as the entire unbranched apical dendrite (gray). (B) The diagram illustrates the developmental pattern of PSD+Cs during maturation of adult-generated GCs. (C) Mean PSD+C density at different stages (d.p.i.) during the maturation of new GCs generated in newborn animals. (D) The diagram illustrates the developmental pattern of PSD+Cs during maturation of neonatal-generated GCs (Kelsch *et al.*, 2008).

Maturation of abGCs is a continuous rather than a discrete process, which varies considerably from cell to cell. So the stages of morphological development above are valid for most of the abGCs, but they partially overlap and exception is allowed. For instance, by 9 dpi, a few neurons with elaborated dendritic arbors (stage 4) are found, while most of the

cells at this age are still migrating radially towards surface of the OB (stage 2) (Petreanu & Alvarez-Buylla, 2002). In most case, by 13 dpi, the majority of the cells are in stage 4 with relatively immature dendrites. In the next 2 days, cells dramatically mature. By 15 dpi, most of the cells develop considerable spines and by 22 dpi, they are in stage 5 (Petreanu & Alvarez-Buylla, 2002). This suggests that abGCs develop their mature morphology dramatically within about one week after their cell bodies settle down in the granule cell layer.

2.2 FUNCTIONAL MATURATION OF ADULT-BORN GRANULE CELLS

Stage 1 and stage 2 (2-7 dpi) cells show relatively high membrane resistance and a complete absence of voltage-gated Na⁺ conductance, accompanied by a lack of spiking. In addition, no spontaneous postsynaptic currents (sPSCs) were found in cells of these two stages (Carleton *et al.*, 2003) (Figure 6). Use of receptor antagonists and agonists showed that a majority of granule cells in stage 1 expressed functional GABA_A receptors, while functional AMPA receptors were found in a small proportion of these cells. However, no cell was found to respond to NMDA. Majority of stage 2 cells present functional GABA_A receptors and AMPA receptors. Moreover, functional NMDA receptors were found in half of these cells.

Different from the first two stages, cells in stage 3 and 4 (9-22 dpi) showed a small voltage-gated Na⁺ current (38% of stage 3 cells and 80% of stage 4 cells respectively). Moreover, spontaneous inhibitory (sIPSCs) was found in 79% of stage 3 cells and almost all of the stage 4 cells, whereas excitatory postsynaptic currents (sEPSCs) was found in 35% of stage 3 cells and 77% of stage 4 cells. Furthermore, stage 4 cells fired only occasional spikes.

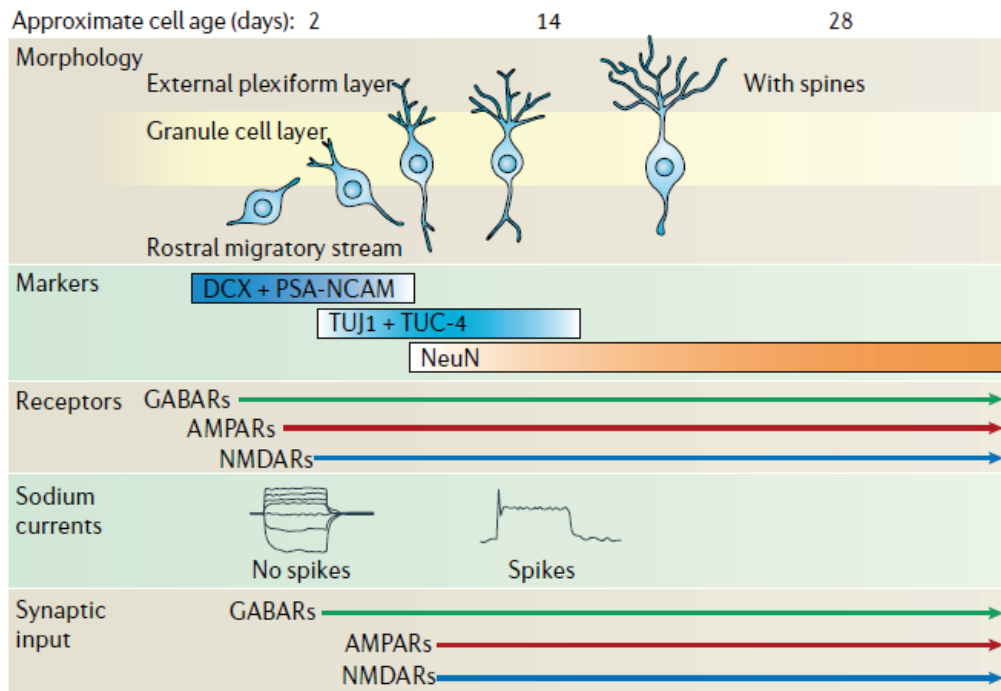


Figure 6. Functional maturation of adult-born granule cells in the OB. AbGCs go through 5 stages in maturation according to their morphological features. Different markers, receptor expression, synaptic input and electrophysiological phenotypes are exhibited along the maturation. It should be noted that maturation stages are continuous but not discrete, and maturation time points of individual cells varies. Therefore, the showed time points in this diagram represent the earliest age at which reported events were observed. Maturation may also last beyond 28 post cell birth (Lledo *et al.*, 2006).

In accordance with their mature morphology, abGCs in stage 5 (15-30 dpi) exhibit mature electrophysiological properties including average rest voltage, membrane resistance, membrane capacitance, adult-like firing frequency and amplitude of IPSCs. Based on these criteria, they are indistinguishable from the older pre-existing granule cells born during ontogenesis. Associated with the ability of firing action potentials in stage 5, voltage-gated Na⁺ channels were found consistently expressed in this type of cells. Moreover, all cells in stage 5 expressed functional NMDA receptors. Therefore, during the maturation of abGCs, the type of expressed receptors can be an indicator of maturation level.

In accordance with a decrease of input membrane resistance along maturation, abCGs at the age of 2 weeks (stage 3 or stage 4) showed significant long-term potentiation (LTP, lasting at least 1 hour) induced by theta burst stimulation of the granule cell layer. This effect was still observed in most abGCs at the age of 8 weeks but in very few by the age 12 weeks and not in pre-existing granule cells (Nissant *et al.*, 2009). Since somata, basal and proximal dendrites

are main targets of centrifugal inputs, and plus LTP is one of predominant mechanisms underlying learning and memory (Kandel, 2001), it is likely that abGCs show a specific sensitivity and plasticity to top-down control from centrifugal innervation by cortical areas and neuromodulatory systems.

In conclusion, adult-born neurons show specific morphological and functional features depending on the level of maturation. Continuous but sequential development of morphology, electrophysiological responses and synapses formation are observed in the adult-born neurons after arriving at the OB, which provide these new neurons with specific plasticity during maturation and integration. Moreover, axo-dendritic inputs develop before the dendro-dendritic outputs, and formation of synaptic connection precedes full development of voltage-gated sodium currents responsible for spiking in the abGCs. This sequential development allows the control of new neuron's activity before it can affect the whole network function, therefore, they integrate into the pre-existing circuitry without or at least minimally disrupting the functioning circuit.

2.3 NEUROGENESIS ALLOWS REPLACEMENT OF PRE-EXISTING AND ADULT-BORN CELLS

Even though a high number of neuroblasts migrate into OB and differentiate into granule cells every day, the size of the OB is quite stable during adulthood (Rosselli-Austin & Altman, 1979; Petreanu & Alvarez-Buylla, 2002). Therefore, new comers or the pre-existing granule cells are supposed to be eliminated every day. Indeed, dUTP-nick end labeling (TUNEL) revealed that bulbar areas receiving numerous adult-born neurons also contained high numbers of apoptotic cells (Biebl *et al.*, 2000).

Regarding adult-born neurons in mice, half of them die between day 15 and day 45 after cell birth. The remaining cells then survive during the following 3 months (Petreanu & Alvarez-Buylla, 2002; Mandairon *et al.*, 2006c). After 1 year, nearly one third of radiolabelled granule cells were still detected in the OB (Petreanu & Alvarez-Buylla, 2002). A study in rat also showed that 40% of BrdU-positive cells survived for 19 months. It seems thus that once they reached the age of 3 months, abGCs (and periglomerular cells) remained stable up to 19 months (Winner *et al.*, 2002).

However, do these surviving adult-born neurons only replace the mature adult-born neurons, or do they replace neurons born during ontogenesis (pre-existing cells)? Does this replacement occur in specific bulbar areas? Using genetic labeling of adult-born cells, Imayoshi and his colleagues found that in 12 months old mice, up to 60 % of granule cells in the granule cell layer of the OB are adult-born, suggesting that they replaced pre-existing ones in adulthood (Imayoshi *et al.*, 2008). In addition, when considering only the deep granule cell layer, after 12 months, nearly 90 % of granule cells are adult-born ones. So, in this region, almost all pre-existing granule cells are probably replaced by adult-born neurons. This phenomenon was confirmed by the genetic ablation of neurogenesis, following which a considerable number of granule cells in the deep region was lost after 12 weeks of neurogenesis deprivation, whereas many granules cells in the superficial region were preserved (Imayoshi *et al.*, 2008). Consistently, the granule cells which are born in early postnatal period predominantly migrate into the superficial region, whereas the abGCs preferentially settle into deep region (Lemasson *et al.*, 2005; Imayoshi *et al.*, 2008). Therefore, different from the more stable neurons which were born in the neonatal period, adult-born neurons are probably replaced by more recently born neurons. Further evidence for adult-born cell replacement came from a study in which abGCs could be prematurely suppressed after learning by an extinction procedure and in which apoptosis blockade prevented behavioral extinction (Sultan *et al.*, 2011b). These observations suggested that learning and forgetting induced a turn-over of adult-born cells (see below for detailed description of the effects of learning on adult-born neurons). To conclude, if 30-40% of abGCs survive more than 12 months in the OB as suggested (Petreanu & Alvarez-Buylla, 2002; Winner *et al.*, 2002), and if the volume of the OB doesn't change with aging and finally if a majority of granule cells are adult-born after approximately one year (Imayoshi *et al.*, 2008), then it is likely that adult-born cells undergo a turn-over that could well be modulated by experience.

In conclusion, numerous abGCs are added in the OB every day leading to replacement of pre-existing granule cells. However, adult-born cells also face a fate of apoptosis throughout life. The lifespan of these neurons may thus highly vary. If eliminated neurons have encoded some olfactory information, their elimination may result in a loss of this information (Sultan *et al.*, 2011b). Therefore, the survival of adult-born neurons does matter for the learning and

memory. Why do some adult-born cells survive while others will die shortly after arriving in the OB? One of the most important factors affecting the survival is the olfactory experience, which will be introduced and discussed in the coming chapters. But before this, below is an overview of the developmental profile of adult-born periglomerular cells.

2.4 ADULT-BORN PERIGLOMERULAR CELLS: ANOTHER TYPE OF ADULT-BORN CELL IN OB

Compared to the number of abGCs, only a minority of neuroblasts migrates from specific areas of the SVZ to the glomerular layer and becomes periglomerular cells. Among them, many are GABAergic, accompanied by some other subtypes of cells which are defined by the specific molecules they express (Kosaka *et al.*, 1995; Winner *et al.*, 2002; Ming & Song, 2011). One study also found a very small percentage of adult-born cells differentiated into glutamatergic juxtglomerular cells (Brill *et al.*, 2009).

Time-dependent precise maturation of adult-born periglomerular cells was not investigated as much as that of abGCs. However, a few of studies could send us some insights regarding the morphological and functional development of these cells.

GABA-induced currents were found in all ages of adult-born periglomerular cells, whereas glutamate-induced responses did not appear until 4 weeks after viral infection in SVZ. Adult-born periglomerular cells can fire as soon as they arrived in the glomerular layer but functional glutamatergic receptors appear much later. This developmental sequence is opposite to what is observed in abGCs, which get functional synaptic inputs before developing the voltage-gated sodium currents and the capacity to fire spikes. (Belluzzi *et al.*, 2003). This difference may suggest that these two types of adult-born interneurons present distinct functional integration patterns depending on the local network and their own morphological properties. Although much is to be learned on this issue.

2.5 ROLE OF ADULT-BORN NEURON IN ODOR DETECTION AND SPONTANEOUS DISCRIMINATION

Ongoing neurogenesis contributes to a continuous renewing circuitry in the OB. Since the OB is the first central processor of sensory information, questions about functions of adult-born neurons in olfaction arose since adult neurogenesis was discovered. Among many essential

roles of olfaction in rodents, odor detection and discrimination are vital for survival. It is thus instinctive to question if adult-neurogenesis contributes these olfactory functions. Neurogenesis-impaired mice (by knocking out polysialylated-neural cell adhesion molecule, PSA-NCAM), showed an impairment of spontaneous discrimination (Gheusi *et al.*, 2000). PSA-NCAM ablation leads to a defect in rostral migration of adult-born cells, resulting in a size reduction of the OB (Rousselot *et al.*, 1995; Gheusi *et al.*, 2000). However, although the total number of abGCs was inferred to be reduced by 35% in the KO mice, BrdU labeling revealed that the percentage of abGCs among the total granule cells showed no difference between wild-type mice and PSA-NCAM knockout mice. It is thus difficult to conclude on the specific role of adult-born cells in spontaneous discrimination in this model. In addition, other studies showed that ablation of neurogenesis during adulthood had no effect on olfactory spontaneous discrimination (Imayoshi *et al.*, 2008; Breton-Provencher *et al.*, 2009). Interestingly, detection thresholds for odors and habituation to odors remained unaltered in the PSA-NCAM knockout mice (Gheusi *et al.*, 2000). Intact habituation (requiring detection and short term memorization of an odorant) was also found after pharmacological ablation of neurogenesis (Moreno *et al.*, 2009) suggesting normal odor detection (and short-term memory).

In conclusion, ablation of adult-born neurons in the OB does not seem to affect odor detection or spontaneous discrimination. These basic and vital functions may rely mostly on the neurons that persist throughout life. Therefore, transiently surviving adult-born neurons showing some plasticity in their immature state, are more likely to be involved in plastic changes in perception according to environmental diversity and olfactory learning during the life. They may allow the animals to adapt to new environment. This hypothesis is nowadays supported by a wealth of data that is discussed in the following chapters.

3. EXPERIENCE-DEPENDENT REGULATION OF ADULT NEUROGENESIS

Adult brain plasticity relies on structural and functional plasticity of specific circuits which are shaped by experience (Holtmaat & Svoboda, 2009). Experience-dependent synaptic plasticity in adult neural circuits involves the strengthening or weakening of connections at

individual synapses through long term potentiation and depression (Kandel, 2001), but also synapse formation, elimination and stabilization (Holtmaat & Svoboda, 2009). In the OB, in addition to these classical synaptic plasticity processes, functional adult-born neurons are continuously added and integrated into existing circuits (Abrous *et al.*, 2005; Doetsch & Hen, 2005; Aimone *et al.*, 2006; Kempermann, 2008; Zhao *et al.*, 2008; Lepousez *et al.*, 2015). A central issue for understanding the role of adult-born neurons is thus to investigate whether experience affect their number, destiny, shape or function and what could be their specific contribution to plasticity, compared to neurons born during ontogenesis. In the olfactory system, more and more studies reveal the crucial roles of adult-born neurons in learning suggesting that they have specific features providing the olfactory network with the ability to elaborate reliable but adaptive representation of olfactory information.

Studies in aged mice also support the function of adult-born neurons in olfactory learning. Aging mice show a progressive decrease in adult-born neurogenesis initially due to a decrease in proliferation of precursors in the SVZ and later on to a decrease in neuronal differentiation of adult-born cells (Rey *et al.*, 2012). Aged mice exhibit impairment in fine olfactory discrimination learning after reward/punishment associative training (Enwere *et al.*, 2004) One possible factor contributing to this impairment is the low level of adult neurogenesis in these aged mice. Interestingly, mice heterozygote for the leukemia inhibitory factor receptor ($Lifr^{+/-}$) and showing a decreased rate of neurogenesis in the OB compared to the wide type littermates exhibited intact olfactory detection but degraded performance at fine olfactory discrimination learning (Enwere *et al.*, 2004). We could thus infer that reduced neurogenesis might be the crucial factor weakening performance in olfactory discrimination learning in the aged mice.

In the following chapters, the modulation by experience of the rate of neurogenesis, the mode of integration of adult-born neurons in the pre-existing network and how they sustain olfactory perception in various learning paradigms will be described and discussed.

3.1 OLFATORY EXPERIENCE PROMOTES SURVIVAL OF ADULT-BORN NEURONS

Many olfactory manipulations have been investigated for their effect on adult-born neurons survival, including olfaction deprivation, olfactory enrichment, associative and perceptual learning.

3.1.1 DEPRIVATION

Studies using transgenic anosmic mice (mice knock-out of the olfactory cyclic nucleotide gated channel) or naris closure found that olfactory deprivation significantly decreased the number of abGCs in the OB (Corotto *et al.*, 1994; Petreanu & Alvarez-Buylla, 2002; Mandairon *et al.*, 2006c). Numbers of adult-born neurons were found dramatically decreased after 4 weeks of naris closure, and this effect was maintained and progressed during the whole period of naris closure (16 weeks) (Corotto *et al.*, 1994). Interestingly, the decreased in cell survival induced by naris closure was reversed after reopening the naris, in other words, the number of abGCs significantly increased after naris reopening (Cummings *et al.*, 1997).

3.1.2 ENRICHMENT

It has been well accepted that environmental enrichment can induce notable functional and anatomical modification in the brain (van Praag *et al.*, 2000). Making no exception, olfactory enrichment consisting in a daily exposure to different odorants during several weeks almost doubled the number of surviving abGCs in the OB compared to a non-enriched group (Rocheffort *et al.*, 2002). Moreover, the increase of survival of abGCs is accompanied by longer short-term olfactory memory (Rocheffort *et al.*, 2002; Veyrac *et al.*, 2009; Rey *et al.*, 2012) and improvement in fine discrimination (Rey *et al.*, 2012). Cell proliferation in the SVZ was not affected by enriched olfactory environment, indicating that cell proliferation and survival of adult-born neurons can be independently regulated. This finding is consistent with studies on hippocampal neurogenesis, in which enriched environment only affected adult-born neuron survival (Kempermann *et al.*, 1997). Interestingly, a daily exposure to a mix of 20 odorants instead of an exposure to a single but different odorant every day during several weeks, did not alter the survival of abGCs in the OB and caused no improvement to olfactory memory (Veyrac *et al.*, 2009). Exposure to ecological stimuli such as lamb odor in sheep was also found to interact with adult neurogenesis (Brus *et al.*, 2014; Corona *et al.*, 2016).

Based on these results, we can infer that olfactory enrichment increasing the level of exposure to novelty is efficient to improve adult-born neuron survival, olfactory short term memory and fine discrimination.

3.1.3 LEARNING

ASSOCIATIVE

The effect of associative learning on adult-born neuron survival in the OB has been investigated by several studies in which animals were conditioned to respond to a reinforced odor (Alonso *et al.*, 2006; Mouret *et al.*, 2008; Sultan *et al.*, 2010; Mandairon *et al.*, 2011; Sultan *et al.*, 2011b). They yield convergent finding indicating an increase in adult-born neuron survival induced by learning. Moreover, abGCs were found to be activated by the learned odorant (Alonso *et al.* 2006, Sultan *et al.* 2010). The increased number of adult-born neurons was temporally correlated to memory of the associative task. First, adult-born neurons were induced to survive in the OB precisely when animals' performances diverged from those of control, pseudo-conditioned animals (Sultan *et al.*, 2011a). Second, as memory of the task decays with time, the level of neurogenesis comes back to control value (Sultan *et al.*, 2010). Third, pharmacological manipulation increasing memory retention also increases neurogenesis (Sultan *et al.*, 2010). Finally, altering the associative memory trace behaviorally through an extinction procedure suppressed adult-born neurons previously saved by learning (Sultan *et al.*, 2011b). In line with this, another study showed that spaced associative learning, occurring along several days and allowing consolidation of the memory trace increased neurogenesis and was subject to long-term retention while massed learning (over one day) induced no increase in neurogenesis and exhibited only a short-term retention of the task (Kermen *et al.*, 2010). Interestingly, increased survival of adult-born neurons occurred in restricted areas in the OB (Alonso *et al.*, 2006; Mandairon *et al.*, 2006b; Sultan *et al.*, 2010). This phenomenon suggests that learning adjusts the distribution of abGCs, resulting in an enhanced and uniquely contrasted signal evoked by learned odors. It is then possible that increased survival of adult-born neurons in specific regions changes the spatial distribution of the activity of mitral/tufted cells, therefore modifying the odor representation transmitted to olfactory cortices in function of the behavioral significance acquired by the odorant. Interestingly, only adult-born neurons in the deep but not superficial granule cell layer are sensitive to olfactory associative learning (Mouret *et al.*,

2008) This result is consistent with other studies which showed that neural replacement mainly takes place in the deep rather than in the superficial area of the granule cell layer (Lemasson *et al.*, 2005; Mandairon *et al.*, 2006c; Imayoshi *et al.*, 2008). Moreover, tufted and mitral cells respond differently to odorants. Tufted cells show lower threshold for spiking in response to olfactory input (Nagayama *et al.*, 2004) than mitral cells. Therefore, they might be able to detect low concentration of odorants. Mitral cell discharge in the other hand is modulated by the learned significance of odorants (Kay & Laurent, 1999; Doucette *et al.*, 2007). Therefore, the spatial recruitment of adult-born neurons might be crucial for odor detection or coding during olfactory learning.

PERCEPTUAL LEARNING

The studies presented above used associative (reinforced) discrimination learning in which the animal learns to discriminate a rewarded from a non-rewarded odor. However, in such task, it remains very difficult to figure out the role of adult-born neurons in olfactory discrimination learning *per se* from their role in the process of association learning between an odor and a reward (Doucette *et al.*, 2007; Doucette *et al.*, 2011). A model allowing a more selective assessment of the role of neurogenesis in discrimination learning is perceptual learning (Gilbert *et al.*, 2001). Perceptual learning is defined as the improvement in discrimination between perceptually similar odorants after non-reinforced repeated exposures to these odorants. Perceptual learning plays crucial roles in daily life to constantly adjust discrimination abilities to the environment. Are adult-born neurons in the OB involved in olfactory perceptual learning? Olfactory perceptual learning increased survival of abGCs (that was not observed for periglomerular cells) aged between 8 and 17 days (Moreno *et al.*, 2009). In this behavioral paradigm, daily odor exposure is of one hour per day, which may allow the enriched odors remaining novel or at least salient every day even though they are presented daily. Accompanying data shed light on the possible mechanism of action of adult-born neurons to support perceptual learning. Indeed, immunohistochemistry of GAD65/67, the GABA synthesizing enzymes, showed increased GAD65/67 expression the OB following perceptual learning (Moreno *et al.*, 2009), strongly supporting increased GABA synthesis. Increased GABA transmission may reinforce inhibition onto mitral cells. This was confirmed by the paired-pulse stimulation of the lateral olfactory tract. Increased paired-pulse ratio in the odor-enriched group was indicative of strengthened granule-to-mitral

inhibition. Since adult-born neurons are more plastic and efficient to respond to new signals (Magavi *et al.*, 2005; Nissant *et al.*, 2009; Breton-Provencher *et al.*, 2014) than mature cells, one may thus propose that increased inhibition in the OB arises from newly formed granule cells and underlies learned discrimination in perceptual learning. This result may support the assumption that activated adult-born neurons participate in tuning bulbar activity to facilitate separation of initially overlapping evoked activity patterns and allow olfactory discrimination as proposed by computational studies (Cecchi *et al.*, 2001; Mandairon *et al.*, 2006d).

3.2 EFFECT OF MANIPULATION OF ADULT-BORN NEURONS ON PERCEPTION AND LEARNING

Data on modulation of the rate of adult-born neurogenesis induced by experience remain correlative. To further explore the causal role of adult-born neuron in olfactory perception and learning, one needs to experimentally manipulate the adult-born neurons and analyze how these manipulations affect perception and learning.

In associative learning, ablation and optogenetic manipulations of adult-born neurons have been proposed to decipher the role of adult neurogenesis. Impaired neurogenesis induced by irradiation or antimetabolic drug administration resulted in deficits in long-term memory (Lazarini *et al.*, 2009; Sultan *et al.*, 2010) without affecting the learning *per se*. Two other studies reported no effect on memory of neurogenesis ablation. However, they used a non-operant associative task (Imayoshi *et al.*, 2008; Breton-Provencher *et al.*, 2009) that was found to have no positive effect on neurogenesis and was consequently not affected by its ablation (Mandairon *et al.*, 2011). Limited temporal selectivity of antimetabolic compounds and post-irradiation neurogenesis rebound and inflammation however hampered the power of these approaches. Using optogenetic allowing temporally targeted and adult-born neuron-specific manipulations in an associative go/no-go operant task, it was found that activation of abGCs at the age of 12 weeks facilitated associative olfactory discrimination learning (Alonso *et al.*, 2012). Interestingly, this effect existed only when the discriminative task was difficult involving highly similar odorants pairs, whereas the effect failed to appear when the odor pair was easy to be distinguished. This supports the hypothesis mentioned earlier that abGCs are crucial in mediating learning-induced plasticity to clarify ambiguous signals in the

OB. Moreover, optogenetic-activation of early postnatal neurons did not improve learning in either the easy or the difficult discrimination learning tasks. All together, these data suggest that the plasticity underlying difficult learning was mainly due to the abGCs but not the pre-existing neurons.

At the cellular level, *in vivo* electrophysiological recordings revealed that optogenetic activation inhibited odor-evoked firing activity in weakly activated mitral cells, while the highly activated ones resisted light-evoked inhibition. Activation of adult-born neurons could thus raise the signal-noise ratio during learning and enhance the signal contrast between similar odorants. Interestingly, the dramatic learning improvement described above was induced apparently by the activation of a small proportion of virally transduced neurons as measured by c-fos expression (Alonso *et al.*, 2012). This suggests that activation of few abGCs can lead to a major behavioral change. Computational model supports this idea by showing that small changes in granule cell activation yield widespread changes in inhibition onto mitral cells, therefore remarkably modifying the output from the OB (Mandairon *et al.*, 2006d; Koulakov & Rinberg, 2011). In odor fear conditioning, a different associative paradigm involving a well-known specific circuit, neurogenesis ablation with irradiation induced diminished fear response to a fear-conditioned odor, even though the association between the odor and food shock is formed successfully (Valley *et al.*, 2009).

In perceptive learning, ablation of neurogenesis during acquisition of learning prevented learning (Moreno *et al.*, 2009).

All together, these studies collectively strongly support a role for adult-born neurons in discrimination learning, both in associative and perceptual tasks.

3.3 CRITICAL PERIOD OF ADULT-BORN NEURONS AND BEHAVIORAL EXPERIENCE

During early development, a restricted “critical period” for experience-dependent plasticity has been clearly proved in the visual, auditory, olfactory and somatosensory systems (Yamaguchi & Mori, 2005). Interestingly, the survival of abGCs seems to be under a similar precise temporal regulation.

Sensory deprivation significantly decreased the number of abGCs aged between 15 and 45 days (Petreanu & Alvarez-Buylla, 2002). Sensory deprivation as mentioned earlier affects

abGCs survival if it performed during the first month of cell life but not later on (Yamaguchi & Mori, 2005). Therefore, lack of olfactory stimulation significantly decreases survival of abGCs at a specific age, which may relate with a special period called “critical period” for adult-born neurons. Regarding spine formation, abGCs dendrites at the age of 15 days were found covered with spines suggesting that early maturation of abGCs might not be affected by olfactory deprivation (Ziv & Garner, 2001; Petreanu & Alvarez-Buylla, 2002).

Odor enrichment also exerts its effect within a critical period. Magavi and his colleague tested the odor-triggered response, using c-fos expression (Magavi *et al.*, 2005), the immediate early gene used as an index of neurons responsiveness to the odorant. This study showed that abGCs in the OB exhibit high response to odors in the age of 2 and 3 weeks, whereas their response to odors massively decreased along their further maturation. Using the same technique, Belnoue and his colleague obtained similar results, in which simple olfactory stimulation preferentially recruited the younger adult-born neurons (2 week-old vs. 5 and 9 week-old) (Belnoue *et al.*, 2011).

Critical period of adult-born neurons is also at play in regulating the effects of associative learning. Indeed, learning was found to increase survival of 15 days old cells and to decrease that of 45 days old cells (Mandairon *et al.*, 2006b). This was confirmed in a go/ no-go olfactory task which significantly increased survival of 18-, 23-, and 30-day-old neurons and massively decreased that of 38-days old neurons (Mouret *et al.*, 2008). Moreover, the number of very young neurons (11-day-old) and fully matured neurons (65-day-old) were not affected. From these results, we could infer that olfactory associative learning may distinctively recruit and shape the adult-born neuron depending on their maturation level and their integration status within local circuit. So, the relative immaturity of the adult-born neurons may well be their most important characteristic, providing them with extra plasticity to respond to and to remember new or behaviorally significant signals more efficiently than fully mature cells. Along development, the immature features of these new neurons fade away, and difference with residential and fully mature neurons vanishes (Nissant *et al.*, 2009). Therefore, time does matter regarding the effect of learning on adult-born neurons.

3.4 MORPHOLOGICAL AND SYNAPTIC TURNOVER OF ADULT-BORN NEURONS DURING LEARNING

There is increasing evidence that adult neural circuitry undergoes various kinds of synaptic plasticity depending on experience, involving synapse formation, elimination and stabilization (Holtmaat & Svoboda, 2009). Will such structural plasticity occur in the OB? Will adult-born neurons show specific structural plasticity compared to pre-existing fully mature neurons?

More and more studies bring evidence that adult-born neurons in OB exhibit substantial and specific structural and functional plasticity during their maturation (Belluzzi *et al.*, 2003; Carleton *et al.*, 2003; Kelsch *et al.*, 2008; Livneh *et al.*, 2009; Nissant *et al.*, 2009; Moreno *et al.*, 2012; Lepousez *et al.*, 2014; Daroles *et al.*, 2016).

Associative learning was found to modify the morphology of adult-born neurons (Lepousez *et al.*, 2014). More specifically, spine density of abGCs were increased in basal, proximal (close to the cell body) and distal (along the primary dendrite), domains (located in the granule cell layer), but not in the apical dendritic domain (in the external plexiform layer). It is well known that centrifugal inputs strongly target granule cell layer. The morphological alterations reported on basal dendrites thus suggest that centrifugal inputs participate into spine growth on adult-born neurons. In turn these neurons may become more sensitive the signal from centrifugal regions. It is very likely that interactions between adult-born neurons and centrifugal inputs, and the following morphological alterations play crucial roles in the learning process.

In the study of Lepousez's, the level of synaptic inputs on the adult-born neurons was also explored. They found that density of PSD95-GFP-positive spines was increased by olfactory learning, specifically in distal, proximal and basal dendritic domains. PSD95 is a key marker of excitatory inputs onto adult-born neurons. This result indicates that the level of excitatory inputs onto abGCs was increased by learning. Authors further showed that these excitatory inputs came from the piriform cortex.

Adult-born neurons also receive inhibitory inputs originating principally from second order interneurons of the OB called deep short-axon cells (Eyre *et al.*, 2008). More gephyrin-positive spines were found on and only on proximal dendrites in this study. Gephyrin is a crucial postsynaptic protein for clustering of GABA_A receptors, and is thus a marker of

inhibitory inputs. This increase in inhibitory inputs was confirmed by electrophysiological recording showing higher spontaneous IPSC after learning (Lepousez *et al.*, 2014). This increase in inhibitory inputs might result from potentiation of cortical glutamatergic inputs on deep short-axon cells. A similar mechanism was found in hippocampal interneurons (Campanac *et al.*, 2013). This increased inhibition may help maintaining a balance between excitatory and inhibitory inputs as it occurs in the cortex (House *et al.*, 2011).

Even though the spine density in apical dendrites was not found modified by olfactory associative experience in Lepousez's study, perceptual learning was found to increase spine density on the apical dendrites of abGCs (the basal domain has not been investigated) (Daroles *et al.*, 2016). Morphological changes of abGCs thus seem to be highly dependent on the task as each task may require specific modulations of odorant processing in the OB.

In addition to changes in spine number, recent evidence argued that spine dynamics is involved in olfactory experience-dependent plasticity (Livneh & Mizrahi, 2012). Using two-photon *in vivo* imaging Livneh and his colleagues found that both 2-day-long and 10-day-long odor enrichment decreased the turnover of the postsynaptic marker PSD95-GFP in the dendrites lying in the EPL, which may suggest that odor enrichment tends to stabilize synapse dynamics at least on the apical dendrites located in the EPL. Therefore, we may infer that in the apical dendritic domain, modification of spine dynamics serves as a potential mechanism in olfactory experience-induced plasticity. On the other side, limited by the technique, synapse dynamics in the basal and proximal dendritic domain is beyond the observation and no data from *in vivo* imaging has been gained from these dendritic regions so far.

Several questions remain regarding experience-dependent changes in synaptic inputs onto abGCs. To what extent these changes are specific to adult-born cells or occur also on pre-existing cells is currently unknown. Whether the increase in cortical inputs to abGCs could also be observed for neuromodulatory centrifugal fibers such as noradrenergic, cholinergic or serotonergic inputs, which are also involved in learning is another important unsolved issue.

3.5 DIFFERENTIATION OF ADULT-BORN NEURONS AND OLFACTORY EXPERIENCE

Even though studies yield some debated results on the effects of olfactory learning on adult-born neurons, the data about the influence of experience on neuronal differentiation were quite consistent, showing that neural differentiation of adult-born cells is free from learning or odors exposure influences (Mouret *et al.*, 2008; Mouret *et al.*, 2009a; Veyrac *et al.*, 2009; Belnoue *et al.*, 2011). This suggests that the process of differentiation is programmed when the cells were born. Indeed, the early arborization and synaptic formation during neural development seem to be independent from experience signal transmission (Verhage *et al.*, 2000). Moreover, the geographical origin of these migrating cells in the SVZ seems to determine their differentiation and final settling location in the OB (Figure 3) (Merkle *et al.*, 2007).

3.6 SUMMARY OF DIFFERENCES BETWEEN EARLY-BORN AND ADULT-BORN NEURONS

It is likely that a sub-population of early-born (neonatal) granule cells survive until adulthood (Lemasson *et al.*, 2005). However only around half of adult-born granule cells survive for one month, and only one third remains in the OB one year after cell birth (Petreanu & Alvarez-Buylla, 2002). According to this phenomenon, long-term surviving of early-born neonatal granule cells likely serves to maintain the vital and long-lasting olfactory behavior, while the short-term surviving abGCs may rather provide crucial plasticity to register olfactory information under new or specific external and internal contexts. The regulation of survival and maturation of adult-born neurons by sensory input and olfactory learning during crucial period of their integration supports this theory (Petreanu & Alvarez-Buylla, 2002; Yamaguchi & Mori, 2005; Mouret *et al.*, 2008; Lepousez *et al.*, 2014). This view also emphasizes that the adult OB permanently contains a certain proportion of plastic neurons among more mature neurons. How do these two types of granule cells respond to behavioral process and new environment respectively? Does the learning process shape these different neurons in a different manner? The answers to these questions are not clear yet, but few studies can give us some insights. Optogenetic-activation of adult-born neurons facilitated olfactory discrimination learning, whereas activation of early born neurons failed to improve learning (Alonso *et al.*, 2012). This might suggest that the plasticity induce by olfactory learning was mainly due to the abGCs but not to the early born neurons.

Another distinct difference between early-born neurons and adult-born neurons is their location in the granule cells. Soma of early-born neurons mainly situates in the superficial region, whereas the majority of adult-born neurons in the deep region (Lemasson *et al.*, 2005; Imayoshi *et al.*, 2008). Such location difference may lead to functional differences in responding to olfactory signals between early-born neurons and adult-born neurons. Indeed, bulbar granule cells located in different layer likely have different dendritic distribution, thus results in distinguished synaptic inputs and outputs (Orona *et al.*, 1983; Orona *et al.*, 1984; Carleton *et al.*, 2003). The secondary dendrites of the mitral cells predominantly ramify in deep EPL, while tufted cells mainly ramify in the superficial EPL, therefore, the superficial granule cells (mainly early-born neurons) and superficial granule cells (mainly adult-born neurons) may modulate the outputs of tufted cells and mitral cells through reciprocal dendro-dendritic synapses, respectively (Orona *et al.*, 1983; Orona *et al.*, 1984; Mori, 1987). Since tufted cells and mitral show different response to odors and projection pattern to the olfactory cortices (Nagayama *et al.*, 2004), it is very likely that granule cells in different layer contacting either tufted cells or mitral cells process or project distinct olfactory information. In line with the results mentioned above, electrophysiological study also shows that mature adult-born neurons in deep layer exhibit distinguished electrophysiological property comparing the ones in superficial layer, in which IPSC decay times are slower in the superficial granule cells (Carleton *et al.*, 2003). Furthermore, it seems that only adult-born neurons in the deep layer are sensitive to learning process (Mouret *et al.*, 2009b).

3.7 CONCLUDING REMARKS

The adult brain provides the organism a flexible ability to adapt new environment, as well as stable ability to keep the learned and familiar information. Maintaining these two abilities is a challenge. Learning/storing new information and forgetting the old one are twin functions of a brain. Adult neurogenesis seems to be an exquisite system rendering flexibility to the brain, in which new cells afford an extra and specific plasticity to adjust to changing environment.

Even though many features of bulbar adult-born neurons have been revealed during the passing decade, the functions of adult-born neurons in learning remain unclear. More

specifically, it appears that the mechanisms linking experience to change in adult-born survival and morphology are unknown. Notably, granule cells are targeted by dense from neuromodulatory systems origin and these systems are heavily involved in learning processes. However, very little is known about the innervation by centrifugal adult-born cell fibers and how it evolves with learning.

Our work focused on the Locus Coeruleus noradrenergic system which is heavily involved in olfactory learning a potential regulator of adult-born neurons as presented below.

4. NORADRENERGIC INPUTS TO THE OLFACTORY BULB

Locus Coeruleus (LC) which is located in the pons is the main source of noradrenergic inputs to the cortex (but see also (Robertson *et al.*, 2013) for recent evidence of non-LC noradrenergic neurons projecting to the prefrontal cortex). LC is consisting of ~1,500 neurons in rats (Marien *et al.*, 2004) and sends projections to the cortex and to most brain regions, including the brainstem, the cerebellum, the diencephalon (Jones *et al.*, 1977; Jones & Moore, 1977; Sara, 2009; Schwarz *et al.*, 2015) (Figure. 7). It is noteworthy that the basal ganglia are the main brain regions free of innervations from the LC.

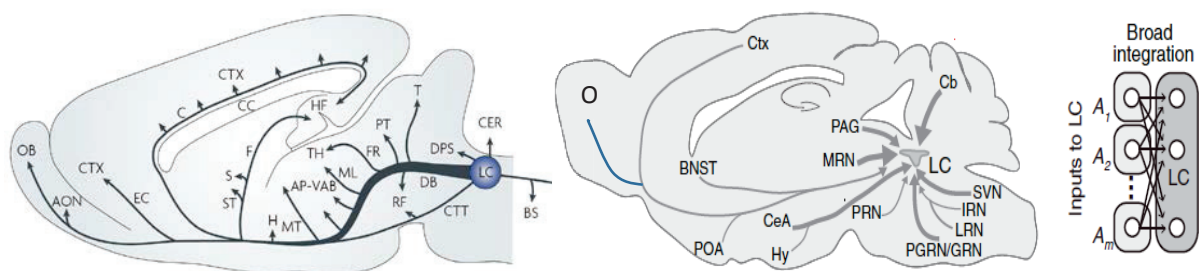


Figure 7. Inputs and outputs of LC-noradrenergic neurons. Left, sagittal schematic rodent brain illustrating the location of the LC in the brainstem (dark blue), providing noradrenergic projections (arrows) throughout the rat brain. Diagram in the middle shows the largest fraction of direct inputs to LC neurons (arrows), determined by trans-synaptic rabies tracing. The thickness of the arrows represents the relative fraction of input neurons contributed by each region. Note that a single LC neuron can send its own terminals to diverse brain regions, and inputs from many brain regions converge onto individual LC- neurons (right). Abbreviations: AON, anterior olfactory nucleus; AP-V AB, ansa peduncularis-ventral amygdaloid bundle system; BS, brainstem nuclei; BNST, bed nucleus of the stria terminalis; C, cingulum; Cb, cerebellum; CC, corpus callosum; CER, cerebellum; CeA, central amygdala; CIT, central tegmental tract; Ctx, cortex; DPS, dorsal periventricular system; DTB, dorsal

catecholamine bundle; EC, external capsule; F, fornix; FR, fasciculus retroflexus; Hi, hippocampus; HF, hippocampal formation; Hy, hypothalamus; IRN, intermediate reticular nucleus; LC, locus coeruleus; LRN, lateral reticular nucleus; ML, medial lemniscus; MRN, midbrain reticular nucleus; MT, mammillothalamic tract; OB, OB; PAG, periaqueductal gray; PGRN/GRN, paragigantocellular/gigantocellular nucleus; POA, preoptic area; PRN, pontine reticular nucleus; PT, pretectal area; RF, reticular formation; S, septal area; SC, spinal cord; SM, stria terminalis; SVN, spinal vestibular nucleus; T, tectum; Th, thalamus (Sara, 2009; Schwarz & Luo, 2015).

Effects of the noradrenergic LC system on brain function and behavior have been largely studied during passed decades through multiple approaches with the help of lesions, pharmacological and transgenic manipulations in targeted brain region or neurons. A founder finding is that tonic LC neurons respond by phasic discharges to new or salient sensory stimuli (Foote *et al.*, 1980; Aston-Jones & Bloom, 1981; Vankov *et al.*, 1995; Bouret & Sara, 2005). Based on this initial discovery, LC has been proposed to play a major role in arousal (Berridge & Waterhouse, 2003; Sara & Bouret, 2012), and from there in memory consolidation (McGaugh & Roozendaal, 2009; McIntyre *et al.*, 2012), working memory (Arnsten *et al.*, 2012) and more generally in cognition (Sara, 2009). Importantly, The LC not only sends axons to the whole cortical sheet, but it receives reciprocal inputs from the regions it innervates. As such, it is a highly integrative structure able to modulate brain activity and dynamics, setting the functional state of the brain according to environmental or internal cues (Bouret & Sara, 2005). Accordingly, LC neurons fire tonically when animals are awake, while low firing rates are detected when they are asleep or during low vigilance behavioral states like grooming (McLean *et al.*, 1989). In behaving animals, LC neurons exhibit pronounced responses to non-noxious environmental stimuli.

The cellular effects of noradrenaline (NA) include modulation of neuronal activity and synaptic plasticity through the action of NA on a diversity of noradrenergic receptors (Schwarz & Luo, 2015).

About 40% of LC neurons are reputed to project to the OB (ShIPLEY *et al.*, 1985). This massive LC innervation of the OB is not the sole centrifugal inputs to the OB. The next part will be focused on the crucial roles these centrifugal projective systems play in olfaction and olfactory learning with a focus on the LC system.

4.1 DISTRIBUTION OF NORADRENERGIC FIBERS AND RECEPTORS IN THE OB

OB receives noradrenergic inputs from the LC, which are mainly targeting the granule cell layers, with lower density of NA axon terminals in mitral cell, glomerular, and external plexiform layers (Shiple *et al.*, 1985; McLean *et al.*, 1989) (Figure 8). In accordance with the wide innervation of NA fibers in the OB, NA receptors exist in multiple OB layers and are expressed by mitral cells, granule cells and periglomerular cells and each cell type seems to be expressing multiple receptors.

There are four major types of NA receptors, namely $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ receptors, in the OB, and different distributions of these receptors were revealed in post-natal animals. Binding studies revealed highest density of $\alpha 1$ receptors in the external plexiform layer than in deeper bulbar layers (Young & Kuhar, 1980). It is interesting that taking the whole brain into account, external plexiform layer in the bulb is found to be one of the regions exhibiting the highest level of $\alpha 1$ receptors. The densest distribution of $\alpha 2$ receptors is in the granule cell layer, whereas $\alpha 2$ receptors are sparse in the external plexiform layer and scarce in the glomerular layer (Holmberg *et al.*, 2003). $\beta 1$ adrenoceptors are primarily expressed in the mitral cell layer, with faint expression in the granule cell and periglomerular layers (Yuan *et al.*, 2003). $\beta 2$ adrenoceptors seem to be localized more widely. Study of *in situ* hybridization revealed that $\beta 2$ adrenoceptors are well expressed in the granule cell and glomerular layers (Nicholas *et al.*, 1993). However, another study using receptor autoradiography (Woo & Leon, 1995) suggested that $\beta 1$ and $\beta 2$ adrenoceptors might be present throughout the whole OB, while periventricular core and external plexiform layer contain relatively rare $\beta 1$ receptors, and mitral cell layer had low density of $\beta 2$ receptors. These partly inconsistent studies reveal that until now, the distribution of adrenoceptors in the OB is not known precisely but it seems that all receptors subtypes are more or less represented in each layer of the OB.

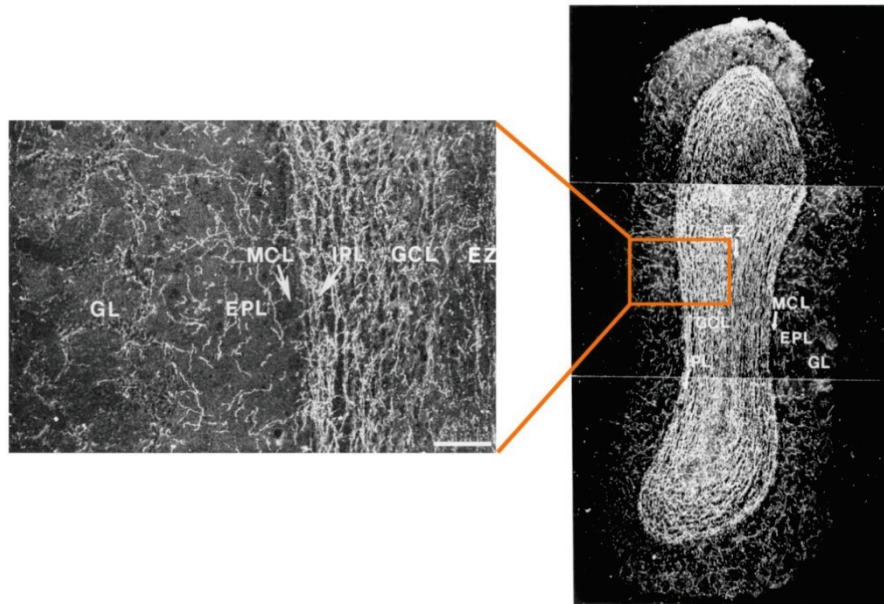


Figure 8. Heterogeneous distribution of noradrenergic fibers in the OB. Images show dopamine beta-hydroxylase (DBH)-immunoreactive (IR) fibers in the main OB. Noradrenergic terminals densely project in the OB, and preferentially in the internal plexiform and granule cell layers, with lower density in mitral cells layer and periglomerular layer, and external plexiform layer. Abbreviations: EPL, external plexiform layer; EZ, subependymal zone; GCL, granule cell layer; GC, glomerular layer; IPL, internal plexiform layer; MCL, mitral cell layer (McLean *et al.*, 1989).

4.2 EFFECTS OF NORADRENALINE ON OLFACTORY PERCEPTION IN ADULT

4.2.1 ODOR DETECTION

LC neurons discharge when novel or significant sensory stimuli are detected (Foote *et al.*, 1980; Vankov *et al.*, 1995), and NA system plays a crucial role in the regulation of vigilance (Aston-Jones *et al.*, 1999; Berridge & Waterhouse, 2003). Therefore, NA system is believed to be critical to odor detection. Injection of NA in the OB dramatically decreases the threshold of odor detection (Escanilla *et al.*, 2010). Activation of α_1 receptor seems to be crucial for detection, as blocking α_1 receptor alone impaired odor detection, while detection still remains when α_2 and β are blocked separately. Moreover, NA-induced improvement of detection is maintained when OB receives mixed injection of β and α_2 blocker, while either α_1/β blockade or α_1/α_2 blockade will fail to improve odor detection (Escanilla *et al.*, 2010), further confirming the key role of α_1 receptor in odor detection.

4.2.2 SPONTANEOUS DISCRIMINATION

Spontaneous odor discrimination is highly related with activation of noradrenergic receptors (Mandairon *et al.*, 2008). Interestingly, blockade of α or β receptors elicits opposite behavioral outputs. Blocking α_1 receptors (but not α_2 receptors) impairs spontaneous odorant discrimination, whereas blocking β receptors enhances discrimination. These effects are dependent on the dosage of noradrenergic receptor antagonist infused in the OB. Low but not high dose of β receptor antagonist enhances discriminative ability, whereas both low and high doses of α_1 receptor antagonist block discrimination, high dose inducing stronger effect.

4.2.3 ASSOCIATIVE LEARNING

The general feature of LC neuron discharge in response to odorants and how they evolve with the behavioral context are well illustrated by a series of experiences done using an olfaction-related Go-No go learning task. In this task, a reinforced odor (odor associated to a reward, CS+ odor) elicits a burst of LC neuron firing (Figure. 9). It is noteworthy that LC neuron activity seems to be more aligned with the behavioral response (nose poke to rewarded port, Go response) than with the odor delivery (Bouret & Sara, 2005). Moreover, it is the behavioral response, but not motor behavior (behaviors such as grooming and promenade in the experimental apparatus) that triggers the LC neuron activation because a non-rewarded odor (CS-) delivery does not have such an effect on LC neurons. However, the interpretation of phasic activity of LC neuron during this behavior needs more investigation. It could be interpreted as recognition of CS+ odor, reward expectation, or decision making (Doucette *et al.*, 2011).

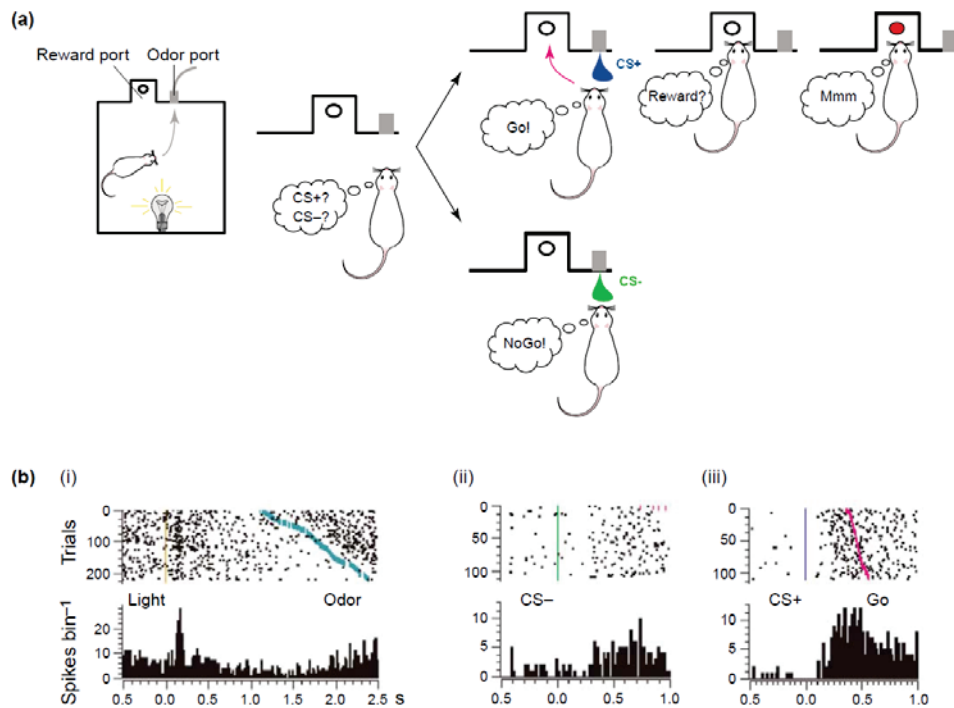


Figure 9. Schema of Go-No go task and LC neuron activities in the task. (a) Schematic representation of the Go-No go task. Before the task and during the interval between trials, the light is off. Each trial is initiated by light onset, which triggers the attention of the animal and leads the animal to move to the odor port. Shortly, a stream of air with either CS+ (blue, top) or CS- (green, bottom) odor is released through the odor port. In the Go trials (top), when the CS+ odor is given, the well-trained animal quickly heads to the reward port (Go response), waiting for the reward (red circle). In the No-go trials (bottom), when the CS- odor is given, the animal refrains from going to the rewarded port (No go response). (b) Raster (top) and peristimulus time histogram (PSTH) (bottom) displays of activity of a single LC neuron during the task. In the raster display, each dot represents an action potential (spike) and each line represents a trial showing the distribution of spikes over time with presence of light and odor. In the PSTH displays, each column (bin) represents a cumulative count of spikes of all the trials during 50 ms (bin size, 50 ms). In (i), yellow line represents the light onset and blue short lines represent the odor delivery. In the raster displays, the intervals between light onset and odor delivery are increased along trials. Light onset triggers a rapid and phasic activation of LC neuron, followed by a long lasting inhibition until odors delivery. If CS- odor is released (ii, green line), activity of LC neuron returns back to the baseline within a short latency (proximately 300 ms). If CS+ odor (iii, purple line) is released, the firing rate of LC neuron is massively increased. It is noteworthy that LC neuron activity is more tightly related to behavioral action (nose poke to reward port, red line in raster displays of iii) than the odor stimulus (Bouret & Sara, 2005).

Another interesting phenomenon about LC activation is the timing shift of LC neurons activity during associative learning (Figure. 10). In the beginning (early trials) of an olfactory associative learning, increased firing rate of LC neurons is observed after reward delivery. However, after the animal learned the association, the firing burst of LC neurons occurs immediately before the behavioral response (nose poke to the reward port, Go response) (Bouret & Sara, 2005).

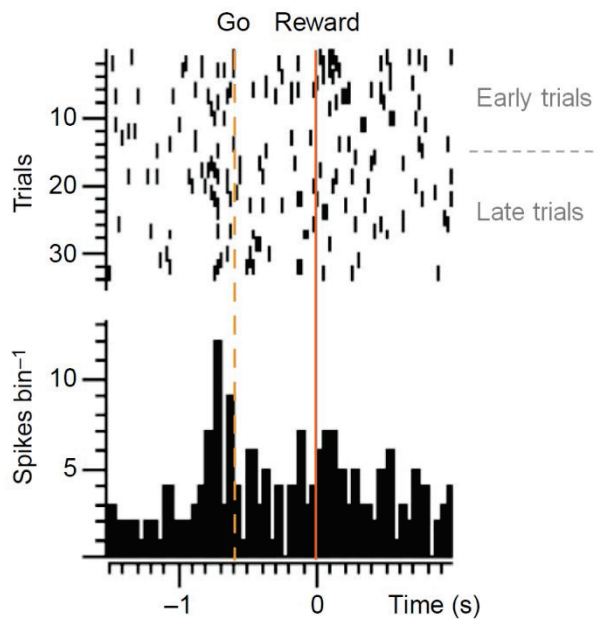


Figure 10. Time shift of LC neuron response during a Go-No go training task. Raster and (top) and peristimulus time histogram (PSTH) (bottom) displays of activity of a single LC neuron after delivery of a rewarded odor. In the raster displays (top), each dot represents a spike and each line represents a trial. Yellow dotted line indicates the average time to visit reward port (Go response), and the yellow solid line indicates the reward delivery, which is defined as time 0. In the early trials (first 15 trials), the animal does not expect the reward, and increased firing rate of LC neuron occurs when the animal starts to consume the reward. In the late trials (from 15th trial), the animal has learned the association between the odor and reward, and increased activity of LC neuron occurs immediately before the behavioral response. (Bouret & Sara, 2005)

In addition to the Go/No Go task presented above, several other models have been used to investigate the role of NA in olfactory associative learning.

Actions of NA in the OB are crucial for ewe to learn and recognize the odor of its own lamb from an alien lamb (Levy *et al.*, 1990). In a standard associative task in rodent, presentation of conditioned odor (associated with a food reward), but not the non-conditioned odor, results in a significant increase in NA release in the OB (Brennan *et al.*, 1990) and modulations in γ frequency (40-100 Hz) oscillations in the local field potential (Gray *et al.*, 1986). This oscillatory modulation is believed to be crucial for olfactory learning (Martin *et al.*, 2006), and was abolished by blocking β noradrenergic receptor. In line with this, synchronization of mitral cells has also been shown to be different in response to rewarded versus non rewarded odors, differences that were reduced under noradrenergic blockade (Doucette *et al.*, 2011).

Blocking β or α noradrenergic receptor alone does not affect associative olfactory discrimination learning, whereas bulbar injection of both β and α receptor antagonists blocks discrimination learning between similar odors (Doucette *et al.*, 2007). It is noteworthy that blockade of both β and α receptors in adult animals does not impair the discrimination learning between dissimilar odors (Doucette *et al.*, 2007). Moreover, in adult animals, blockade of both β and α receptors only before the test, but not during training, does not

affect the expression of discrimination learning, which is in accordance with the finding in neonates (Sullivan & Wilson, 1991a; b). These pharmacological results are consistent with findings of *in vivo* electrophysiological recording on LC neurons: detection of rewarded odor, but not of the unrewarded odor, generates a phasic increase in LC neuron firing rate (Bouret & Sara, 2004).

Additional argument in favor of a role for NA in the build-up of the behavioral significance of an odor came from an intriguing study in anesthetized animals. Indeed, in ketamine/xylazine-anesthetized mice, pairing between LC neuron activation and odor presentation produces a specific long-lasting suppression of responses of mitral cells to the paired odor, but not to unpaired odors (Shea *et al.*, 2008). Moreover, LC activation paired to odors in anesthetized mice produces behavioral changes resembling habituation, which last at least 24 hours after pairing (Shea *et al.*, 2008). This may indicate that LC stimulation paired to odor presentation is sufficient to trigger gene expression and/or morphological restructuring which underlie formation and maintenance of odor memory (Reymann & Frey, 2007).

Shea and his Colleague found that spontaneous activity of mitral cell is not altered by LC neuron activation (Shea *et al.*, 2008), which is different than another previous finding that activation of LC could transiently but robustly decrease spontaneous discharge of mitral cells (Jiang *et al.*, 1996). These conflicting results may result from the different protocols used to stimulate the LC (electrical versus chemical stimulation) and to stimulate the olfactory system (odor versus electrical activation of the olfactory epithelium). The anesthesia may also be a possible factor affecting the results, since activity of mitral cell is highly related to anesthetics used. In addition, as described below, the cellular effects of NA are complex and dose-dependent, making very difficult the emergence of a simple and clear-cut effect of NA on mitral cells, as well as on other OB cell types.

4.2.4 PERCEPTUAL LEARNING

The LC system is highly involved in olfactory perceptual learning. Labetalol (α_1 and β receptor antagonist) administration during olfactory perceptual learning (enrichment with a perceptually similar odorant pair) massively impairs acquisition of learned odor discrimination (Moreno *et al.*, 2012). Accordingly, the daily administration of dexefaroxan,

an $\alpha 2$ presynaptic inhibitory receptor antagonist which increases NA release in the OB (Veyrac *et al.*, 2005) for 10 days without odor enrichment, can enhance discriminative ability between perceptually similar odorants and thus mimic the effect of olfactory enrichment (Moreno *et al.*, 2012). Interestingly, both enrichment and dexefaroxan treatments are accompanied by increased survival of abGCs and their increased responsiveness to learned odorants (Moreno *et al.*, 2012). These results indicate the crucial role of NA in this learning process.

As additional evidence of noradrenergic involvement in perceptual learning, expression of dopamine β -hydroxylase (DBH, NA synthetic enzyme) in the LC was found to be increased after perceptual learning (Moreno *et al.*, 2012). Odor-induced activation of the LC system was shown in several other studies, with increased DBH expression in LC (Rey *et al.*, 2012) or enhanced release of NA in the OB (Brennan *et al.*, 1990; Veyrac *et al.*, 2009).

Importantly, the learning-like effect of $\alpha 2$ receptor blockade on behavior can be inhibited by pharmacologically blocking neurogenesis, indicating that $\alpha 2$ receptor blockade induced enhancement of fine discrimination through action on adult-born neurons (Moreno *et al.*, 2012).

4.2.5 SHORT TERM MEMORY

LC system in the OB plays complex roles in short term memory as emerged from studies of odor habituation. A pharmacological study using intra bulbar infusions of noradrenergic drugs showed that blocking the main noradrenergic receptors did not affect habituation (same odor presented repeatedly with short 5-min intervals) (Mandairon *et al.*, 2008). However, pharmacological depletion of NA by the specific noradrenergic neurotoxin N-Ethyl-N-(2-chloroethyl)-2-bromobenzylamine hydrochloride (DSP4), blocked odor habituation (Guerin *et al.*, 2008). NA infusion in the OB restored habituation. These opposite behavioral results may arise from difference in the level of pharmacological depletion, DSP-4 depleting up to half of the NA fibers while the actual level of receptors blockade in pharmacological experiments is difficult to assess. The effect of NA system on short-term odor memory was confirmed in an odor recognition test, similar to the classical object recognition task. In this task, dexefaroxan ($\alpha 2$ presynaptic inhibitory receptor antagonist) can prolong odor retention (from 15 to 30 min in this paradigm), accompanied by enhanced level of normetanephrine

(extracellular metabolite of NA, index of NA release) (Veyrac *et al.*, 2007). Moreover, in the same study, mice treated by with propranolol (β -adrenoceptor antagonist) or prazosin (α 1-adrenoceptor antagonist) showed impaired short-term odor memory.

Taking all these results together, NA activity in the OB does affect short-term odor memory but probably in a very much dose-dependent manner.

4.3 EFFECTS OF NORADRENALINE IN NEONATES

NA release and activation of NA receptors in the OB has been believed to play crucial role in olfactory learning during sensitive periods in neonatal animals (Rangel & Leon, 1995). In the neonatal rats (the first postnatal week), β -receptor activation in the OB paired with odor produces a subsequent preference to the paired odor (Sullivan *et al.*, 1989; Sullivan & Wilson, 1994; Sullivan *et al.*, 2000; Harley *et al.*, 2006). Isoproterenol, used as a β -receptor agonist in this experiment, displays a dose-dependent effect on the odor preference showing an inverted-U curve (Sullivan *et al.*, 1989; Sullivan & Wilson, 1991b).

Interestingly, dose-dependent curve of isoproterenol can be shifted by manipulations of 5-HT activity in the OB. Lesions of 5-HT innervation and pharmacological activation of 5-HT system in the OB shifts the effect curve to the right and left respectively (Langdon *et al.*, 1997; Price *et al.*, 1998), even though activation of 5-HT(2A/2C) receptor alone is not sufficient to induce odor-related learning (Price *et al.*, 1998). Interestingly, β -receptor antagonist treatment does not affect the expression of learned olfactory preference (Sullivan & Wilson, 1991b). Furthermore, odor preference learning in the neonatal can be facilitated by infusing acetylcholine in the LC, which will strongly activate LC neurons, resulting in NA release in the OB (Moriceau & Sullivan, 2004). Putative LC stimulation (cholinergic stimulation in the LC) in rat pups paired with odor exposure could produce a learned-approach response, whereas this effect could be blocked by a pre-training injection of the β -receptor antagonist (Sullivan *et al.*, 1989). However, the effect of LC activation by cholinergic stimulation in the LC on odor-preference learning only exists during very early neonatal age (postnatal ~9 days), which is defined as sensitive period of rapid learning. After this sensitive period, LC activation could no longer induce odor-preference learning (Moriceau & Sullivan, 2004). This phenomenon seems to be caused by developmental changes in LC autoreceptors (both α 1

and $\alpha 2$ autoreceptors) functional changes rather than OB changes (Moriceau & Sullivan, 2004).

4.4 CELLULAR EFFECTS OF NORADRENALINE

4.4.1 BI-DIRECTIONAL EFFECT OF NA ON GRANULE CELLS

NA actions on granule cells in the OB are highly dependent on the concentration of NA (Nai *et al.*, 2010). Low and high levels of NA lead completely opposite effects on the granule cells by activating different types of noradrenergic receptors. Low concentrations of NA hyperpolarize granule cells through activating $\alpha 2$ receptors. In contrast, relatively high concentrations of NA depolarize granule cells through acting on $\alpha 1$ receptors. Dominant activation of specific receptor at different concentrations of NA is likely due to their differential affinity to NA and their distribution in the OB. $\alpha 2$ receptors show highest affinity to NA ($\alpha 2 > \alpha 1 > \beta$) (Mandairon *et al.*, 2008), therefore low levels of NA mainly activate $\alpha 2$ receptors, followed by an inhibition of granule cells. However, dense distribution of $\alpha 1$ receptors than $\alpha 2$ receptors, leads to a dominant activation of $\alpha 1$ receptors at higher dose, followed by excitation of granule cells (Figure 11).

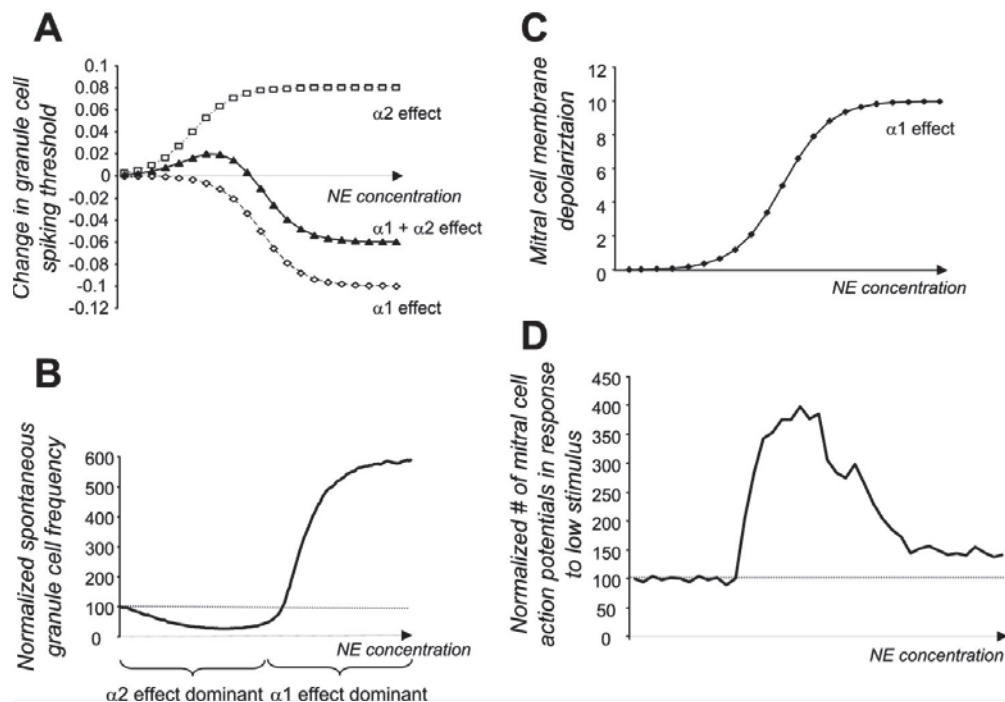


Figure 11. Effects of noradrenaline (NA) modulation on cellular parameters. *A:* NA modulates granule cell spontaneous firing (Nai et al. 2010). The measured effects of NA activation of α 1- and α 2-receptors on granule cell-mediated spontaneous inhibitory postsynaptic currents (IPSCs) was simulated by changes in granule cell firing thresholds. α 1- and α 2-receptor activation modulates spontaneous IPSCs in opposite directions, each with their own concentration dependency. The summated effect is a non-monotonic modulation of firing threshold. *B:* effect of threshold modulation on granule cell spontaneous firing. The size of the effect was adjusted to data reported by Ennis and colleagues (Nai et al. 2009, 2010). *C:* modulation of membrane potential by NA in model mitral cells. Based on data by Hayar et al. (2001). In the model, spontaneous activity was not affected by this effect, but responses to low-threshold stimuli were modulated. *D:* modulation of response probability to low-threshold stimuli in mitral cells. Both modulation of inhibition by α 1- and α 2 effects on granule cell firing and direct modulation of mitral cell membrane potential were taken into account. Mitral cell responses to low-amplitude stimuli were modulated in a non-monotonic manner by simulated NE due to the additive effects of NA receptor activation (Linster *et al.*, 2011).

4.4.2 TRI-PHASIC INHIBITORY EFFECT OF NA ON MITRAL CELLS

NA exhibits a dose-dependent tri-phasic effect of on both spontaneous and miniature IPSCs (sIPSCs and mIPSCs) frequency of mitral cells as shown *in vitro* (Nai *et al.*, 2009) (Figure 12). Specifically, low concentrations of NA reduce both sIPSCs and mIPSCs frequency, whereas intermediate concentrations massively enhance the frequency of IPSCs and a relative suppression of IPSCs is seen at high concentrations of NA. This tri-phasic effect of NA highly relates to affinity difference among receptors subtypes and distinct functions of these receptors on granule cells and mitral cells.

-Alpha2 receptor activation disinhibits mitral cells at low concentration of NA

Low doses of NA suppress IPSCs of mitral cells. This excitatory effect is mediated by α 2 receptors activation since it is present when α 1 and β receptors are blocked and α 2 receptor agonist alone mimic the effect of the low doses of NA (Nai *et al.*, 2009). Due to higher affinity for NA, α 2 disinhibiting effect dominates at low doses of NA.

-Alpha1 receptor activation increases inhibition on mitral cells at medium to high NA concentration

The enhancement of IPSCs at intermediate doses of NA is mainly induced by activation of α 1 and β receptors. At high dose, it is mediated by activation of α 1 receptors since β receptor agonist has no effect on mIPSCs.

-Beta receptor activation inhibits mitral cells through an indirect mechanism

β receptor agonists increase spontaneous IPSCs of mitral cells but not if spiking is blocked by TTX (Nai *et al.*, 2009), indicating an indirect effect. β receptors are well expressed in mitral

cells, NA may thus activate mitral cells which release glutamate onto granule cells. Granule cells in turn inhibit Mitral cell by releasing GABA, allowing an enhanced GABAergic inhibition onto mitral cells.

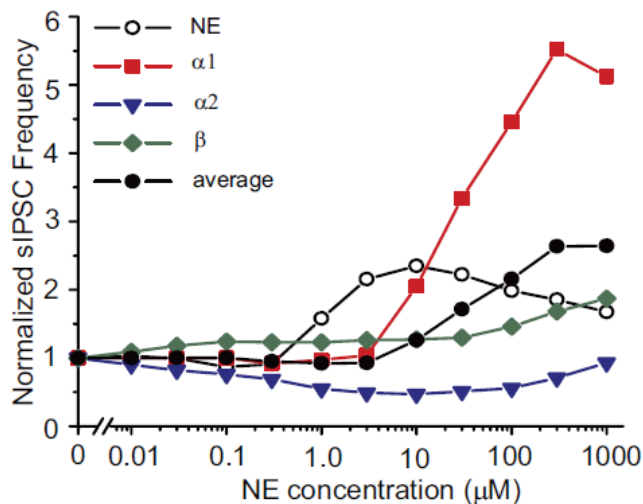


Figure 12. A summary plot of dose-dependent effects of NA on mitral cell sIPSCs. Recording done in normal artificial cerebrospinal fluid (ACSF) with or without presence of noradrenergic antagonists. Each line represents effect of NA alone or single subtype of noradrenergic receptor activation on sIPSCs of mitral cell, and average effect of NA is showed by averaging effects of three individual noradrenergic receptor activation (Nai *et al.*, 2009)

In other words, intermediate concentrations of NA first excite mitral cells through direct action on their own α_1 receptors. Then, activated mitral cells release glutamate on granule cells. Activated granule cells in turn inhibit mitral cells through GABAergic release. This sequence may explain the positive effect of NA on detection and discrimination: activation of mitral cells may render them more sensitive to weak odors by decreasing their activation threshold and then, mitral cell inhibition may facilitate the precision of odor coding. Another non-exclusive possibility is that NA-induced increased excitation and then inhibition of mitral cells may contribute to increasing oscillatory synchronization in the mitral/granule cell network, therefore enhancing the contrast and precision of the output from OB (Linster & Cleland, 2001; Cleland & Linster, 2002). This oscillatory synchronization theory is partially supported by a study which indicated that NA application induced a long-term increase in gamma frequency (30–70 Hz) synchronized oscillations (Gire & Schoppa, 2008).

5. CHOLINERGIC INPUTS TO THE OLFACTORY BULB

Beside the LC, the OB receives centrifugal inputs from many brain regions, including cortical regions and sub-cortical regions among which the neuromodulatory cholinergic nuclei of the

horizontal limb of the diagonal band of Broca and serotonergic raphe nuclei (Shipley & Adamek, 1984; Kunze *et al.*, 1992; Dugue & Mainen, 2009). Although these are important for olfaction and olfactory learning, detailed description of them is somewhat out of the focus of the present thesis. However, since we have obtained some preliminary data regarding the cholinergic system, a brief up-date on cholinergic inputs to the OB is provided.

Cholinergic fibers from the horizontal limb of the diagonal band of Broca target neurons throughout the OB, and are predominantly distributed in the granule cell layer and glomerular layer, and a less extent in external plexiform layer (Heimer *et al.*, 1990; D'Souza & Vijayaraghavan, 2014). Via acting on multiple receptors and different cell types, acetylcholine contributes to various effects on the olfactory function. It decreases excitability of granule cells through acting on muscarinic receptor and enhancing GABA release. On the contrary, periglomerular neurons and mitral cells are excited when their nicotinic receptors activated by acetylcholine (Castillo *et al.*, 1999). At the behavioral level, cholinergic manipulations alter many olfactory performances, like perceptual learning (Fletcher & Wilson, 2002), olfactory discrimination and short-term memory (Roman *et al.*, 1993; Ravel *et al.*, 1994). What's more, several evidences are found in favor of the regulation by the cholinergic system of adult neurogenesis in the OB. Mice lacking $\beta 2$, which is the main subunit of nicotinic acetylcholine receptors, exhibit nearly 50% more newborn neurons and significantly fewer apoptotic cells than wide type mice (Mechawar *et al.*, 2004). Conversely, chronic nicotine administration in subcutaneous manner significantly decreased the number of abGCs in wild-type mice. Of particular interest, intraperitoneal administrations of donepezil, which is a selective acetylcholinesterase inhibitor and is used for therapy in Alzheimer's disease, profoundly increase the survival of newborn neurons, but not proliferation of neural progenitor cells in SVZ (Kaneko *et al.*, 2006), while cholinergic forebrain lesion in the adult rat impairs neurogenesis in the OB (Cooper-Kuhn *et al.*, 2004).

These results led to the hypothesis that activation of different subtypes of acetylcholine receptors may trigger diverse modifications on adult-born neurons. Although little is known about how cholinergic inputs regulate neurogenesis, formation of synapses between cholinergic fibers and new-born neurons during the very early period after cell birth provides encouraging evidences that cholinergic inputs have the potential to control survival and maturation through synaptic communication (Whitman & Greer, 2007b; a).

6. INTERACTIONS BETWEEN LC CENTRIFUGAL INPUTS AND ADULT-BORN NEURONS

On one hand, olfactory learning induces changes in the rate of neurogenesis that are clearly related to the learning process (Moreno *et al.*, 2009; Mouret *et al.*, 2009a; Sultan *et al.*, 2010; Mandairon *et al.*, 2011; Alonso *et al.*, 2012). More recently, learning was also shown to induce structural remodeling of adult-born neurons (Lepousez *et al.*, 2014; Daroles *et al.*, 2016). In the other hand, centrifugal inputs and among them noradrenergic inputs exert a top-down modulation on the OB which is essential to learning (Moreno *et al.*, 2012). Since granular interneurons, which are the main targets of neurogenesis in the OB, receive dense neuromodulatory projections, it can be hypothesized that neurogenesis-associated plasticity may be regulated by feedback neuromodulatory activities. This is reinforced by the positive action of NA newborn neuron survival (Bauer *et al.*, 2003; Veyrac *et al.*, 2007). In addition, antidepressants among which some of them target noradrenergic transmission stimulate neurogenesis and these changes in neurogenesis may support at least some of the beneficial cognitive and emotional effects of anti-depressants (Miller & Hen, 2015)

The model of perceptual learning shows clear evidence for NA regulation of adult-born neurogenesis has been obtained. Daily blockade of $\alpha 2$ presynaptic receptor (enhancing NA release) for a certain period can mimic the effect of perceptual learning, inducing enhanced discriminative ability between a pair of very similar odorants, accompanied by increase in abGCs and of their responsiveness to the learned odors (Moreno *et al.*, 2012; Vinera *et al.*, 2015). Ablation of neurogenesis can completely suppress the beneficial effect of $\alpha 2$ receptor blockade on discrimination learning (Moreno *et al.*, 2012). Adult-born neurons and NA thus interact in perceptual learning to allow the improvement in discrimination. Electrophysiological study with adult born neurons at age of 12-18 days showed that NA significantly alters the membrane resistance (Moreno *et al.*, 2012), indicating that these young adult-born neurons are actually sensitive to NA.

The enhanced neural plasticity brought by adult-born neurons combined to the synaptic inputs they get from centrifugal system, and particularly from the LC system, likely provides a sensitive mean to integrate the sensory environment and internal information such as

sleep/arousal, anxiety/stress, attention, and motivation (Lepousez *et al.*, 2015). These internal contextual states might modify the proliferation, survival, development, and integration of adult-born neurons into neural network, thus supporting an impressive level of metaplasticity responding to internal and external information.

In conclusion, interactions between adult born neurons and centrifugal NA inputs likely play crucial roles in olfactory behavior. However, the substratum for these interactions is poorly investigated. In this thesis, the experiments we have performed unmask at least partly the neural basis of the modulation by noradrenergic inputs of abGCs integration during perceptual learning.

7. OBJECTIVES OF THE THESIS

Mammalian OB receives continuous influx of new cells every day during adulthood (Altman, 1969). These newly generated cells differentiate into interneurons and integrate into the local circuitry, providing irreplaceable flexibility in coding olfactory information (Lledo *et al.*, 2006). It has been believed that these adult-born neurons are highly involved in the olfactory learning and memory (Mouret *et al.*, 2008; Breton-Provencher *et al.*, 2009; Sultan *et al.*, 2010; Mandairon *et al.*, 2011; Peretto *et al.*, 2014), especially in hard tasks related with discrimination among perceptually similar odorants. Furthermore, specific plasticity of adult-born neurons during maturation allows them to easily adjust to the inputs from local circuitry, resulting in mega-plasticity of the OB to adapt to new environment and tasks (Lledo *et al.*, 2006; Nissant *et al.*, 2009). The main inputs that the abGCs receive are from mitral cells and centrifugal afferents. Among various centrifugal inputs in the OB, noradrenergic inputs from the LC play distinctive roles on regulating bulbar signal processing (Nai *et al.*, 2009; Nai *et al.*, 2010). Noradrenergic terminals are densely distributed in the OB, especially in the granule cell layer and internal plexiform layer (Shipley *et al.*, 1985; McLean *et al.*, 1989), where soma and basal dendrites of granule cells are lying. LC system plays crucial roles in many behaviors, including arousal (Berridge & Waterhouse, 2003), memory consolidation (McGaugh & Roozendaal, 2009; McIntyre *et al.*, 2012) and working memory (Arnsten *et al.*, 2012) etc. Without exception, as a main target of the LC, OB receives high modulation from this system, resulting in altered behavioral performance by manipulating it (Sullivan *et al.*, 1989; Sullivan & Wilson, 1994; Sullivan *et al.*, 2000; Harley *et al.*, 2006; Escanilla *et al.*, 2010; Devore & Linster, 2012; Moreno *et al.*, 2012). Furthermore, a previous study in our lab revealed that NA was required for olfactory perceptual learning, and intriguingly NA action in this learning is through adult-born neurons (Moreno *et al.*, 2012).

However, many questions remain regarding the interaction between NA and adult-born neurons under perceptual learning. How does the LC system interact with the maturation and integration of adult-born neurons during learning, and what is the time course of these interactions? Therefore, the work presented in thesis is based on is using the model of olfactory perceptual learning to unmasking the interactions

between the LC system and adult-born neurons that are underlying perceptual learning. The questions asked in this thesis, hypothesis made and tools used are as followed:

1. What is the time course of the innervation of adult-born neurons by LC inputs? To answer this question, we injected lentiviruses tagged with GFP (green fluorescent protein) in the SVZ to label adult-born cell precursors which will migrate into the OB, and then analyzed the contacts established between LC noradrenergic fibers; identified thanks to the expression of norepinephrine transporter (NET) and adult-born neurons. With 3-dimension views using ImageJ software, the number of appositions between NET-positive fibers and GFP-positive neurons was recorded. An apposition is recognized when GFP and NET signals are close and proximity is confirmed by different views by rotating the neurons in ImageJ. Moreover, three time points were used to detect the development of noradrenergic inputs on maturing adult-born neurons, aged 8, 18 and 38 d. To confirm if the contacts between GFP and NET signals are actual synaptic inputs onto adult-born neurons, a presynaptic marker, synaptophysin, was used in immunohistochemical co-labeling with NET and GFP.

2. Since the adult-born neurons undergo morphological maturation after arriving in the OB, we then question if the development of noradrenergic inputs on adult-born neurons was accompanied by changes of spine density in these new neurons. The software NeuronStudio was used to generate 3-dimension images of adult-born neurons, allowing a precise analyze of spine density. Spine density of basal and apical dendritic domains was analyzed for the 3 time points, 8, 18 and 38 days post viral injection. Moreover, the spines were divided into 4 subtypes according to their appearance (Yuste & Bonhoeffer, 2004; Tronel *et al.*, 2010). The density and proportion of each type of spine were also analyzed (however, the data obtained are too preliminary to be presented in the frame of this thesis).

3. After establishment of the developmental pattern of LC innervation onto adult-born neurons, then we asked if learning affected this developmental process. The learning model we chose is that of perceptual learning, which is dependent on both adult-born neurons and NA. Perceptual learning is defined as an improvement of discrimination between a pair of perceptually similar odorants after repetitive exposure to these odorants for a certain period, which is called enrichment. We

chose (+)Limonene and (-)Limonene as enriched odors. Enrichment began 8 days after virus injection, the time allowing adult-born cells labelled by the virus injection to reach the OB and start to integrate the local circuitry (Petreanu & Alvarez-Buylla, 2002; Carleton *et al.*, 2003; Lledo *et al.*, 2006; Moreno *et al.*, 2009). The enrichment lasts for 10 days, and after behavioral assessment of discrimination abilities using a habituation/ dishabituation task (Moreno *et al.*, 2009; Moreno *et al.*, 2012), mice were sacrificed 18 days (D18) or 38 days (D38) after the first enrichment day respectively. Note that at these two time points, D18 and D38, adult-born neurons were 25 and 45 days old respectively. Both noradrenergic inputs (contacts between NET-positive and GFP-positives profiles) and spine density of adult-born neurons were analyzed using the same protocol and criteria as described above.

4. It has been revealed that adult-born neurons show specific functional plasticity and response patterns compared to the pre-existing neurons (Magavi *et al.*, 2005; Nissant *et al.*, 2009; Alonso *et al.*, 2012). Therefore, we asked question if the effect of learning on LC fibers contacts and spine density was specific to adult-born neurons. To label neurons formed during ontogenesis and compare their innervation pattern and structural plasticity to that of adult born neurons, neonatal mice were injected with lentiviruses tagged by GFP, in the ventricle immediately after they were born (P0 mice). After they grew up (2 months old), these P0 mice were tested in the same behavioral protocols and cellular analysis was performed as in the mice injected during adulthood.

5. Pharmacological blockade of the NA system during enrichment impairs the learning process (Moreno *et al.*, 2012). This suggests that NA is important to install the learning-dependent plasticity. However, if the function of the NA system is impaired during testing, specifically when the mice are sniffing, will the learned discrimination be blocked? To test this question, we used optogenetic manipulation. The mice received injection of lentivirus tagged with Halorhodopsin (light-gated chloride ion channel) in the LC and optic fibers implantation in the OB. Thus, when the Halorhodopsin was well expressed in the noradrenergic terminals in the OB (two months after virus injection), we could temporally reduce noradrenaline release in the OB. The optogenetic manipulation was only conducted when mice are exploring the odorant and only during the test trial in the habituation/ dishabituation task. The

cellular effect of light stimulation was investigated by immunohistochemistry of the expression of immediate early gene *zif268* and BrdU (injected 8 days before the enrichment).

6. After all, since cholinergic system is another important neuromodulatory system strongly regulating learning processes (Linster *et al.*, 2011), we looked at the developmental pattern of cholinergic inputs onto adult-born neurons. In preliminary experiments, the contacts between cholinergic fibers and adult-born neurons had been visualized by immunohistochemistry of GFP and choline acetyltransferase (ChAT). With 3-dimension view using ImageJ, the number of contacts between GFP-positive and CHAT-positive profiles was recorded at 0 day (D0), 18 days (D8) and 38 days (D38) from the beginning of enrichment. Afterwards, the number of NET-positive and ChAT-positive appositions had been compared to reveal the different innervation patterns and probably regulatory roles of these two major centrifugal inputs in the OB.

8. RESULTS

8.1 ARTICLE IN PREPARATION : INCREASED DENSITY IN SYNAPTIC CONTACTS FROM LOCUS COERULEUS FIBERS TO ADULT-BORN BUT NOT PRE-EXISTING NEURONS UNDERLIES LEARNED DISCRIMINATION IN THE OLFACTORY BULB.

Publication en préparation

Résumé

L'apprentissage olfactif de discrimination est sous-tendu par une augmentation du nombre de fibres noradrénergiques se connectant spécifiquement et fonctionnellement aux neurones néoformés du bulbe olfactif.

Contexte

Les neurones noradrénergiques en provenance du Locus Coeruleus (LC) se projettent sur l'ensemble des aires corticales et sont connus pour répondre aux stimuli sensoriels saillants. L'organisation et le mode de réponse de ces neurones font de ce système neuromodulateur un élément clef des processus d'éveil comportemental, d'attention mais également d'apprentissage et de mémoire et donc de plasticité. L'implication large des neurones du LC dans la cognition pourrait expliquer leurs rôles potentiels dans certaines pathologies telles que la dépression ou l'anxiété. En effet, certaines classes d'antidépresseurs ciblent plus particulièrement le système noradrénergique. Par ailleurs, la neurogenèse adulte serait atteinte lors de la dépression et est stimulée par les antidépresseurs. Ces données soulèvent la question de l'interaction physique et fonctionnelle entre les fibres du LC et des neurones néoformés dans les processus de plasticité et d'apprentissage.

Objectif

Il s'agira ici d'étudier si, dans un contexte d'apprentissage, les projections noradrénergiques sont 1- indispensables à l'apprentissage perceptif grâce à une expérience d'optogénétique, 2- capables de plasticité et 3- peuvent être reliés aux modifications de la morphologie des cellules granulaires du bulbe olfactif (BO), cibles importantes des fibres issues du LC.

Méthodes

Dans une première expérience, un virus halorhodopsine (pLenti-hSyn-eNpHR) a été injecté dans le LC de souris C57Bl6J adultes et des fibres optiques ont été implantées dans les BO afin d'inhiber les retours noradrénergiques spécifiquement sur le BO au moment où l'on teste les animaux sur leur capacité à discriminer des odeurs apprises. L'apprentissage perceptif consistait à enrichir les souris pendant 10 jours avec une paire d'odorants très similaires d'un point de vue perceptif et de tester leurs performances de discrimination à l'issue de la période d'enrichissement.

Dans une deuxième expérience, un virus Lenti-PGK-GFP a été injecté à des souris C57Bl6J dans le ventricule latéral au premier jour post-natal pour marquer les cellules granulaires issues de l'ontogenèse ou dans la zone sous-ventriculaire de souris âgées de 2 mois pour marquer les cellules granulaires néoformées. L'effet de cet apprentissage olfactif sur le nombre de contacts noradrénergiques sur les cellules granulaires préexistantes et néoformées ainsi que sur les modifications morphologiques des nouveaux neurones a été évalué.

Résultats

Grâce à la manipulation optogénétique, nous avons premièrement montré que l'inactivation spécifique des fibres noradrénergiques présentes dans le BO altérait l'expression de l'apprentissage perceptif et réduisait sélectivement l'activation des cellules granulaires néoformées. Deuxièmement, grâce à une étude de la plasticité structurale, nous avons révélé que les fibres noradrénergiques contactaient les cellules granulaires néoformées précocement même si ces contacts restaient plus denses sur les cellules granulaires préexistantes. L'apprentissage perceptif change cet équilibre et induit une augmentation des contacts uniquement sur les cellules granulaires néoformées, phénomène accompagné d'un changement de la morphologie des néo-neurones.

Conclusion

Ces données révèlent que le système noradrénergique en provenance du LC est capable de réorganiser ses terminaisons spécifiquement en direction des neurones néoformés afin de permettre l'apprentissage. Cette plasticité structurale des terminaisons du LC offre de nouvelles perspectives pour la compréhension des mécanismes d'action des voies neuromodulatrices noradrénergiques dans l'apprentissage et démontre un rôle spécifique des néo-neurones comparés aux neurones issus de l'ontogenèse dans l'action de la noradrénaline lors de l'apprentissage.

Increased density in synaptic contacts from *Locus Coeruleus* fibers TO adult-born but not pre-existing neurons underlies learned discrimination in the olfactory bulb.

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Abstract

Noradrenergic fibers originating from the *Locus Coeruleus* project massively onto the olfactory bulb which is the target of adult neurogenesis. Dysfunctions of both neurogenesis and noradrenaline have been involved in psychiatric pathologies such as depression and anxiety. These evidences raised the question of the pattern of innervation by noradrenergic fibers of adult-born cells and its potential learning-dependent plasticity. To address this issue, we studied the effect of olfactory perceptual learning which depends both on noradrenaline release and adult-born neurons in the olfactory bulb on the interaction between these two systems. We found (1) that optogenetic inactivation of noradrenergic fibers specifically in the olfactory bulb abolished perceptual learning and selectively reduced adult-born granule cells activation, (2) that noradrenergic fibers which innervate pre-existing granule cells to a greater extent than adult-born granule cells in basal conditions, are able to show specific remodeling only on adult-born cells in response to learning and that (3) this plasticity of noradrenergic fibers induced by learning is accompanied by changes in adult-born cell morphology. All together these findings provide original evidence for a structural plasticity of the noradrenergic system and its functional contribution to learning.

Introduction

The noradrenergic neuromodulatory system originating in the *Locus Coeruleus* (LC) projects to and receives inputs from virtually all cortical areas (Schwarz & Luo, 2015). This integrative mode of organization together with the responsiveness of LC neurons to salient sensory stimuli (Sara, 2009) makes the LC system a key modulator of arousal and attention orientation (Sara & Bouret, 2012). In addition to these on-line immediate effects, the LC system is also essential to learning and memory and modulates functional synaptic plasticity (Sara & Bouret, 2012). The broad involvement of the LC system in cognition and learning accounts for the number of conditions, including aging (Mather & Harley, 2016) neurological (Chalermphanupap *et al.*, 2013) and psychiatric pathologies in which its dysfunction is suspected. More specifically, in depression or anxiety (Willner *et al.*, 2013) LC involvement is reinforced since several classes of antidepressants target the noradrenergic system (Belzung *et al.*, 2015). Interestingly, a neurogenic theory of depression and anxiety is currently under investigation since in one hand antidepressant treatments stimulate adult neurogenesis and in the other hand there are some evidence in animal models and humans that adult neurogenesis is altered in depression and anxiety (Miller & Hen, 2015). These evidences converge to raising the issue of the interaction between LC noradrenergic fibers and adult-born neurons. Elucidating this interaction requires to decipher the spatio-temporal pattern of LC fibers connections onto adult-born neurons as well as their potential learning-dependent plasticity. To address these issues, we took advantage of a relatively simple olfactory discrimination learning paradigm which is dependent on both noradrenaline release and adult-born neurons in the olfactory bulb (OB) (Mandairon *et al.*, 2006c; Moreno *et al.*, 2012; Vinera *et al.*, 2015). The main target of adult neurogenesis in the OB are the inhibitory interneurons granule cells. They are functionally polarized. Their basal dendritic domain receives dense inputs from various origins including cortical areas and neuromodulatory systems including LC projections (Shepherd *et al.*, 2007). Their apical domain similarly receives some centrifugal inputs but also forms the granule cells output through reciprocal synapses with the relay cells of the OB (mitral and tufted cells) (Shepherd *et al.*, 2007). By this mean, granule cells compute olfactory afferents and context-dependent centrifugal inputs to shape the mitral cell discharge. As such, they play a crucial role in experience-related changes in odor representation (Devore & Linstner, 2012). We first optogenetically assessed whether the activity of the noradrenergic fibers projecting from the LC to the OB (Shipley *et al.*, 1985) is required for learned olfactory discrimination. Second, we investigated the

spatio-temporal pattern of innervation by LC terminals of OB adult-born granule cells and compared this innervation to that of granule cells formed during ontogenesis (and thereafter called pre-existing granule cells). Third, we investigated whether noradrenergic fibers are able of structural plasticity by analyzing how LC fibers synaptic contacts onto dendritic domains of these two categories of granule cells evolved with learning. We show (1) that optogenetic inactivation of LC fibers spatially limited to the OB and temporally restricted to behavioral odor exploration, abolished learned discrimination and selectively reduced adult-born granule cells activation and (2) that while LC fibers innervate pre-existing granule cells to a greater extent than adult-born granule cells, they responded to learning by an increase in synaptic contacts onto the basal and to a lesser extent, the apical dendritic domains of adult-born but not pre-existing cells. Finally, (3) this plasticity of LC fibers induced by learning was accompanied by changes in adult-born cell morphology. All together these findings reveal that the LC system can undergo a previously unknown reorganization of its terminals that selectively targets adult-born neurons to support learning. This structural plasticity of LC terminals offers a new insight into the mechanisms of action of the LC fibers in learning and demonstrates a very specific role of adult-born neurons compared to pre-existing ones in mediating LC influence in learning.

Material and Methods

Ethical statement

All experiments using mice 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 3-4 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).

Olfactory perceptual learning.

Perceptual learning allows discrimination between similarly perceived odorants. It is induced by non-reinforced repeated exposure to these odorants (enrichment) and tested using a habituation/dishabituation test. For enrichment, swabs containing 100 µl of pure odorant were placed

in 2 tea balls hanging from the cover of the standard breeding cages for 1 hour daily during 10 days. In the control group, the mice were housed under the same conditions except that the 2 tea balls contained 100 μ l of mineral oil. To assess discrimination abilities modified by enrichment, we used a habituation/dishabituation task. The odorants were all diluted in mineral oil proportionally to their vapor pressure to reach a pressure of 1 Pa (Cleland *et al.*, 2002; Mandaïron *et al.*, 2006c). Habituation experiments were performed in standard home cages, and odorants were presented by placing 60 μ l of odor stimulus onto a filter paper (Whatman). The filter paper was presented in a tea ball hanging from the cover of the cage. A test session consisted of one 50-second presentation of mineral oil, then four 50-second odor presentations of the first odorant (Hab1-4) at 5-minute intervals, followed by one 50-second presentation of the second odorant of the pair (Test). Investigation was defined as active sniffing within 2 cm of the tea ball. Each odorant of each pair was alternatively used as habituation or test odor. Odor investigation was recorded manually for each trial (Moreno *et al.*, 2009; Moreno *et al.*, 2012). Decreased investigation time over the first four trials reflects habituation to the presented odorant. Increased exploration time between the fourth habituation trial and the test trial reflects discrimination between the two odorant of the pair.

Optogenetic experiment

Experimental design. 2 months after injection of an halorhodopsin-expressing lentivirus into the LC and implantation of optical fibers in the OB, mice were tested on spontaneous discrimination between pairs of perceptually similar odorants (+limonene, (+Lim, 0.204%)/-limonene (-Lim, 0.204%) and between perceptually dissimilar odorants (anisole (AN, 0.052%)/amyl acetate (AA, 0.072%)) with or without light stimulation. Mice were injected with Bromo-deoxy-uridine (BrdU, 50 mg/KG, 3 injections spaced by 2 hours) 8 days before being subjected to perceptual learning i.e enriched 1 hour daily during 10 days with +Lim and Llim. After the enrichment period, mice were tested again on spontaneous discrimination among the two odor pairs with or without light stimulation.

Surgery. 18 Adult mice (Charles River laboratory, L'Arbresles, France) were anaesthetized (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). 300 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 LC (with respect to the bregma: AP, -5.4 mm; ML, \pm 0.9 mm; DV, -4.0 mm) at a rate of 150 nl/min (n=10 and n=8 for NpHR and control group respectively). Following virus infusions, a dual optical fiber (200-nm core diameter, 0.22 N.A.; Doric Lenses) was implanted into the OB (with respect to the bregma: AP, +4.3 mm; ML, \pm 0.75 mm; DV, -2.0 mm). The pLenti-hSyn-eNpHR 3.0-EYFP was a gift from Karl Deisseroth 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) (Kermen *et al.*, 2016).

Optical stimulation during habituation/dishabituation test. Habituation/dishabituation tests as described previously were performed except that a bilateral continuous light stimulation (Crystal Laser, 561 nm, 10–15 mW) was automatically triggered during the dishabituation test trial when the mouse's nose came within 1 cm around the tea ball (VideoTrack, Viewpoint) and stopped automatically when the nose exited the zone.

Control of light-triggered inhibition. Mice were sacrificed 1 day after post-enrichment test. One hour before sacrifice, mice were light-stimulated (2 s of light stimulation every 6 s, mimicking the light stimulation received during the test trial, repeated during one hour; 450 repetitions). Sacrifice and brain treatments are described below (histology) and immunohistochemistry performed as previously (Moreno *et al.*, 2012). For Zif268/NeuN/BrdU labeling (Zif268/NeuN/BrdU) olfactory bulb slices were incubated overnight at 25°C in rabbit anti-Zif268 (1:500, Santa Cruz) and mouse anti-NeuN (1:500, Millipore) and rat anti-BrdU (1:100, AbCys). The appropriate secondary antibodies coupled to Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 633 (Molecular Probes) were used.

Image acquisition and analysis. Images were analyzed by pseudo-confocal microscopy (Zeiss microscope equipped with an Apotome) using 63x objective. Lateral and z-axis resolutions were 100 and 200 nm respectively.

Morphology experiment.

Experimental design. At the beginning of the experiment, mice were tested on spontaneous discrimination between the two perceptually similar odorants +Lim and -Lim using the

habituation/dishabituation task. This first step was done before virus injection except for the P0 group. Mice were enriched 1 hour daily during 10 days with these two odorants or non-enriched for the control group. After the enrichment period, mice were tested again on spontaneous discrimination. To label neurons born during ontogenesis or in adult, mice were injected with a Lenti-PGK-GFP lentivirus respectively in the lateral ventricle at P0 or in the sub-ventricular zone 8 days before the enrichment period.

Animals. 21 C57BL/6J mice born and raised in our animal facility as well as 25 adult male C57BL/6J mice (aged 2 months) from Charles River (L'Arbresles, France) were used.

Adult-born granule cell labeling. Prior to surgery, adult mice were anaesthetized and secured in a stereotaxic instrument as previously. 150 nl of Lenti-PGK-GFP lentivirus ($3,8 \times 10^7$ IU/ml) was injected bilaterally into the sub-ventricular zone (with respect to the bregma: AP, +1.0 mm; ML, $\pm \pm 1.0$ mm; DV, -2.3 mm) at a rate of 150 nl/min.

Preexisting granule cell labeling. P0 mice were anesthetized in ice and injected in the right lateral ventricle with the Lenti-PGK-GFP lentivirus. The injection (1 μ l at a rate of 0.2 μ l/s) was done using a glass-micropipette linked to a Hamilton syringe.

Histology. Adult mice were sacrificed 8, 25 or 45 days after virus injection, i.e on Day0, Day18 and Day38 of the behavioral protocol (Figure 2A). Mice injected at P0 were sacrificed at the same ages. Mice 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. Brains were then sectioned (40 μ m-thickness) with a cryostat. For norepinephrine transporter/GFP-tagged adult-born neuron double labeling (NET/GFP), olfactory bulb sections were incubated after blockade during 72 hrs at 4°C in a rabbit anti-GFP (1:750, Millipore) and mouse anti-NET (1:1000, Mab Technologies). The appropriate secondary antibodies coupled to Alexa Fluor 488 and Alexa Fluor 546 (Molecular Probes) were used. For Norepinephrine transporter/GFP-tagged adult-born neuron/synaptophysin labeling (NET/GFP/SYN), olfactory bulb slices were incubated 72 hrs at 4°C in mouse anti-NET (1:1000, Mab Technologies), chicken anti-GFP (1:1000, Anaspec/Tebu) and rabbit anti-synaptophysin (1:200, SySy). The appropriate secondary antibodies coupled to Alexa Fluor 488, Alexa Fluor 546 and Alexa Fluor 633 (Molecular Probes) were used. For dopamine beta-

hydroxylase/GFP-tagged adult-born neuron labeling (DBH/GFP), brain slices were incubated 48 h at 4°C in rabbit anti-DBH (1:200, Santa Cruz) and chicken anti-GFP (1:1000, Anaspec/Tebu). The appropriate secondary antibodies coupled to Alexa Fluor 488 and Alexa Fluor 546 (Molecular Probes) were used.

Simple, double- and triple-labeling were analyzed by pseudo-confocal microscopy (Zeiss microscope equipped with an Apotome). For analysis of the spine density and morphology of granule cells, images were taken with a 100x objective (lateral and z-axis resolutions were 60 and 200 nm respectively). Dendritic processes and spines were then analyzed using NeuronStudio software. This software allows for automated detection of three dimensional (3D) neuronal morphology (dendrites and spines) from confocal z-series stacks on a spatial scale. ImageJ software was used to analyze the contacts between GFP labeled adult-born neurons (expressing or not the synaptophysin) and the noradrenergic fibers. Photos of DBH/GFP were taken at 40x objective. Between 4 and 8 neurons were analyzed per animal.

Statistical analyses

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 Kruskal-Wallis ANOVA, *post-hoc* multiple comparisons or Mann-Whitney tests for unpaired data or Wilcoxon tests for paired data (Statistica) group comparisons. Data are showed as mean \pm SEM

Results

LC fiber optogenetic inhibition in the OB blocks learned olfactory discrimination

Similar to discrimination learning described in other sensory modalities (Gilbert *et al.*, 2001), olfactory perceptive learning is defined as an improvement of discrimination ability between perceptually close odorants following repeated exposure to these odorants (Mandairon *et al.*, 2006c; Moreno *et al.*, 2009). Here, adult animals were beforehand injected in the LC with a lentivirus allowing the expression in neurons (cell bodies and axons) of the light-sensitive inhibitory HaloRhodopsine channel (lenti-hSyn-NpHR-EYFP, eNpHR) or an empty lentivirus (lenti-hSyn-EYFP, control) and implanted bilaterally with

optic fibers in the OB (Figure 1 A and B). Two months later, mice were submitted to perceptive learning through exposure to the two enantiomers of limonene (+Lim and -Lim). Odor exposure (enrichment) consisted in one-hour exposure per day for 10 days to the two odorants. Pre-tests performed before the enrichment period confirmed that the two odorants were not spontaneously discriminated (Supplementary Figure 1). Assessment of discrimination between +Lim and -Lim was done thanks to a habituation/dishabituation test. In this test, the animal is allowed to explore one odorant of the pair across four trials (habituation trials) and on the fifth trial (Test), the other odorant is presented. The time spent by the animal exploring the odorant was recorded for all trials. Increased exploration in the test trial compared to the last habituation trial indicates discrimination of the two odorants of the pair. To assess whether LC system is required for expression of learning, post-enrichment, optogenetic inhibition of LC fibers was applied during the test trial and triggered automatically only when the animal explored the odorant. Both eNpHR and control groups were tested with and without light stimulation. As expected, exploration time decreased along the first four trials, reflecting habituation to the odorant for both eNpHR and Control groups (Figure 1 C and D, Hab1 to Hab4). An increased exploration time was observed for the fifth trial compared to the last habituation trial, indicating that eNpHR animals could discriminate +Lim from -Lim with the light off. This learned discrimination was abolished by light stimulation as evidenced by the exploration that remained low, similar to that of the fourth habituation trial (Figure 1C, Test). Control animals could discriminate +Lim from -Lim with both the light on and off indicating that the light itself had no detrimental effect on learned discrimination (Figure 1D, Test). To assess that inhibitory light stimulation did not impair general processes of discrimination, an additional control was conducted using a pair of perceptually dissimilar odorants that the animals could spontaneously discriminate (Anisol and Amyl Acetate). Both eNpHR and control groups discriminated these two odorants under light stimulation (Figure 1E) indicating no impairment in basic discrimination abilities.

Post-mortem count of LC fibers in the OB, identified by the co-expression of the noradrenaline transporter (NET) and EYFP (indicative of transduction by the lentivirus) revealed that more than half of the NET-positive fibers expressed the HaloRhodopsine channel (Figure 1B lower panel) ($52,3 \pm 3$ for control and $61,2 \pm 12$ for eNpRH, mean \pm sem, $n=6$ and $n=5$ respectively, Mann-Whitney, $p=0.57$).

Optogenetic inhibition of LC fibers selectively affects adult-born granule cell activation

To further document the cellular effect of the optogenetic inhibition, animals were submitted before sacrifice to a light stimulation consisting in a repeated pattern (number of lightening and total duration) similar to that received by the animals during discrimination testing, allowing measuring the expression of the immediate early gene Zif268 as an index of cell activation. The total number of Zif268-positive granule cells was similar between eNpHR and control groups (Figure 1 F). In contrast, when considering only adult-born granule cells as co-labelled with the cell division marker Bromodeoxyuridine (BrdU, injected 8 days before the enrichment period to allow adult-born neuron to be present in the OB during learning) and Zif268, it was found that their number was significantly reduced by the light stimulation in eNpRH compared to control group (Figure 1 G).

Altogether, this finding indicated that the optogenetic inhibition of LC fibers was able to suppress learned discrimination and to selectively reduce adult-born neurons activation. In order to further investigate the structural basis of this selective effect, we then looked at the innervation pattern of adult-born neurons by LC fibers and compared it to that of pre-existing granule cells in the OB.

LC fibers innervation of adult-born versus pre-existing granule cells and effect of learning

For the purpose of analyzing the effect of perceptive learning on LC fibers organization in the OB, new groups of animals were injected with a lentivirus allowing GFP expression under a PGK promotor (lenti-PGK-GFP) either at post-natal day 0 (PN0) to label granule cells born during ontogenesis (pre-existing granule cells) or at the age of 2 months to label granule cells born in the adult brain (adult-born granule cells). Virus infusion in adult was done 8 days before odor enrichment, to allow GFP-positive neuroblasts to migrate from the subventricular zone to the OB before the onset of learning. Control groups were similarly injected but not submitted to learning (Figure 2 A). Animals were tested shortly after learning (D18) and a longer time point (D38) to assess retention of their ability to discriminate +Lim from -Lim using the same habituation/dishabituation test as in the previous experiment. At both delay, learning groups were able to discriminate +Lim from -Lim indicating long-term retention of the learned discrimination, while the control groups were not (Figure 2 B).

For morphological analysis, groups of animals were sacrificed before learning (D0) and behaviorally tested animals were sacrificed at D18 and D38. At each time point, we searched for evidence of contacts between NET-positive LC fibers and GFP-positive neurons in the form of LC fibers close appositions onto GFP-positive granule cell dendrites (Whitman & Greer, 2007). These appositions

were counted based on visual inspection of 3D pictures and data were analyzed separately for basal dendrites and apical dendrites (Figure 1C).

On the apical side, the density of NET-positive appositions was similar between the 3 time points analyzed for pre-existing and adult-born neurons (Kruskall-Wallis for control groups $p < 0.00001$ but $p > 0.05$ for the comparison between the 3 delays within pre-existing and adult-born cells). Interestingly, this density, is higher in pre-existing than in adult-born granule cells (Figure 2D) (Kruskall-Wallis for controls $p < 0.00001$, pre-existing versus adult-born: D0, $p = 0.02$; D18, $p = 0.000001$; D38, $p = 0.00008$). In animals submitted to perceptive learning, the number of NET-positive appositions on pre-existing as well as on adult-born granule cells was unchanged compared to controls (Kruskall-Wallis, $p = 0.25$ and $p = 0.11$ respectively) (Figure 2D). Thus, on the apical side of granule cells, LC fibers appositions show no increase with the age of the adult-born cells, were more numerous onto pre-existing than adult-born granule cells and were not sensitive to learning.

On the basal side, the density of NET-positive appositions was again similar between the 3 time points analyzed for pre-existing and adult-born neurons (Kruskall-Wallis for controls $p = 0.0001$ but $p > 0.05$ for comparison between the 3 delays within pre-existing and adult-born cells) and were overall larger on pre-existing than on adult-born granule cells (Kruskall-Wallis for controls $p = 0.0001$, pre-existing versus adult-born: $p = 0$. D18, $p = 0.00$; D38 0.005) (Figure 2E). Learning induced a large increase in the density of NET-positive appositions specifically on adult-born cells (adult-born: Kruskall Wallis, $p < 0.00001$, control versus learning, D18 $p = 0.004$, D38, $p = 0.0005$; pre-existing: Kruskall Wallis $p = 0.06$). This learning-induced increase brought the density of LC contacts on adult-born cells to a level similar to that of control pre-existing granule cells. (Figure 2E). On the basal domain, LC appositions were thus more numerous on pre-existing than on adult-born granule cells but their density on adult-born granule cells increased markedly with learning.

To further characterize the synaptic identity of these contacts, we used triple labeling to detect NET-positive contacts onto GFP-positive dendrites that expressed the pre-synaptic marker Synaptophysin. The percentage of Synaptophysin-positive contacts among the NET-positive appositions on granule cell dendrites reached ~20% with no difference between conditions (control or learning), age of cells (pre-existing or adult-born) or dendritic domains (apical or basal) ($p > 0.05$) except for contacts on adult-born granule cells at the earliest delay. At this time point, the percentage of synaptophysin-positive

contacts was lower at D0 compared to D18 ($p < 0.000003$ and $p = 0.000001$ for apical and basal domains respectively) (Supplementary Figure 2).

The density of synaptic contacts between LC fibers and granule cells dendrites was then calculated as the density of NET-positive * percentage of synaptophysin- and NET-positive apposition.

On apical dendrites of control adult-born cells, no synaptic contact was found at D0. At later delays, synaptic contact density increased (Kruskall Wallis for controls, $p < 0.00001$, D0 versus D18 $p = 0.000001$, D0 versus D38 $p < 0.0000001$) but remained lower than on pre-existing (adult-born versus pre-existing, control, D18, $p = 0.001$; D38, $p = 0.001$) (Figure 2 F). Learning increased synaptic contact on apical dendrites of adult-born cells (Kruskall Wallis, $p < 0.00001$, control versus learning D18 $p = 0.02$; D38 $p = 0.0009$) but induced no change on apical dendrites of pre-existing granule cells (Kruskall Wallis, $p = 0.07$) (Figure 2F).

On the basal compartment, a low density of contacts was found at D0 on control adult-born dendrites (Kruskall Wallis for controls, $p < 0.00001$, D0 different from all other groups). It increased at later delays (D0 versus D18 $p = 0.000004$; D0 versus D38 $p < 0.000001$) and reached the same level as on the dendrites of control pre-existing granule cells ($p > 0.05$ at D18 and D38). Learning increased the density of LC fiber synaptic contacts on adult-born (Kruskall Wallis, $p < 0.00001$, control versus learning D18 $p = 0.003$, D38 $p = 0.001$) but not on pre-existing granule cell dendrites (Kruskall Wallis, $p = 0.07$). Finally, after learning, the density of contacts was higher on adult-born than on pre-existing basal dendrites (D18 $p = 0.02$; D38 $p = 0.001$) (Figure 2G).

To sum up, while no change was observed in the number of apposition, a developmental increase in LC fiber synaptic contacts on adult-born granule cells was observed. LC fiber synaptic contacts were absent or few respectively on the apical and basal dendritic tree of adult-born granule cells aged 8 days (D0). Density of these contacts increased thereafter but remained stable for granule cells at D18 and D38 (cells aged 28 and 48 days respectively). Importantly, learning increased LC fiber synaptic contact density on apical and basal dendritic domains of adult-born granule cells but had no effect on pre-existing granule cells. The learning-induced increase in LC fiber synaptic contacts lead to a higher density of these contacts on the basal domain of adult-born granule cells compared to pre-existing.

Learning induced changes granule cell spines in response to learning.

In a last step, we asked whether the number of spines beard by the granule cells were modified accordingly to the changes in LC fibers synaptic contacts. The apical domain of pre-existing granule cells showed no change in spine density with learning (Kruskall Wallis $p=0.1$). In adult-born cell, apical spine density increased with the age of the cells (Kruskall Wallis, $p<0.00001$, D0 versus D18 $p=0.000001$; D0 versus D38 $p=0.02$) and stabilized (D18 versus D38 $p=0.17$). In addition, learning increased apical spine density in adult-born granule cells (control versus learning, D18 $p=0.002$; D28 $p=0.00009$) (Figure 3 A).

Spine density on basal dendrites decreased with learning in pre-existing cells (Kruskall Wallis, $p=0.02$, control versus learning D18 $p=0.002$, D28 $p=0.02$). In adult-born cells, spine density increased between D0 and D18 (Kruskall Wallis $p<0.00001$, D0 versus D18 $p=0.0001$, D0 versus D38 $p=0.0005$) and stabilized (D18 versus D38 $p=0.5$). In contrast to pre-existing cells, learning increased basal spine density in adult-born cells (Control versus learning, D18 $p= 0.00001$, D38 $p=0.004$) (Figure 3 B).

In summary, spine density on apical and basal domains of adult-born cells increased between D0 and D18 when it reached its maximum value in controls. Learning increased apical and basal spine density in adult-born cells and reduced basal spine density in pre-existing cells.

Discussion

Original findings reported in the present study include the increase in LC fiber synaptic contacts onto adult-born granule cells, following learning in the OB, providing evidence for a previously unknown structural plasticity of LC fiber terminals in physiological condition. Second, this massive reorganization targeted only adult-born while leaving unchanged the anatomical contacts between LC fibers and pre-existing neurons, shedding new light on the unique properties of adult-born in neuronal networks. In addition, the study provides evidence for a crucial functional role played by these supplementary synapses in the expression of the learned behavior. Finally, our experiments also reveal the precocity of the innervation by LC fibers of developing adult-born OB granule cells, opening a large time window during the critical period of adult-born neuron development for LC system to influence adult-born neuron morphology and physiology.

LC fibers plasticity: de novo connections

Learning led to an increased density in synaptic contacts tagged by the expression of both NET and synaptophysin, on adult-born cells at D18 and D38. This increase paralleled that of close appositions, suggesting that learning induced *de novo* contact formation rather than functional maturation of existing appositions. While axonal sprouting of LC axons in the central nervous system has been described following LC lesioning (Marien *et al.*, 2004), the demonstration that such process occurs in physiological learning reveals a novel mechanism by which the LC system may influence learning. Indeed, while modulation of post-synaptic plasticity has been classically ascribed to LC fibers, our finding suggest that it would also rely on the enhancement of the LC fiber pre-synaptic weight onto cellular targets.

Adult-born granule cells are selectively targeted by LC fiber remodeling

In contrast to adult-born cells, pre-existing cells showed no change in their pattern of LC innervation. This raises the question of the mechanisms that are able to attract and/or stabilize into synaptic contacts LC terminals selectively on adult-born granule cells. Difference in responsiveness to learned odors have been reported in adult-born neurons after perceptual learning (Moreno *et al.*, 2009; Moreno *et al.*, 2012). Adult-born neurons are also prompted to long term potentiation (Nissant *et al.*, 2009). These properties may underlie or promote activity-dependent changes in spine morphology and/or stability in adult-born cells (Bernardinelli *et al.*, 2014) accounting for the selectivity of the LC fiber remodeling. Interestingly and in line with this hypothesis, adult-born but not pre-existing adult-born cells showed a learning-induced increase in dendritic spines, forming the post-synaptic compartment of centrifugal fiber-to-granule cell synapses. Furthermore, in the basal domain, spine density decreased on pre-existing neurons. Learning may thus be able to synergistically lower centrifugal load on pre-existing granule cells and increase it on adult-born cells.

Whether or not LC innervation remodeling on adult-born neurons is a specific feature of the LC system is currently unknown. Indeed, experience-dependent remodeling of pre-synaptic contacts from cortical origins onto adult-born neurons in the dentate gyrus of the hippocampus (Bergami *et al.*, 2015) and the OB (Lepousez *et al.*, 2014) have been recently reported but not compared to that of contacts onto pre-existing neurons.

Synaptic contacts between LC fibers and adult-born neurons are necessary to the expression of perceptive learning

The optogenetic approach used in this study selectively targeted LC fiber in the OB, overcoming the diffuse nature of LC projection to cortical areas encountered in the case of LC manipulation. Observed effects can thus be ascribed to local changes in LC fibers activity. In our experimental conditions, we observed that following enrichment, animals lost their ability to discriminate two perceptually close odorants under light inhibition of LC fibers while they were perfectly able to do so in the absence of light stimulation. Appropriate controls further showed that light by itself, in the absence of HaloRhodopsine expression, did not impair learned discrimination. The finding that only adult-born granule cells were affected by LC fiber optogenetic inhibition suggest is consistent with the increase in LC fiber contacts on these cells. So, LC fiber inhibition had more impact on adult-born than on pre-existing cells. Finally, our previous works showed that the acquisition of perceptive learning depends on noradrenaline (Moreno *et al.*, 2012; Vinera *et al.*, 2015). The present finding further indicate that LC fibers are needed not only for acquisition but also for expression of perceptual learning.

The LC fiber load is stronger on basal than on apical dendritic domains of adult-born cells.

The noradrenergic synaptic contacts showed higher density on the basal compared to the apical dendritic domain of adult-born cells. This finding is consistent with previous description of a higher density of noradrenergic fibers in the granule cell layer (harboring basal dendritic domain of granule cells) compared to that in the external plexiform layer (containing the apical domain of granule cell dendrites) (Shiple et al 1985). Also, while the synaptic contacts were already present on basal dendrites of adult-born neurons aged 8 days, none were seen on apical dendrites at the same age. Then on the basal side, the density of synaptic contacts reached that of pre-existing neurons in 28 days old neurons while it remains below pre-existing cells on the apical side up to 38 days' post cell birth. The potential influence of LC fibers is thus more precocious and globally heavier on the basal than on the apical dendritic domains of adult-born granule cells. Furthermore, the effect of learning was larger in the basal compared to the apical domain with density of LC synaptic contacts on adult-born neurons overcoming that of pre-existing cells after learning.

Deciphering the exact influence of LC contacts on granule cells activity and ultimately mitral cells is a complex issue given the concentration-dependent effect of noradrenaline in the OB and the existence of noradrenergic receptors on mitral cells as well as on granule cells. Noradrenaline action depends on subtle concentration changes and divergent action on granule and mitral cells. In this context, it is

likely that the drastic changes LC synaptic load on adult-born cells with their specific properties in terms of excitability and plasticity impacts the network. This aspect will need further investigation but one should keep in mind that the final output of this remodeling allows discrimination and thus produces disambiguation of similar odor-evoked patterns and thus of the messages exiting the OB.

Role of noradrenaline released by LC fibers

The decreased activity in adult-born granule cells following light inhibition of LC fiber is consistent with the excitatory action of noradrenaline released by LC fibers on these cells. Indeed, noradrenaline is mostly excitatory on granule cells through activation of alpha1 noradrenergic receptors. When the concentration of noradrenaline is lowered, as can be expected from LC fiber optogenetic inhibition, high affinity inhibitory alpha 2 receptors activity may dominate, resulting in granule cell inhibition (Devore & Linster, 2012). Although implication of co-transmitters released by LC fibers cannot be excluded in our experiment (Schwarz & Luo, 2015), the current finding is consistent with a crucial of noradrenaline for the expression of learned discrimination.

Our previous studies have demonstrated the prominent role of noradrenaline in supporting acquisition of perceptual learning. Indeed, pharmacological blockade of noradrenaline during enrichment prevented learning (Moreno *et al.*, 2012). In addition, perceptive learning was shown to increase the expression of Dopamine Beta hydroxylase, the synthetic enzyme for noradrenaline, in noradrenergic LC neurons suggesting that enrichment repeatedly solicited LC neurons. These data suggest that noradrenaline release triggered by olfactory stimulation may be important to promote the interplay between adult-born cells and LC fibers eventually leading to the structural plasticity of both adult-born cells and LC terminals observed after learning.

Relevance to pathologies

We provided here strong evidence that experience is able to increase the density LC contacts specifically on adult-born cells, reinforcing a functional link between the LC system and the modulation of neurogenesis (Miller & Hen, 2015). This phenomenon could be related to the positive effect of antidepressants increasing the synaptic level of noradrenaline. They may act in a way similar to experience and enhance synaptic contacts between the LC system and adult-born cells. The learning-dependent LC terminals plasticity reported in this study may also be relevant for the understanding of

the build-up of the brain cognitive reserve, defined as the beneficial effect of cognitive activity to prevent cognitive decline in normal or pathological aging. Noradrenaline has been proposed as a mediator of the imprint in the brain of experience and cognitive activity (Wilson et al 2013). Our findings further suggest that increase in LC fibers contacts on newly-generated neurons could participate into structural plasticity underlying at least partly the cognitive reserve with implication in normal (Mather and Harley, 2016) or pathological (Chalermphanupap *et al.*, 2013) cognitive aging.

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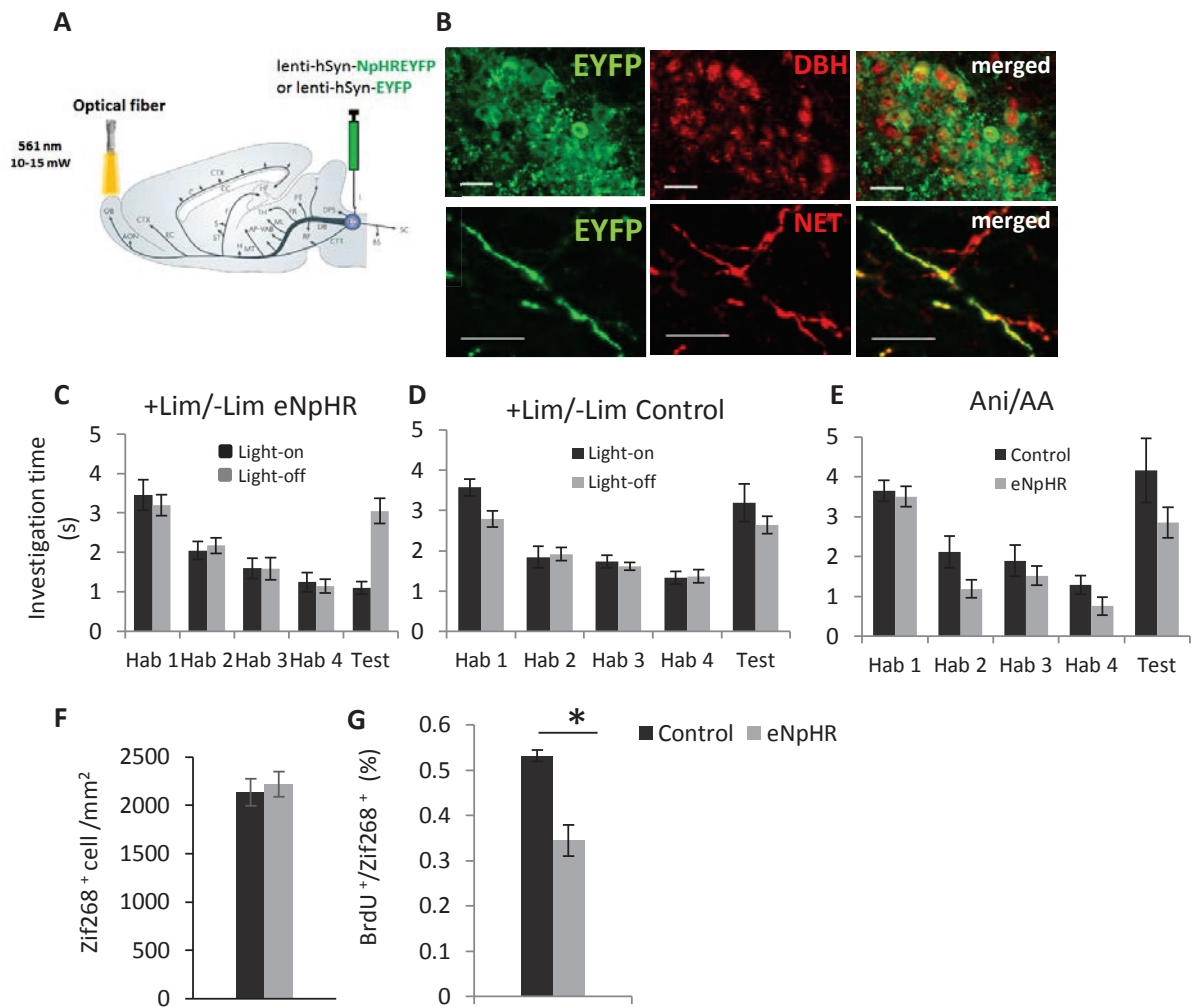


Figure 1. Optogenetic LC fiber inhibition prevents expression of perceptual learning

A. 2 months before the experiment, lentiviruses expressing NpHR-EYFP (eNpHR) or control (EYFP) are injected in the LC and optical fibers are implanted in the olfactory bulb. **B.** Up: EYFP+ neurons at the viral injection site in NpHR mice co-localize with DBH+ LC cells (bar =25 μ m); down: EYFP expressing NET-positive fibers in the OB (Bar=10 μ m). **C.** Habituation/cross habituation task indicated that +Lim and -Lim are not discriminated in eNpHR animals when light in on (n=10; Friedman test for Habituation p=0.001; Wilcoxon test for discrimination p=0.59) while it is when light is off (n=5; Friedman test for Habituation p<0.0001; Wilcoxon test for discrimination p=0.005). **D.** In control EYFP animals, light has no effect on +Lim/-Lim discrimination performance (light on: n=6; Friedman test for Habituation p<0.0001; Wilcoxon test for discrimination, p=0.02 and light off: n=10; Friedman test for Habituation p<0.0001; Wilcoxon test for discrimination p=0.003). **E.** Light stimulation does not impair discrimination of odorants that are perceptual dissimilar in eNpHR (n=5; Friedman test for Habituation p=0.001; Wilcoxon test for discrimination, p=0.005) and control animals (n=6; Friedman test for Habituation p=0.0002; Wilcoxon test for discrimination, p=0.002). Ani= Anisol; AA= Amyl Acetate. **F.** The density of Zif268-positive cell is similar between control and eNpHR mice under light stimulation (control n=6 and eNpHR n=5; Mann-Whitney p=0,31). **G.** Light stimulation decreases the percentage of BrdU-positive cells expressing Zif268 in eNpHR (n=5) compared to control animals (n=6; Mann-Whitney p=0,01). *:p<0,05

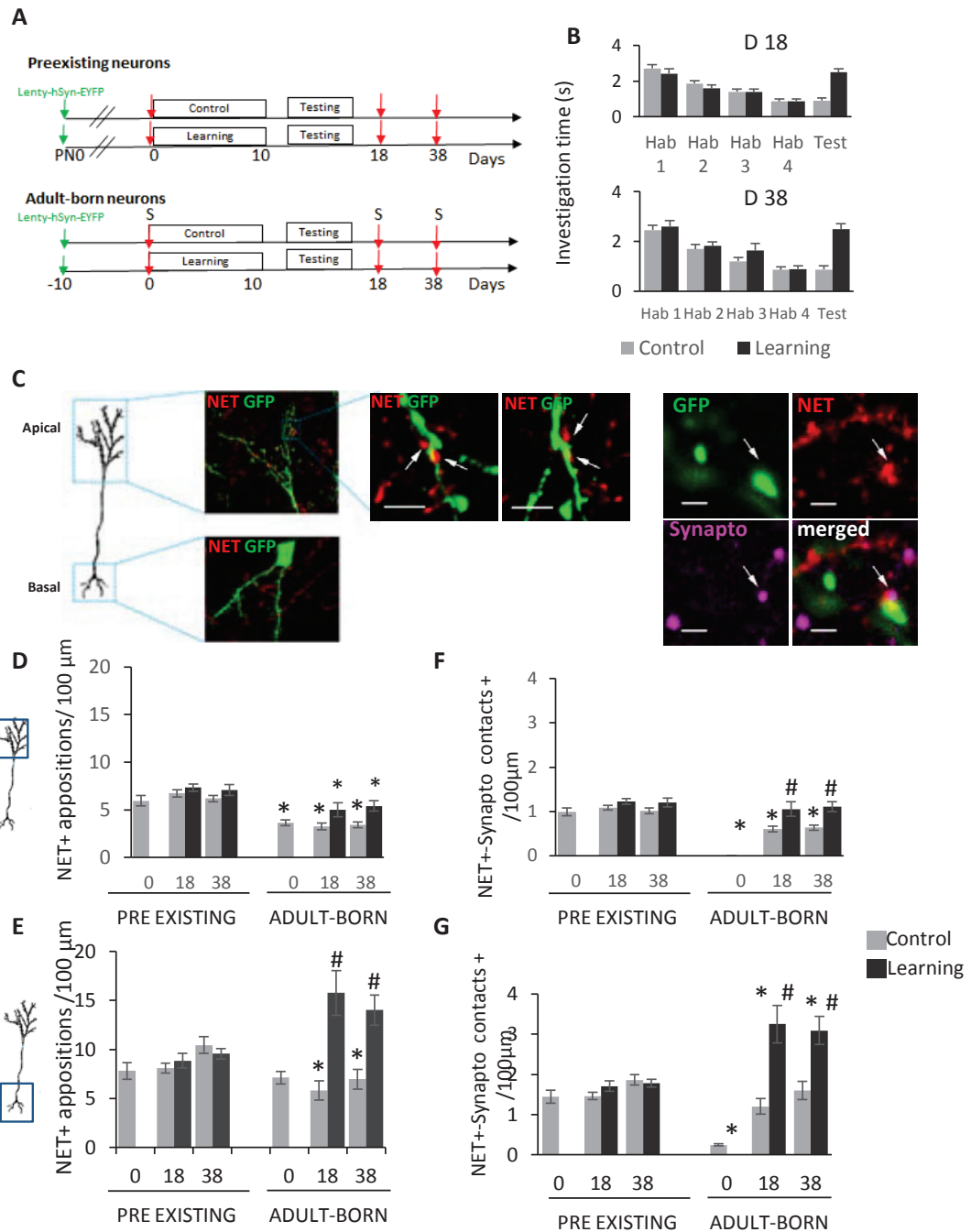


Figure 2. Perceptual learning increases LC fiber synaptic contacts specifically on adult-born neurons

A. Experimental design. **B.** Habituation/cross habituation task indicated that +Lim and -Lim are discriminated 18 (n=18; Friedman test for Habituation $p < 0.0001$; Wilcoxon test for discrimination $p = 0.0001$) and 38 days after the beginning of the learning period (n=18; Friedman test for Habituation $p < 0.0001$; Wilcoxon test for discrimination $p = 0.0001$). No such effect is observed in control non-enriched animals (after 18 days: n=18; Friedman test for Habituation $p < 0.0001$; Wilcoxon test for discrimination $p = 0.84$ and after 38 days: n=18; Friedman test for Habituation $p < 0.0001$; Wilcoxon test for discrimination $p = 0.86$). **C. Left,** Schematic drawing of an adult-born granule cell. **Middle,** NET-positive LC fibers making appositions with GFP-positive adult-born neurons. Scale bar, 8 μ m. **Right,** NET and synapophysin-positive contact on an adult-born GFP positive granule cell, scale bar = 2 μ m. Density of Net-positive appositions on apical dendrites (**D**) and basal dendrites (**E**) of pre-existing and adult-born neurons before (D0) and 18 or 38 days after enrichment. Density of NET- and synapophysin-positive contacts on the apical (**F**) and the basal (**G**) dendritic compartments of pre-existing and adult-born cells. * $p < 0,05$ for difference between corresponding groups of pre-existing and adult-born granule cells.; #: $p < 0,05$ for difference between enriched and corresponding control group. Detailed statistics are given in the text.

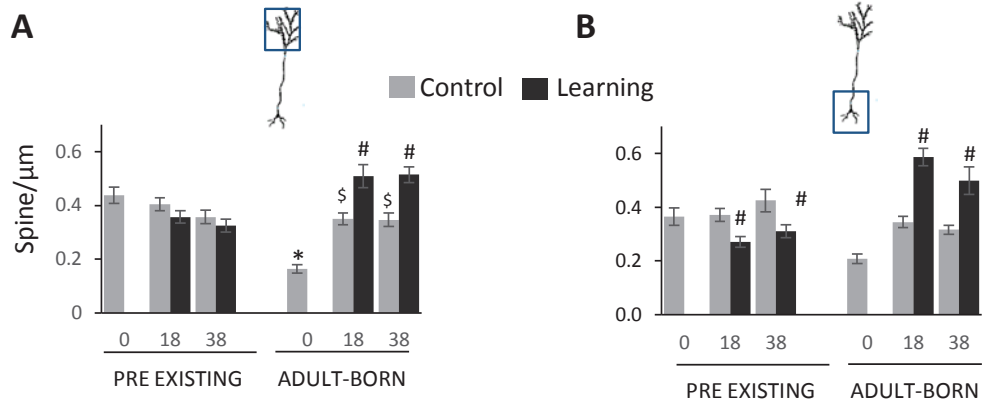
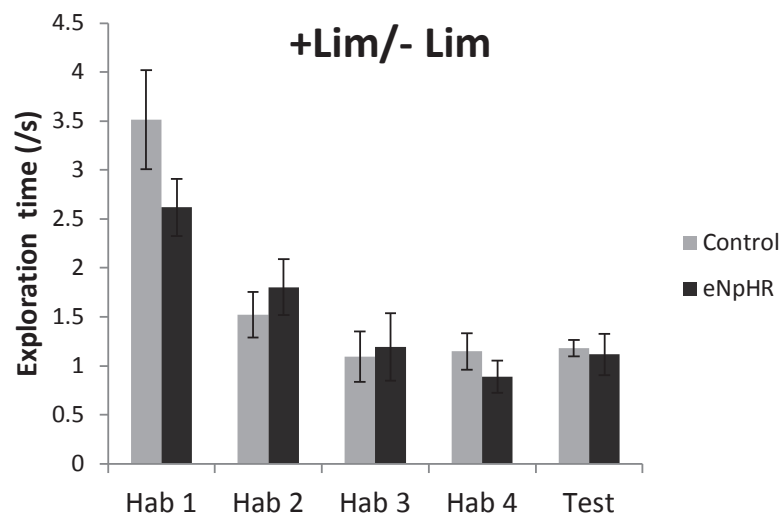
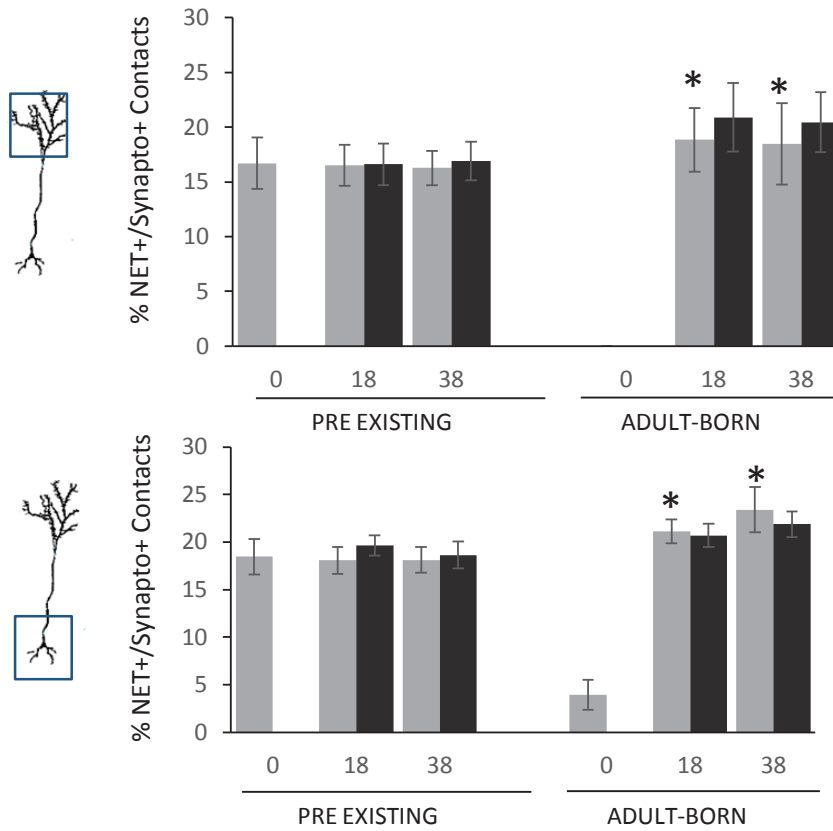


Figure 3. Spine density on the apical (A) and basal domain (B) of pre-existing and adult-born neurons in control and learning.

* $p < 0,05$ for difference between corresponding groups of pre-existing and adult-born granule cells.; #: $p < 0,05$ for difference between enriched and corresponding control group. \$, $p < 0,05$ for difference between D0 and D18 and D38 in control, adult-born groups. Detailed statistics are given in the text. N similar to Figure 2



Supplementary Figure 1: Habituation/dishabituation pre-tests for animals included in the optogenetic experiment. Both group showed habituation (ANOVA Friedman, $p = 0,0006$) but no discrimination (Wilcoxon, Hab 4 versus test $p=0,75$) ($n= 6$ in control and $n=5$ in eNpHR). No light stimulation was applied.



Supplementary Figure 2: Percentage of NET- and synaptophysin-positive contacts. On D0 (cells aged D8), the percentage of Synaptophysin-positive contacts among NET-positive apposition on GFP-positive neurons are lower than at later delay. For all other groups this percentage is similar. *:p<0,05 for difference from D0 Adult-born.

Cholinergic fibers from the horizontal limb of the diagonal band of Broca project throughout the olfactory bulb, and predominantly distributed in the granule cell layer and glomerular layer, and a less extent in the external plexiform layer (Shiple & Adamek, 1984; Kunze *et al.*, 1992; Mouret *et al.*, 2009b; D'Souza & Vijayaraghavan, 2014). Activating the cholinergic system generates multiple and complex modulations of olfactory processing in the olfactory bulb. Nicotinic acetylcholine receptors excite the granule OB, whereas muscarinic receptors activation exerts inhibitory effect on the firing rate of granule cells (Castillo *et al.*, 1999). Moreover, mice with nicotinic acetylcholine receptors ablation show nearly half more abGCs in the olfactory bulb, whereas chronic nicotine exposure significantly decreased the number of newborn granule cells (Mechawar *et al.*, 2004). But little is known about the role of the cholinergic system on the maturation of adult-born neurons in the olfactory bulb during olfactory learning.

Similarly, to what we have done for the LC system, we investigated the pattern of cholinergic inputs onto developing abGCs pattern during maturation of adult-born neurons. The same mice as those used in the previously presented experiments were used. Based on 3D view of neurons, close appositions between cholinergic fibers and abGCs were measured with the help of immunohistochemistry of choline acetyltransferase (ChAT) labling cholinergic terminals. Interestingly, unlike the findings in the noradrenergic inputs, the density of ChAT-positive appositions on the basal dendrites showed an inverted-U curve shape along maturation, namely increased on D18 (25 days after cell birth) and then decreased on D38 (45 days after cell birth) (Kruskal-Wallis, $p=0.001$: D0 vs. D18, $p=0.049$; D18 vs. D38, $p=0.0009$), even though these differences were not found in the apical dendritic domain (Kruskal-Wallis, $p=0.240$) (Figure 1).

To further investigate the plasticity of the cholinergic innervation on abGCs, we analyzed and compared the densities of ChAT-positive appositions between perceptive learning group and control group (naive mice). Interestingly, learning significantly decreased the density of ChAT-positive appositions on D18 on the basal dendrites (Kruskal-Wallis, $p<0.0001$, Mann

Whitney learning vs. control, $p=0.0005$), whereas the decreased effect switched to a tendency to increase on D38 (Mann Whitney learning vs. control $p=0.0824$). Moreover, no significance between groups was found in the apical dendritic compartment. This complex time-dependent effect was distinctive from what we observed between LC fiber and adult-born neurons.

Comparison between ChAT-positive and NET-positive appositions indicated that NET-positive are denser than ChAT-positive apposition on the apical dendrites of abGCs and that learning increased NET-positive apposition whose density then reached a level similar to the of ChAT-positive ones (Figure 2 A). On the basal side, ChAT-positive and NET-positive appeared of similar density but learning increased NET-positive apposition density to a level significantly higher than that of ChAT-positive ones (Figure 2 B). Normalized data for each category of apposition help visualizing the fact that the effect of learning is strikingly stronger on NET-positive than on ChAT positive appositions onto abGCs (Figure 2, C and D).

Although preliminary (data regarding how many of these apposition express synaptic marker is missing), these data strongly suggest that the two neuromodulatory system have distinct temporal and spatial pattern of development onto abGCs and importantly that they are not influenced in the same way by learning. Here, in the perceptual learning task, only the LC system is affected. This is in accordance with the lack of effect of pharmacological action of the cholinergic system on this task (Vinera et al 2015). Whether another olfactory task such as an associative conditioning would trigger plastic changes of the contacts made by the cholinergic fibers in the OB is unknown but certainly deserves further investigation.

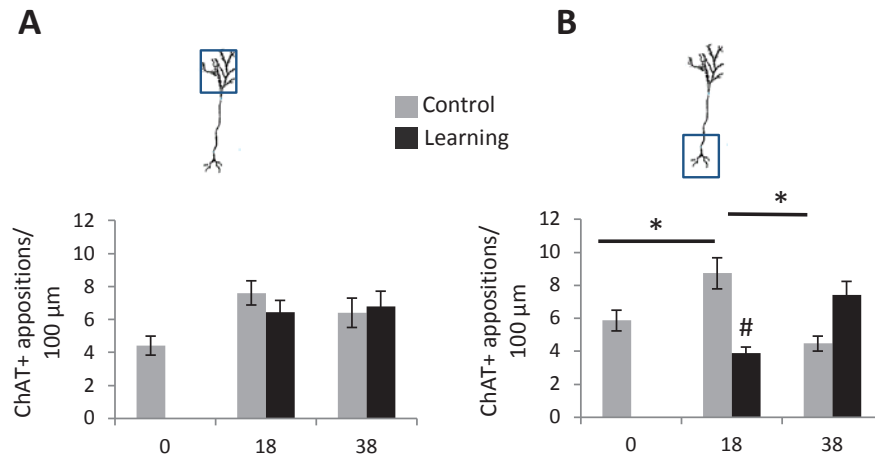


Figure 1: Cholinergic-positive profile density on abGCs and effect of learning. Analysis were performed before (D0) and after (D18 and D38) perceptual learning. Density of ChAT-positive appositions on apical (A) and basal (B) dendritic compartments. On the basal dendrites, the density of ChAT-positive appositions dendrites increased on D18 and then decreased on D38 (Kruskal-Wallis, $p=0.001$: D0 vs. D18, $p=0.049$; D18 vs. D38, $p=0.0009$). * $p<0,05$ for difference between control groups; #: $p<0,05$ for difference between enriched and corresponding control group.

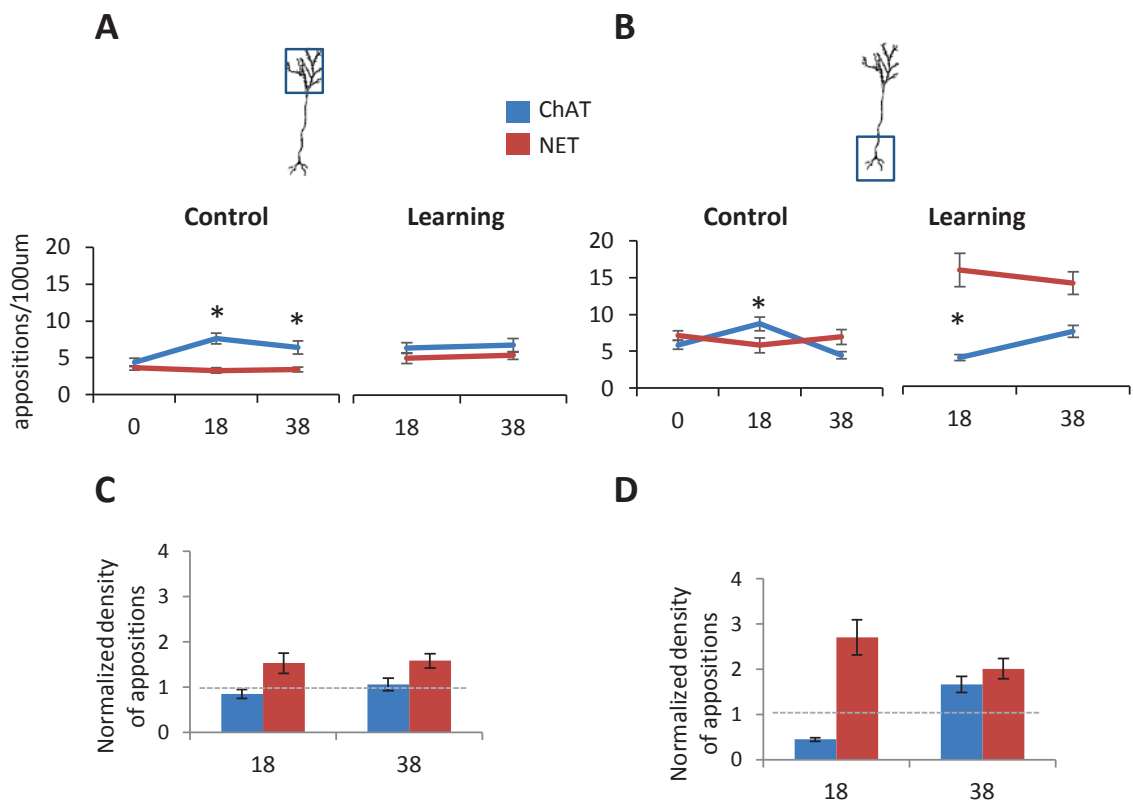


Figure 2: Comparisons of the densities of ChAT-positive and NET-positive appositions on abGCs and effect of learning. **A** and **B**, density of appositions on abGCs apical (**A**) and basal (**B**) domains at D0 (before enrichment) or at D18 and D38 post enrichment (See figure 1, article in preparation for details on the protocol). Kruskal Wallis for controls NET vs ChAT, apical, $p < 0,0001$; basal, $p = 0,003$, *: $p < 0,05$ for paired comparisons NET versus ChAT. **C** and **D**: effect of learning at D18 and D38 shown after normalization of ChAT-positive and NET-positive appositions to each corresponding control (no learning) group. Significant changes were found on the basal dendritic domain with increased density for NET-positive appositions and decreased density for ChAT-positive (see Figure 1, this chapter, for statistics)

9. DISCUSSION AND PERSPECTIVES

In present thesis, we report the pattern of LC inputs onto abGCs during their maturation and we provide evidence for structural plasticity of LC fibers in the OB. Indeed, we report that the pattern of innervation by LC fibers of abGCs is highly affected by olfactory perceptual learning. These findings have several important implications. First, it contributes to the elucidation of the cellular basis of the unique part played by LC innervation in olfactory perceptual learning. Second, it provides an original evidence for pre-synaptic axonal plasticity of LC fibers under physiological learning conditions. Third, learning-induced structural plasticity of the LC innervation occurs on adult-born, but not on pre-existing neurons. This straightens out the distinctive role of adult-born neurons in perceptual learning. By analyzing the structural plasticity of abGCs themselves in response to learning, we also found that they respond differently from pre-existing neurons to learning in term of structural plasticity. More specifically, changes in spine density of abGCs, but not of pre-existing neurons at least partly mirrored LC fiber remodeling. These changes occur during a critical period of adult-born cell development (from 8 to 45 days after cell birth) which provides a time window for LC system to modulate morphology and function of adult-born neurons. Moreover, inhibiting LC fibers activity when the animals explore the learned odor is sufficient to block the learned discrimination, indicating the precise timing of the influence of the LC system on expression of learned discrimination and emphasizes the functional importance of the plasticity revealed by our experiment. Finally, cholinergic innervation onto adult-born neurons shows a different pattern from the one of the LC system, enlightening the disparate roles of these two systems on adult-born neurons under learning.

9.1 THE PATTERN OF ABGCS NEURONS INNERVATION BY LC FIBERS

To our knowledge, our data are the first reporting on the developmental pattern of LC innervation on adult-born neurons. Based on the 3D view, close appositions between NET-positive fibers and GFP-positive adult-born neurons were measured on neurons of three different ages (8, 25 and 45 days old). We found no change of NET-positive appositions with

time neither on the basal nor on the apical dendritic domains. However, the density of appositions was higher on basal dendrites compared to apical. This is in line with previous description of LC terminals in the OB being dominantly distributed in the granule cell layer and internal plexiform layer, with much less in the external plexiform layer (Shiple *et al.*, 1985; McLean *et al.*, 1989). Using synaptophysin, a protein expressed by synaptic vesicles, as a pre-synaptic marker, combined to immunohistochemistry of GFP and NET, we found that in very young adult-born cells (8 days old), none of the physical appositions seemed to form synaptic contacts between LC fibers and adult-born neurons on apical dendrites, while a few of them were present on the basal dendrites. These results suggest that synapses between LC terminals and adult-born neurons first contact basal dendrites around 8 dpi. This timing is consistent with that of the formation of glutamatergic inputs on the basal dendritic pole preceding the appearance of inputs (GABAergic) on the apical dendrites (Whitman & Greer, 2007b; Kelsch *et al.*, 2008). Altogether, this reinforced the precocious establishment of basal inputs on abGCs as a general feature of their development. This timing offers the opportunity to LC fibers to influence abGCs development as early as they reach the position in the OB. On 25 dpi, both basal and apical dendrites receive considerable synaptic LC innervation, which remains at the same level up to at least 45 dpi. One can propose that the development of LC innervation on adult-born neurons is accomplished on 25 dpi and remain stable afterwards, at least to 45 dpi.

9.2 LC FIBERS ACTIVITY DO MATTER IN LEARNED OLFACTORY DISCRIMINATION

A previous study revealed that pharmacological blockade of NA inputs (labetalol, $\alpha 1$ and β antagonist administration) impaired discrimination learning, while enhanced NA release (dexefaroxan, $\alpha 2$ antagonist administration) enhanced the discrimination between perceptually similar odorants, mimicking the effect of perceptual learning (Moreno *et al.*, 2012). These pharmacological results revealed the crucial role of LC activity in the acquisition of olfactory discrimination learning. However, the NA dependency of the expression of perceptual learning could not be easily tested with these approaches. Indeed, pharmacological manipulations did not allow exploring the need for LC fiber activity over a short, behaviorally defined period, such as during discrimination testing. To investigate the role of LC system on learned discrimination in a precisely controlled manner, we used an

optogenetic manipulation. Through optogenetic manipulation, we could inhibit the release of NA from LC- terminals selectively in the OB, and only when the mice were exploring the learned odorants during the test trial of the habituation/dishabituation task. This precise manipulation showed us that normal activity of LC fibers in the OB is required for the discrimination of the learned odorants. Indeed, optogenetic inhibition of NA release completely abolished learned discrimination in the group injected with virus encoding Halorhodopsin, while the control group showed intact discrimination. Implication of co-transmitters released with NA by LC fibers cannot be ruled out in our experiment (Schwarz & Luo, 2015). At any rate, the finding presented here demonstrates the key role of LC-to-OB projections in the expression of learned discrimination.

Moreover, our experiment reveals that the optogenetic inhibition of LC fibers selectively inactivated adult-born granule cells. The reason why granule cells would be inhibited when reducing LC fibers activity can be envisioned based on our knowledge about noradrenergic synapses in the OB. Indeed, at high concentration, NA excites granule cells through $\alpha 1$ receptors (Nai *et al.*, 2009). At lower concentration, the low affinity $\alpha 2$ inhibitory receptors dominate the action of NA (Devore & Linster, 2012). Consistent with this, inhibitory optogenetic stimulation of LC fibers which theoretically reduces NA release would result in prominent activation of inhibitory $\alpha 2$ receptors. This interplay could be at least partly responsible for the observed inhibition of granule cells by light stimulation. The reason why abGCs, and not pre-existing cells were the target of this effect of light stimulation can be speculated based on the present finding. Due to the fact that innervation from LC fibers on abGCs is greatly increased after learning and becomes denser than on pre-existing neurons, abGCs are likely to be exceptionally sensitive to change in NA concentration. However, this remained to be further substantiated.

There are some technical limitations to the optogenetic experiment we conducted. First, the breadth of changes in NA concentration induced by the light-stimulation is unknown. Actual concentrations of NA before and after light stimulation could be recorded by the microdialysis technique, even though, its use is complicated by the small size of the OB in mice. Measuring actual concentration of NA thus remains a technical challenge.

Another technical limitation should also be considered. Based on the laser strength we used in this experiment and the physical features of rodent brain, it cannot be excluded that the

light reaches neighboring structures outside the OB. Observations in the brain of animals injected in the LC with the Halorhodopsine-EYFP expressing lentivirus revealed NET-positive fibers expressing Halorhodopsin in the accessory olfactory bulb and in cortex areas situated near the OB. Turning the light on in the OB may thus possibly affect LC fibers in these neighboring structures. Therefore, it cannot be excluded that a component of the effect of optogenetic manipulation on learned discrimination arose from inhibition of LC fibers in adjacent brain regions. However, we do not favor this hypothesis since light may not keep enough power to significantly activate distant fibers as it spreads over some distances from the source. Technical improvements are needed in order to be able to measure the light power decay around a brain area targeted by optogenetic stimulation.

9.3 PERCEPTUAL LEARNING ENHANCES LC SYNAPTIC INPUTS ONTO ADULT-BORN NEURONS

After shedding light on the crucial role of LC fibers on adult-born neurons to support learned discrimination, we investigated the development of LC innervation during the maturation of abGCs under perceptive learning. Perceptive learning led to an increase in the density of synaptic contacts between LC fibers and abGCs in basal and apical dendritic domains on D18. This increase was long-lasting, until at least D38, is in line with the behavioral results showing that the animals were still able to discriminate the learned odorants at this delay. It could be interesting to study an additional later delay at which the learning would be forgotten. Our prediction is that by then, LC fiber contact density may be returned to control level.

An important issue regarding the increase in synaptic contacts from LC fibers onto abGCs is to find out whether they derive from functional maturation of pre-existing appositions or if they are entirely *de novo* contacts. Our data give some insight onto this question. The changes in density of synaptic contacts between LC fibers and abGCs follow the evolution of the physical appositions. Given that at D18 and D38 the percentages of synaptic contacts among appositions were similar between control and learning groups, one may argue that learning mainly induced the formation of new appositions, a constant proportion of which expressed synaptic markers. The observed effect would thus be caused by profound remodeling of LC fiber connections onto abGCs, instead of a transformation into a functional synaptic connection of already present physically close, non-synaptic appositions.

For the most part, the action of NA in learning and memory is thought to be exerted through direct modulation of activity or functional plasticity of the neurons targeted by LC fibers. Indirect action through acetylcholine release (or interactions with other neurotransmitters) has also been proposed (Marien *et al.*, 2004). Importantly, our finding reveals a novel mechanism underlying the influence of LC system on learning involving LC pre-synaptic plasticity in response to learning. So far, in the brain, axonal sprouting of LC fibers was demonstrated only as a post-lesion compensatory phenomenon (Marien *et al.*, 2004).

Our results rely on the use of synaptophysin, a protein expressed by synaptic vesicles, as a pre-synaptic marker, combined to immunohistochemistry of GFP in abGCs and of NET in LC fibers. Due to the limits in the sensitivity of immunohistochemical labeling, we may have underestimate the number of synaptic contacts between LC fibers and abGCs. In addition, we did not label the post-synaptic compartment facing the synaptophysin-positive appositions. Therefore, we could not really make the distinction between synaptic and non-synaptic NA releasing sites. Indeed, the NA system is known to exhibit varicosities making “en passant” contacts, lacking the specialized synaptic structures, suggesting a paracrine action for NA (Marien *et al.*, 2004). Our approach does not allow finely investigating this specific aspect of the LC system morphological organization. Due to difficulty inherent to multiple immunohistochemical labeling, a promising way to distinguished between conventional synaptic structures and varicosities would be to use a virus encoding a post-synaptic protein tagged by GFP, allowing direct visualization of the post-synaptic protein (Kelsch *et al.*, 2008; Lepousez *et al.*, 2014).

9.4 PERCEPTIVE LEARNING INCREASES SPINE DENSITY OF ADULT-BORN NEURONS

Since we found that perceptual learning increased synaptic contacts of LC-NE fibers onto adult-born neurons, we then explored if this enhancement of inputs on adult-born neurons was accompanied by an increase in spine density. Indeed, spines receive majority of synapses in adult-born neurons. We found that spine densities were increased markedly in the learning group, both on basal and apical dendritic domains. This increase lasted at least until D38, which is consistent in the behavioral expression and the pattern of LC inputs on the

adult-born neurons. This finding suggest that additional LC contacts on adult-born neurons are made on abGCs spines rather than dendritic shaft for instance, even though the latter cannot be excluded considering that the increased spine density may be overcome by the increase in LC contacts, particularly on the basal dendritic domain.

Structural plasticity has been shown to be required for perceptual learning (Daroles *et al.*, 2016). Denser spines in adult-born neurons thus at least partially encode the learned in our behavioral model and store it at least for 28 days after learning.

Our finding is partially consistent with a previous work in which reinforced odor learning increased spine density in distal, proximal and basal dendritic domain of abGCs (Lepousez *et al.*, 2014). However, in this study, no change on the apical side was reported. While further investigations will clarify this point, it should be emphasized that the task used are quite different (perceptual implicit, versus reinforced explicit learning). Different tasks probably require different types of modulation of the odor representation and may thus involve distinct modes of plasticity (Mandairon *et al.*, 2011).

Our findings are also consistent with those obtained on adult-born neurons in the hippocampus. Indeed, spatial learning massively increases the number of dendritic spines of adult-born neurons in the dentate gyrus, accompanied by increased spine width and spine length (Tronel *et al.*, 2010).

The brain bases its function on billions of neurons through trillions of synapses. Spines are the main fundamental material supporting these synaptic connections. It has been postulated that dendritic spines underlie a storage locus for synaptic strength, allowing both short-term and long-term memories embedding in relatively stable substrates. Moreover, activity-dependent alteration in spine number and morphology may be directly related with the changes of synaptic transmission. Spine density changes in response to sensory experience or environmental changes have been extensively studied. For instance, neuronal spine density evolves in response to light deprivation of stimulation during the development of the visual system (Valverde, 1971; Parnavelas *et al.*, 1973). In adults also, many example can be given of structural neuronal plasticity. Spine density modulations have been related to singing in adult birds (Rausch & Scheich, 1982), to the retention of an associative task in chick (Patel *et al.*, 1988), or environmental conditions in hibernating squirrels in which the

number of spines in hippocampal CA3 pyramidal neurons massively but reversibly decreases in winter (Popov & Bocharova, 1992; Popov *et al.*, 1992). Environmental enrichment also increased dendritic spine density (Rampon *et al.*, 2000; Johansson & Belichenko, 2002).

In this context, it is of particular interest that fully mature neurons, those that were born during ontogenesis did not show much learning-induced structural plasticity in our model. They showed no increase in dendritic spine density and were not targeted by more LC contacts after learning compared to control. So, in the OB, pre-existing granule cells seem to be structurally very stable compared not only to their adult-born counterparts; but also compared to other neurons born during ontogenesis elsewhere in the brain. Whether this applies to other OB cell populations such as mitral cells for instance is unknown. It is also possible that even if the total spine density is not affected by learning in pre-existing neurons, the proportion of the morphological subtypes of spines reflecting different maturation states (Caroni *et al.*, 2012) may be affected by learning in pre-existing and adult-born neurons. This has been reported in the dentate gyrus for adult-born neurons showing more mushroom-shaped spines than thin spines after learning (Tronel *et al.*, 2010). Some of our data are currently being analyzed in order to get more information on this aspect.

Following lentivirus injection in the SVZ, some labeled adult-born neuroblasts can continue to leave the SVZ and migrate to the OB for a few weeks (Alonso *et al.*, 2012). We cannot thus eliminate the possibility that some neurons that we analyzed might have arrived in the OB during learning and did not go through the whole 10-day-learning period. In some extreme cases, the analyzed neuron might even arrive in the OB after learning. However, since we analyzed the neurons at relatively short delays after learning, the number of neurons present in the OB and born after learning is likely to remain low. Moreover, the few neurons encountered which showed a very immature morphology were not included in the analysis. At any rate, this bias would lead to an underestimate of the number of LC contacts since younger neurons exhibit less of them and thus to an underestimate of the effect we report. An improved technic might be to give BrdU injection the same day of virus injection in the SVZ. Therefore, we could make sure to analyze neurons born the same day.

9.5 LC SYSTEM PREFERENTIALLY ACTS ON ADULT-BORN NEURONS IN LEARNING

Many differences between early-born pre-existing granule cells and abGCs have been revealed regarding maturation, lifespan and location in the OB (Lemasson *et al.*, 2005; Kelsch *et al.*, 2008). However, very few studies have addressed the different role of these two populations of granule cells in olfactory-guided behaviors or learning, or the distinct effect of olfactory experience on them. In this thesis, newborn mice were injected with lentivirus in the ventricle and exposed to the behavioral experiments after they grew to adulthood. Our original finding indicates that experience shapes very differently pre-existing and adult-born cells. First, in contrast to abGCs, early-born neurons show no differences of LC innervation between learning and control group, neither at D18, nor D38, in line with the lack of change in spine density. Second, learning provides abGCs with a much higher LC innervation than that of pre-existing neurons on the basal dendrites. These results suggest that LC plasticity induced by learning selectively occurs on abGCs. This suggests very specific mechanisms attracting or stabilizing LC contacts on abGCs that remained to be explored. NA itself or neurotransmitters co-released with NA may play a part as well as the abGCs themselves, through increased excitability or activity. Indeed, adult-born neurons go through special period called “critical period”, during which they show specific sensitivity to sensory inputs (Petreanu & Alvarez-Buylla, 2002; Yamaguchi & Mori, 2005; Mouret *et al.*, 2008; Lepousez *et al.*, 2014). Interestingly, learning decreased the spine density of pre-existing neurons on the basal dendrites, suggesting the intriguing possibility that LC fibers may re orient from pre-existing granule cells toward abGCs. However, this might be difficult to further investigate with current state-of-the-art techniques.

Early-born and adult-born granular neurons differ in their location in the granule cell layer and their main interacting cell types. Soma of early-born neuron mainly situates in the superficial region, whereas the majority of adult-born neurons are sitting in the deep granule cell layer (Lemasson *et al.*, 2005; Imayoshi *et al.*, 2008). Moreover, most superficial granule cells extend their apical dendritic arbor to the superficial EPL where tufted cells mainly ramify, whereas, apical dendrites of deep granule cells are mostly restricted in the deep EPL where secondary dendrites of the mitral cells predominantly ramify in deep EPL (Orona *et al.*, 1983; Orona *et al.*, 1984; Mori, 1987). It has been known that mitral cells and tufted cells are distinctive in their response to odorants and to odorant concentration. They also have different projection patterns to the olfactory cortices (Scott *et al.*, 1980; Nagayama *et al.*,

2004). Therefore, these two populations reacting differently to learning and having distinct connection to higher projection areas might play dissimilar role in olfactory behaviors.

9.6 COMPARISON BETWEEN THE NORADRENERGIC AND THE CHOLINERGIC SYSTEM

Although preliminary, our data suggest that both the developmental pattern and the effect of learning is different for the two neuromodulatory systems. In the task used in our study, the cholinergic contact on abGCs did not seem to be affected. This may suggest that the present task solicits and relies on the noradrenergic system more than on the cholinergic system in line with previous pharmacological studies (Vinera *et al.*, 2015). Nevertheless, the cholinergic function might be affected. The modification of the noradrenergic load on abGCs may affect responses to acetylcholine in abGCs due to changes in excitability or to direct interaction of NA with cholinergic fibers who often bear noradrenergic receptors (Marien *et al.*, 2004). The modulation of the NA load by learning may thus affect the balance between centrifugal inputs on abGCs. This type of questions could probably be addressed by *in vitro* slice electrophysiology.

Finally, as neurogenic response has been shown to be task specific, other olfactory learning task, such as an associative task, may induce cholinergic fiber pre-synaptic plasticity as perceptual learning did for the LC fibers. This remains an open question.

CONCLUSION

Our work provides the first evidence that learning can trigger remodeling of LC fibers inputs. Importantly, our finding also showed that this plasticity is specific for adult-born neurons.

Our finding are original since previous evidence for pre-synaptic plasticity of adult-born neurons reported in the OB (Lepousez *et al.*, 2014) and the dentate gyrus of the hippocampus (Bergami *et al.*, 2015) regarded cortical feed-back on adult-born neurons but no comparison with pre-existing neurons was provided.

NA system plays various even opposite role on the activity of granule cells depending on the

concentration of NA and the activation of subtypes of noradrenergic receptors. Therefore, it would be exciting to investigate the response and distribution pattern of these receptor subtypes during the maturation of adult-born neuron in perceptive learning. Will learning preferentially build the contacts with specific receptor subtypes? Will learning change the distribution of these receptors on the adult-born neurons which are recruited in the learning? Moreover, we found that LC plasticity occurs on the adult-born neurons, but not the pre-existing neurons. What is the cellular mechanism underlying this phenomenon? Are noradrenergic receptors distributed differently between these two populations of granule cells? All these important questions remain to be explored.

Another interesting issue raised from this thesis relates to the notion of critical period and to what makes this period critical. A period from 14 to 28 days after cell birth was revealed as essential for survival of abGCs (Yamaguchi & Mori, 2005). Learning can save abGCs during this time window while suppressing slightly older adult-born neurons (Mandairon *et al.*, 2006b; Mouret *et al.*, 2008). We showed here also that structural plasticity is induced during this critical period. What cellular factors do influence survival and structural plasticity of adult-born during this time window? Does learning induced LC fiber plasticity that was revealed in our work, contribute to survival and plasticity of adult-born neurons? NA has neuro-protective effects (Marien *et al.*, 2004), and even more specifically, studies in our lab revealed that NA promotes survival of adult-born neurons (Veyrac *et al.*, 2007; Moreno *et al.*, 2009; Veyrac *et al.*, 2009). Future studies using optogenetic to promote NA release and subsequent analysis of adult-born survival rate and morphology should help in going further on this track and progressively solve some of the questions about the precise role of LC system on the olfactory learning and adult neurogenesis.

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