Mécanismes neuronaux sous-tendant l'apprentissage perceptif olfactif chez la souris adulte

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TITRE :

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Abréviations

AMPA	Alpha-amino-3-hydroxy-5-méthylisoazol-4-propionate
AraC	Cytosine Arabinoside
BO	Bulbe olfactif
BDNF	Brain derived neurotrophic factor
BrDU	5-Bromo-2-déoxyuridine
DBH	Dopamine beta-hydroxylase
GABA	Acide gamma-aminobutyrique
Gad65/67	1-Glutamic acid decarboxylase
GFP	Green Fluorescence Protein
GL	Couche glomérulaire
GrL	Couche granulaire
LC	Locus Coeruleus
LTP	Potentialisation à long terme
NET	Transporteur de la noradrénaline
NMDA	N-Méthyl-D-aspartate
ZSV	Zone sous ventriculaire
2-DG	2-DéoxyD-glucose
5-HT	5-Hydroxytryptamine ou sérotonine

RESUME

Le bulbe olfactif est le siège d'une neurogenèse adulte importante et est une structure clé des apprentissages olfactifs. Nous nous sommes intéressés au rôle de cet apport permanent de nouveaux interneurones inhibiteurs dans un type d'apprentissage particulier qu'est l'apprentissage perceptif. Cet apprentissage, très écologique pour l'animal reflète un processus permanent d'assimilation de l'environnement sensoriel. C'est un apprentissage de type implicite qui se définit comme une amélioration des performances de discrimination suite à une exposition répétée du stimulus.

Dans une première étude, nous avons trouvé que l'apprentissage perceptif augmentait non seulement la survie des neurones granulaires néoformés mais grâce au blocage de la neurogenèse nous avons également mis en évidence le caractère indispensable des nouveaux neurones à l'acquisition de cet apprentissage. Cette augmentation de la neurogenèse a pour conséquence une augmentation du niveau d'inhibition dans le bulbe olfactif à l'origine de l'amélioration des performances de discrimination.

Les mécanismes gouvernant l'intégration et la survie des neurones néoformés pendant l'apprentissage étant très mal connus, nous avons fait l'hypothèse d'un rôle de la noradrénaline dans le contrôle de l'intégration des nouveaux neurones car elle se projette massivement sur les interneurones bulbaires et est très impliquée dans les apprentissages olfactifs. Dans une deuxième étude, nous avons trouvé que la manipulation directe de la transmission noradrénergique affectait significativement la survie des nouveaux neurones et les performances dans l'apprentissage perceptif. En effet, l'augmentation de la libération de noradrénaline conduit à une amélioration des performances de discrimination ainsi qu'à une augmentation de la survie des néo-neurones. Inversement, le blocage des récepteurs noradrénergiques altère l'amélioration des performances olfactives et n'augmente pas la neurogenèse. Finalement, nous avons montré que les fibres noradrénergiques se projetaient sur les nouveaux neurones qui répondaient à la noradrénaline et que la présence des nouveaux neurones était indispensable pour que la noradrénaline puisse améliorer les performances de discrimination.

Enfin, dans une troisième étude nous nous sommes intéressés à l'effet du vieillissement sur l'apprentissage perceptif. Nous avons trouvé un défaut d'apprentissage perceptif chez la souris âgée associé à une diminution de la neurogenèse. En revanche, une stimulation noradrénergique permet de restaurer l'apprentissage perceptif sans moduler la neurogenèse bulbaire suggérant l'existence de mécanismes compensatoires.

Ces travaux ont permis de mieux comprendre le rôle des nouveaux neurones dans le cerveau adulte, comment ils sont recrutés et comment ils agissent sur le réseau préexistant pour moduler le comportement.

Préambule : de la découverte de la neurogenèse adulte à l'étude au rôle des néo neurones dans l'apprentissage olfactif

Nous interagissons de façon permanente avec notre environnement. En effet, des stimuli sensoriels sollicitent nos sens : la vision, l'odorat, le goût, l'ouïe et le toucher influençant ainsi notre comportement. Parmi nos 5 sens, l'olfaction, pourtant bien souvent négligée, est très importante. En effet, elle est essentielle au comportement de recherche et de prise de nourriture mais également à l'évitement des dangers comme par exemple des incendies ou des prédateurs et enfin à la reproduction. Les odeurs guident ainsi notre comportement pour assurer les besoins essentiels de survie et les plaisirs.

Pour analyser ces milliers d'odeurs que nous percevons tous les jours nous disposons d'un système olfactif capable de détecter, identifier et discriminer les odeurs. Le traitement sensoriel des odeurs est un processus dynamique tout au long de notre vie et qui nécessite des apprentissages et donc met en jeu des mécanismes de plasticité cérébrale. Il existe dans le système olfactif une forme particulière de plasticité appelée neurogenèse adulte et qui consiste en l'apport permanent de nouveaux neurones dans le circuit neuronal préexistant.

En 1906, le neurobiologiste espagnol Santiago Ramon y Cajal (1852 – 1934) obtient le prix Nobel pour ses travaux sur l'histologie du cerveau. Un des nombreux postulats qu'il a formulé affirme que le cerveau adulte est un organe incapable de se régénérer: '*Une fois le développement terminé, la pousse et la régénération axonale et dendritique s'arrêtent de manière irrévocable. Dans le système nerveux central adulte, le réseau nerveux est fixe, terminé et immuable'*. Ainsi, à la naissance, nous disposons d'un stock de neurones qui établissent entre eux de nouvelles connexions mais il n'y aurait pas d'apparition de nouveaux neurones chez l'adulte. De part la très grande influence de Ramón y Cajal en neurosciences, cet énoncé s'érigea en dogme. Cependant, depuis les années 60, beaucoup de certitudes concernant la neurogenèse se sont effondrées. En effet, tout d'abord, un chercheur américain, Joseph Altman, décrivit grâce à l'injection de thymidine tritiée, des cellules en division ayant une morphologie de neurone dans le cortex, le bulbe olfactif et le gyrus denté de rat adulte (Altman and Das, 1965). Dans les années 80 et grâce à la microscopie électronique, Michel Kaplan rapporte des données allant dans ce sens en montrant des cellules nouvellement formées très ressemblantes aux cellules voisines dans le cerveau de rat et de souris (Kaplan and Hinds, 1977). Fernando Nottebohm a également contesté ce dogme en montrant de la neurogenèse adulte mais cette fois-ci chez l'oiseau (Goldman and Nottebohm, 1983). En effet, il met en évidence l'arrivée de nouveaux neurones dans une région cérébrale importante pour la production du chant (Goldman and Nottebohm, 1983). Parce que ces découvertes, et particulièrement chez le

mammifère, ont été longtemps contredites par Pasko Rakic, neuroscientifique très influent à l'époque (Rakic, 1985), il a fallu attendre les années 90 et les travaux de Brent Reynolds et Samuel Weiss pour confirmer l'existence d'une neurogenèse. En effet, ces chercheurs ont mis en évidence des cellules souches dans le cerveau adulte (Reynolds and Weiss, 1992). C'est à la fin des années 90 qu'Elizabeth Gould, Fred Gage et Peter Eriksson publièrent une série de papiers qui ont initié une explosion de la recherche sur l'existence et la fonction de la neurogenèse adulte chez le mammifère adulte (Gould and Gross, 2002).

Cette neurogenèse a été décrite chez de nombreuses espèces comme le rongeur (Corotto et al., 1993; Kuhn et al., 1996; Lois and Alvarez-Buylla, 1994; van Praag et al., 2002) mais encore le primate (Gould et al., 1997, 1999; Kornack and Rakic, 1999) et enfin chez l'homme dans l'hippocampe (Eriksson et al., 1998) et dans le bulbe olfactif (Curtis et al., 2007; Sanai et al., 2004) bien qu'une étude récente suggère qu'elle soit limitée (Bergmann et al., 2012). D'autres niches neurogéniques ont été trouvées dans le cerveau comme au sein du complexe vagal dorsal du tronc cérébral (Bauer et al., 2005; Moyse et al., 2006), dans le cortex piriforme (Seki and Arai, 1991), l'amygdale (Bernier et al., 2002), l'hypothalamus (Huang and Sato, 1998; Kokoeva et al., 2005) ou encore le néocortex (Ehninger and Kempermann, 2003; Gould, 2007; Kornack and Rakic, 2001). Quoiqu'il en soit, la neurogenèse adulte dans ces structures, lorsqu'elle n'est pas remise en question, est observée à un taux beaucoup plus faible.

Ainsi, au terme de ces années de recherche, la communauté neuroscientifique s'accorde à dire que les deux sites principaux de neurogenèse adulte sont l'hippocampe et le bulbe olfactif. Concernant l'hippocampe, les cellules souches se situent dans la zone sous granulaire du gyrus denté (Figure 1) et les neuroblastes issus de ces cellules souches vont migrer au sein même de la couche granulaire pour donner des cellules granulaires néo-formées. La neurogenèse bulbaire quant à elle est issue de cellules souches qui prolifèrent au sein de la zone sous-ventriculaire des ventricules latéraux. Ces cellules souches vont donner des neuroblastes qui migrent sur une longue distance le long du flux rostral migratoire jusqu'au bulbe olfactif (Figure 1).

La neurogenèse adulte, même restreinte dans le cerveau de mammifère semble fortement impliquée dans les processus d'apprentissage et de mémoire, aussi bien dans l'hippocampe (Glasper et al., 2012) que dans le bulbe olfactif (Lazarini and Lledo, 2011). Elle constituerait un processus de plasticité accrue dans des structures clés de l'apprentissage et de la mémoire en permettant l'intégration des neurones dans un réseau préexistant complexe. Mon travail s'est plus particulièrement intéressé à la neurogenèse dans le système olfactif et à son rôle dans l'apprentissage olfactif. La suite du document présente donc l'organisation anatomo-fonctionnelle du système olfactif, sa plasticité en lien avec l'expérience sensorielle puis les principaux éléments

de la littérature concernant la neurogenèse olfactive et son implication dans la fonction olfactive. Les résultats sont présentés sous la forme de 3 articles dont 2 sont publiés et un soumis, qui décrivent le rôle de la neurogenèse dans l'apprentissage perceptif, l'implication de la noradrénaline dans la modulation de la neurogenèse nécessaire à l'apprentissage perceptif et enfin le processus de vieillissement des capacités d'apprentissage perceptif et certains de ces mécanismes. L'ensemble de ces résultats est ensuite discuté.

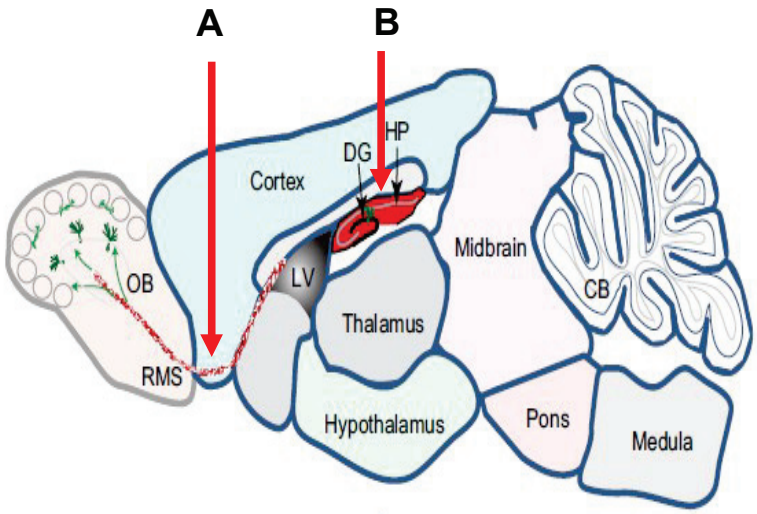

Figure 1 : La neurogenèse adulte bulbaire et hippocampique.
A. La neurogenèse bulbaire débute dans la zone sous ventriculaire des ventricules latéraux (LV) puis les neuroblastes migrent le long du flux rostral migratoire (RMS) pour atteindre le bulbe olfactif (OB). **B**. La neurogenèse adulte a également été mise en évidence dans l'hippocampe (HP) avec des zones de prolifération, migration et différentiation contiguës aboutissant à la formation de nouveaux neurones granulaires dans le gyrus denté (DG). (D'après Ming and Song, 2011).

INTRODUCTION

I/ ORGANISATION ANATOMO-FONCTIONNELLE DU SYSTEME OLFACTIF

La perception olfactive se fait schématiquement en trois étapes. Tout d'abord, un odorant, molécule chimique volatile, va atteindre la cavité nasale grâce à la respiration. Cet odorant va alors se fixer sur les récepteurs olfactifs présents sur les neurones sensoriels de l'épithélium au fond de la cavité nasale. La reconnaissance odorant-récepteur va initier la transduction du message chimique en un message électrique.
Ensuite, via les axones des neurones sensoriels, le message olfactif est acheminé jusqu'au bulbe olfactif, premier relais cérébral de l'information olfactive. Au sein du bulbe olfactif, les axones des neurones sensoriels vont faire synapses avec les dendrites des neurones relais, les cellules mitrales. C'est dans le bulbe olfactif que l'information olfactive va subir un premier traitement avant d'être transmise aux centres olfactifs supérieurs que sont les cortex piriforme et entorhinal, l'amygdale, le tubercule olfactif et le noyau olfactif antérieur.

I. 1. Étage périphérique : l'épithélium olfactif

I. 1. 1. Les neurorécepteurs olfactifs

Le fond de la cavité nasale est tapissé d'un épithélium olfactif sur lequel vont se fixer les molécules odorantes. Cet épithélium olfactif est constitué par les neurones sensoriels olfactifs (les neurorécepteurs), les cellules de soutien et les cellules basales (Figure 2).
Les neurones sensoriels, directement en contact avec le milieu extérieur, sont des cellules bipolaires présentes en grand nombre (12 millions chez l'homme, 20 millions chez la souris, (Breer, 2003; Moran et al., 1982)) et dont la durée de vie est limitée à un mois environ. En effet, elles sont en permanence renouvelées à l'âge adulte grâce aux cellules souches basales (Schwob, 2002). Le rôle des neurones sensoriels dans la perception olfactive est essentiel puisque sur leurs dendrites ciliées sont présents les récepteurs moléculaires aux odeurs. Le message olfactif naît de l'interaction des molécules odorantes avec ces récepteurs olfactifs.

Les récepteurs olfactifs, découverts en 1991 chez le rat par Linda Buck et Richard Axel, sont issus d'une superfamille multigénique répartie sur de nombreux chromosomes et codant des récepteurs à sept domaines transmembranaires couplés aux protéines G (Buck and Axel, 1991).
Ces protéines ont une partie fixe caractéristique et une région hyper-variable. Cette région pourrait correspondre au site de fixation des molécules odorantes d'autant plus que les odorants sont des molécules aux caractéristiques physico-chimiques très variables et diversifiées.

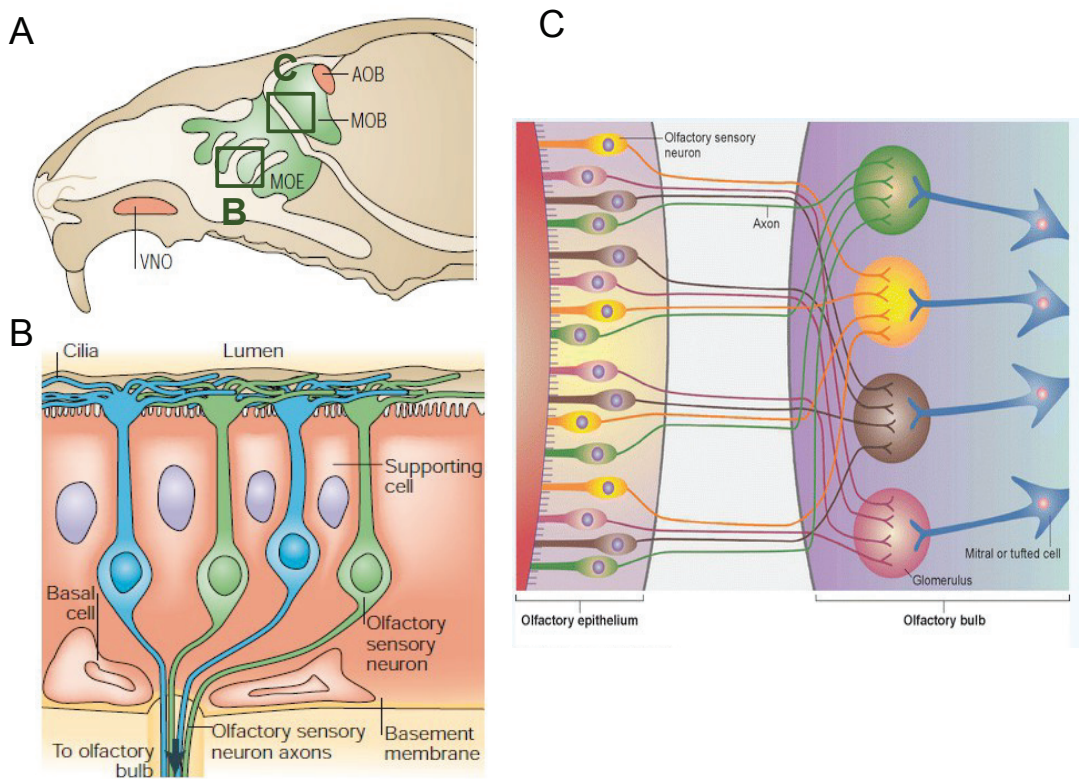

Figure 2 : L'épithélium olfactif et encodage périphérique de l'odeur
A. L'épithélium olfactif (MOE), situé au fond de la cavité nasale, est le siège de la liaison des molécules odorantes. (D'après Mombaerts, 2004). **B.** En effet, les neurones sensoriels situés au sein de l'épithélium olfactif présentent des récepteurs olfactifs sur leurs nombreux cils qui assurent la transduction du signal chimique en signal électrique. L'épithélium olfactif est également constitué de cellules de soutien et de cellules basales qui lui permettent de maintenir son intégrité (D'après Mombaerts, 2004). **C**. Chaque neurone sensoriel n'exprime qu'un seul type de récepteur olfactif et les neurones exprimant un même récepteur se projettent sur 1 à 4 glomérules donnés dans le bulbe olfactif (MOB). Une telle détermination spatiale des projections des neurones sensoriels permet un premier encodage de l'odeur. (D'après Mombaerts, 2006).

Le nombre de récepteurs olfactifs fonctionnels exprimés dans l'épithélium olfactif est actuellement estimé à 390 chez l'homme et 1035 chez la souris (Saito et al., 2009; Zhang and Firestein, 2002). Ce nombre est inférieur au nombre d'odorants pouvant exister et être identifié, le système olfactif met donc en place d'autres mécanismes pour augmenter le nombre d'odeurs discriminées.

Grâce à des techniques de RT-PCR effectuées sur des neurones sensoriels olfactifs de mammifère, il a été montré qu'un seul type de récepteur olfactif s'exprimait par cellule de manière mono-allélique (Chess et al., 1994; Malnic et al., 1999; Touhara et al., 1999). Le fait que le neurone sensoriel présente un seul type de récepteur conditionne le profil de réponse bulbaire puisque les axones d'une population neuronale exprimant un même récepteur olfactif convergent dans le bulbe

olfactif sur une structure appelée glomérule (Mombaerts et al., 1996). Un glomérule est donc innervé par des neurones sensoriels exprimant le même récepteur olfactif ((Treloar et al., 2002), Figure 2).

I. 1. 2. Codage combinatoire de l'odeur par les neurones sensoriels

Le profil de réponse des neurones sensoriels à un odorant va dépendre de l'affinité de la molécule odorante pour le récepteur olfactif. En effet les récepteurs olfactifs ont des spectres de ligands relativement larges (Duchamp-Viret and Duchamp, 1997; Duchamp-Viret et al., 1999; Firestein, 2001; Gesteland et al., 1965; Hallem and Carlson, 2006). Un récepteur olfactif reconnaît donc plusieurs molécules odorantes (Malnic et al., 1999) qui constituent son champ récepteur qui lui est propre. Les caractéristiques des champs récepteurs varient selon les récepteurs olfactifs. Certains récepteurs sont plutôt sélectifs et d'autres généralistes (Araneda et al., 2004; Keller and Vosshall, 2007; Saito et al., 2009). Ainsi chaque odorant active une combinaison donnée de récepteurs olfactifs et donc une population spécifique de neurones sensoriels. Ce processus est désigné sous le nom de « code combinatoire » de l'odorant (Malnic et al., 1999). Grâce à ce « code combinatoire », un plus grand nombre d'odeurs peuvent être discriminées seulement avec quelques centaines de récepteurs olfactifs. L'intensité d'une odeur serait codée par l'augmentation de la fréquence de décharge des neurones sensoriels et par l'augmentation du nombre de neurones sensoriels activés par l'odorant au fur et à mesure que sa concentration augmente, devenant ainsi moins sélectif (Malnic et al., 1999).

I. 2. Le bulbe olfactif : premier relais central de l'information olfactive

I. 2. 1. Organisation histologique et synaptique du bulbe olfactif

Le bulbe olfactif est un paléocortex paire de forme ovale, situé en avant des hémisphères cérébraux chez le rongeurs et reposant sur la lame criblée de l'ethmoïde qui le sépare de la cavité nasale au travers de laquelle passent les axones des neurones sensoriels formant le nerf olfactif (Figure 3).

Le bulbe olfactif comporte sept couches histologiques distinctes et concentriques (Figure 3) décrites ci-dessous de la plus superficielle à la plus profonde :

- La couche la plus périphérique est la couche des nerfs olfactifs provenant de la cavité nasale.
- La couche glomérulaire : les glomérules sont des structures neuropilaires sphériques

dans lesquelles les axones des neurones sensoriels font des synapses excitatrices avec les dendrites des cellules relais du bulbe olfactif que sont les cellules mitrales et les cellules à panache.

Récemment, il a été suggéré que l'activation des cellules mitrales serait produite efficacement aussi et peut-être surtout de manière indirecte par une catégorie de cellules à panache, très facilement activées par l'input sensoriel et capable d'activer à leur tour les cellules mitrales (Gire et al., 2012).
Dans la couche glomérulaire se situent également les interneurones périglomérulaires. Ces interneurones sont présents en plusieurs sous-types selon les neuromédiateurs et/ou neuropeptides qu'ils expriment : GABA, Dopamine, Calbindine, Calrétinine. Ils vont avoir une action inhibitrice sur les cellules mitrales avec lesquelles ils font des synapses dendro-dendritiques.

- La couche plexiforme externe : comportant les dendrites des cellules mitrales et les dendrites des interneurones granulaires avec lesquels elles établissent des synapses réciproques.
- La couche des cellules mitrales : les cellules mitrales, dont les corps cellulaires se situent dans cette couche, sont des neurones de projection glutamergique transmettant l'information des neurones sensoriels reçue par leur dendrite au niveau des couches glomérulaires jusqu'aux différents centres olfactifs supérieurs grâce à leur long axone.
- La couche plexiforme interne : comportant les dendrites des cellules granulaires ainsi que les axones des cellules mitrales.
- La couche granulaire : elle est constituée par l'ensemble des cellules granulaires. Ce sont des inter-neurones inhibiteurs GABAergiques faisant des synapses dendro-dendritiques avec les cellules mitrales dans la couche plexiforme externe, afin d'en réguler l'activité.
- La partie bulbaire du flux rostral migratoire par laquelle arrivent les nouveaux neurones

La transmission verticale du signal olfactif par les cellules mitrales est modulée à deux niveaux du bulbe olfactif: au niveau des entrées sensorielles par les interneurones périglomérulaires et au niveau de la sortie du bulbe olfactif par les interneurones granulaires (Schoppa, 2006; Shepherd et al., 2007).

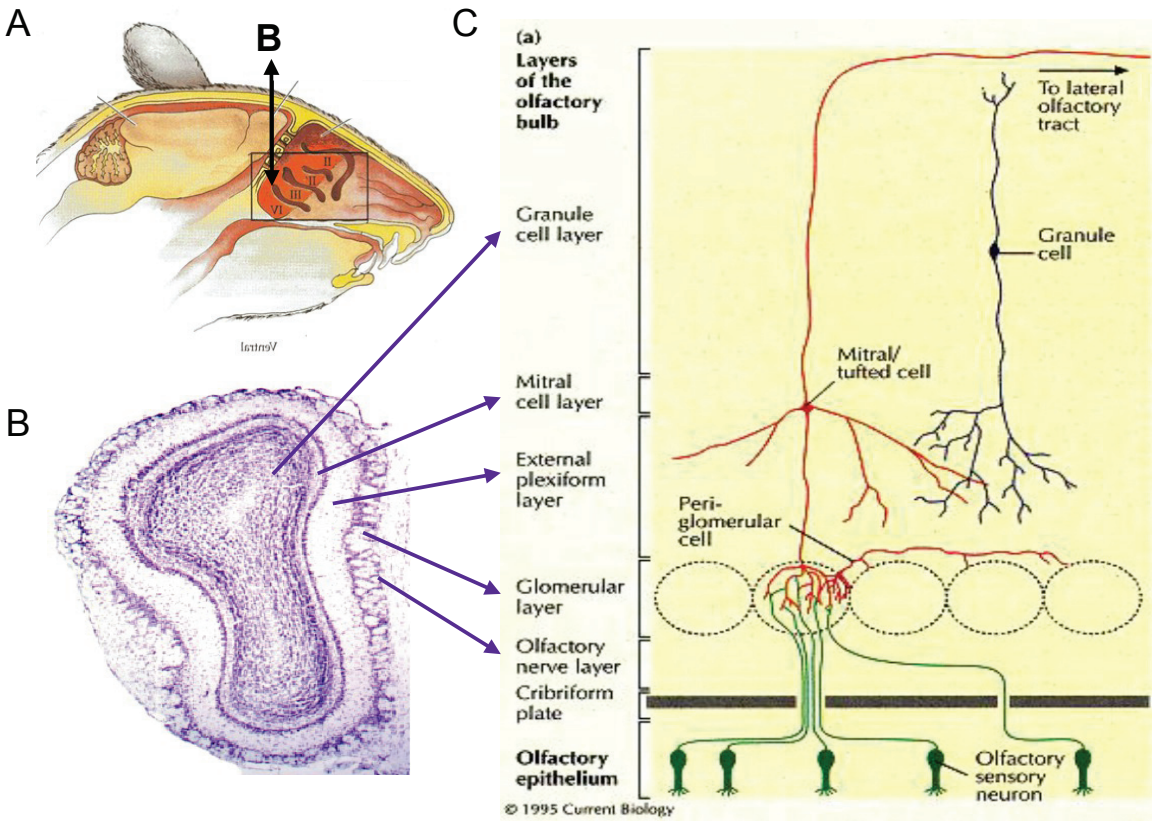

Figure 3 : Le bulbe olfactif et son organisation histologique.
A. Le bulbe olfactif est situé en avant des deux hémisphères cérébraux chez le rongeur, et en arrière de la lame criblée par laquelle passent les axones des neurones sensoriels de la cavité nasale**. B**. Coupe transversale du bulbe olfactif (marqué par un trait en A) montrant après coloration au violet de Crésyl, l'organisation laminaire du bulbe olfactif. **C**. Les axones des neurones sensoriels se projettent sur les glomérules après le franchissement de la lame criblée et font synapses avec les cellules mitrales qui transmettent l'information olfactive vers les centres supérieurs. L'activité des cellules mitrales est modulée à l'entrée par les cellules périglomérulaires et à la sortie par les cellules granulaires. (D'après Efstratiadis,1995).

I. 2. 2. Codage bulbaire de l'odeur

Comme nous l'avons vu, les axones des neurones sensoriels font synapses avec les cellules mitrales au sein des glomérules (Figure 2). Les neurones sensoriels ayant un même récepteur olfactif se projettent en miroir sur un nombre donné de glomérules spécifiques. La stimulation par un odorant va donc activer une combinaison de récepteurs olfactifs qui eux-mêmes vont se projeter et activer une combinaison spécifique de glomérules dans le bulbe (Johnson and Leon, 2007; Murthy, 2011) donnant lieu ainsi à un patron d'activation glomérulaire spécifique et reproductible de l'odeur (Imai et al., 2010; Soucy et al., 2009). Le patron d'activité glomérulaire peut être révélé par différentes techniques comme le 2-déoxyglucose (2DG) (Jourdan et al., 1980; Royet et al., 1987), l'imagerie optique (Friedrich and Korsching, 1997; Vincis et al., 2012) l'analyse de l'expression des gènes précoces comme le Zif 268 (Inaki et al., 2002; Knapska and Kaczmarek, 2004; Mandairon et al., 2006a, 2008a) ou encore l'imagerie par résonance magnétique fonctionnelle

(Schafer et al., 2006; Xu et al., 2003). Pour des odorants naturels susceptibles d'activer plusieurs classes de récepteurs olfactifs, le patron d'activation peut inclure un grand nombre de glomérules (Vincis et al., 2012).

Le pattern spatial d'activité à l'entrée du bulbe olfactif participe au codage de l'odeur. En effet, il a été montré que les cartes d'activation glomérulaire sont spécifiques à chaque odorant et prédictives de la perception de l'odeur (Ho et al., 2006; Linster et al., 2001). Deux odorants ayant de fortes similarités physico-chimiques vont se lier à des récepteurs semblables donnant des patrons d'activation glomérulaire proches rendant les odeurs peu discriminables par l'animal. C'est par exemple le cas des énantiomères du limonène (Figure 4). Les cartes d'activation glomérulaire en 2DG du (+) et (-) limonène sont superposables (http://leonlab.bio.uci.edu). On constate que ces deux odeurs ne sont pas spontanément discriminées par le rat (Mandairon et al., 2006b). Au contraire, des molécules odorantes comme le (+) limonène et le décanal qui évoquent des cartes glomérulaires en 2DG très différentes (Figure 4), sont facilement discriminées spontanément par le rongeur (Mandairon et al., 2006c, 2008a).

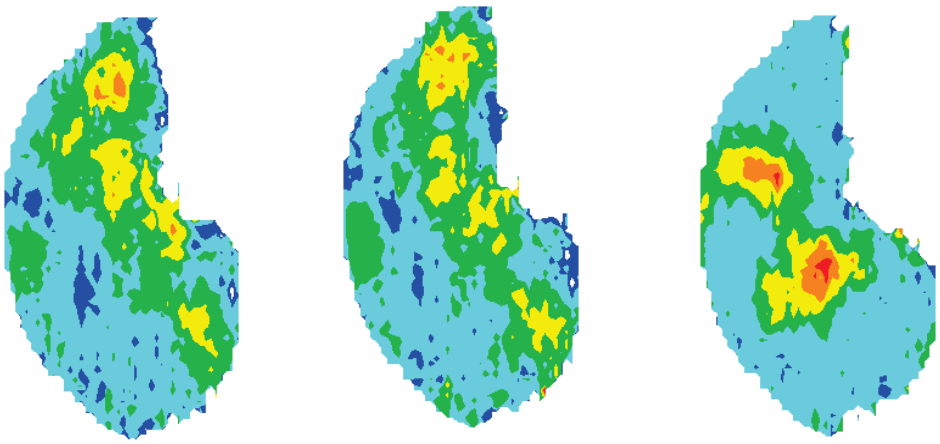

Figure 4 : Les cartes d'activation glomérulaire sont prédictives de la similarité perceptuelle des odorants.
Le marquage au 2DG permet de voir l'activation des glomérules en réponse à une odeur. Les patrons d'activation glomérulaire du + et – limonène sont hautement similaires ce qui correspond à leur similarité perceptuelle. Au contraire les patrons d'activation glomérulaire du limonène et du décanal sont différents et ces deux odeurs sont discriminées spontanément par le rongeur. (D'après http://gara.bio.uci.edu et Linster et al., 2001).

En accord avec la population de glomérules activés, l'enregistrement de la réponse aux odeurs de cellules mitrales chez le lapin montre qu'une odeur donnée active des populations spécifiques de cellules mitrales (Imamura et al., 1992; Katoh et al., 1993). En particulier, chez le rat, il a été montré que ce sont les cellules mitrales proches des glomérules activés par l'odeur (donc potentiellement connectées à ce glomérule) qui sont préférentiellement activées (Buonviso et al., 1991). L'activité glomérulaire serait donc transmise aux cellules mitrales à l'aplomb des glomérules activés suggérant une transmission verticale de l'information olfactive (Figure 3).

Le message olfactif est modulé dans le bulbe par les interneurones périglomérulaires et granulaires. Les cellules périglomérulaires, reçoivent des synapses axo-dendritiques des neurones sensoriels et établissent des synapses dendro-axoniques avec ces mêmes récepteurs. Elles établissent également des synapses dendro-dendritiques avec les cellules mitrales. Elles sont donc activées par les neurones sensoriels afin d'inhiber l'activité des cellules mitrales selon les entrées sensorielles et également inhiber en retour les neurones sensoriels (Ennis et al., 2001). Cette modulation de l'activité des cellules mitrales en fonction des entrées sensorielles contribuerait à affiner la représentation spatiale glomérulaire des odeurs (Murthy, 2011). Les connexions excitatrices au sein du glomérule serviraient à amplifier le signal et l'inhibition intra-glomérulaire serait un mécanisme de contrôle du gain (Murthy, 2011). Les cellules périglomérulaires activées peuvent aussi inhiber l'activité des glomérules voisins des glomérules activés afin de renforcer le contraste d'activité entre chaque glomérule (Olsen et al., 2010; Wilson and Mainen, 2006).
Ainsi la représentation spatiale d'une odeur va être affinée grâce à une augmentation du contraste entre glomérule activé/glomérule inhibé et ce d'autant plus que le signal est faible (Cleland, 2010; Cleland and Sethupathy, 2006). Cet ajustement global du signal olfactif lors de son arrivée dans le bulbe olfactif serait nécessaire à l'identification de l'odeur (Aungst et al., 2003; Cleland and Sethupathy, 2006; Cleland et al., 2007).

Plus en profondeur dans le bulbe olfactif, l'activité des cellules mitrales est également modulée par les cellules granulaires. Ces interneurones GABAergiques ont leurs corps cellulaires dans la couche granulaire et par l'intermédiaire d'une unique dendrite qui se ramifie dans la couche plexiforme externe, ils établissent des synapses réciproques dendro-dendritiques avec les dendrites latérales des cellules mitrales (Figures 3). Les cellules mitrales libèrent du glutamate au niveau de ces synapses réciproques ce qui excite les cellules granulaires par le biais de récepteurs NMDA et AMPA (Chen et al., 2000). En retour les cellules granulaires libèrent du GABA pour inhiber les cellules mitrales (Chen et al., 2000; Schoppa and Urban, 2003; Schoppa et al., 1998).

Les cellules granulaires sont donc activées par l'odeur par l'intermédiaire des cellules mitrales et leur activation va donc également se faire selon un patron d'activation spécifique de

l’odeur (Guthrie et al., 1993; Sallaz and Jourdan, 1993). Cependant, le patron d'activité granulaire pour une odeur donnée est plus diffus que dans la couche glomérulaire (Busto et al., 2009; Mandairon et al., 2006c; Tan et al., 2010) du fait de la projection à longue distance des dendrites latérales des cellules mitrales sur les cellules granulaires. De plus, par sa dendrite ramifiée, une même cellule granulaire va pouvoir établir des synapses avec plusieurs dendrites latérales appartenant à différentes cellules mitrales connectées à des glomérules voisins. Ainsi l'activation descendante d'une cellule mitrale par son glomérule entraînerait l'activation d'un ensemble de cellules granulaires qui inhiberait ensuite les cellules mitrales adjacentes et donc contribuerait à l'inhibition latérale de l'activité des cellules mitrales (Mori and Yoshihara, 1995; Shepherd et al.,2007).
L'inhibition latérale des cellules mitrales par les inter-neurones inhibiteurs granulaires contribue à la mise en forme spatiale du signal olfactif.

L'enregistrement de potentiels de champs locaux montre que le bulbe olfactif est le siège d'activités oscillatoires provenant de la synchronisation de l'activité des neurones bulbaires (Gervais et al., 2007). La fréquence de ces oscillations varie entre 1 et 100 Hz. Il y a trois régimes oscillatoires principaux : une bande d’ondes lentes thêta (entre 1 et 15 Hz), une bande bêta (entre 15 et 40 Hz) et une bande gamma (entre 60 et 90 Hz). Les ondes thêta appelées également oscillations respiratoires coïncident avec le rythme respiratoire. Les ondes bêta sont plus courtes et calées dans 2 à 4 cycles de respiration; elles sont associées à plusieurs types d’apprentissage olfactif et de sensibilisation à l’odeur. Les ondes gamma sont renforcées par un stimulus olfactif et calées dans un seul cycle inspiration-expiration (Buonviso et al., 2003; Kay et al., 2009; Neville and Haberly, 2003). Ces dernières oscillations seraient dues à l’interaction granule-mitrale puisque la perturbation de l'inhibition par les cellules granulaires dans le bulbe olfactif altère ces oscillations (Halabisky and Strowbridge, 2003; Lagier et al., 2004, 2007) contrairement aux oscillations lentes qui proviendraient elles du retour du cortex olfactif (Buonviso et al., 2003; Neville and Haberly, 2003). La synchronisation des cellules mitrales semble importante d'un point de vue perceptif car la perturbation de l'activité oscillatoire par des antagonistes des récepteurs GABAergiques chez l'insecte est associée à une diminution spécifique de la discrimination fine des odeurs similaires (Stopfer et al., 1997; Cenier et al., 2008). Cependant, des études suggèrent également que le taux de décharge des cellules mitrales en réponse à l’odeur, et pas seulement l’organisation temporelle de leurs décharges, est crucial pour le codage de l’identité de l’odeur (Bathellier et al., 2008; Gschwend et al., 2012).

I. 3. Vieillissement du système olfactif

Les pertes structurales dans le bulbe olfactif débutent plus tôt que les déficits sensoriels chez l'homme (Hummel et al., 2007; Kovács, 2004) ce qui suggère qu'il existerait une période de compensation des altérations du système olfactif au cours du vieillissement débutant. De même, ce constat suggère également que les premiers effets du vieillissement seraient des modifications subtiles, ne touchant pas l'ensemble des structures olfactives, permettant ainsi de jouer sur d'autres mécanismes non encore altérés pour compenser les premières altérations liées au vieillissement.

Une idée répandue concernant le vieillissement du système olfactif est que le bulbe olfactif va présenter à terme une atrophie avec conservation de son architecture chez l'homme (Bhatnagar et al., 1987; Kovács, 2004; Meisami et al., 1998) mais aussi le rongeur vers 24 mois (Hinds and McNelly, 1977; Mirich et al., 2002). Cependant, une étude récente montre que l'organisation et la densité cellulaire des différentes couches du bulbe olfactif semblent relativement stables jusqu'à un âge avancé chez le rongeur (Richard et al., 2010; Rey et al., 2012a). L'étude de Richard et al., (2010) suggère plutôt que des modifications plus subtiles des réseaux neuronaux, au niveau des connections synaptiques, se produiraient au cours du vieillissement plutôt qu'une simple dégénérescence globale et homogène du bulbe olfactif. Des études concernant d'autres cortex sensoriels retrouvent de la même manière des altérations synaptiques liées à l'âge plutôt qu'une dégradation globale de la structure corticale (Burke and Barnes, 2010; Dickstein et al., 2007; Livneh and Mizrahi, 2011).

Ainsi, des modifications structurales fines se produiraient lors du vieillissement induisant un potentiel déséquilibre dans l'organisation du réseau bulbaire responsable des déficits cognitifs observés mais qui pourrait être compensé pendant une certaine période. Une étude sur le vieillissement de la drosophile montre qu'avec l'âge apparaît une altération sélective de certains types de neurones et que, grâce à une modification de l'excitabilité des autres neurones préservés, il est possible de restaurer un apprentissage (Tonoki and Davis, 2012).

Le réseau bulbaire que l'on vient de décrire n'est pas statique mais est hautement plastique (Mandairon and Linster, 2009). En effet, l'expérience olfactive, qu'elle soit une privation, un enrichissement sensoriel ou un apprentissage olfactif, est capable de modifier le réseau bulbaire et par conséquent la perception et le comportement de l'animal face aux odeurs.

II/ PLASTICITE BULBAIRE EN LIEN AVEC L'EXPERIENCE SENSORIELLE

II. 1. Modulation du réseau bulbaire par les entrées sensorielles

La privation olfactive, par occlusion d'une narine, induit une augmentation de l'activité glomérulaire (observée grâce à un marquage 2DG) ainsi qu'une augmentation de la proportion de cellules mitrales répondant à l'odeur (Guthrie et al., 1990; Tyler et al., 2007) augmentant ainsi la sensibilité aux odorants (Leon, 1998). La transmission synaptique entre les neurones sensoriels et les cellules mitrales est donc renforcée pour compenser la diminution des entrées sensorielles.

Il a été également montré que des expositions à des odeurs permettaient d'améliorer les capacités de discrimination (Mandairon et al., 2006b, 2006d, 2008a) et la mémoire des odeurs (Rochefort et al., 2002; Veyrac et al., 2007; Rey et al., 2012a). Au niveau bulbaire, l'exposition répétée à des odeurs est associée à une modification du champ récepteur des cellules mitrales (Chaput and Panhuber, 1982; Fletcher and Wilson, 2003), à une diminution du nombre de cellules mitrales activées et à une augmentation du nombre de cellules mitrales inhibées par l'odeur d'enrichissement (Buonviso and Chaput, 2000; Buonviso et al., 1998). Une modulation de la réponse unitaire des cellules mitrales est aussi observée suite à une simple exposition olfactive (Spors and Grinvald, 2002). Ces modulations pourraient contribuer à l'amélioration des performances olfactives suite à l'expérience olfactive. De même, l'exposition à une odeur induit une augmentation spécifique de l'expression des gènes précoces dans certaines régions du bulbe olfactif. Une exposition préalable avec la même odeur entraîne une diminution de l'expression de ces gènes précoces dans les mêmes régions conduisant à une modification du patron granulaire d'expression des gènes précoces de manière spécifique de l'odeur (Montag-Sallaz et al., 2002). Au contraire, un enrichissement olfactif de 10 jours induit une augmentation du nombre de cellules exprimant le gène précoce Zif268 dans la couche granulaire en réponse à l'odeur d'enrichissement et module également les patrons d'activation dans la couche glomérulaire (Mandairon et al., 2008a; Woo et al., 2007). L'ensemble de ces données indiquent que la réponse du réseau bulbaire est restructurée suite à une exposition répétée à des odeurs ce qui pourrait rendre compte des changements de comportement.

II. 2. Modulation du réseau bulbaire par l'apprentissage associatif

L'apprentissage associatif olfactif, associant une odeur à un renforcement positif ou négatif, entraîne des modifications comportementales vis-à-vis de l'odeur apprise, et également des modifications de la représentation de l'odeur dans le bulbe olfactif.

Un premier travail par Freeman et collègues montrait en EEG que la dynamique de réponse aux odeurs était modulée par l'apprentissage associatif (Freeman and Schneider, 1982; Grajski and Freeman, 1989). Une modulation des oscillations de potentiels de champs locaux bulbaires a été observée chez le rat lors d'un apprentissage associatif (Beshel et al., 2007; Kay et al., 1996; Martin et al., 2004, 2006). L'établissement de carte d'activité en réponse à l'odeur grâce aux gènes précoces, au 2DG ou à l'imagerie intrinsèque met aussi en évidence une modification de la représentation spatiale bulbaire de l'odeur suite à l'apprentissage associatif (Busto et al., 2009; Fletcher, 2012; Salcedo et al., 2005; Wilson and Sullivan, 1994; Yuan et al., 2002; Mandairon et al., 2006a). L'activité unitaire des cellules mitrales répondant à l'odeur est également modifiée par l'apprentissage (Doucette and Restrepo, 2008; Doucette et al., 2011; Kay and Laurent, 1999). Ces changements de réponse des cellules mitrales pourraient être le substrat du codage de l'odeur selon sa signification.

L'apprentissage associatif olfactif est ainsi associé à un remaniement important du fonctionnement du réseau bulbaire. Ces modifications bulbaires pourraient sous-tendre à la fois l'association odeur-renforcement (Freeman and Schneider, 1982; Kendrick et al., 1992; Wilson and Stevenson, 2003; Wilson and Sullivan, 1994) mais aussi les changements d'acuité olfactive (Wilson and Stevenson, 2003).

II. 3. Modulation du réseau bulbaire par l'apprentissage perceptif

L'apprentissage perceptif consiste en une amélioration des performances de discrimination suite à une exposition répétée du même stimulus, sans renforcement. Cette simple exposition olfactive est associée à une modulation durable du réseau bulbaire contrairement à la sensibilisation ou l'habituation synaptique à court terme (Wilson and Stevenson, 2003).

Plus précisément, il a été montré qu'une activation non spécifique du bulbe olfactif par l'administration quotidienne intra-bulbaire de NMDA améliore les capacités de discrimination chez le rat de manière similaire à l'apprentissage perceptif (Mandairon et al., 2006e). Cette manipulation du réseau bulbaire est suffisante pour induire des modifications de la perception de manière durable. De plus, le blocage des récepteurs au NMDA annule les effets de l'enrichissement olfactif sur la discrimination (Mandairon et al., 2006e) et diminue l'inhibition des cellules mitrales par les cellules granulaires (Wilson, 1995). En effet, les récepteurs NMDA sont nécessaires à la genèse de l'inhibition dendro-dendritique (Isaacson and Strowbridge, 1998) et leur blocage par un antagoniste durant l'enrichissement olfactif empêche l'amélioration des capacités de discrimination (Mandairon et al., 2006e). La modulation de l'activité bulbaire via les récepteurs NMDA lors de l'apprentissage perceptif apparaît donc comme nécessaire pour ce type d'apprentissage (Mandairon et al., 2006c). Une modélisation du réseau bulbaire suggère qu'une plasticité synaptique activité-dépendante suffit

à induire des changements dans le fonctionnement du réseau bulbaire avec une augmentation de l'activité des cellules granulaires (Mandairon et al., 2008a) et donc une augmentation du niveau d'inhibition, qui pourrait expliquer les changements de perception induits par l'apprentissage perceptif (Mandairon et al., 2006c).

II. 4. Effet du vieillissement sur la plasticité olfactive et le comportement

L'olfaction est un bon témoin et sujet d'étude du vieillissement puisqu'elle est altérée à la fois dans le vieillissement normal et pathologique. Des études chez l'homme ont montré une forte altération de l'olfaction avec l'âge (Boyce and Shone, 2006) associée avec une perte du goût, des difficultés d'alimentation et des déficits en détection de nuisances comme la fumée par exemple (Hummel and Nordin, 2005; Schiffman, 1997). Les déficits olfactifs sont également associés à un plus haut risque de mortalité chez l'homme (Gopinath et al., 2012). Le vieillissement olfactif inclut des déficits en détection, identification, discrimination, mémoire et jugement hédonique des odeurs. Les troubles de la détection évoluent de manière non linéaire avec un début généralement vers 60 ans et une accentuation après 80 ans (Wysocki and Gilbert, 1989). Cette cinétique suggère une période de compensation du vieillissement débutant du système olfactif. Pour la discrimination, un déficit s'installe à partir de 60 ans et est d'autant plus marqué que la tâche de discrimination est difficile (Hummel et al., 2007; Kaneda et al., 2000; Schiffman et al., 1979). Ces déficits sont accentués dans certaines pathologies comme la maladie d'Alzheimer ou de Parkinson (Kovács, 2004).

Chez l'animal l'évolution de la perception avec l'âge semble se faire de manière similaire bien que relativement peu d'études se soient intéressées à ces altérations. Les souris âgées de 25 mois ont un seuil de détection des odeurs augmenté par rapport à de jeunes adultes (2 mois) (Patel and Larson, 2009). La discrimination semble également affectée mais de manière variable selon la difficulté de la tâche. La discrimination entre deux odeurs bien distinctes est conservée mais la discrimination fine est plus précocement altérée (Enwere et al., 2004; Rey et al., 2012a). Des études sur le rats ont montré une altération de l'apprentissage associatif olfactif chez l'animal âgé (Brushfield et al., 2008; LaSarge et al., 2007; Prediger et al., 2005; Roman et al., 1996) dont un déficit d'acquisition (Dardou et al., 2008; Patel and Larson, 2009; Roman et al., 1996). La rétention d'une odeur est également altérée avec l'âge ainsi que l'effet de l'enrichissement sur la mise en mémoire (Rey et al., 2012a). Les déficits olfactifs liés au vieillissement semblent se faire de manière non linéaire avec une première phase de déficits « modérés », pouvant correspondre à une période de compensation, suivi d'un déclin plus important traduisant de possibles altérations irréversibles. Des altérations lors du vieillissement pathologique ont aussi été rapportées dans des modèles murins de la maladie d'Alzheimer (Guérin et al., 2009; Wesson et al., 2010; Rey et al.,

2012b).

Ces altérations de la perception pourraient être reliées aux altérations même fines du réseau bulbaire que l'on a décrites plus haut et pourraient expliquer l'apparition progressive des altérations des capacités olfactives.

III/ RÔLE DES VOIES NEUROMODULATRICES DANS LES APPRENTISSAGES OLFACTIFS

Comme vu plus haut, le bulbe olfactif est une structure essentielle pour la mise en place des apprentissages olfactifs. Les systèmes neuromodulateurs sont connus pour avoir un rôle important dans les apprentissages et se projettent massivement sur le bulbe olfactif et en particulier sur les interneurones inhibiteurs (Matsutani and Yamamoto, 2008).

Les trois principaux systèmes neuromodulateurs se projetant sur le bulbe sont les systèmes cholinergique, noradrénergique et sérotoninergique, chacun ayant leurs noyaux dans des régions cérébrales différentes (Figure 5).

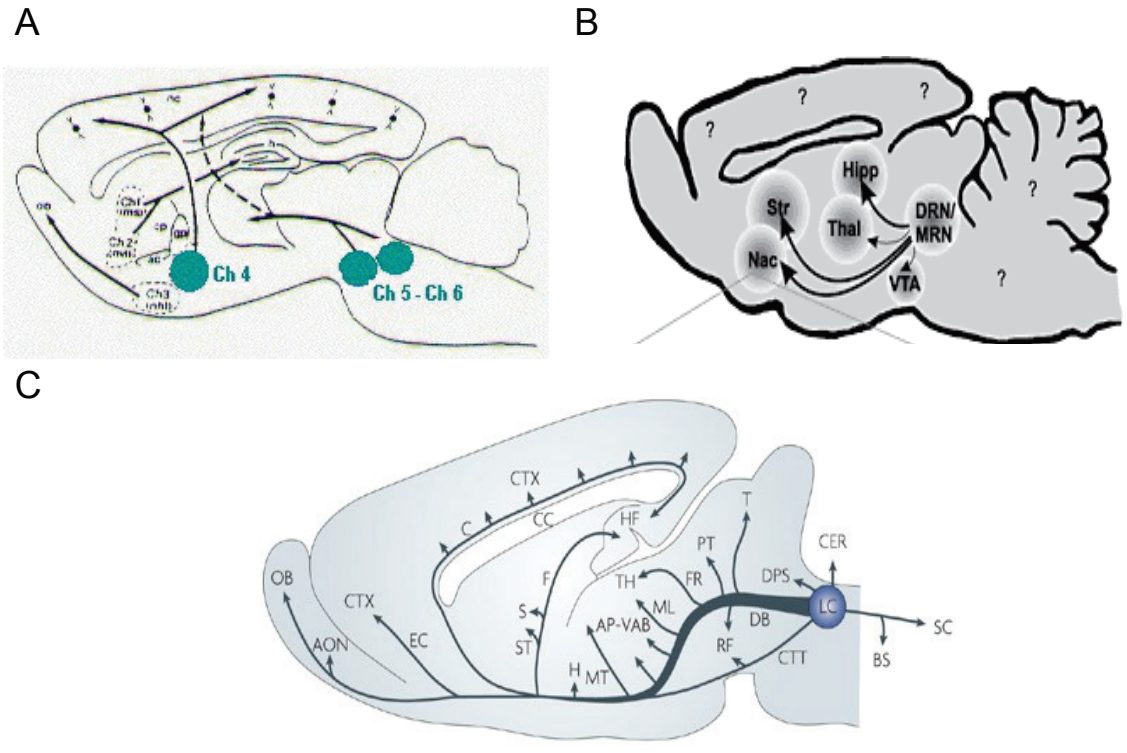

Figure 5 : Anatomie des systèmes neuromodulateurs
A. Le système cholinergique : noyaux basal de Meynert (Ch4) et méso-pontins (Ch5-6) (D'après http://neurobranches.chez-alice.fr/sommeil/cartoneuromed.html)
B. Projections du système sérotoninergique (D'après Müller et al., 2003)
C. Projections du système noradrénergique (D'après Sara, 2009)

III. 1. Le système cholinergique

Les corps cellulaires des neurones cholinergiques sont situés dans plusieurs noyaux regroupés en deux régions. Il y a un premier regroupement de noyaux dans le télencéphale (noyau septal médian, la bande diagonale et le noyau basal de Meynert) qui se projettent vers les régions corticales et limbiques et vers l'hippocampe et le striatum. Le second regroupement de noyaux se situe dans le mésencéphale (noyaux pédonculo-pontin et tegmental latero-dorsal) et leurs projections se font vers les ganglions de la base (Figure 5). L'innervation cholinergique vers le bulbe olfactif se fait depuis la bande diagonale de Broca (Luskin and Price, 1982; Macrides et al., 1981; Záborszky et al., 1986). Grâce à l'utilisation de marqueurs histologiques, il a été montré que les fibres cholinergiques se projettent majoritairement dans la couche glomérulaire mais aussi dans la couche granulaire. Les fibres cholinergiques font synapses avec les dendrites des cellules périglomérulaires et granulaires (Le Jeune et al., 1995; Macrides et al., 1981). Les récepteurs nicotiniques et muscariniques sont présents dans le bulbe olfactif.

Les effets cellulaires de l'acétylcholine dans le bulbe olfactif sont complexes en lien avec la diversité des récepteurs et leur répartition (Castillo et al., 1999). Par le biais des récepteurs nicotiniques, l'acétylcholine facilite la transmission de l'information olfactive en excitant directement les cellules mitrales de manière paracrine. L'activation des récepteurs nicotiniques induit également une augmentation d'activité des cellules périglomérulaires qui en retour inhibent les cellules mitrales et donc conduit à un effet opposé à l'action directe de l'acétylcholine sur les cellules mitrales. Via les récepteurs muscariniques, l'acétylcholine exerce également deux actions distinctes sur les cellules granulaires. En effet, l'acétylcholine réduit le taux de décharge des cellules granulaires en se liant aux récepteurs présents sur leurs corps cellulaires ce qui entraîne une désinhibition des cellules mitrales. Par contre, en agissant au niveau des récepteurs pré-synaptiques sur les dendrites des cellules granulaires, l'acétylcholine induit une libération de GABA sur les cellules mitrales renforçant ainsi leur inhibition. Ainsi, grâce à ses différents récepteurs et en fonctions de leur localisation, l'acétylcholine peut réguler les signaux d'entrée et de sortie du bulbe olfactif et est ainsi essentielle dans la modulation des processus olfactifs.

Un modèle des modulations cholinergiques dans le réseau bulbaire suggère que les afférences cholinergiques pourraient affiner le patron d'activité des cellules mitrales et ainsi permettre l'amélioration des capacités de discrimination (Linster and Cleland, 2002). La manipulation du réseau bulbaire par des modulations du système cholinergique entraînerait des modifications dans les capacités des rongeurs à discriminer des odeurs perceptuellement similaires (Linster et al., 2001; Mandairon et al., 2006e), ce qui est observé après administration intra-bulbaire d'un antagoniste nicotinique (Mandairon et al., 2006e). Plus généralement, la manipulation de

l'activité cholinergique dans le bulbe olfactif conduit à des changements dans la réponse des cellules mitrales à l'odeur qui sont prédictifs des changements dans les capacités de discrimination (Chaudhury et al., 2009). L'apprentissage associatif quant à lui, ne semble pas affecté par les modulations cholinergiques intra-bulbaires (Mandairon et al., 2006e). Enfin, la mémoire à court terme de l'odeur est altérée par le blocage de la transmission muscarinique (Ravel et al., 1992, 1994).

III. 2. Le système sérotoninergique

Le bulbe olfactif reçoit une innervation dense de fibres sérotoninergiques depuis le noyau du raphé (Figure 5, (McLean and Shipley, 1987)). Les fibres sérotoninergiques sont distribuées de manière non homogène dans le bulbe olfactif du rongeur. La densité de fibres est plus importante dans la couche glomérulaire que dans les autres couches du bulbe olfactif (Won et al., 1998). Plusieurs sortes de récepteurs sérotoninergiques sont exprimées dans le bulbe olfactif comme le 5HT1A, 5HT1B et 5HT2C. Les récepteurs 5HT2 sont principalement situés dans les couches glomérulaire, granulaire et mitrale. En revanche les récepteurs 5HT1A et 5HT1B se situent respectivement dans la couche plexiforme externe et dans la couche granulaire (Wright et al., 1995). La sérotonine inhibe certaines cellules mitrales par un mécanisme indirect impliquant une libération de GABA par les interneurones périglomérulaires ou granulaires. Cette inhibition peut être modulée par l'état de vigilance de l'animal. Au contraire, d'autres cellules mitrales sont directement dépolarisées par l'action de la sérotonine sur les récepteurs 5HT2A. De plus, la sérotonine entraîne une dépolarisation de quelques cellules périglomérulaires par le biais des récepteurs 5HT2C (Hardy et al., 2005).

Les récepteurs 5HT2 semblent requis pour les tâches d'acquisition mais pas pour les tâches de consolidation ou de rappel (McLean et al., 1996). La déplétion de fibres sérotoninergiques induit une perte des capacités de discrimination et des mécanismes de base de la reconnaissance olfactive (Moriizumi et al., 1994). La sérotonine agirait également de manière synergique avec la noradrénaline dans les apprentissages olfactifs même si la noradrénaline seule semble pouvoir compenser les déficits sérotoninergiques dans certaines conditions d'apprentissages olfactifs (McLean and Harley, 2004; Yuan et al., 2003).

III. 3. Le système noradrénergique

Les fibres noradrénergiques proviennent du *Locus Coeruleus* situé dans le tronc cérébral, de part et d'autre du quatrième ventricule (Figure 5). Une grosse partie des fibres noradrénergiques (40%, (Shipley et al., 1985)) se projettent sur le bulbe olfactif. Au sein du bulbe olfactif, les fibres

noradrénergiques se projettent majoritairement sur les couches granulaire et plexiforme externe (McLean et al., 1989; Shipley et al., 1985). De façon intéressante, il existe au niveau des terminaisons axonales noradrénergiques, des varicosités suggérant un possible rôle paracrine de la noradrénaline sur les cellules gliales et les vaisseaux sanguins. L'action de la noradrénaline passe par trois types principaux de récepteurs à la noradrénaline, α1, α2 et β, les trois étant présents sur les cellules granulaires et mitrales (McCune et al., 1993; Pieribone et al., 1994; Rosin et al., 1996; Talley et al., 1996; Woo and Leon, 1995). Les synapses dendro-dendritiques entre cellules mitrales et granulaires sont la cible de la noradrénaline pouvant ainsi moduler le message olfactif (Jahr and Nicoll, 1982; Trombley and Shepherd, 1992).

L'activation des récepteurs noradrénergiques entraîne des réponses cellulaires différentes et complexes, en fonction de la nature des récepteurs mis en jeu et de leur localisation. Les récepteurs inhibiteurs α2 pré-synaptiques interviendraient dans la modulation de la libération de noradrénaline puisque leur blocage par un antagoniste, comme le dexefaroxan, conduit à une augmentation de la libération de noradrénaline (Marien et al., 2004). Par ailleurs, il a été montré en culture cellulaire que la fixation de la noradrénaline sur des récepteurs α2, sur les cellules granulaires, conduit à la désinhibition des cellules mitrales alors qu'au contraire une fixation sur les récepteurs α1 active les cellules mitrales directement (Hayar et al., 2001). Cependant, des études s'intéressant aux potentiels de champs locaux suggèrent que la noradrénaline, par le biais des récepteurs α1, pourrait dépolariser les cellules granulaires ce qui entraînerait une inhibition des cellules mitrales. Enfin, chez le rat, l'activation du *Locus Coeruleus* diminue les décharges spontanées des cellules mitrales mais augmente leur réponse à un bref signal d'entrée olfactif. La stimulation du *Locus Coeruleus* entraînerait une diminution initiale suivie d'une augmentation de la libération de glutamate par les cellules mitrales sur les cellules granulaires par le biais du récepteur β dans le bulbe olfactif (Okutani et al., 1998).

Ainsi, grâce à ces trois récepteurs, la noradrénaline peut conduire à une grande variabilité de réponses sur les cellules mitrales et granulaires en jouant sur la balance excitation/inhibition.

Un autre effet potentiel du système noradrénergique sur les neurones bulbaires, pouvant contribuer à la mise en mémoire des odeurs, est le rôle facilitateur de la noradrénaline sur la potentialisation à long terme (Bliss et al., 1983) et sa synergie avec le système cholinergique pour favoriser la potentialisation à long terme (Bröcher et al., 1992).

La littérature soutient également un rôle de la noradrénaline dans les mécanismes attentionnels (Berridge and Waterhouse, 2003) et ceux sous-tendant la réponse à la nouveauté ou au stress (Abercrombie and Jacobs, 1987; Foote et al., 1983). Le taux de décharge du *Locus Coeruleus* montre une augmentation en réponse à des stimuli nouveaux ce qui pourrait moduler l'éveil comportemental (Aston-Jones and Bloom, 1981; Foote et al., 1980). Grâce à la sélection de stimuli

pertinents dans un environnement nouveau, la noradrénaline pourrait contribuer à favoriser leur mise en mémoire (Berridge and Waterhouse, 2003; Sara, 2009). Dans des paradigmes de conditionnement olfactif, une augmentation du taux de noradrénaline dans le bulbe olfactif est observée (Bouret and Sara, 2004) suggérant un rôle du système noradrénergique dans ce type de conditionnement bien que son rôle précis demeure méconnu. La noradrénaline est aussi importante pour l'acquisition de la valeur hédonique d'une odeur. En effet, à la naissance, le système noradrénergique joue un rôle dans l'établissement des préférences olfactives chez le raton (Wilson et al., 1987) et dans la reconnaissance maternelle entre l'agneau et la brebis (Kendrick et al., 1992).
Le récepteur α1 serait parallèlement critique pour la discrimination olfactive chez l'adulte (Doucette et al., 2007; Mandairon et al., 2008b). En effet, un blocage des récepteurs α1 à la noradrénaline induit une altération de l'apprentissage associatif par une diminution de la discrimination qui est d'autant plus importante que les odeurs sont similaires.
La noradrénaline semble donc essentielle pour les apprentissages olfactifs. Cependant les mécanismes exacts d'action du système noradrénergique sur le bulbe olfactif pouvant sous-tendre cette action demeure mal connus bien qu'il soit clairement établi que la noradrénaline est à l'origine de nombreuses modifications du fonctionnement bulbaire.

En résumé, la noradrénaline, par son action complexe sur la synapse mitrale-granulaire, module le ratio inhibition/excitation bulbaire qui semble être critique pour la discrimination et la mise en mémoire des odeurs. L'importance de la noradrénaline dans les apprentissages olfactifs est fortement suggérée par de nombreuses études. En effet la noradrénaline aurait un rôle central dans l'apprentissage olfactif en permettant d'extraire les informations pertinentes de l'environnement et de favoriser leur mise en mémoire par modification du réseau inhibiteur bulbaire (Berridge and Waterhouse, 2003; Sara, 2009). Cependant peu de travaux se sont attachés à étudier les mécanismes d’action précis de la noradrénaline lors d'apprentissages olfactifs.

III. 4. Vieillissement des voies centrifuges

Les systèmes neuromodulateurs sont également affectés par le vieillissement. En effet, il a depuis longtemps été observé une diminution de la quantité d'acétylcholine au niveau du cortex cérébral et de l'hippocampe chez le rongeur âgé (Gibson et al., 1981; Takei et al., 1989) mais également une diminution de la densité des récepteurs muscariniques dans le cortex (Gurwitz et al., 1987) suggérant une altération partielle de la voie cholinergique pouvant concerner également le bulbe olfactif. Peu d'études se sont intéressées aux conséquences fonctionnelles de ces altérations, particulièrement dans le système olfactif.

L'étude du système sérotoninergique au cours du vieillissement montre une réduction de la

5-HT dans l'hippocampe, le cortex, le striatum (Esteban et al., 2010; Nakai et al., 2006a). Ces études ont également permis d'observer un très intéressant phénomène compensatoire en réponse à l'altération des fibres sérotoninergiques qui est le sprouting des projections sérotoninergiques. Les mécanismes de sprouting dépendraient du système noradrénergique et du BDNF (Mamounas et al., 2000; Nakai et al., 2006b). Le cerveau vieillissant serait donc bien capable de compenser certaines altérations progressives et subtiles pendant un certain temps expliquant l'évolution non linéaire des déficits cognitifs mais aucune étude dans le bulbe olfactif n'a encore été menée.

Le système noradrénergique, dont nous avons vu l'importance pour les tâches comportementales est également touché par le temps. Les modifications du système noradrénergique au cours du vieillissement affectent à la fois le *Locus Coereleus* ainsi que les projections et la qualité du message noradrénergique. En effet, il existe chez l'homme une dégénérescence des fibres du *Locus Coeruleus* (Lee et al., 2001) ainsi qu'une diminution du nombre de neurones noradrénergiques. Chez la souris également, le nombre de fibres noradrénergique est diminué (Allard et al., 2011; Ishida et al., 2001) y compris dans le bulbe olfactif (Rey et al., 2012b). Cependant il existerait un mécanisme compensatoire à cette diminution numérique des fibres puisqu'il est observé une augmentation du nombre de fibres noradrénergiques dans le cortex, chez la souris âgée de 15 mois (Ishida et al., 2000) pouvant permettre de maintenir la force du signal noradrénergique (Ishida et al., 2001) pendant quelques temps.
La qualité de la transmission noradrénergique est également affectée par le vieillissement puisque dans le cortex ont été rapportées une diminution de la liaison de la noradrénaline à son transporteur ainsi qu'une diminution de la densité des récepteurs noradrénergiques α1 et α2 (Haapalinna et al., 2000; Hamilton et al., 1984; Kalaria and Andorn, 1991; Qi and Nomura, 1988; Viticchi et al., 1989). Le vieillissement conduit donc à une altération de la régulation noradrénergique.

Nous venons de voir que le bulbe olfactif, premier relais central de l'information olfactive, était une structure clé de l'apprentissage olfactif. Son réseau et notamment l'activité de ses cellules principales et de ses interneurones est largement modulée lors d'apprentissages. Mais le bulbe olfactif fait preuve d'une plasticité structurale encore plus importante : les interneurones sont la cible d'une neurogenèse adulte. Cette autre forme de plasticité apporterait au système olfactif un avantage adaptatif important par rapport aux autres systèmes sensoriels pouvant permettre de faire face à la complexité et la diversité des signaux olfactifs et à l'évolution de leur signification (Goldstone, 1998).

IV/ LA NEUROGENESE BULBAIRE CHEZ LA SOURIS ADULTE

IV. 1. Description de la neurogenèse bulbaire

La neurogenèse bulbaire comporte trois étapes principales (Figure 6).

Tout d'abord, des cellules souches prolifèrent dans la zone sous-ventriculaire des ventricules latéraux pour donner des neuroblastes. Puis ces neuroblastes vont migrer d'abord tangentiellement le long d'un chemin bien stéréotypé appelé le flux rostral migratoire pour atteindre le bulbe olfactif, puis au sein du bulbe olfactif, les neuroblastes migrent radialement pour atteindre les couches granulaire et glomérulaire. Dans le bulbe olfactif les neuroblastes se différencient enfin en interneurones granulaires et périglomérulaires.

On estime que 80 000 nouveaux neurones granulaires atteignent chaque jour le bulbe olfactif chez le rongeur soit environ 1% des cellules granulaires déjà présentes. En 18 mois, la neurogenèse bulbaire permet un renouvellement de 60% de la couche granulaire (Imayoshi et al., 2008).

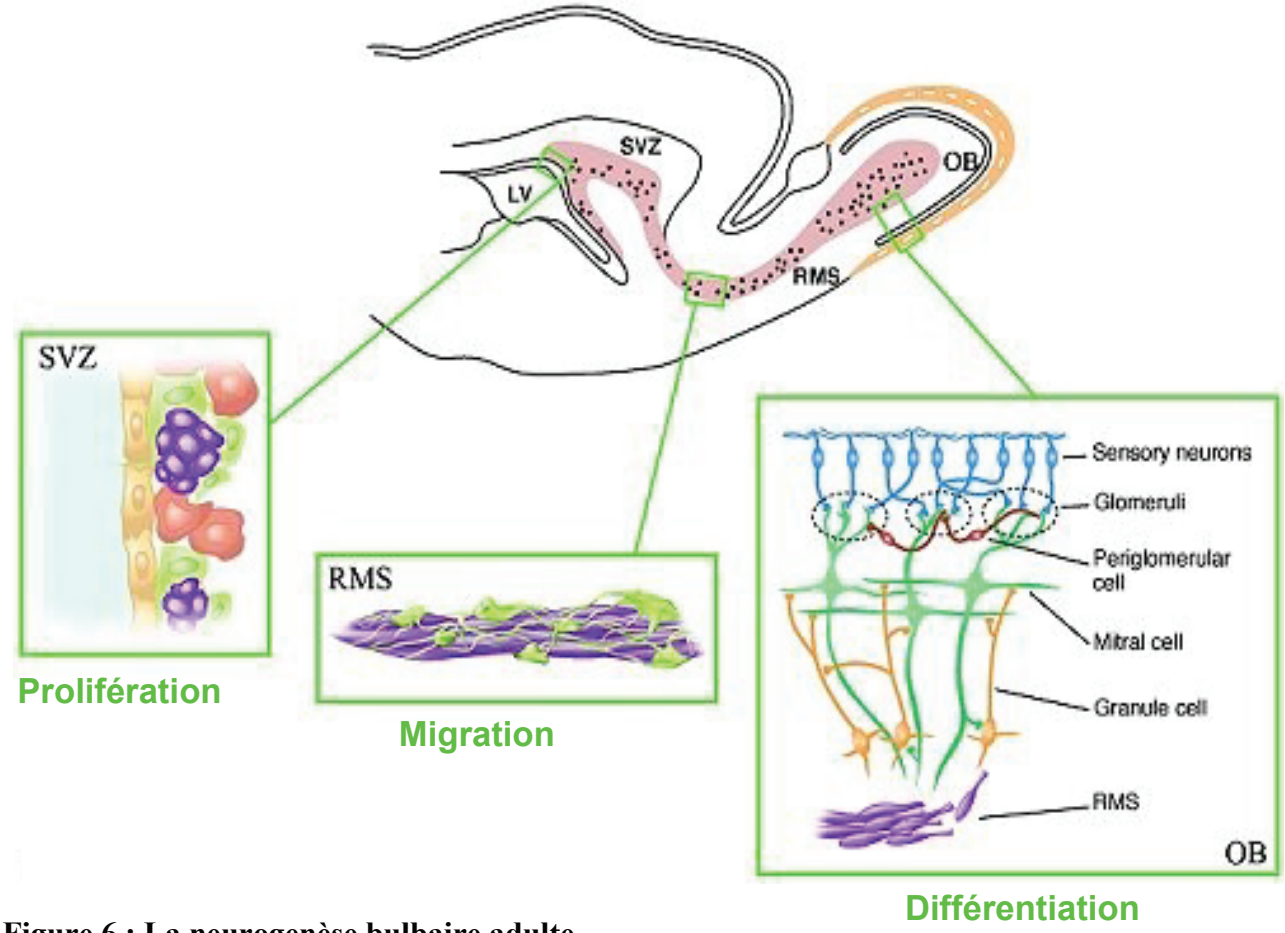

Figure 6 : La neurogenèse bulbaire adulte.
La neurogenèse bulbaire débute dans la zone sous-ventriculaire des ventricules latéraux (SVZ) avec la prolifération des cellules souches puis les neuroblastes migrent le long du flux rostral migratoire (RMS) pour atteindre le bulbe olfactif (OB) en son centre. Ces neurones nouvellement formés vont alors achever leur maturation fonctionnelle et s'intégrer au sein du bulbe olfactif dans les couches granulaire et glomérulaire.
(http://www.crulrg.ulaval.ca/fr/recherche/axes/chercheurs/armen-saghatelyan.php

IV. 1. 1. Prolifération et migration des neuroblastes

La zone sous-ventriculaire est une véritable niche neurogénique très vascularisée et dont l'architecture joue un rôle dans la détermination du devenir des précurseurs neuronaux (Alvarez-Buylla and Garcia-Verdugo, 2002) (Figure 7). Elle est constituée principalement de trois types de cellules (Doetsch et al., 1997; Peretto et al., 1999). Les cellules souches B, sont des cellules multipotentes (Merkle et al., 2004, 2007). Elles ont une activité mitotique lente et assurent le renouvellement de la niche et la formation des cellules C. Les cellules C ont un fort pouvoir mitotique ce qui permet d'amplifier le pool de précurseurs neuronaux. Elles vont générer des neuroblastes, les cellules de type A. Les cellules A sont des neuroblastes car elles expriment des marqueurs de neurones immatures : la double cortine (DCX) qui est spécifique des cellules en cours de migration et la PSA-NCAM qui est une molécule d'adhésion cellulaire (Lois and Alvarez-Buylla, 1994) (Figure 7). Ce sont les cellules de type A qui migrent jusqu'au bulbe olfactif et se différencient en interneurones, majoritairement granulaires.

La position des progéniteurs neuronaux dans la zone sous-ventriculaire va conditionner leur devenir au sein du bulbe olfactif (Lledo et al., 2006). En effet, les progéniteurs présents dans les régions dorsales de la zone sous-ventriculaire vont donner plutôt des cellules granulaires superficielles tandis que les cellules ventrales vont donner plutôt des cellules granulaires profondes (Merkle et al., 2007; Lledo et al., 2008).

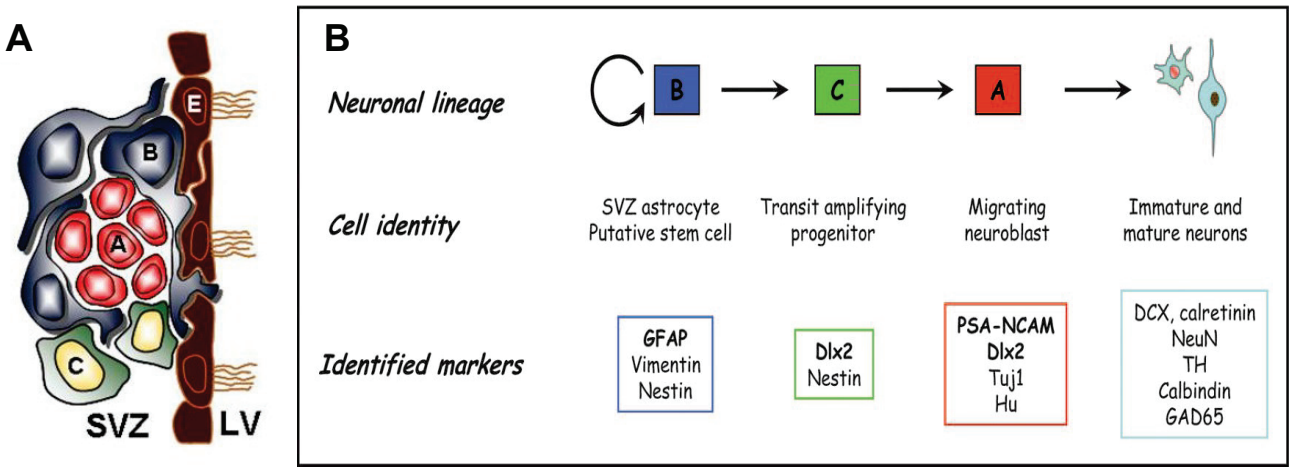

Figure 7 : Organisation de la zone sous ventriculaire : niche neurogénique.
A. La zone sous-ventriculaire (SVZ) présente une architecture bien définie assurant la prolifération des cellules souches et la formation des neuroblastes. Elle contient trois types cellulaires, les cellules A, B et C organisées autour de la paroi du ventricule. LV : Ventricule latéral; E : cellule épendymaire. (D'apres Arias-Carrion, 2008). **B.** Les cellules B sont les cellules souches, à activté mitotique faible, permettant le renouvellement de la SVZ. Les cellules C, issues de la division des cellules B, sont des progéniteurs avec un fort potentiel mitotique permettant l'amplification de la production de neuroblastes. Les cellules A, issues des cellules C, sont les neuroblastes qui vont initier la migration le long du flux rostral migratoire. Ils présentent des marqueurs caractéristiques de neurones immatures comme le PSA-NCAM. (D'après Abrous et al., 2005).

Les neuroblastes entament leur migration tangentielle en se regroupant en avant de la zone sous-ventriculaire et en formant une voie de migration, le flux rostral migratoire, afin d'atteindre le bulbe olfactif en son centre (Curtis et al., 2007; Kam et al., 2009; Lois et al., 1996). Cette migration en chaîne se fait grâce à la présence d'astrocytes qui permettent la constitution d'un tube glial étroitement lié aux vaisseaux sanguins et qui servirait de guide à la migration (Snapyan et al., 2009; Whitman and Greer, 2009). Les interactions avec les vaisseaux sanguins, la matrice extra-cellulaire et les astrocytes contribuent au bon déroulement de cette étape (Bovetti et al., 2007).

Une fois le bulbe olfactif atteint, les neuroblastes vont se détacher du flux rostral migratoire pour réaliser une migration radiale (Petreanu and Alvarez-Buylla, 2002) vers les couches glomérulaire et granulaire. Les vaisseaux sanguins du bulbe olfactif pourraient servir de guides aux neuroblastes (Bovetti et al., 2007). Il a été montré que des souris déficientes en Ténascine-R présentaient une diminution de 40% de la densité de cellules granulaires nouvellement formées. Cette réduction serait due à une altération de la migration radiale suggérant un rôle important de la Ténascine-R dans cette étape (Saghatelyan et al., 2004). D'autres molécules comme les protéines slit et reelin, sécrétées par les cellules mitrales, seraient essentielles pour assurer la transition de la migration dans le flux rostral migratoire à la migration radiale individuelle (Nguyen-Ba-Charvet et al., 2004; Ramos-Moreno et al., 2006).

C'est au sein du bulbe olfactif que les neuroblastes vont initier leur maturation et s'intégrer au sein du réseau bulbaire.

Les neuroblastes se différencient majoritairement en inter-neurones inhibiteurs (90%) et en cellules gliales (10%) (Lledo et al., 2006; Lois and Alvarez-Buylla, 1994). 80% des inter-neurones vont donner des cellules granulaires et 20% vont donner des cellules périglomérulaires (Lledo et al., 2006; Lois and Alvarez-Buylla, 1994).

IV. 1. 2. Intégration et survie des néo-neurones au sein du bulbe olfactif

La maturation des neuroblastes en cellules granulaires est décrite en 5 étapes et prend environ quatre semaines partiellement concomitante de la migration radiale (Carleton et al., 2003; Petreanu and Alvarez-Buylla, 2002) (Figure 8). Les neuroblastes qui arrivent dans le bulbe olfactif sont sous la forme de cellules bipolaires sans capacité de faire des potentiels d'action. Dès le début de la migration radiale elles vont développer des courants GABA. Puis, une fois la migration radiale terminée, le développement synaptique débute au sein de la couche plexiforme externe. L'arborisation dendritique progresse en extension avec également une augmentation des connexions. Le développement des épines dendritiques, l'apparition de potentiels synaptiques (AMPA, NMDA, GABA) et de potentiels d'action viennent clore le processus de maturation. L'arbre dendritique des cellules granulaires reste plastique au-delà de ce processus de maturation

(jusqu'à 90 jours après leur naissance) (Mizrahi, 2007).
Concernant les cellules périglomérulaires, le processus semble similaire (Belluzzi et al., 2003) et la capacité à générer des potentiels d'action précèdent les contacts synaptiques mettant en relief une probable différence fonctionnelle de ces deux types d'interneurones inhibiteurs néoformés.

Les néo-neurones inhibiteurs développent, grâce au processus de maturation, des propriétés électrophysiologiques semblables à celles des inter-neurones matures (Carleton et al., 2003; Petreanu and Alvarez-Buylla, 2002). Le processus de maturation serait dépendant des inputs GABAergiques sur les nouveaux neurones. En effet, le GABA aurait un rôle critique sur la maturation structurelle des nouveaux neurones et la formation des synapses glutamatergiques (Pallotto et al., 2012). Cependant des particularités électrophysiologiques les distinguent des cellules matures. En effet, les nouvelles cellules granulaires présentent une excitabilité et des propriétés membranaires différentes (Belluzzi et al., 2003; Carleton et al., 2003) et ont une propension à développer de la potentialisation à long terme (Nissant et al., 2009). Elles sont distribuées plus en profondeur que les cellules préexistantes (Mandairon et al., 2006a).
Les cellules nouvellement formées vont s'intégrer au réseau bulbaire de manière fonctionnelle (Belluzzi et al., 2003; Bovetti et al., 2009; Carlén et al., 2002; Magavi et al., 2005) et grâce à leurs caractéristiques spécifiques, elles confèrent au système olfactif une plasticité unique et originale qui présente un avantage devant la diversité et la complexité du milieu olfactif.

Cependant, au cours des premiers mois suivant leur naissance, seules 50% des cellules granulaires néoformées survivent, le reste entrant en apoptose dans les 15 à 45 jours après leur naissance (Mandairon et al., 2006a; Petreanu and Alvarez-Buylla, 2002; Winner et al., 2002). Cette régulation se produit majoritairement au moment du développement des connexions synaptiques, c'est à dire au moment de l'intégration fonctionnelle des néo-neurones (Carleton et al., 2003).
De nombreux facteurs moléculaires tels que des facteurs de transcription ou des neurotransmetteurs, sont susceptibles de jouer sur l'entrée ou non en apoptose des néo-neurones (Gao et al., 2009; Kuhn et al., 1997; Lim et al., 2000). Par exemple, le BDNF et son récepteur, TrKB, sont largement présents dans le bulbe olfactif et sur les précurseurs neuronaux, ce qui suggère un rôle du BDNF dans la régulation de la survie des néo-neurones (Mackay-Sima and Chuahb, 2000; Zigova et al., 1998). De plus des injections de BDNF augmentent la survie des néo-neurones sans augmenter la prolifération des précurseurs (Galvão et al., 2008). Ces données ont été confirmées par la réalisation de souris transgéniques KO pour le récepteur TrKB, qui montrent un déficit de neurogenèse (Bath et al., 2008).

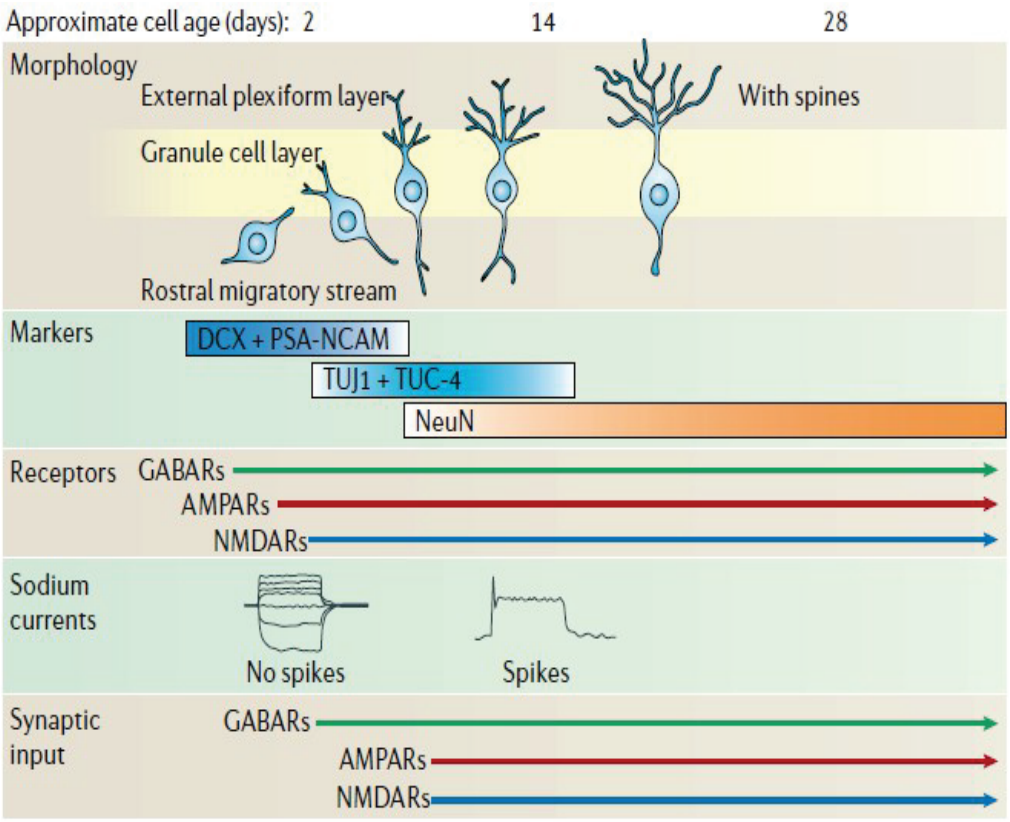

Figure 8 : Maturation des cellules granulaires néo-formées.
Les cellules granulaires néoformées, arrivant par le flux rostral migratoire au centre du bulbe olfactif, vont entamer une migration radiale au sein de la couche granulaire. Cette migration s'accompagne d'une maturation des néo-neurones. Les dendrites des cellules granulaires vont se développer jusqu'à obtenir un arbre dendritique ramifié dans la couche plexiforme externe réalisant des contacts synaptiques avec les cellules mitrales. En parallèle, les néo-neurones acquièrent de nouvelles caractéristiques comme la capacité à produire des potentiels d'action, assurant ainsi leur intégration fonctionnelle et leur plasticité. (D'après Lledo et al., 2006).

La régulation de la survie des néo-neurones constitue donc une étape clé dans l'intégration fonctionnelle des inter-neurones nouvellement formés. D'autant plus que la modélisation du réseau bulbaire suggère que l'incorporation des précurseurs neuronaux se fait de manière continuelle et non régulée tandis que la survie des néo-neurones serait l'étape importante de la régulation et dépendante de l'activité du réseau bulbaire donc des entrées sensorielles et de l'expérience olfactive (Cecchi et al., 2001; Lepousez et al., 2013).

IV. 2. Neurogenèse et plasticité fonctionnelle du bulbe olfactif

De nombreuses études suggèrent un lien étroit entre expériences sensorielles et modulation de la neurogenèse bulbaire (Mandairon et al., 2006c; Rochefort et al., 2002; Yamaguchi, 2005). En effet des manipulations des entrées olfactives (privation, stimulation) ou encore certains

apprentissages olfactifs sont liés à une modulation de la survie des néo-neurones granulaires. Ainsi la neurogenèse serait un élément clé de la plasticité du bulbe olfactif pour répondre à la complexité du milieu olfactif.

IV. 2. 1. Neurogenèse et entrées sensorielles

IV. 2. 1. 1. Effet de la privation olfactive

L'occlusion de la narine de rongeur entraîne une diminution du nombre de cellules granulaires néo-formées. Cette réduction de la survie des néo-neurones n'est observée que si la privation sensorielle est réalisée pendant une période critique correspondant à la maturation des cellules nouvellement formées (15-30j, (Mandairon et al., 2006a; Yamaguchi, 2005)) . Le début de cette phase critique de modulation de la survie par les entrées sensorielles coïncide avec le développement des premiers contacts synaptiques glutamatergiques (Carleton et al., 2003; Kelsch et al., 2008).

La réduction de l'activité neuronale bulbaire, induite par la privation olfactive, influence également le développement des connexions synaptiques des cellules granulaires nouvellement formées (Saghatelyan et al., 2005) et diminue le nombre d'épines dendritiques des néo-neurones (Kelsch et al., 2009). De plus, la privation olfactive entraîne une augmentation d'excitabilité des cellules granulaires pré-existantes (Saghatelyan et al., 2005). Cette hausse de l'excitabilité pourrait permettre de compenser la perte d'inhibition causée par la réduction de la survie des néo-neurones inhibiteurs (Saghatelyan et al., 2005). Enfin, l'activité sensorielle serait essentielle pour l'acquisition des caractères dopaminergique et GABAergique des interneurones périglomérulaires (Bastien-Dionne et al., 2010; Bovetti et al., 2009).

IV. 2. 1. 2. Effet de l'enrichissement olfactif

Un enrichissement olfactif augmente la survie des néo-neurones bulbaires (Mandairon et al., 2006c; Rochefort et al., 2002) et est sans effet sur leur prolifération dans la zone sous-ventriculaire (Rochefort et al., 2002; Veyrac et al., 2009). Cette hausse du nombre des néo-neurones inhibiteurs est durable puisqu'elle persiste 30 jours après la fin de l'exposition (Rochefort and Lledo, 2005). L'enrichissement olfactif a un effet spécifique sur la neurogenèse bulbaire et n'agit pas sur la neurogenèse hippocampique (Rochefort and Lledo, 2005).

Cette hausse de cellules granulaires nouvellement formées pourrait contribuer à augmenter l'inhibition du réseau bulbaire (Buonviso and Chaput, 2000).

Ainsi, les néo-neurones pourraient participer à la plasticité du réseau bulbaire grâce à la modulation par les entrées sensorielles de leur survie et du développement de leur arbre dendritique et grâce à

leurs propriétés fonctionnelles originales (Lledo and Gheusi, 2003).

IV. 2. 2. Neurogenèse et apprentissages olfactifs

L'apprentissage associatif de discrimination module la survie des nouveaux neurones (Alonso et al., 2006; Mandairon et al., 2006a). Plus particulièrement, ces deux études montrent que l'apprentissage olfactif module la neurogenèse, mais de façons différentes en fonction de l'âge des neurones néoformés. En effet, l'apprentissage favorise la survie des neurones âgés de 30 jours ou moins, alors que le nombre de neurones âgés de 45 jours est diminué après apprentissage. Il existe donc des périodes successives de sensibilité des nouveaux neurones à l'apprentissage, en fonction de leur âge (Mouret et al., 2008). Enfin, la survie des nouveaux neurones semble être également dictée par la difficulté de la tâche à effectuer, avec une augmentation du nombre de néo-neurones observée lorsque la discrimination est difficile (Mandairon et al., 2006a).

Dans l'apprentissage associatif olfactif, l'augmentation de survie des cellules granulaires néo-formées est corrélée avec les capacités mnésiques des rongeurs (Alonso et al., 2012; Lazarini et al., 2009; Sultan et al., 2010). En effet, la survie des néo-neurones est augmentée dans des régions de la couche granulaire spécifiques de l'odeur apprise (Sultan et al., 2011a) et la persistance de l'apprentissage est superposable à la persistance des néo-neurones néoformés recrutés durant la tâche mnésique (Sultan et al., 2010). De plus, la trace mnésique disparaît avec l'apoptose des nouveaux neurones (Sultan et al., 2011b). Ces données suggèrent fortement un rôle de la neurogenèse comme support de la trace mnésique olfactive. Dans ce sens, une étude récente utilisant l'optogénétique a montré que l'activation artificielle des nouveaux neurones pendant l'apprentissage associatif accélérait celui-ci et renforçait la mémoire à long-terme (Alonso et al., 2012). Les processus de consolidation semblent jouer un rôle majeur dans cette augmentation du nombre des nouveaux neurones par l'apprentissage (Kermen et al., 2010).

Ainsi, la neurogenèse bulbaire, par l'ajout permanent de nouveaux interneurones inhibiteurs, confère au bulbe olfactif une plasticité étonnante qui semble jouer un rôle fondamental dans la mémoire olfactive. Afin de mieux comprendre les intérêts de cette neurogenèse et la part qu'elle apporte au fonctionnement du réseau bulbaire, plusieurs études ont réalisé des blocages de la neurogenèse bulbaire. Une première étude utilisant l'injection intra-ventriculaire d'un anti-mitotique, (AraC) pour bloquer l'apport en nouveaux neurones pendant la période d'apprentissage associatif, conduit à une altération de la mémoire à long terme de la tâche associative mais pas de son acquisition (Sultan et al., 2010). De même, après irradiation de la zone sous-ventriculaire, qui bloque la neurogenèse bulbaire, des résultats similaires ont été obtenus (Lazarini et al., 2009). Une approche transgénique, permettant d'induire la mort neuronale des cellules souches chez la souris, présente quant à elle des résultats contraires. En effet après altération de la neurogenèse bulbaire

aucun déficit olfactif de discrimination, d'association ou de rétention (Imayoshi et al., 2008) n'est observé. Dans ce modèle de blocage à long terme de la neurogenèse, la mise en place de mécanismes compensatoires permettant de rétablir une fonction olfactive correcte ne peut être exclue. Enfin, une autre manipulation de la neurogenèse bulbaire par l'AraC ne montre pas de déficit de mémoire olfactive à long terme mais un déficit en mémoire de reconnaissance à court terme (Breton-Provencher et al., 2009).

Les contradictions entre ces différentes études pourraient être expliquées d'une part par la différence de technique de blocage de la neurogenèse qui se produit parfois longtemps avant les tests de performances et qui peuvent induire des mécanismes de compensation. D'autre part, les apprentissages olfactifs réalisés diffèrent d'une étude à l'autre et ne mettent pas forcement en jeu les mêmes mécanismes. Par exemple, les études d'Imayoshi et al (2008) et Breton-Provencher et al (2009) ont utilisé un apprentissage non-opérant alors que Sultan et al (2010) et Lazarini et al (2009) ont utilisé un apprentissage opérant, ce qui pourrait expliquer les différences neurogéniques observées. En fait, il a été montré que le conditionnement olfactif opérant augmentait le nombre de cellules nouvellement formées dans le bulbe olfactif contrairement au conditionnement non opérant (Mandairon et al., 2011).

Ainsi, l'implication de la neurogenèse semble être différente en fonction de la tâche olfactive à effectuer et ces différentes études ne permettent pas de déterminer un rôle unique de la neurogenèse dans l'apprentissage olfactif.

IV. 3. Discrimination et neurogenèse

IV. 3. 1. Introduction

Évoluer dans un environnement riche et changeant nécessite que l'on puisse de façon permanente discriminer et se souvenir des expériences de tous les jours même si elles sont similaires. Être capable de produire et de maintenir des représentations neurales distinctes même pour des événements similaires est critique pour la discrimination et pour éviter les interférences avec la mémoire à long-terme. La notion de séparation de patterns est un processus qui transforme des représentation similaires en représentations différentes, non-superposées (Aimone et al., 2011; Sahay et al., 2011a). Cette fonction est une composante clé de l'hippocampe et du bulbe olfactif, les deux sites de neurogenèse du cerveau adulte.

Dans le débat actuel, s'appuyant principalement sur des approches de modélisation, les chercheurs proposent que les nouvelles cellules hippocampiques et bulbaires pourraient permettre à des patterns d'activité fortement recouvrants de devenir plus distincts (Aimone et al., 2011; Bakker et al., 2008; Barnes et al., 2008; Sahay et al., 2011a, 2011b). Ce processus reposerait sur une

augmentation de l'inhibition. L'hippocampe doit encoder des expériences très similaires en représentations neurales différentes pour permettre une mémoire spatiale et épisodique bien distincte. Récemment, il a été suggéré que ces nouveaux neurones pourraient jouer un rôle dans ce processus. En effet, la capacité des souris à discriminer entre deux contextes très proches est altérée lors du blocage de la neurogenèse (Clelland et al., 2009). Quoiqu'il en soit, le rôle exact des nouveaux neurones dans le réseau responsable de la discrimination reste à déterminer.

Comme on l'a dit précédemment, le bulbe olfactif doit discriminer des centaines de molécules odorantes ou des mélanges et a besoin pour cela de produire des patterns d'activation différents pour chacune de ces molécules qu'il faut discriminer. Chaque odeur évoque un pattern spatial spécifique d'activation et la similarité entre les patterns se corrèle de façon inverse aux performances de discrimination (Linster et al., 2002; Rubin and Katz, 2001). L'expérience module les patterns d'activation et la discrimination. En effet, l'exposition répétée aux mêmes stimuli en absence de récompense améliore la discrimination, un processus appelé apprentissage perceptif (Gilbert et al., 2001; Mandairon and Linster, 2009; Mandairon et al., 2006b, 2006c, 2006d, 2008a). Pendant cet apprentissage perceptif le niveau d'inhibition est augmenté dans le bulbe olfactif (Mandairon et al., 2008a). Cette augmentation d'inhibition pourrait être dépendante de la neurogenèse. Ainsi, l'apprentissage perceptif olfactif semble être un modèle très approprié pour étudier le rôle de la neurogenèse dans le processus de séparation de patterns.

IV. 3. 2. L'apprentissage perceptif

Le premier concept d'apprentissage perceptif est défini en 1961 par Eleanor et James Gibson comme étant l'extraction d'informations de l'environnement préalablement non utilisées (Gibson, 1963; Gibson et al., 1981). Il rend compte du fait que l'exposition à des éléments de son environnement peut en changer leur perception de manière passive. Puis Goldstone en 1998 précise la définition de cet apprentissage implicite comme une modification spécifique et relativement permanente de la perception et du comportement en réponse à une expérience sensorielle (Goldstone, 1998).

L'apprentissage perceptif correspond à une amélioration de la discrimination suite à l'exposition répétée dans le temps de certains stimuli sensoriels. L'apprentissage perceptif s'oppose à l'apprentissage associatif par son côté passif dans le sens où il n'implique pas de conditionnement et pas d'action comportementale ciblée de la part de l'animal.

Malgré l'importance du système olfactif dans les comportements sociaux et alimentaires et la facilité d'étude du système olfactif chez le rongeur, peu d'études se sont attachées à décrire l'apprentissage perceptif olfactif et à en comprendre les mécanismes. L'apprentissage perceptif

olfactif est classiquement présenté par la capacité des amateurs de vins à discriminer différents composants des crus. En effet l'expérience sensorielle chez les amateurs de vins, œnologues ou encore parfumeurs va améliorer leur capacité de discrimination olfactive (Melcher and Schooler, 2004; Wilson and Stevenson, 2003; Jehl et al., 1995). Plus précisément, la seule répétition du stimulus sensoriel suffit à améliorer la discrimination des vins (Owen and Machamer, 1979; Peron and Allen, 1988). Ces données ont été confirmées plus largement par une expérience montrant que la présentation de différents esters à des sujets permettait d'en améliorer la discrimination (Fletcher and Wilson, 2002).

Chez les rongeurs, l'apprentissage perceptif olfactif a également été étudié (Mandairon et al., 2006b). Les rongeurs ont été exposés à deux odeurs chimiquement et perceptuellement proches (les énantiomères du limonène). La discrimination spontanée entre les deux odeurs a été évaluée grâce à un test d'habituation/déshabituation. Les auteurs observent que le (+) et (–) limonène ne sont pas discriminés spontanément par les rongeurs. Cependant l'enrichissement quotidien de vingt jours avec ces deux odeurs améliorent leur discrimination. Ainsi, la manipulation de l'expérience sensorielle par une simple exposition passive à des odeurs induit des changements dans la discrimination olfactive spontanée (Mandairon et al., 2006b). De manière plus intéressante, l'enrichissement avec du (+)/(-) limonène va également améliorer la discrimination d'autres paires d'odeurs proches comme le pentanol et butanol sans exposition préalable à celles-ci. Ainsi l'apprentissage perceptif olfactif est transférable à des odeurs proches ; des odeurs dont les patterns d'activation évoqués dans le bulbe olfactif sont partiellement recouvrant avec ceux des odeurs utilisées pour l'enrichissement. Le bulbe olfactif est une structure clé de l'apprentissage perceptif puisque le blocage des récepteurs NMDA dans le bulbe olfactif lors de l'apprentissage altère la discrimination (Mandairon et al., 2006e) et qu'une augmentation de l'activité des interneurones bulbaires a été observée suite à l'apprentissage (Mandairon et al., 2008a).

IV. 4. Modulation de la neurogenèse bulbaire par les systèmes neuromodulateurs

Les systèmes neuromodulateurs cholinergique et noradrénergique, en se projetant sur le bulbe olfactif, ont non seulement une action sur le réseau bulbaire préexistant mais également sur la neurogenèse bulbaire.

Concernant le système cholinergique, il a été montré que la lésion partielle des neurones cholinergiques du septum médian entraînait une diminution du nombre de nouveaux neurones dans la couche granulaire et une augmentation dans la couche glomérulaire (Cooper-Kuhn et al., 2004). De manière similaire, un traitement par un inhibiteur de l'acétylcholine estérase, qui va donc renforcer la transmission cholinergique, conduit à une augmentation de la densité de néo-neurones

granulaires au sein du bulbe olfactif et de l'hippocampe sans modifier la prolifération (Kaneko et al., 2006; Kotani et al., 2008). Cette modulation pourrait passer par l'activation des récepteurs nicotiniques B2 (Mechawar et al., 2004). Ces données sont appuyées par le fait que les fibres cholinergiques feraient des synapses avec les cellules granulaires et pourraient ainsi agir sur leur maturation et survie (Whitman and Greer, 2007).

Concernant le système sérotoninergique, une large littérature imlique le système sérotoninergique comme modulateur de la neurogenèse hippocampique (Sahay and Hen, 2007) et bulbaire (Samuels and Hen, 2011). Par contre, à ce jour, le rôle du système sérotoninergique dans la modulation de la neurogenèse en situation d'apprentissage n'est pas documenté.

Enfin, le système noradrénergique semble être impliqué dans la modulation de la neurogenèse. En effet, la stimulation des récepteurs pré-synaptiques α2, augmentant la libération de noradrénaline (Dennis et al., 1987), entraîne une augmentation de la neurogenèse bulbaire (Bauer et al., 2003) par diminution de l'apoptose plutôt que par des modifications de prolifération (Bauer et al., 2003). Ces effets sont compatibles avec les effets anti-apoptotiques connus de la noradrénaline sur les neurones dans des modèles lésionnels comme l'ischémie, l'excitotoxicité et la dévascularisation corticale (Marien et al., 2004). De même, dans un modèle de mort neuronale induite par axotomie, l'utilisation d'agonistes adrénergiques α2 limite la mort neuronale des nouveaux neurones (Veyrac et al., 2005). La noradrénaline, par son rôle favorisant la survie des néo-neurones bulbaires et compte tenu de son implication dans les apprentissages olfactifs pourrait contribuer à réguler la neurogenèse dans divers apprentissages olfactifs.

En résumé, nous pouvons dire que le bulbe olfactif est une structure corticale très plastique pour faire face aux changements de l'environnement olfactif grâce à la modulation de son réseau par divers acteurs. Plus précisément, la modulation de l'inhibition bulbaire semble avoir un rôle primordial dans la perception olfactive et la neurogenèse est un des éléments pouvant jouer sur le taux d'inhibition bulbaire et conférer une grande plasticité structurale et fonctionnelle au système olfactif.

IV. 5. Vieillissement et neurogenèse bulbaire

La neurogenèse bulbaire est également soumise aux effets du temps. En effet, une diminution du volume de la zone sous-ventriculaire est observée au cours du vieillissement (Luo et al., 2006). Elle serait due à une diminution de la prolifération des progéniteurs (Maslov et al., 2004; Tropepe et al., 1997) de l'ordre de 86% pour des souris âgées de 24 mois (Shook et al., 2012). Cette diminution de la prolifération serait due à une augmentation de la durée du cycle cellulaire ainsi qu'à une hausse de l'apoptose des progéniteurs (Luo et al., 2006; Tropepe et al., 1997). Au sein du

bulbe olfactif, les cellules néoformées sont de moins en moins nombreuses avec une réduction progressive allant jusqu'à 75% à 23 mois (Enwere et al., 2004; Rey et al., 2012a). Cette réduction du nombre de neurones néoformés dans le bulbe olfactif suit deux phases : une première de baisse de la prolifération qui intervient entre 2 et 12 mois, suivie d'une baisse du taux de survie des néo-neurones dans le bulbe, intervenant entre 12 et 24 mois (Rey et al., 2012a). La conséquence du défaut de neurogenèse au cours du vieillissement est très peu étudiée mais semble se corréler au moins partiellement temporellement avec les déficits cognitifs observés (Enwere et al., 2004; Rey et al., 2012a). La diminution du nombre de nouveaux neurones au sein du bulbe olfactif pourrait entraîner une diminution de la plasticité synaptique bien que le nombre de cellules soit stable et donc conduire à des déficits de perception olfactive.

Au cours du vieillissement, le nombre total de cellules granulaires est stable (Richard et al., 2010) malgré la baisse de neurogenèse alors que l'ablation génétique de la neurogenèse conduit à une diminution du nombre de neurones granulaires (Imayoshi et al., 2008). Ceci suggère que le taux de renouvellement des neurones granulaires est diminué avec l'âge et que probablement, les fonctionnalités associées aux néoneurones ne sont plus présentes. La stabilité dans le nombre de neurones au sein du bulbe olfactif suggère cependant qu'il existe des mécanismes compensatoires à la diminution drastique de neurogenèse chez le rongeur vieillissant. Il a notamment été montré que pendant un certain temps, la diminution de la prolifération dans la zone sous ventriculaire était compensée par une hausse de la survie des nouveaux neurones bulbaires (Sui et al., 2012) et pourrait également être accompagnée d'une hausse de survie des anciennes cellules granulaires pour maintenir un équilibre au sein du bulbe olfactif.

La réduction de la neurogenèse bulbaire au cours du vieillissement participe à l'altération progressive du réseau et de sa plasticité. Elle est intriquée avec les déficits fonctionnels olfactifs sans que son implication précise dans ces déficits soit clairement établie.

V/ OBJECTIFS DE LA THESE

Le rôle du système olfactif est d'analyser et de traiter des centaines d'odorants. Pour cela, il dispose d'une plasticité très importante. En effet, en plus des processus classiques de plasticité synaptique, le système olfactif et plus particulièrement le bulbe olfactif dispose d'un apport permanent de nouveaux neurones (Alvarez-Buylla and Garcia-Verdugo, 2002). Le rôle de cette neurogenèse a été largement étudié, principalement dans le contexte d'apprentissage associatif, et pourtant de nombreuses questions restent encore en suspens.

L'objectif général de cette thèse est donc d'étudier le rôle des nouveaux neurones en utilisant le modèle comportemental de l'apprentissage de type perceptif. En effet, cet apprentissage est un modèle idéal pour étudier le rôle des nouveaux neurones dans les processus de séparation de

patterns.

La poursuite de cet objectif a été permise grâce au développement d'un ensemble de techniques (implantation de canules chez la souris, injection intracérébrale de virus, implantation de micro-pompes chez la souris) et à l'utilisation de techniques déjà présentes dans l'équipe (immunohistochimie, neuropharmacologie, imagerie cellulaire, analyse de la neurogenèse, microscopie confocale, tests comportementaux : voir Mandairon et al., 2009 en annexe)). Mes travaux se sont déroulés selon trois axes :

-Nous avons cherché à caractériser le rôle des nouveaux neurones dans l'apprentissage perceptif. Pour cela, nous avons soumis des souris adultes à un enrichissement par une paire d'odeurs similaires une heure par jour pendant dix jours. L'effet de cette expérience olfactive sur la survie des nouveaux neurones a été analysé. Nous avons également mesuré le niveau d'implication fonctionnel de ces nouveaux neurones en utilisant comme marqueur d'activité l'expression du gène Zif268. L'impact sur le réseau inhibiteur bulbaire de cet apport de nouveaux neurones a été évalué grâce à deux techniques, l'électrophysiologie et l'analyse du niveau d'expression de l'enzyme de synthèse du GABA dans la couche granulaire. Enfin, afin de révéler le rôle des nouveaux neurones dans cet apprentissage, nous avons bloqué la neurogenèse bulbaire et étudié les conséquences du blocage sur les performances comportementales et sur le niveau d'inhibition dans le bulbe (Étude 1 publiée).

-Les systèmes neuromodulateurs se projettent massivement sur le bulbe olfactif et contribuent aux processus d'apprentissage. Plus particulièrement, le système noradrénergique joue un rôle majeur dans la discrimination (Doucette et al., 2007; Guérin et al., 2008; Mandairon et al., 2008b) mais la façon dont ce système neuromodulateur interagit avec la neurogenèse est méconnue. Dans cette partie nous avons recherché l'impact de la modulation du système noradrénergique sur la neurogenèse lors de l'apprentissage perceptif. Pour répondre à ce point, nous avons manipulé pharmacologiquement la transmission noradrénergique et observé les conséquences de cette manipulation sur les performances comportementales et sur le réseau bulbaire chez des souris dont la neurogenèse était intacte ou bloquée. Grâce à l'injection de lentivirus GFP et de l'électrophysiologie, nous avons également recherché si les fibres noradrénergiques se projetaient directement sur les nouveaux neurones (Étude 2 publiée).

-Enfin, le vieillissement normal du système olfactif semble correspondre à une intrication de modifications structurales et fonctionnelles subtiles et progressives touchant à la fois la neurogenèse et les systèmes neuromodulateurs et conduisant à terme à des déficits dans les tâches de perception et de mémoires olfactives. Ces modifications liées au vieillissement sont largement méconnues et

font appel à des schémas mécanistiques encore mal compris. Nous avons étudié l'impact du vieillissement sur la neurogenèse et sur l'apprentissage perceptif grâce aux analyses comportementale et cellulaire chez la souris âgées (Étude 3, soumise).

RESULTATS

Etude 1 :

Olfactory perceptual learning requires adult neurogenesis

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Olfactory perceptual learning requires adult neurogenesis

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Perceptual learning is required for olfactory function to adapt appropriately to changing odor environments. We here show that newborn neurons in the olfactory bulb are not only involved in, but necessary for, olfactory perceptual learning. First, the discrimination of perceptually similar odorants improves in mice after repeated exposure to the odorants. Second, this improved discrimination is accompanied by an elevated survival rate of newborn inhibitory neurons, preferentially involved in processing of the learned odor, within the olfactory bulb. Finally, blocking neurogenesis before and during the odorant exposure period prevents this learned improvement in discrimination. Olfactory perceptual learning is thus mediated by the reinforcement of functional inhibition in the olfactory bulb by adult neurogenesis.

discrimination | mice | enrichment | olfactory bulb

Perceptual learning is an implicit (nonassociative) form of learning in which discrimination between sensory stimuli is improved by previous experience (1). For instance, animals trained on a tactile discrimination task improve their behavioral performances and in parallel, the neural representation of the stimuli is sharpened (2, 3). In the olfactory modality, perceptual learning has been shown to occur in humans (4), and an experimental model of olfactory perceptual learning has recently been proposed in rats (5). Olfactory perceptual learning is crucial for basic olfactory functions because it sets the degree of discrimination between stimuli, and thus contributes to the perceptual representation of the environment, which guides the animal's behavior. However, neural mechanisms underlying such changes of perception remain elusive. We here show that a modulation of newborn cell survival in the olfactory bulb (OB) underlies olfactory perceptual learning. We show that neurogenesis is not only involved in, but necessary for perceptual learning to occur.

We have shown that odor enrichment enhances rats' ability to discriminate between chemically similar odorants in a relatively odor-unspecific manner (5, 6). Indeed, the discrimination of a pair of similar odorants is improved by enrichment with the same odorants or with other odorants that activate regions of the OB partially overlapping with the regions activated by the discriminated pair. Even if the mechanisms underlying this learning remain unclear, it has been shown that infusions of NMDA into the OB improves odor discrimination in a manner similar to odor enrichment indicating that changes in OB processing contribute at least partially to the perceptual plasticity (5). A computational model proposed that activation of OB neurons produces widespread changes in inhibitory processing, which can underlie the observed improvement of odor discrimination (5). In support to this model, odor exposure has been shown to increase inhibition of mitral cells (7) and to increase the responsiveness of the inhibitory granule cells to odorants, as measured by expression of an immediate early gene (8).

Inhibitory neurons in the OB are continuously generated in adulthood. They are formed from neural stem cells located in the subventricular zone (SVZ) of the lateral ventricle (9), and migrate from the SVZ to the OB where they functionally integrate into the neuronal network as granule and periglomerular interneurons (10–13). Modulation of the rate of newborn neurons formation parallels changes in discrimination induced by sensory experience (14) but the exact role of OB neuronal renewal is still unclear (15).

In this work, we studied the neural mechanisms underlying perceptual learning in mice. The experiments reported here showed that (*i*) daily odor enrichment improves behavioral discrimination between odorants when there is spatial overlap between the bulbar areas activated by enrichment and test odors; (*ii*) odor enrichment increases the survival of the newborn neurons involved in processing of experienced odors; (*iii*) inhibition in the OB network increases in response to odor enrichment; and (*iv*) newborn cells are necessary for the improvement of olfactory discrimination in response to odor enrichment. Our results reveal increased network inhibition in the OB due to OB neurogenesis as the cellular mechanism underlying olfactory perceptual learning.

Results

Perceptual Learning in Response to Enrichment Improves Discrimination. In this experiment, we used a habituation/dishabituation test (four habituation trials with the same odorant followed by a test trial with a different odorant, see *Materials and Methods*) to assess olfactory discrimination between three pairs of chemically similar odorants: +/−limonene, pentanol/butanol, and decanal/dodecanone. Discrimination was assessed before and after a 10-day enrichment period (Fig. 1*A*). Enrichment consisted of exposure to a pair of similar odorants (+/−limonene or decanal/dodecanone) for 1 h daily in the home cage. At the end of the enrichment period, mice were tested for discrimination between the two odorants of each of the three tested pairs. Tested pairs of odorants (+/−limonene, pentanol/butanol, and decanal/dodecanone) exhibit various degree of response overlap (as measured by 2-deoxyglucose activation maps; http://leonlab.bio.uci.edu) with enrichment odors (6) (Fig. 1*A*). Overlaps of the activation pattern of +limonene and pentanol (pairwise correlation coefficient, $r = 0.28$) are larger than overlaps between +limonene and decanal (pairwise correlation coefficient, $r = -0.06$) (5).

Significant habituation, evidenced by the reduction in investigation of the odorant across repeated presentations, was observed in preenrichment and postenrichment conditions (Fig. S1). We confirmed that before enrichment none of the three test odor pairs (+/−limonene, pentanol/butanol, and decanal/dodecanone) was discriminated by the mice ($P > 0.05$ for difference between Hab4 and Otest, Fig. 1*Bi*).

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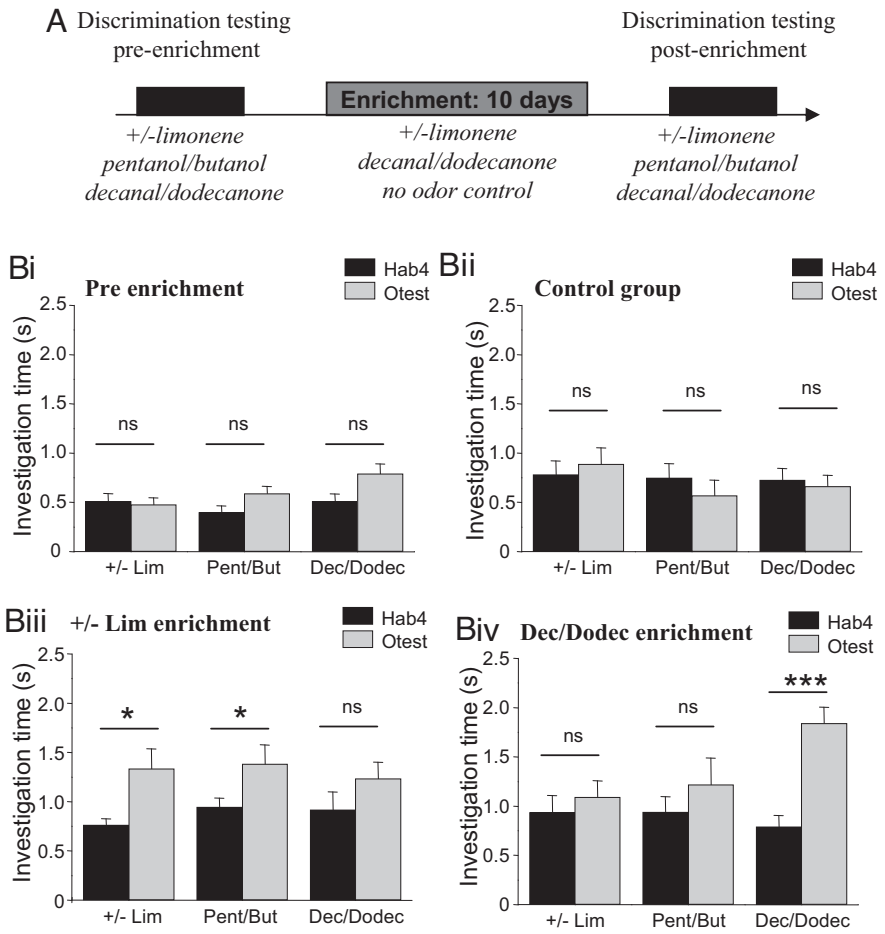

Fig. 1. Olfactory enrichment improves discrimination. (*A*) Design of the experiment. Spontaneous discrimination between +/−limonene, pentanol/butanol, and decanal/dodecanone was tested before and after an odor enrichment period. Experimental groups were enriched by introducing +/−limonene or decanal/dodecanone into the home cage for 1-h periods over 10 days. A control group was not enriched (no odor). (*B*) Behavioral discrimination before (*Bi*) and after (*Bii-Biv*) enrichment. The two odorants of each pair are confused before the enrichment period (*Bi*), and in the control nonenriched group (*Bii*). After enrichment with +/−limonene, +/−limonene and pentanol/butanol are discriminated (*Biii*), and after enrichment with decanal/dodecanone, only this pair of odorants is discriminated (*Biv*). [*, $P < 0.05$, ***, $P < 0.0001$, in response magnitude between trials 4 (Hab4) and 5 (Otest)]. The data are expressed as mean values ± SEM.

Mice were then exposed to +/−limonene ($n = 10$), decanal/dodecanone ($n = 10$), or no odor (Control group, $n = 10$) and subsequently tested on the same discrimination task again. Significant differences were found between experimental groups [$F(2, 475) = 15.33$, $P < 0.0001$]. As expected, control mice showed no improvement of discrimination after the enrichment phase (exposure to mineral oil only) (ANOVA followed by Fisher post hoc comparison between Hab4 and Otest; $P > 0.05$ for all three odor pairs) (Fig. 1*Bii*). In contrast, the +/−limonene enriched group displayed a significant improvement of discrimination between +limonene and −limonene or between pentanol and butanol ($P < 0.05$), but not between decanal and dodecanone ($P > 0.05$; Fig. 1*Biii*). In the decanal/dodecanone enriched group the discrimination between decanal and dodecanone was improved ($P < 0.001$) but not the discrimination between the enantiomers of limonene or between pentanol and butanol ($P > 0.05$; Fig. 1*Biv*).

These results show that odor enrichment improves olfactory discrimination in adult mice, when the OB activation patterns of enrichment odors and test odors partially overlap.

Newborn Cell Survival Is Increased During Perceptual Learning. To test the effect of olfactory enrichment on OB neurogenesis, the DNA synthesis marker Bromodeoxyuridine (BrdU) was injected 8 days before behavioral training and 25 days before sacrifice (Fig. 2*A*). Because newborn neurons reach the OB ≈8–10 days after their birth and then start to differentiate (15, 16), this injection protocol allows to label the newborn neurons arriving in the OB at the beginning of the enrichment period and integrating in the network during the enrichment phase.

In the granule cell layer (GrL), the density of newborn cells differed significantly between experimental groups (+/−limonene or decanal/dodecanone enriched and no odor group) [$F(2, 10) = 5.344$; $P = 0.026$]. Enrichment with +/−limonene (Fisher; $P < 0.05$) and decanal/dodecanone Fisher; $P < 0.05$) increased the density of BrdU-positive cells compared to control (Fig. 2*B*). In contrast, there was no effect of enrichment on BrdU-positive cell density in the glomerular layer (GL) [ANOVA, $F(2, 8) = 0.753$, $P = 0.502$] (Fig. 2*B*). Enrichment had no effect on the GrL [$F(2, 9) = 0.669$; $P = 0.452$] or the GL volume [$F(2, 8) = 1.511$; $P = 277$] (Fig. S2).

By using BrdU/NeuN double-labeling in the GrL (Fig. 2*C* and Fig. S3*A*) and BrdU/Calbindin double-labeling in the GL (Fig. 2*D* and Fig. S3*B*), we did not observe any effect of enrichment on the level of neuronal differentiation of newborn cells neither in the GrL ($P > 0.05$, Fig. 2*C*) nor in the GL ($P > 0.05$, Fig. 2*D*).

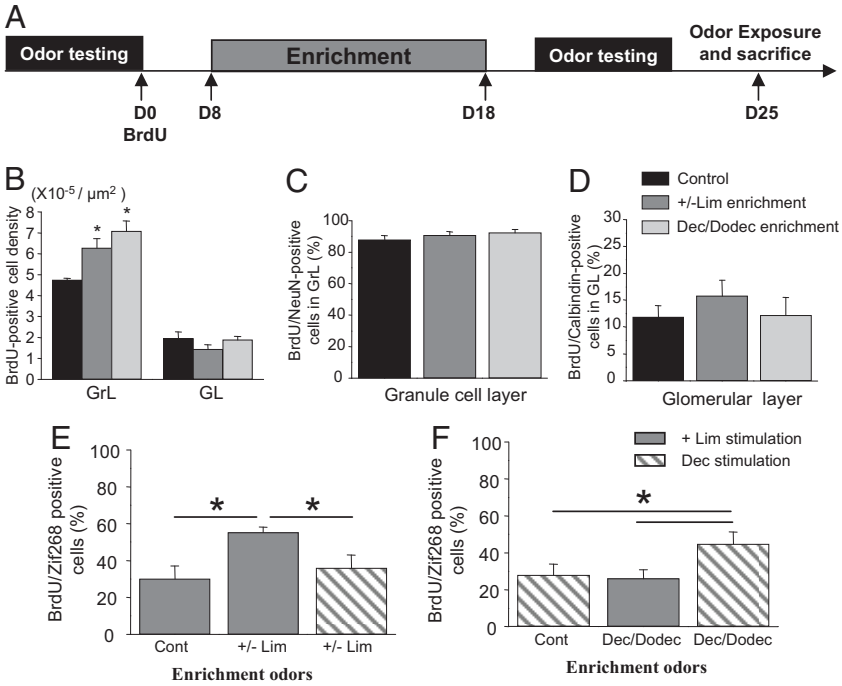

Fig. 2. Olfactory enrichment improves the survival of newborn cells involved in odor processing. (*A*) Experimental paradigm. BrdU was administered 8 days before the enrichment period and mice were killed 25 days after administration of BrdU. Animals were exposed to + limonene or decanal 1 h before sacrifice. (*B*) Olfactory enrichment induces an increase of BrdU-positive cell density in the granule (GrL) but not glomerular (GL) cell layers of the OB (*, $P < 0.05$). (*C*) Quantification of BrdU/NeuN double-labeling in the GrL showed no effect of the enrichment on the neuronal fate of newborn cells. (*D*) By using BrdU/Calbindin double-labeling in the GL, no effect of the enrichment on the phenotype of newborn cells in the GL was found. (*E*) Enrichment with +/−limonene increases the percentage of newborn neurons responding to +limonene compared to newborn neurons responding to decanal or to newborn neurons that respond to +limonene in control non enriched animals. (*F*) Similarly, enrichment with decanal/dodecanone increases the percentage of newborn neurons responding to decanal compared to newborn neurons responding to +limonene or to newborn neurons that respond to decanal in control non enriched animals. (*, $P < 0.05$). The data are expressed as mean values ± SEM.

Newborn Neurons Are Preferentially Involved in Processing of the Learned Odor. Because the density of newborn granule but not periglomerular cells is increased by perceptual learning, we further examine the involvement of newborn granule cells in the plasticity induced by odor enrichment. We assessed the percentage of newborn granule cells expressing Zif268 as an index of cellular activation in response to odor stimulation (Fig. 2 *E* and *F* and Fig. S3*C*) (8).

Control and enriched animals (+/−limonene or decanal/dodecanone) were stimulated with +limonene or decanal on the day of sacrifice (Table 1) to assess the responsiveness of newborn neurons to the odorant used for the enrichment or to a different odorant. +/−limonene enriched animals displayed more newborn neurons expressing Zif268 in response to +limonene than in response to decanal more Zif268-positive cells than nonenriched animals stimulated with +limonene [$F(5, 24) = 3.026$, $P = 0.029$, Fisher $P < 0.05$] (Fig. 2*E*). Similarly, in the decanal/dodecanone enriched animals, there was an increase of the percentage of BrdU/Zif268 double-labeled cells after decanal stimulation compared to +limonene stimulation and to nonenriched animals stimulated with decanal (Fisher $P < 0.05$, Fig. 2*F*).

Taken together, these results indicate that adult-born granule neurons are preferentially recruited in the processing of the enrichment odor.

Table 1. Experimental groups

Experimental groups	Odors used for the enrichment	Odor used for the stimulation the day of sacrifice
1	No odor	+ limonene
2	No odor	decanal
3	+/− limonene	+ limonene
4	+/− limonene	decanal
5	Decanal/dodecanone	+ limonene
6	Decanal/dodecanone	decanal

Perceptual Learning Increases GAD65/67 Expression. A computational model proposed that perceptual learning produces widespread increase in inhibitory processing that could underlie the observed increase in odor discrimination (5). To test this hypothesis, we assessed the level of GAD65/67 expression, the GABA synthesizing enzymes, by using optical density measurements on OB sections treated for immunohistochemistry of GAD65/67 and calculated a labeling index (Fig. 3*A*, see *Materials and Methods*). In both groups of enriched animals, the labeling index was increased compared to control animals [$F(2, 21) = 10.885$, $P = 0.001$; Fisher $P = 0.001$; Fig. 3*B*] indicating that olfactory enrichment increases GAD65/67 expression in the OB. These data suggest that odor enrichment induces an increase in GABA synthesis in the OB and could thereby increase the network inhibition.

Inhibition in the Olfactory Bulb Is Increased During Perceptual Learning. To confirm that the increase of GAD65/67 induced by perceptual learning is accompanied by an actual increase of inhibitory activity in the OB, in a separate group of mice we used paired-pulse stimulation of the lateral olfactory tract (LOT) to estimate the relative strength of granule-to-mitral inhibition in the OB (17, 18) after enrichment with +/−limonene and in control mice (Fig. 3*C*). ANOVA with experimental group (control and enriched) and Inter Stimulus Interval (ISI: 10, 20, 40,

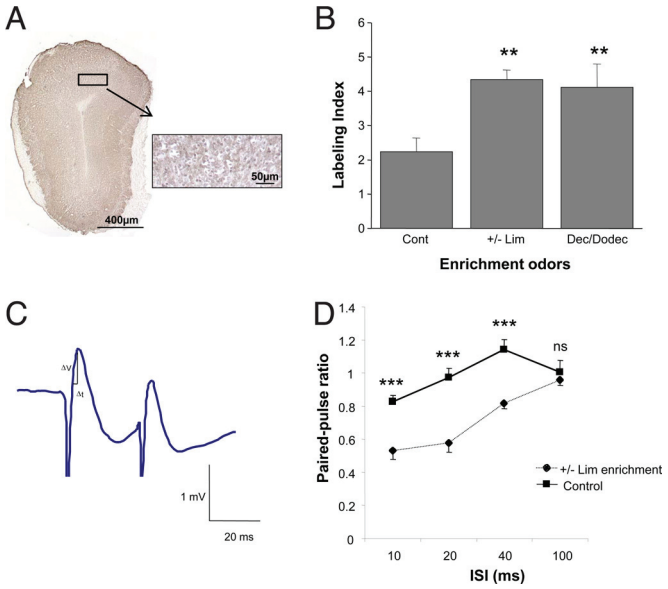

Fig. 3. Olfactory enrichment increases inhibition in the olfactory bulb. (*A*) Example of GAD65/67 immunolabeling in the OB. (*B*) Enrichment with +/−limonene or decanal/dodecanone increases the expression of GAD65/67 in the granule cell layer of the OB compared to control nonenriched animals. (**, $P <$ 0.001 for difference from control group). (*C*) Paired-pulse stimulation. The graph shows an example of population EPSPs in the granule cell layer in response to two LOT stimulations separated by 20 ms. The response to the second pulse is significantly decreased. (*D*) Paired-pulse inhibition quantified by the ratio between the rising slopes of responses to the second and first LOT pulse in control and +/−limonene enriched mice. The data are expressed as mean values ± SEM.

and 100 ms) as main factors showed a significant effect of group [F (1, 142) = 39.296, $P < 0.001$], ISI [F (3, 142) = 9.895, $P < 0.001$] and a significant interaction between group and ISI [F (3, 142) = 5.188, $P < 0.01$]. Paired-pulse inhibition was significantly larger in enriched as compared to control mice at 10, 20, and 40-ms delay ($P < 0.001$) but not at 100-ms delay ($P > 0.7$) (Fig. 3 *C* and *D*) indicating stronger inhibitory processes in the enriched OB. Before the electrophysiology experiment, we verified that enriched mice used in this experiment were able to discriminate the +/−limonene [F (1, 72) = 6.864, $P < 0.02$] whereas control mice were not [F (1, 32) = 2.393, $P > 0.05$]. Significant habituation for both groups was observed (difference between the first and the last habituation trial; $P < 0.05$ in all cases).

These data strengthen previous results on GAD65/67 expression and show an increase of functional inhibitory activity accompanying the improvement of olfactory discrimination after enrichment.

Neurogenesis Is Necessary for Perceptual Learning. Perceptual learning induces an increase of neurogenesis and an increased level of inhibition in the OB. Next, we tested how essential neurogenesis is for perceptual learning. For that purpose, we infused the mitotic blocker cytosine arabinoside (AraC) in the SVZ, which has been shown to block the division of constitutively proliferating cells (19) and as a consequence to inhibit neurogenesis. The AraC treatment was started 10 days before the enrichment and was maintained during the whole enrichment period (Fig. 4*A*).

After +/−limonene enrichment, AraC-treated mice had a strong reduction of BrdU-positive cell density in the OB compared to saline injected control mice [F (1, 5) = 8.838, $P = 0.031$] (Fig. 4*B*). A nonsignificant trend toward a reduction of BrdU-positive cell density was observed in the dentate gyrus of the hippocampus in AraC-treated, +/−limonene enriched mice (Fig. S4). The volume of the OB was not significantly modified by the treatment (Fig. S5). The strong reduction of OB neurogenesis induced by the AraC treatment had no effect on habituation memory, as evidenced by the habituation curves obtained in both groups (Fig. S6 *Ai* and *Aii*). However, inhibition of OB neurogenesis blocks perceptual learning (Fig. 4*C*). Indeed, Saline and AraC-treated groups behaved differently [F (1, 177) = 14.89, $P < 0.0001$]. Enriched mice treated with AraC did not discriminate any of the two odor pairs tested after the enrichment period [F (4, 47) = 5.97, $P < 0.0001$, Fisher between Hab4 and Otest, $P = 0.87$ for +/−limonene; F (4, 39) = 2.71, $P < 0.05$, Fisher $P = 0.48$ for pentanol/butanol; Fig. 4*Ci*], whereas saline treated enriched mice did [F (4, 45) = 11.89, $P < 0.0001$ Fisher $P = 0.004$ for +/−limonene; F (4, 51) = 3.69; $P < 0.001$; Fisher $P = 0.025$ for pentanol/butanol; Fig. 4*Cii*]. This result indicates that neurogenesis is required for perceptual learning. Saline and AraC treated mice significantly discriminated between mineral oil and limonene (Fig. S6*B*) indicating the absence of nonspecific effects of AraC. In addition, AraC treatment did not affect locomotor's activity as measured during a 2-min trial on an open field [F (1, 15) = 1.429, $P = 0.25$].

To further investigate whether newborn neurons support the increase of inhibition in the OB, we analyzed the expression of GAD65/67 in the OB of enriched mice after neurogenesis inhibition. GAD65/67 expression is lower in the AraC group compared to saline [F (1, 14) = 15.789, $P = 0.001$, Fig. 4*D*] indicating that increased neurogenesis contributes to the enhancement of bulbar inhibition during perceptual learning.

Discussion

Perceptual learning is evidenced by an improvement of discrimination due to prior experience and is a crucial feature of sensory system, which adjusts the level of discrimination between stimuli to a changing environment (4). We show here that the integration of newborn cells into the OB neural network is necessary for perceptual learning to occur in the olfactory system. The increase in newborn granule cell survival observed after enrichment is necessary for the increase in inhibitory activity in the OB, as evidenced by GAD65/67 immunohistochemistry and electrophysiology, and ultimately leads to better discrimination of highly similar odorants.

We confirmed that, similar to our previous observations in rat (5, 6), odor enrichment in mice affects the perception of odorants that activate at least partially overlapping regions of the OB.

Our computational model of the OB predicted that the perceptual effects of olfactory enrichment depend on strengthening of inhibitory inputs onto mitral cells. Because mitral cells are modulated by granule and periglomerular interneurons in the OB, which are the target of adult neurogenesis, we assessed the effect of a 10-day odor enrichment on OB neurogenesis. We found an increase in the number of BrdU-positive cells in the granule cell layer of odor enriched groups compared to the control group. Due to our BrdU protocol, this increase likely reflects an enhancement of cell survival rather than an increase in proliferation or migration (20, 21). Furthermore, newborn neurons respond preferentially to the odorant used for the enrichment. This result is in agreement with a previous study showing that the response of newborn granule cells is altered by olfactory experience in a stimulus-specific manner (13) and further suggests that newborn neurons support the learning-induced improvement of discrimination.

Correlative studies have suggested a role of neurogenesis in associative discrimination learning (14, 22, 23), as well as an impairment of spontaneous discrimination due to a low rate of neurogenesis (19, 24). However, a recent study reported no effect of blockade of neurogenesis on olfactory discrimination learning (25). Those contradictory results may be related to the

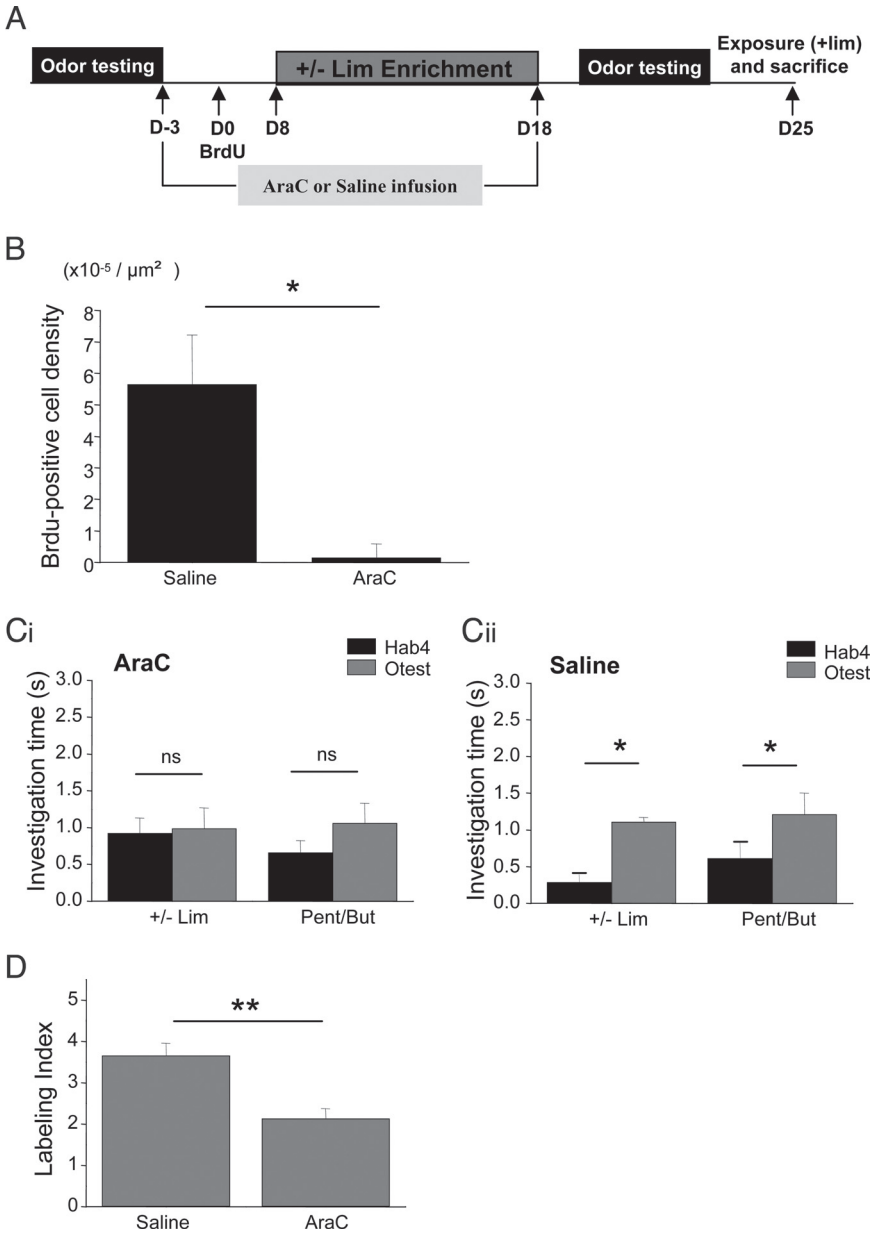

Fig. 4. Neurogenesis is necessary for perceptual learning. (*A*) Experimental design. Saline or AraC was locally infused 3 days before the administration of BrdU and lasting for 21 days. (*B*) +/−limonene enriched mice that received AraC have a significant reduction of BrdU-positive cell density in the granule cell layer of the OB. (*C*) The strong reduction of bulbar neurogenesis in the AraC group blocks the enrichment-induced improvement of discrimination that occurs in the saline group (*, $P < 0.05$; **, $P < 0.001$, ***, $P < 0.0001$). (*D*) The expression of GAD65/67 in the granule cell layer of the OB is decreased in the AraC group compared to the saline group. (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). The data are expressed as mean values ± SEM.

difficulty of the discrimination task used. Indeed, the more difficult the task, the more it seems to require modulation of newborn neuron survival (14). An easy task (25) is performed with a low level of neurogenesis, in accordance with our data in AraC treated animals. Our experiment, by using an ecological model of learning and testing discrimination of odorants with differential degrees of similarity, allows a fine and precise evaluation of olfactory discrimination and unravels the important role of neurogenesis in olfactory learning and perception. We further found that perceptual learning is accompanied by increased inhibition evidenced by increased in GAD65/67 expression and paired-pulse inhibition. Paired-pulse inhibition and GAD65/67 expression assessment suggest an overall increase of inhibition within the granule cell layer. This observation is compatible with the specific improvement of discrimination for the enriched odorants: the network involved in processing of individual odorants is specific but largely distributed across the OB (26) due to 1- activation of several glomeruli and 2- lateral dendrites of mitral cells running several hundred micrometers across the granule cell layer (27). As a consequence, even relatively localized changes in granule cell activation can create widespread changes in inhibitory inputs to mitral cells, as shown by computational modeling (5). This is in line with previous work showing a widespread increase in responsiveness of granule cells after perceptual learning (8).

We now propose that continuous integration of new GABAergic interneurons in the OB provides a way by which learning increases the inhibitory activity by increasing the density of

newborn interneurons. Modulation of neurogenesis thus adjusts bulbar odor representation, which in turn allows an improvement of olfactory discrimination.

In conclusion, relevant sensory input during learning allows an odor-specific increase of survival of newborn neurons. Neurogenesis sustains the ability of the adult network to adapt information processing to relevant ethologic needs.

Materials and Methods

Animals. Sixty adult male C57BL/6J mice (8 weeks old, Charles River, L'Arbresles, France) were used in this study. Thirty mice were involved in the first experiment including behavioral testing, quantification of newborn cells and assessment of GAD65/67 expression. A second cohort of 10 mice was used in the electrophysiology experiment. Finally, 20 more mice were used in the AraC experiment. All behavioral training was conducted in the afternoon (14:00–17:00). All efforts were made to minimize the number of animals used and their suffering during the experimental procedure in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the French Ethical Committee.

Behavioral Experiments

Enrichment. Swabs containing 100 μL of pure odorant were placed in tea balls hanging from the cover of the cage. Odorants were presented daily for 1 h during 10 days. Ten mice were enriched with +/−limonene and 10 other mice with decanal/dodecanone. Control mice were housed under the same conditions except that tea balls contained mineral oil ($n = 10$).

Discrimination Testing. We tested the discrimination of +/−limonene, pentanol/butanol, and decanal/dodecanone by using an olfactory habituation/dishabituation task (Table S1). A test session consisted of one presentation of mineral oil then four odor presentations of the habituation odor, followed by one presentation of test odor. Investigation time of the Otest significantly different from that of Hab4 indicated discrimination (*SI Methods*).

Newborn Cells in the OB. ***Bromodeoxyuridine (BrdU) administration.*** Mice were injected with BrdU (Sigma) (50 mg/kg in saline, 3× at 2-h intervals), 8 days before the beginning of the enrichment period (25 days before sacrifice).
Sacrifice and BrdU immunohistochemistry. Mice taken randomly from each experimental group were stimulated with 100 μL of pure +limonene ($n = 5$) or decanal ($n = 5$) before sacrifice and BrdU immunochemistry was carried on as described in *SI Methods* and (28).

Morphometry and BrdU-positive cell quantification. BrdU-positive cell densities were assessed in the GL and GrL of 24 sections of the left OB in all experimental groups and on five sections of the dentate gyrus. Cell counting procedures are detailed in *SI Methods*.

Double Labeling Immunohistochemistryand Analysis. BrdU, NeuN, calbindin, and Zif268-positive cells were detected by using rat anti-BrdU (1:100, Harlan Sera lab), mouse anti-NeuN (1:500; Chemicon), rabbit anti-calbindin (1:500; Chemicon), and rabbit anti-Zif268 antibody (1:1000; Santa Cruz Biotechnology) (*SI Methods*).

GAD65/67 Expression in the GrL. GAD65/67 immunohistochemistry (anti-rabbit GAD65/67 (1/750, Chemicon), was performed as described in *SI Methods*, by using optical density measurements (Morpho Expert, Explora Nova).

Paired-Pulse Inhibition. Electrophysiology was performed on control and +/−limonene enriched mice ($n = 2$ per group). Bipolar stimulation electrodes (5–10 mOHms, A-M Systems) were placed in the LOT (4.7 mm anterior; 3.4 mm lateral; 5.2 ventral from bregma). For recording of field potentials in the OB, a monopolar tungsten electrode (5–10 MOhms) was lowered into the approximate center of the OB until the population EPSP typical in the granule cell layer in response to LOT stimulation was observed. For paired-pulse stimulation, two 0.3-ms pulses were delivered at interstimulus intervals of 10, 20, 50, and 100 ms. Paired-pulse stimulation was quantified by measuring the ratio of the rising slope of the evoked potentials in response to the second and first pulse. Analysis was done on the paired pulse ratio of individual stimulations with experimental group (control and enriched) and interstimulus interval (10, 20, 50, and 100 ms) as main factors. For more details see *SI Methods*.

Neurogenesis Blockade. Mice were stereotaxically implanted with an osmotic pump (Alzet; anteroposterior, +1.2 mm; lateral, +0.9 mm, dorsoventral, −3 mm). Mice were injected in the SVZ with cytosine arabinoside AraC (4% in 0.9% saline, Sigma) ($n = 10$) or saline solution ($n = 10$) at a flow rate of 0.25 μL/h (*SI Methods*).

Statistical Analysis. Results were expressed as mean ± SEM. Differences between groups were assessed by using ANOVAs followed by *posthoc* comparisons with Fisher least significant difference test when appropriate (Systat statistical software). For all comparisons, values of $P < 0.05$ were considered significant.

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Supporting Information

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SI Methods

Behavioral Experiments. ***Discrimination testing.*** We tested in mice the discrimination of +/−limonene, pentanol/butanol, and decanal/dodecanone by using an olfactory habituation/dishabituation task (Table 1). Pentanol or butanol in one hand and decanal or dodecanone however elicit activation patterns in the OB that are respectively partially overlapping or distinct from +/−limonene (http://leonlab.bio.uci.edu/). All habituation experiments took place in clean cage similar to the home cage. Odors were presented by placing 60 μL of the odorant onto a filter paper (Whatman #1). The filter paper was put into a tea ball hanging from the cover of the cage. A test session consisted of one 50-s presentation of plain mineral oil then four 50-s odor presentations of the habituation odor at 5-min intervals (Hab1–4), followed by one 50-s presentation of test odor (Otest). Each odorant of each pair was used alternatively as habituation or test odorant. Animal groups were encoded for the test so that the experimenter was unaware of the identity of the animal group. The amount of time that the mice investigated the odorant was recorded during all trials. Investigation was defined as active sniffing within 1 cm of the odorant stimuli. The duration of investigation in response to the test odorant is a measure for discrimination between the habituated and test odorant.

Data analysis. Investigation time was averaged within groups for each trial. All data analyses on time spent sniffing during odor presentation trials were performed with Systat statistical software. Only mice that investigated the habituation odor for at least 1 s during its first presentation were included in the analysis. Results are presented as group mean ± SEM. After ANOVA testing for the main effects of group (+/−limonene enriched, decanal/dodecanone enriched or control nonenriched), test (pre or postenrichment) and trial number, post hoc pairwise comparisons (Fisher) were performed to determine (*i*) whether investigation time during the last habituation trial (Hab4) was significantly lower than during the first habituation trial (Hab1) (habituation) and (*ii*) whether the investigation responses to the presentation of the test odor (Otest) were significantly different in duration from that elicited by the habituation odor during its fourth presentation (Hab4). The level of significance was set to 0.05.

Newborn Cells in the OB. ***Sacrifice.*** Ten mice taken randomly from each experimental group were put in a clean cage during 1 h, and then stimulated during 1 h by hanging in the cage a tea ball containing 100 μL of pure +limonene ($n = 5$) or decanal ($n = 5$). One hour after the end of the odor stimulation, mice were deeply anesthetized (Urethane, 2g/kg) and were killed by intracardiac perfusion of 50 mL of cold fixative [paraformaldehyde 4% in PBS, pH 7.4]. Brains were removed, post fixed, cryoprotected in sucrose (20%), frozen rapidly and then stored at −20 °C before sectioning with a cryostat.

BrdU immunohistochemistry. Briefly, 14 μm-thick sections were incubated in Target Retrieval Solution (Dako) for 20 min at 98 °C. After cooling, they were treated with pepsin (0.43 U/mL in 0.1N HCl, Sigma) for 3 min. Sections were transferred to a blocking solution (5% normal horse serum (Sigma) with 5% BSA and 0.125% Triton X-100), and were then incubated overnight at 4 °C with a mouse anti-BrdU antibody (1/100, Chemicon), followed by a biotinylated anti-mouse secondary antibody (1/200, Vector Laboratories) for 2 h. The sections were then processed through an avidin–biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). After dehydration in graded ethanols, the sections were defatted in xylene and cover-slipped in DPX (Fluka, Sigma).

Morphometry and BrdU-positive cell quantification. Data were collected with the help of mapping software (Mercator Pro, Explora Nova), coupled to a Zeiss microscope. The surfaces of the GL and the GrL were measured on 24 sections at 70-μm intervals and the volumes of the two layers were calculated. In addition, all BrdU-positive cells were counted in the GL and GrL of the 24 sections of the left OB in all experimental groups ($n = 5$ per group). Density of BrdU-positive cells (number of labeled profiles/μm^2) was calculated for each layer. The density of BrdU-positive cells was also assessed in the granule and subgranular layers of the dente gyrus (sample of five sections at 350-μm intervals).

Double labeling immunohistochemistry. To determine the phenotype of BrdU-positive cells in the OB, double-labeling was performed by using a rat anti-BrdU (1:100; Harlan Sera Laboratory) and a mouse anti-NeuN (1:500; Chemicon) or a rabbit anti-calbindin (1:500; Chemicon). For the Zif268/BrdU double labeling, a rabbit anti-Zif268 antibody (1:1000; Santa Cruz Biotechnology) was used. The appropriate secondary antibodies, coupled to Alexa 633 (Molecular Probes) for revelation of BrdU and Alexa 488 (Molecular Probes) for revelation of the different markers, were used.

Double labeling analysis. On five animals of each experimental group, 30 BrdU-positive cells per animal were examined for colabeling with NeuN, Calbindin or Zif268. Double-labeling was analyzed by confocal scanning microscopy (Zeiss). Each labeled cell was examined along the *z*-axis to ensure proper identification of double labeled cells. A percentage of double labeled cells was calculated for each group ($n = 5$ animals per group) and compared by using ANOVA followed by Fisher post hoc test.

GAD65/67 Expression in the GrL. ***GAD65/67 immunohistochemistry.*** Sections were transferred to a blocking solution [5% normal goat serum (Sigma) with 2% BSA and 0.5% Triton X-100], and were then incubated overnight at 4 °C in an anti-rabbit GAD65/67 (1/750; Chemicon), followed by a biotinylated anti-rabbit secondary antibody (1/200; Vector Laboratories) for 2 h. The sections were then processed through an avidin–biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). After dehydration in graded ethanols, the sections were defatted in xylene and cover-slipped in DPX (Fluka, Sigma).

Data analysis. By using optical density measurements (Morpho Expert, Explora Nova), we compared the expression level of GAD65/67 between the different experimental groups ($n = 5$ animals per group). All GAD65/67 quantifications were conducted blind with regards to the mouse status. For each animal, the optical density was measured in the GrL on four sections at 300-μm intervals. A threshold was set, similar for all groups and integral optical density was measured in labeled regions. A labeling index was then calculated for each section as follows: integral optical density/labeled surface*labeled surface relative to total surface of the granule cell layer. Labeling index was then averaged across animals within each group and presented in Figs. 4 and 5 as mean ± SEM. Between-groups comparisons were performed by ANOVA followed by Fisher post hoc test.

Paired-Pulse Inhibition. Electrophysiology was performed on control and +/−limonene enriched groups (five mice per group). Mice were anesthetized with urethane (1.5 g/kg, i.p.). Levels of anesthesia were monitored by rate of respiration and foot

withdrawal reflex and supplemented if necessary by i.p injections. Body temperature was maintained at 37 °C with a heating pad. Anesthetized animals were placed in a stereotaxic apparatus and the skull was exposed by scalp incision. Bipolar stimulation electrodes (5–10 mOHms, A-M Systems) were placed in the LOT (4.7 mm anterior; 3.4 mm lateral; 5.2 ventral from bregma). All stimulation currents were delivered by a constant current stimulus isolation unit (Grass Instruments) controlled by a Grass Instrument stimulator. For recording of field potentials in the OB, a monopolar tungsten electrode (5–10 MOhms; A-M Systems) was lowered into the approximate center of the OB until the population EPSP typical in the granule cell layer in response to LOT stimulation was observed. Signals were amplified (1,000×) and filtered between 0.1 and 600 Hz via a Neuralynx differential amplifier. Data acquisition, display and control of stimulus were done by use of computer by using the Spike 2 software (Cambridge Electronics Design). For paired-pulse stimulation, two 0.3-ms pulses were delivered at interstimulus intervals of 10, 20, 50, and 100 ms. Paired-pulse stimulation was quantified by measuring the ratio of the rising slope of the evoked potentials in response to the second and first pulse (Fig. 4*C*).

Analysis was done on the paired pulse ratio of individual stimulations with experimental group (control and enriched) and interstimulus interval (10, 20, 50, and 100 ms) as main factors.

Neurogenesis Blockade. To determine the implication of newborn neurons in the improvement of discrimination due to the enrichment, we used mitotic blocker cytosine arabinoside (AraC) to inhibit neurogenesis. Because newborn cells need ≈10 days to reach and begin to functionally integrate the OB, the treatment began 11 days before the enrichment and went on during all of the enrichment period (total 21 days). A new pool of twenty adult C57BL/6 mice were anesthetized with mixture injection of 50 mg/kg ketamine and 7.5 mg/kg xylazine (i.p.) and secured in a stereotaxic instrument (Narishige Scientific Instruments). All animals were implanted with an osmotic pump (Alzet). The cannula was located anterior to the left ventricle, in the subventricular zone (SVZ) (anteroposterior, +1.2 mm; lateral, +0.9 mm, dorsoventral, −3 mm). Mice were injected with AraC (4% in 0.9% saline, Sigma) ($n = 10$) or saline solution ($n = 10$) at a flow rate of 0.25 μL/h. To assess the level of neurogenesis, BrdU was injected (50 mg/kg, 3× every 2 h) 25 days before the sacrifice (Fig. 5). At the end of the enrichment and before the discrimination testing, all mice were anesthetized and the catheter between the cannulae and the pump was sectioned to stop the infusion. All animals were enriched with +/−limonene and the performances of discrimination were tested on two odor pairs (+/−limonene and pentanol/butanol) and between +limonene and mineral oil. Because we injected the drug in the left SVZ, we decided to perform the olfactory test by using mice with the right naris deprived of olfactory input. To do so, mice infused with saline or AraC were lightly anesthetized with 25 mg/kg ketamine and 3.25 mg/kg xylazine (i.p.) and unilateral olfactory deprivation was achieved by inserting a nose plug into the right external naris. In addition, assessment of locomotor's activity was performed by using a video tracking system (View Point) during one 2-min trial in an open field (40 cm × 40 cm). The recorded parameter was the total distance covered.

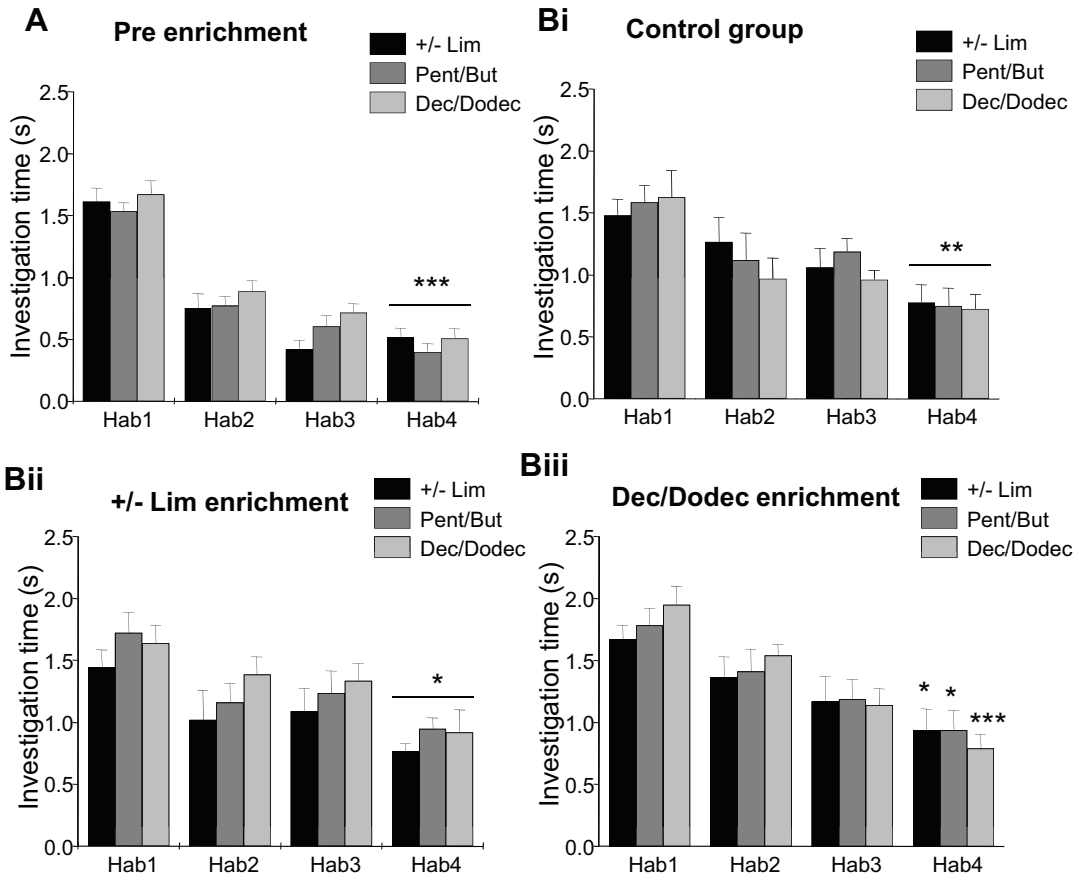

Fig. S1. Behavioral habituation (*A*) before enrichment [F (4, 456) = 84.77, $P < 0.0001$] and after enrichment period in (*Bi*) control group [no odor, F (4, 50) = 3.757, $P = 0.01$] (*Bii*) group enriched with +/−limonene [+/−Lim, F (4, 40) = 2.819, $P = 0.38$] or (*Biii*) group enriched with decanal/dodecanone [Dec/Dodec, F (4, 41) = 2.751, $P = 0.041$]. Asterisks indicate a significant difference in investigation time between Hab1 and Hab4 (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$).

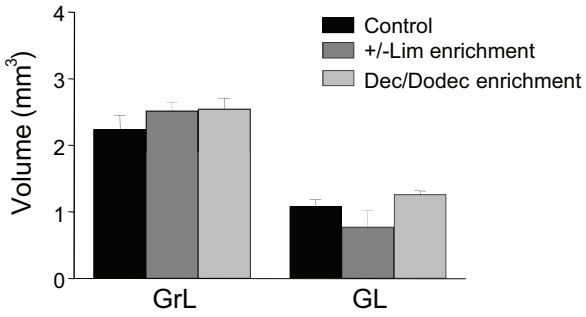

Fig. S2. Olfactory enrichment with +/− limonene (+/− Lim) or decanal/dodecanone (Dec/Dodec) has no effect on granule cell layer (GrL) or glomerular cell layer (GL) volume.

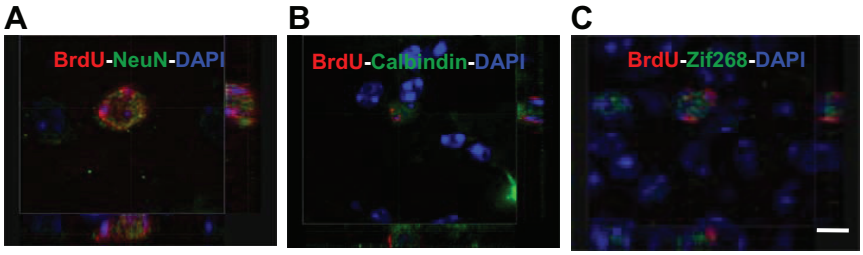

Fig. S3. (*A*) Confocal imaging of BrdU/NeuN double immunolabeling in the GrL. (Scale bar, 5 μm.) (*B*) Confocal imaging of BrdU/Calbindin double immunolabeling in the GL. (*C*) Confocal imaging of BrdU/Zif268 double immunolabeling in the GrL.

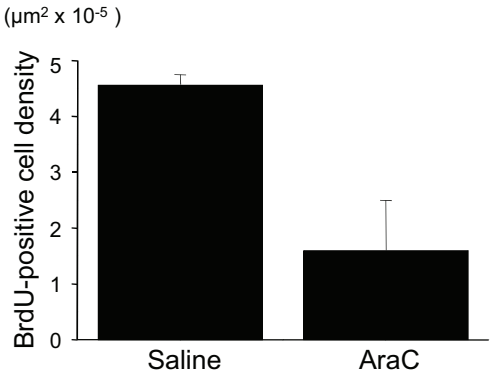

Fig. S4. In the hippocampus, a nonsignificant ($P = 0.059$) reduction in BrdU-positive cell density can be observed in the group infused with AraC during 21 days compared to saline-infused animals.

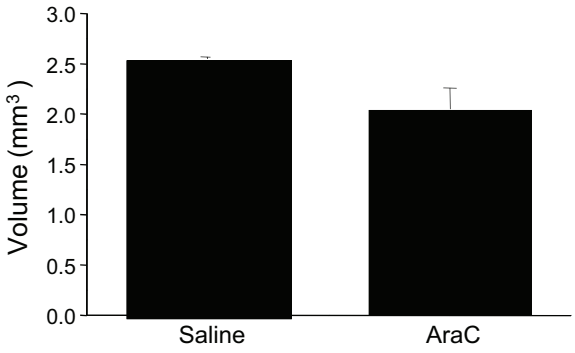

Fig. S5. AraC infusion during 21 days has no effect on granule cell layer's volume compared to the saline infusion.

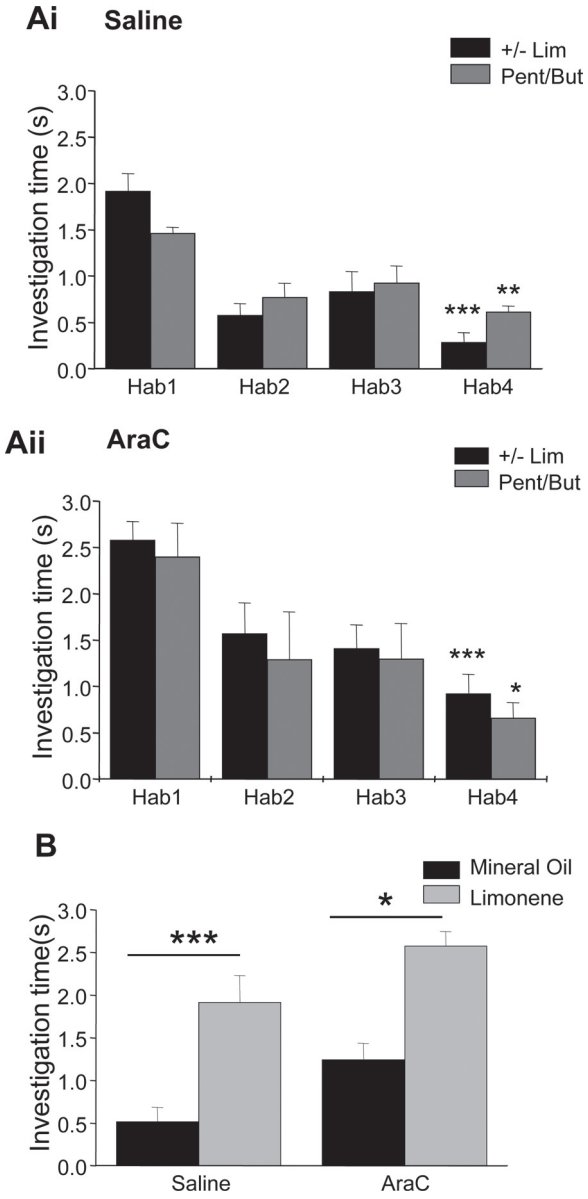

Fig. S6. Behavioral habituation after a 10-day enrichment period with +/− limonene, (*Ai*) in saline infused group [F (4, 45) = 11.889, $P < 0.0001$] and (*Aii*) in AraC infused group [F (4, 47) = 5.97, $P = 0.001$]. Asterisks indicate a significant difference in investigation time between Hab1 and Hab4 (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$). (*B*) AraC infusion does not alter basic olfactory discrimination between mineral oil and limonene (*, $P < 0.05$; ***, $P < 0.0001$).

Table S1. Odor sets used for spontaneous discrimination task with corresponding vol/vol dilutions (1 Pa)

	Odorants	
Odor sets	A	B
1	+ Limonene (0.204%)	− Limonene (0.204%)
2	Butanol (0.021%)	Pentanol (0.074%)
3	Decanal (1.776%)	Dodecanone (124.98%)

Etude 2 :

Action of the noradrenergic system on adult-born cells is required for olfactory learning in mice

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Behavioral/Systems/Cognitive

Action of the Noradrenergic System on Adult-Born Cells Is Required for Olfactory Learning in Mice

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We have previously shown that an experience-driven improvement in olfactory discrimination (perceptual learning) requires the addition of newborn neurons in the olfactory bulb (OB). Despite this advance, the mechanisms which govern the selective survival of newborn OB neurons following learning remain largely unknown. We propose that activity of the noradrenergic system is a critical mediator providing a top-down signal to control the selective survival of newly born cells and support perceptual learning.

In adult mice, we used pharmacological means to manipulate the noradrenergic system and neurogenesis and to assess their individual and additive effects on behavioral performance on a perceptual learning task. We then looked at the effects of these manipulations on regional survival of adult-born cells in the OB. Finally, using confocal imaging and electrophysiology, we investigated potential mechanisms by which noradrenaline could directly influence the survival of adult-born cells.

Consistent with our hypotheses, direct manipulation of noradrenergic transmission significantly effect on adult-born cell survival and perceptual learning. Specifically, learning required both the presence of adult-born cell and noradrenaline. Finally, we provide a mechanistic link between these effects by showing that adult-born neurons receive noradrenergic projections and are responsive to noradrenaline.

Based upon these data we argue that noradrenergic transmission is a key mechanism selecting adult-born neurons during learning and demonstrate that top-down neuromodulation acts on adult-born neuron survival to modulate learning performance.

Introduction

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

In previous work, we have shown that perceptual learning increased the responsiveness of the bulbar inhibitory granule cells to odorants as measured by expression of an immediate early gene (Mandairon et al., 2008b). A computational model (Mandairon et al., 2006c) as well as recent data using GAD65/67 expression and paired-pulse inhibition (Moreno et al., 2009) led us to conclude that perceptual learning produces widespread changes in inhibitory processing which can underlie the observed improvement of odor discrimination. In line with these findings, we have recently shown that inhibitory interneurons, which regulate the activity of mitral cells and are continuously generated in adulthood (Lois and Alvarez-Buylla, 1994; Petreanu and Alvarez-Buylla, 2002), are required for perceptual learning to occur in the olfactory system (Moreno et al., 2009). This suggests that perceptual plasticity relies on long-term changes in odor processing at the level of the olfactory bulb (OB) involving increased survival of adult-born neurons.

However, the mechanisms governing the integration and survival of adult-born neurons during learning are unknown. A potential candidate is noradrenaline (NA). Indeed, the granule cell layer of the OB, the main target of neurogenesis is largely innervated by the noradrenergic system coming from the locus ceruleus (Shipley et al., 1985; McLean et al., 1989). NA plays a critical role in olfactory perception as well as learning (Fletcher and Chen, 2010). For example, in newborn rats, NA is involved in the acquisition of conditioned odor preferences (Sullivan and Wilson, 1994; Moriceau and Sullivan, 2004) and in the learning of an odor-based attachment to the mother (Sullivan et al., 2000). In adult animals, the presence of NA is required for the modulation in the OB of electrophysiological responses associated with olfactory learning (Gray et al., 1986), for long-lasting suppression of odor responses after pairing locus ceruleus stimulation with odor presentation (Shea et al., 2008), for spontaneous discriminations (Mandairon et al., 2008a) and short-term olfactory memory (Veyrac et al., 2007, 2009). Interestingly, olfactory enrichment stimulates the noradrenergic system (Veyrac et al., 2009; Rey et al., 2012) and NA is a positive modulator of newborn neuron survival (Bauer et al., 2003; Bovetti et al., 2011).

In the present study, we were interested in the potential role of NA as a mediating signal for adult-born neuron integration during perceptual learning. We found that the improvement of odor

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Table 1. Odor pairs used for olfactory discrimination testing (cross-habituation testing) and corresponding v/v dilutions (1 Pa)

Odor pairs	Odors
1	+Limonene and −Limonene (0.204%)
2	Pentanol (0.074%) and Butanol (0.021%)
3	Decanal (1.78%) and (2)-Dodecanone (12.5%)
4	(+) Limonene (0.204%) and Decanal (1.78%)
5	Isoamyl acetate (0.05%) and Octanal (0.148%)

Each pair is composed of perceptually similar odorants (1 to 3) or dissimilar odorants (4 and 5) diluted in mineral oil to have similar vapor pressure.

discrimination due to enrichment is under the control of the noradrenergic system. Moreover, we found that the noradrenergic system acted on adult-born cells to enhance the discrimination ability of mice. These data are supported by experiments showing NA projections onto adult-born neurons and an electrical response to NA application. These data provide a significant advancement in our understanding of the role of neurogenesis in the adult brain, its regulation by internal inputs (NA) in response to environmental stimuli, and its contribution to improvements in perceptual ability.

Materials and Methods

Animals

Ninety-seven adult male C56BL/6J mice (Charles River) aged 8-weeks at the beginning of the experiments, were housed under a 12 h light/dark cycle in an environmentally controlled room. All behavioral training was conducted in the afternoon (2:00–5:00 P.M.). All efforts were made to minimize the number of animals used, and the experimental procedures were in accordance with the European Community Directive of 24 November 1986 (86/609/EEC), and the French Ethical Committee.

Experimental design

For all experiments, before any drug treatment and/or environmental manipulations (enrichment with +/−limonene), all groups of animals were tested for their ability to spontaneously discriminate between three pairs of similar odorants: + and −limonene, pentanol/butanol, and decanal/dodecanone using an olfactory cross habituation test (see below). To assess the efficacy of our manipulations to modulate olfactory discrimination abilities, mice were then subjected to an identical post-test, beginning the day after the end of treatment, again using all of the three odorant pairs to assess changes in their discrimination ability.

Olfactory cross-habituation test

We used a cross-habituation test (Cleland et al., 2002; Mandairon et al., 2006c; Imayoshi et al., 2008; Breton-Provencher et al., 2009; Lazarini et al., 2009; Moreno et al., 2009) to assess spontaneous discrimination because it is a naturalistic task which relies upon intrinsic motivation whereas go-no go or other associative tasks rely upon exogenous rewards which can influence learning and engage other systems.

Behavioral testing. Briefly, the olfactory cross-habituation task assesses the degree to which mice are able to spontaneously discriminate between odorants by habituating them to an odorant (Ohab) and measuring their cross-habituation to a second odorant (Otest). If the second odorant is not discriminated from the first, it will not elicit an increased investigation response by the mouse. Each presentation lasts 50 s and is separated by 5 min. Odors are presented using a tea ball hanging on the cover of the cage and containing 60 μl of the diluted odor (1 Pa, Table 1) on filter paper (Whatman No. 1). Odors are renewed between each test. Each odorant of each pair was used alternatively as habituation or test odorant. Animal groups were encoded for the test so that the experimenter was unaware of the identity of the animal group. The amount of time that the mice investigated the odorant was recorded during all trials. Only mice that investigated Ohab for at least 1 s during its first presentation were included in the analysis. Outlier trials that deviated from the mean by more than two SDs were also excluded from analysis (between 2 and 5 outlier trials among 100 trials total per experimental group for one odor pair were excluded). The pairs of similar odorants used for the cross-habituation test were the following: +/−limonene, pentanol/butanol and decanal/dodecanone (Table 1). Odorant pairs were chosen based upon their perceptual similarity and their activation of overlapping regions of the glomerular cell layer. Indeed, butanol and pentanol are partially overlapping with limonene whereas decanal and dodecanone are more distinct from limonene (Mandairon et al., 2006c). All odor pairs were encoded so that the experimenter was unaware of the identity of each odor.

Table 2. Treatments used for the different experimental groups

Experiment	Enrichment odors	Drugs used during the enrichment	Size of group
1	No odor	Saline	10
1	+/−Limonene	Saline	10
1	No odor	Labetalol (25 mg/kg, i.p.)	10
1	+/−Limonene	Labetalol (25 mg/kg, i.p.)	10
1	No odor	Dexefaroxan (0.63 mg/kg, i.p.)	10
2	No odor	Dexefaroxan (0.63 mg/kg, i.p.) + saline intracerebral infusion	15
2	No odor	Dexefaroxan (0.63 mg/kg, i.p.) + AraC intracerebral infusion	15

Experiment 1: Animals were submitted to a 10 d +/−limonene enrichment period or were not enriched. In parallel, to modulate the noradrenergic system, animals were injected with saline solution, dexefaroxan, or labetalol. Experiment 2: Animals infused with AraC or Saline solution in the subventricular zone were treated during a 10 d period with dexefaroxan in the absence of odorant.

Statistical analyses. All data were analyzed using Systat statistical software (Systat Software). The data for each odor pair were analyzed by ANOVA followed by *post hoc* Fisher test to determine (1) if the time of investigation during Ohab4 is significantly lower than during Ohab1 (habituation) and (2) if the time of investigation Otest is significantly higher than the one of Ohab4 (discrimination). The level of significance was set to 0.05.

Drug treatment and/or environmental manipulations

In Experiment 1, mice were randomly assigned to five experimental groups (Table 2).

Odor enrichment and control. Odor enrichment consisted of exposure with + and −limonene for 1 h per day for 10 consecutive days. Odors were presented simultaneously on two separate swabs containing 100 μl of pure odor placed in two separate tea balls and hung from the cover of the animals cage (Mandairon et al., 2006a). Control, non-enriched mice were housed under the same conditions except that the two tea balls were left empty.

Dexefaroxan and labetalol drug treatment. Twenty minutes before each enrichment session, each mouse received a single intraperitoneal injection of 100 μl of freshly prepared drug or saline solution (Table 2; a total of 10 injections). The two noradrenergic agents used were the α2 presynaptic receptor antagonist dexefaroxan hydrochloride (0.63 mg/kg in saline; synthesized and provided by Centre de Recherche Pierre Fabre, Castres, France) promoting noradrenaline release and the α1-β receptor antagonist labetalol hydrochloride (25 mg/kg in saline; (Sigma) (Veyrac et al., 2007).

Cellular analysis

BrdU injections. Newly born cells of the OB require several days to migrate from the subventricular zone to the OB, where they differentiate into mature granule and periglomerular cells. Thus, we injected mice with bromodeoxyuridine (BrdU; Sigma; 50 mg/kg in saline three times daily at 2 h intervals, i.p.) 8 d before enrichment to investigate the survival of BrdU-labeled cells that are in the OB during the period of enrichment. Animals were killed 25 d after the injections (Fig. 1*A*).

Histology. Five mice were randomly chosen from each experimental group and were put in a clean cage for 1 h. To investigate immediate early gene expression in response to odorant exposure, mice were then presented with a tea ball containing 100 μl of pure + limonene or decanal for 1 h. One hour after the end of the odor stimulation, mice were deeply anesthetized (Urethane, 2 g/kg) and were killed by intracardiac perfusion of 50 ml of cold fixative (paraformaldehyde 4% in PBS, pH 7.4). Brains

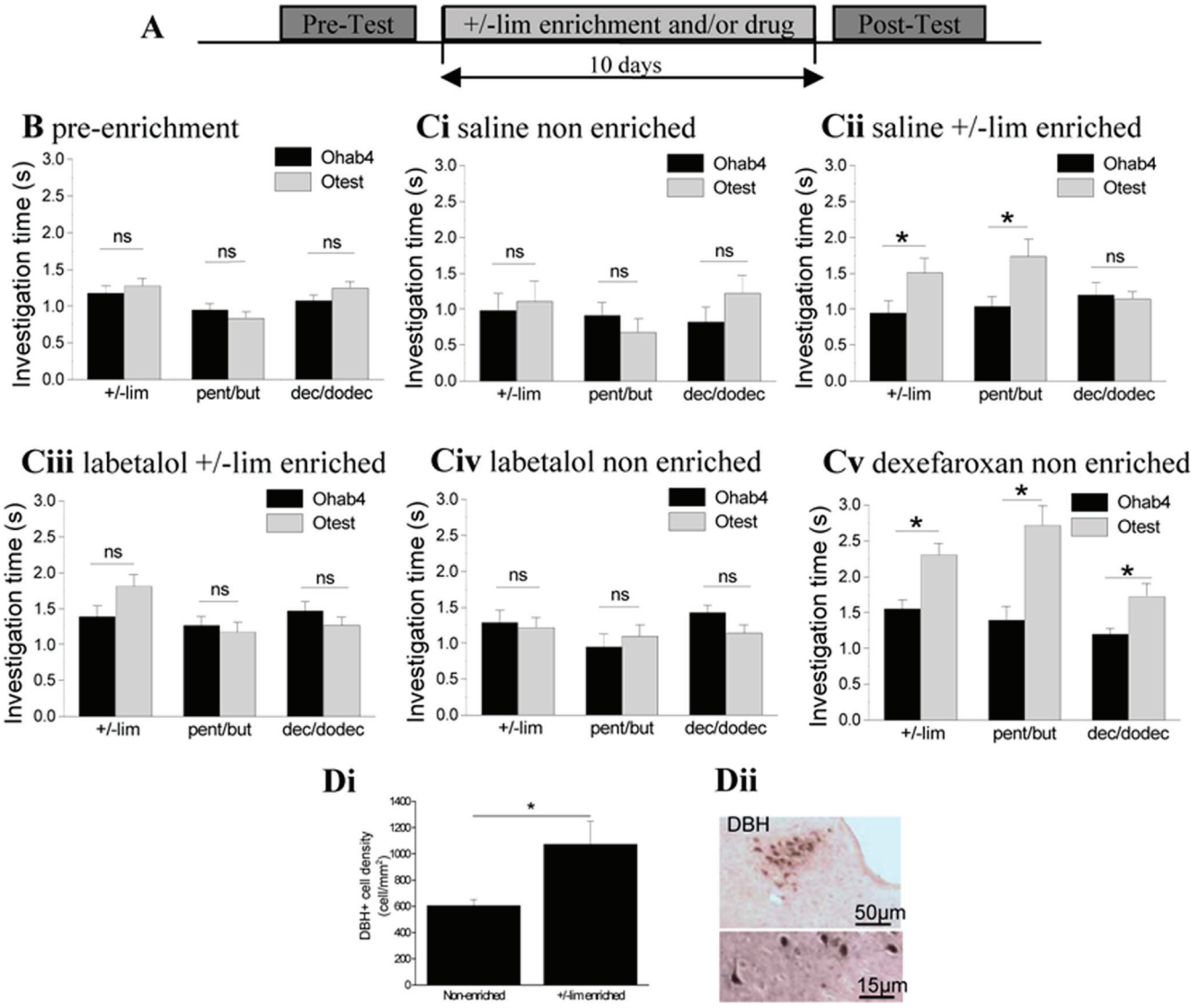

Figure 1. Perceptual learning is under control of the noradrenergic system. ***A***, Time course of the experiment. Spontaneous discrimination between +/−limonene (+/−lim), pentanol/butanol (pent/but), and decanal/dodecanone (dec/dodec) was tested before and after a period of olfactory enrichment and/or drug treatment. Experimental groups were enriched by introducing +/−lim into the home cage for 1 h periods over 10 d and treated during the same period of time with dexefaroxan, labetalol, or saline. *B*, *C*, Behavioral discrimination was tested before (***B***) and after (***C***) the treatment period. The two odorants of each pair are cross-habituated before the enrichment period and thus not discriminated (***B***), as well as in the control non-enriched group (***Ci***). After enrichment with +/−lim, two of the three odor pairs are discriminated (+/−lim and pent/but) (***Cii***). Treatment with labetalol with (***Ciii***) or without (***Civ***) enrichment prevents the improvement of discrimination. In contrast, after treatment with dexefaroxan without any enrichment, all three odor pairs are discriminated (***Cv***). *$p < 0.05$ in response magnitude between trials 4 (Hab4) and 5 (Otest). ***Di***, Quantification of DBH-positive neurons in the locus ceruleus. Odor enrichment increased the density of DBH-positive cells compared with non-enriched animals. *$p < 0.05$. ***Dii***, DBH labeling in the locus ceruleus. The data are expressed as mean values ± SEM.

were removed, postfixed, frozen rapidly and then stored at −20°C before sectioning with a cryostat.

BrdU immunocytochemistry. The protocol has been described in detail previously (Mandairon et al., 2003). Sections were incubated overnight in a mouse anti-BrdU antibody (1:100, Millipore Bioscience Research Reagents) at 4°C followed by a biotinylated anti-mouse secondary antibody (1:200, Vector Laboratories) for 2 h. The sections were then processed through an avidin-biotin-peroxidase complex (ABC Elite Kit, Vector Laboratories). Following dehydration in graded ethanols, the sections were defatted in xylene and coverslipped in DPX (Fluka, Sigma).

Zif268 immunocytochemistry. Sections were incubated overnight in a rabbit anti-Zif268 antibody (1:1000, Santa Cruz Biotechnology) at room temperature for 16 h. Sections were then incubated in a biotinylated anti-rabbit secondary antibody (1:200, Vector Laboratories) for 2 h. The remaining treatments were similar to those for the BrdU labeling.

Data analysis (BrdU and Zif-268 levels). The OB of mice was coronally sectioned (14 μm). Every fifth section was processed for immunostaining (sampling interval = 70 μm). Within each analyzed section, every BrdU- or Zif268-positive cell was counted in the granule layer of the right OB using mapping software (Mercator, Explora Nova, La Rochelle, France) coupled to Zeiss microscope. The mean positive cell density was calculated and averaged within each experimental group. Between-groups comparisons were performed by ANOVA followed by a *post hoc* Fisher test. The level of significance was set to 0.05.

To confirm the link between Zif268 expression and neural activity, the right OB of four additional mice was sensory deprived by naris occlusion using plug insertion as described in our previous report (Mandairon et al., 2006b). Zif268 expression has been assessed on 4 sections per animals in both right and left OBs. Between-OB comparison was performed by paired *t* test.

BrdU- and Zif-268-positive cell mapping. Maps of positive cells were constructed as follows: The layer was divided into 36 sectors of 10° with a reference axis drawn parallel to the most ventral aspect of the subependymal layer of the OB (Mandairon et al., 2006d). The cell density (number

of labeled profiles/μm^2) was calculated for each sector. Measurements were then merged into arrays of 10° × 70 μm bins. The most rostral aspect of the accessory OB served as an anatomical landmark to align the sections across animals (Matlab v.6). For visualization of the positive cell density maps, arrays were averaged across animals within each group, and colored image plot of the data was constructed in Matlab v.6.

Double-labeling immunohistochemistry. To determine the phenotype of BrdU-positive cells in the OB, double-labeling was performed using a rat anti-BrdU (1:100, Harlan Sera-Lab) and a mouse anti-NeuN (1:500, Millipore Bioscience Research Reagents) or a rabbit anti-calbindin (1:500, Millipore Bioscience Research Reagents). For the Zif268/BrdU double labeling, a rabbit anti-Zif268 antibody (1:1000, Santa Cruz Biotechnology) was used. The appropriate secondary antibodies, coupled to Alexa Fluor 633 (Invitrogen) for revelation of BrdU and Alexa Fluor 488 (Invitrogen) for revelation of the different markers, were used.

Double-labeling analysis. On five animals of each experimental group, 20–25 BrdU-positive cells per animal were examined for colabeling with NeuN or Zif268. Double-labeling was analyzed by pseudo-confocal scanning microscopy (apotome, Zeiss). Each labeled cell was examined along the *z*-axis to ensure proper identification of double labeled cells. A percentage of double labeled cells was calculated for each group and compared using ANOVA followed by Fisher *post hoc* test.

AraC treatment and BrdU labeling. To determine the target of the noradrenergic system during perceptual learning, we used mitotic blocker AraC to inhibit neurogenesis (Moreno et al., 2009; Sultan et al., 2010). A new pool of 40 adult C57BL/6 mice were anesthetized with a cocktail injection of 50 mg/kg ketamine and 7.5 mg/kg xylazine (i.p.) and secured in a stereotaxic instrument (Narishige Scientific Instruments) (Table 2, Experiment 2). All animals were implanted with an osmotic pump (Alzet Osmotic pumps 2004). The cannula was located in the left subventricular zone (SVZ) (anteroposterior, +1.2 mm; lateral, +0.9 mm, dorsoventral, −3 mm). Mice were injected with AraC (4% in 0.9% saline, Sigma) ($n = 25$) or saline solution ($n = 15$) at a flow rate of 0.25 μl/h (Table 2, Experiment 2). To assess the level of neurogenesis, BrdU was injected (50 mg/kg, 3 times every 2 h) 3 d after the beginning of AraC infusion. All animals (AraC- or saline-infused mice) were injected with dexefaroxan once a day during 10 d with no odor enrichment. At the end of the treatment by dexefaroxan and before the discrimination testing, the infusion of AraC or saline was stopped by sectioning and plugging the catheter between the pump and the cannulae, under light anesthesia with 25 mg/kg ketamine and 3.25 mg/kg xylazine (i.p.). Then, a day after the end of the drug treatment and AraC/Saline infusion, discrimination performances were assessed on three similar odor pairs (+/−limonene, pentanol/butanol and decanal/dodecanone) and two dissimilar odor pairs (+limonene/decanal and isoamyl acetate/octanal) using a cross-habituation task.

Dopamine β-hydroxylase immunohistochemistry and quantification. To detect noradrenergic neurons in the locus ceruleus, immunohistochemistry of the noradrenaline synthetic enzyme dopamine β-hydroxylase (DBH) was performed on brainstem sections including the locus ceruleus of 5 animals per group (Rey et al., 2012). Brain sections were incubated in Target Retrieval Solution for 20 min at 98°C. After cooling, they were incubated for 90 min in 5% normal serum, 5% BSA, and 0.125% Triton X-100 and then in rabbit anti-DBH primary antibody (1:200; Santa Cruz Biotechnology) for 40 h at 4°C. Sections were then incubated in a biotinylated goat anti-rabbit secondary antibody (1:200; Vector Laboratories) for 2 h. The remaining treatments were similar to those for the BrdU labeling. For each animal, all sections (intersection interval 140 μm) containing DBH-positive cells were analyzed. The area containing DBH-positive cells was delineated and labeled cells (very dark cells with marked key lines) were counted. The number of DBH-positive cells was related to the selected area and data were expressed as the number of positive cells per mm^2 (density). Statistical analysis was performed by an unilateral *t* test comparing non-enriched and enriched animals.

Norepinephrine transporter-newborn GFP cell double-labeling. The subventricular zone of the experimental mice ($n = 3$) was stereotaxically injected with lentiviruses expressing the GFP driven by the PGK promoter (200 nl/injection site) using a Narishige Scientific Instruments. The coordinates were as follows: anteroposterior +0.5 mm, mediolateral ±1.1 mm, dorsoventral −1.7 mm, and anteroposterior +1 mm, mediolateral ±1 mm and dorsoventral −2.3 mm. Mice were then killed 15 d after injection to observe whether NA project on newborn cells during the enrichment period. Brains were removed, postfixed, frozen rapidly and then stored at −20°C before sectioning with a cryostat (coronal sections of 40 μm). Immunohistochemistry of norepinephrine transporter (NET), a marker of noradrenergic terminals was performed in the OB. OB sections were rehydrated and incubated for 30 min in Triton X-100 0.1%, followed by a 90 min incubation in 5% normal horse serum in 5% BSA and 0.1% Triton X-100 to block unspecific binding, and incubated for 72 h at 4°C in mouse anti-rat NET antibody (1:1000; MAb Technologies). Sections were then incubated in horse anti-mouse biotinylated antibody (1:200; Vector Laboratories) for 2 h. They were then incubated in streptavidin-coupled Alexa Fluor 488 (1:1000; Invitrogen). Double-labeling was analyzed by pseudo-confocal microscopy (Zeiss microscope equipped with an apotome).

Electrophysiological experiments

For electrophysiological experiment, eight additional mice were injected with lentiviruses expressing the GFP as described previously. From 12 to 18 d after lentiviruses injections animals were anesthetized with intraperitoneal injection of 50 μl of ketamine (50 mg/ml) and killed by decapitation. The head was quickly immersed in ice-cold (2−4°C) cutting artificial CSF (Cutting-ACSF containing, in mM: 135 Choline chloride, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 1 Na-piruvate, 0.5 $CaCl_2$, 7 $MgCl_2$, 7 glucose). OBs were removed as previously described (Palouzier-Paulignan et al., 2002) and cut in horizontal slices (350 μm thick) using a Leica VT1000s vibratome. Slices were incubated in ACSF (containing, in mM: 125 NaCl, 4 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 0.3 ascorbic acid, 1 Na-piruvate,1.4 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose; pH = 7.4) (oxygenated with 95% O_2/5% CO_2; pH = 7.4) at 32°C for 1 h and then at room temperature (25 ± 2°C). The osmolarity was adjusted to 330 mOsm with sucrose. The slices were transferred into a recording chamber mounted on an upright microscope (Axioskop FS, Zeiss) and were continuously superfused with oxygenated 31 ± 1°C ACSF (4 ± 1 ml/min). Neurons were visualized using a 40× objective with differential interference contrast optics (Nomarski) or infrared illumination (Hamamatsu camera). Whole-cell recordings were performed on GFP-expressing granular neurons visualized by epifluorescence (excitation 395–440 nm, emission 470 nm; Zeiss filter set 05) using an RK-400 amplifier (Bio-Logic). The signal was sampled at 10 kHz and low-pass filtered at 1 kHz. Borosilicate microelectrodes (outer diameter, 1.5 mm; inner diameter, 0.86 mm; Harvard Apparatus) were filled with the following solution (in mM): 121 $KMeSO_4$, 13.5 KCl, 10 HEPES, 1 $MgCl_2$, 0.5 $CaCl_2$, and 5 EGTA, 310 mOsm adjusted with KCl, pH = 7.3. NA was diluted in ACSF at a final concentration of 10 μM and was bath applied.

Results

Perceptual learning depends upon noradrenergic activity

To assess the role of the noradrenergic system during perceptual learning, we investigated the ability of mice to discriminate between perceptually similar odorants with or without noradrenergic manipulation during enrichment.

To assess discrimination, we used a cross-habituation task consisting of 4 habituations trials followed by one test trial. Habituation curves are not presented since all groups of mice from all experiments habituated correctly (Table 3). The discrimination performances are presented Figure 1 (Hab4 compared with Otest).

An overall ANOVA with experimental groups (saline non-enriched, saline enriched, dexefaroxan, labetalol non-enriched and labetalol enriched), test (pre-enrichment test and post-enrichment test), and trial number as main effects showed significant effect of experimental group ($F_{(4,894)} = 12.694$ $p < 0.0001$), of test ($F_{(1,894)} = 9.097$ $p < 0.003$) or of trial number ($F_{(4,894)} =$

Table 3. Habituation and discrimination performances after enrichment and/or drug injections

		ANOVA		*Post hoc* Fisher test	
Groups	Odor pairs	*F* value	*p* value	Habituation	Discrimination
Saline non-enriched	+/− lim	$F_{(4,38)} = 2.668$	$p = 0.047$	$p = 0.008$	$p = 0.754$, no
	Pent/but	$F_{(4,55)} = 6.621$	$p < 0.0001$	$p = 0.001$	$p = 0.328$, no
	Dec/dodec	$F_{(4,47)} = 3.252$	$p = 0.020$	$p = 0.006$	$p = 0.156$, no
Saline +/− lim enriched	+/− lim	$F_{(4,66)} = 4.680$	$p = 0.002$	$p < 0.0001$	$p = 0.029$, yes
	Pent/but	$F_{(4,49)} = 3.209$	$p = 0.021$	$p = 0.006$	$p = 0.038$, yes
	Dec/dodec	$F_{(4,58)} = 6.292$	$p < 0.0001$	$p = 0.001$	$p = 0.782$, no
Labetalol non-enriched	+/− lim	$F_{(4,70)} = 4.024$	$p = 0.005$	$p = 0.002$	$p = 0.787$, no
	Pent/but	$F_{(4,49)} = 8.745$	$p < 0.0001$	$p < 0.0001$	$p = 0.648$, no
	Dec/dodec	$F_{(4,66)} = 6.499$	$p < 0.0001$	$p = 0.004$	$p = 0.096$, no
Labetalol +/− lim enriched	+/− lim	$F_{(4,70)} = 6.189$	$p < 0.0001$	$p < 0.0001$	$p = 0.072$, no
	Pent/but	$F_{(4,63)} = 2.530$	$p = 0.049$	$p = 0.028$	$p = 0.650$, no
	Dec/dodec	$F_{(4,64)} = 6.877$	$p < 0.0001$	$p = 0.001$	$p = 0.393$, no
Dexefaroxan	+/− lim	$F_{(4,72)} = 7.793$	$p < 0.0001$	$p < 0.0001$	$p = 0.003$, yes
	Pent/but	$F_{(4,63)} = 9.116$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$, yes
	Dec/dodec	$F_{(4,71)} = 4.657$	$p = 0.002$	$p < 0.0001$	$p = 0.026$, yes

Pent, Pentanol; but, butanol; Dec, decanal; dodec, (2)-dodecanone; lim, limonene; iso, isoamyl acetate; oct, octanal.

44.253 $p < 0.0001$). An interaction between experimental group and test was observed ($F_{(4,894)} = 5.444$ $p < 0.0001$), indicating that the observed effect of test depends on the experimental groups.

As previously described, all mice failed to spontaneously discriminate between all three odor pairs during pre testing (Fig. 1*B*; see Table 3 for statistics details). Ten days later, mice that did not receive enrichment continued to fail to spontaneously discriminate odorant pairs (Fig. 1*Ci*; Table 3). Mice receiving +/−limonene enrichment showed a significant enhancement in discrimination abilities for +/−limonene and butanol/pentanol (which are partially similar to the limonene) but not for decanal/dodecanone (which are very different from the limonene; Fig. 1*Cii*; Table 3; Mandairon et al., 2006c).

Mice treated during enrichment with labetalol failed to show improvements in discrimination ability (Fig. 1*Ciii*; Table 3) suggesting that perceptual learning depends upon NA.

As a control, an additional group of mice was injected with labetalol without any enrichment. These animals did not discriminate any of the three odor pairs (Fig. 1*Civ*; Table 3). This failure to improve their ability to discriminate between odorants was not likely due to impairments in olfactory sensitivity, novelty detection, or changes in arousal, as labetalol-treated mice did not differ from other groups in the initial level of odorant investigation ($F_{(4,66)} = 2.104$, $p > 0.05$), or basal body weight (an indicator of general health, data not shown).

These initial findings strongly suggest that NA is a mediator of enrichment-induced enhancements in discrimination. To confirm this, we used a similar paradigm, but treated non-enriched animals with dexefaroxan a drug that increases NA neurotransmission (Veyrac et al., 2007), to investigate whether NA stimulation mimics olfactory enrichment. Mice treated with dexefaroxan showed a significant increase in their ability to discriminate between odorant pairs, succeeding in discriminating all 3 odorant pairs (Fig. 1*Cv*; Table 3).

Together, these data suggest that perceptual learning is not only dependent upon increased NA activity, but increased NA activity is sufficient to drive improvements in olfactory discrimination abilities.

To further document the involvement of NA in perceptual learning, we looked at the activation of the noradrenergic system following enrichment. For that purpose, we evaluated the density of DBH-positive cells in the locus ceruleus of non-enriched and enriched animals. We found that the density of DBH-positive cells was increased in the enriched animals compared with the control non-enriched animals ($p = 0.015$; Fig. 1*D*). These results showed that learning raised noradrenergic system activity.

Neurogenesis is spatially enhanced during perceptual learning and regulated by noradrenergic system

We were next interested in understanding the neural substrates of improved performance, both following olfactory perceptual learning as well as following pharmacological stimulation of NA activity. Impairments in adult OB neurogenesis have previously been shown to result in impairments in olfactory perceptual learning. We thus hypothesized that augmentation of NA activity through enrichment or pharmacological manipulation may support improvement in olfactory discrimination ability through positive effects on olfactory neurogenesis. To test this, mice treated with saline, dexefaroxan, or labetalol and subjected or not to olfactory enrichment were injected beforehand with BrdU to track the survival of newly born cells. At the end of testing, mice were killed and the density of newly born cells was assayed in the OB.

We observed significant differences between experimental groups on the density of adult-born cells ($F_{(4,16)} = 4.612$, $p = 0.011$). More specifically, we found an increase in the density of surviving adult-born cells after odor enrichment ($p = 0.002$) or dexefaroxan treatment ($p = 0.004$) compared with saline-non-enriched mice. This indicated to us that in both conditions in which discrimination was improved, bulbar neurogenesis was also enhanced. In contrast, the density of BrdU-positive cells in mice injected with labetalol during enrichment was not different from the saline-non-enriched mice ($p = 0.179$; Fig. 2*A*,*B*). Finally, we found that the treatment with labetalol alone did not modulate the level of neurogenesis ($p = 0.240$ compared with control non-enriched animals; Fig. 2*B*).

Using BrdU/NeuN double-labeling in the granule cell layer (Fig. 2*C*), we found that olfactory enrichment and/or drug treatments did not affect the level of neuronal differentiation of adult-born cells ($p > 0.05$).

Enrichment but not NA stimulation leads to regionalized survival of newly born cells

It has previously been shown that olfactory enrichment leads to selective survival of adult-born cells within the OB, with the greatest increase in neurogenesis occurring in regions that are responsive to odorant stimulation (Moreno et al., 2009). To test whether the distribution of surviving adult-born cells in the granule cell layer in enriched and drug-treated animals is regionally localized, we used a previously established method for newborn cell mapping (Mandairon et al., 2006d; Sultan et al., 2011; see Materials and Methods). We found a nonhomogeneous distribution of BrdU-positive cells in the OB (Fig. 2*D*). More precisely, to understand the relationship between behavioral performances and the spatial distribution of BrdU-positive cell, we first analyzed the similarities between groups of animals showing an enhancement of discrimination abilities (enriched and dexefaroxan-treated groups) versus groups that did not show such improvement (non-enriched and the two labetalol-treated groups, with and without enrichment) (Fig. 2*D*). To do so, we delimited for each map the regions with a high density of BrdU-positive cells (corresponding to values superior to mean +2SD of control map) and compared these regions between the different maps by calculating a percentage of overlap. We found that the maps of enriched and dexefaroxan-treated animals showed more similarities (51.56% of overlap) compared with the other maps (non-

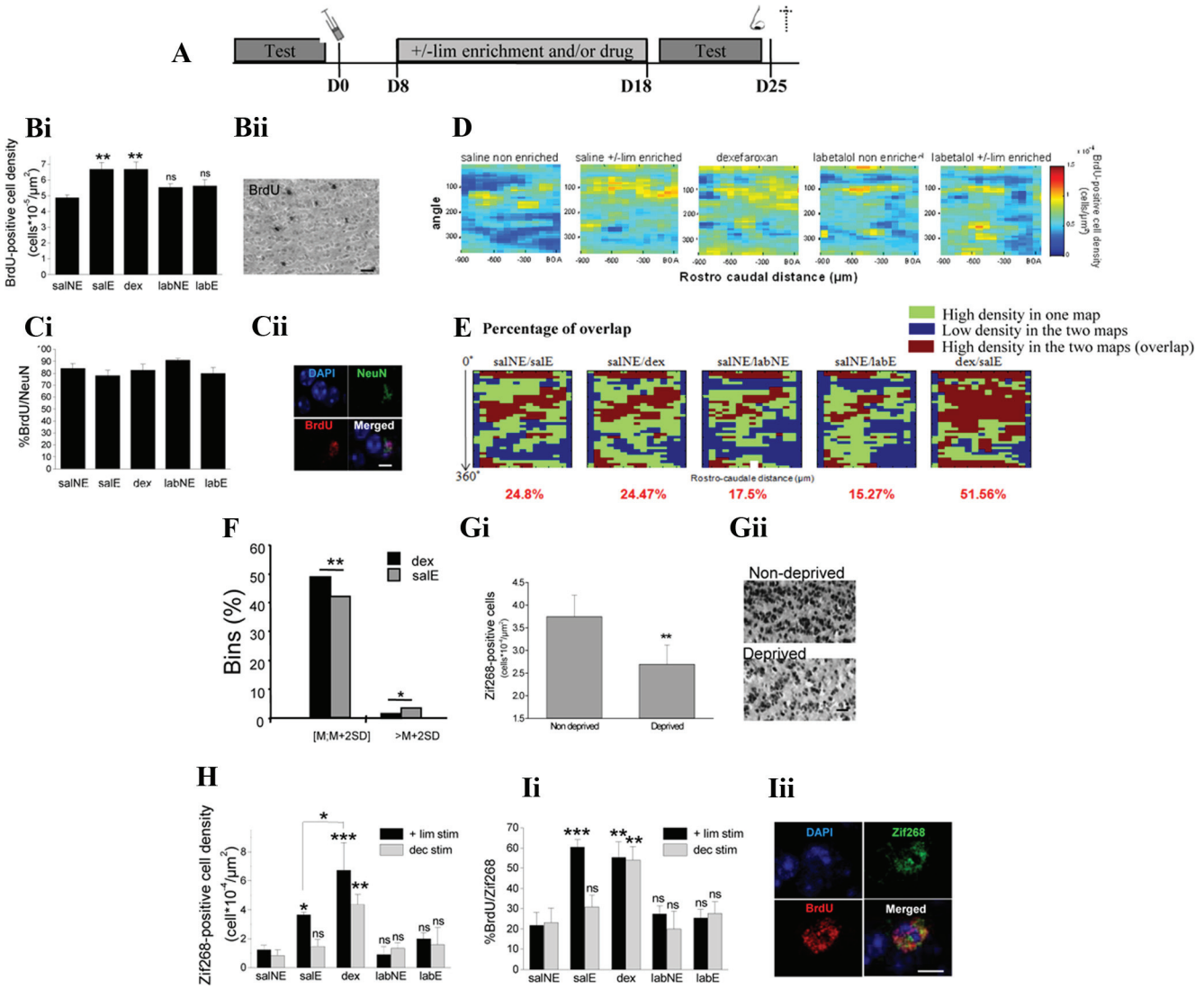

Figure 2. Newborn cell survival is modulated during perceptual learning and regulated by noradrenergic system. ***A***, Experimental paradigm. BrdU was administered 8 d before the enrichment period and mice were killed 25 d after administration of BrdU. Animals were exposed to +limonene or decanal 1 h before they were killed. ***Bi***, Odor enrichment and noradrenergic modulation affect neurogenesis. BrdU-positive cell density in the granule cell layer of the OB is increased in the saline-enriched and dexefaroxan-treated animals compared with the control non-enriched group. In both groups treated with labetalol with or without enrichment, the density of BrdU-positive cells is similar to that of the control group. ***Bii***, Representative image of BrdU labeling. Scale bar, 15 μm. ***Ci***, Quantification of BrdU/NeuN double-labeling in the granule cell layer showed no effect of the enrichment or drug treatment on the neuronal fate of adult-born cells. ***Cii***, Pseudo-confocal imaging of BrdU/NeuN. Scale bar, 4 μm. ***D***, Mapping of BrdU-positive cell density in the granule cell layer of the OB. Average maps of BrdU-positive cell density showed similarities between saline-enriched and dexefaroxan-treated animals compared with the other groups and correspond to animals whose discrimination abilities were improved. ***E***, Overlap maps were constructed by delimitation of regions with a high density of BrdU-positive cells, and these high-density areas were compared between maps and a percentage of overlap was calculated. ***F***, Value of all bins of the enriched and dexefaroxan-treated maps was extracted and grouped in two categories: [mean; mean + 2 SD] representing intervals of mean values and [mean + 2SD; + ∞] representing intervals of more extreme values. The dexefaroxan-treated animals map presents more bins ranged in the interval of [mean; mean + 2SD] and less bins in the interval of [mean + 2SD; + ∞] compared with the enriched-animals map, indicating that adult-born cells are more widely distributed after noradrenergic treatment than after odor enrichment (*t* test for comparison of proportions; $*p < 0.05$; $**p < 0.005$). The data are expressed as mean values ± SEM. ***G***, ***Gi***, Olfactory deprivation decreased the density of Zif268-positive cells in the granule cell layer of the OB compared with the non-deprived side ($**p < 0.005$). ***Gii***, Representative image of Zif268 labeling. Scale bar, 15 μm. ***H***, Enriched and dexefaroxan-treated animals displayed more granule cells expressing Zif268 in response to +limonene than did non-enriched animals or both groups of labetalol-treated animals (black bars). Only dexefaroxan-treated animals displayed an increase of Zif268-positive cells in response to decanal compared with the other groups (gray bars). ***Ii***, In response to +limonene stimulation, enriched and dexefaroxan-treated animals displayed more adult-born neurons expressing Zif268 than did non-enriched animals or both groups of labetalol-treated animals. After decanal stimulation, only dexefaroxan-treated animals displayed more BrdU-positive cells expressing Zif268 compared with the other groups. ***Iii***, Confocal imaging of BrdU/Zif268. Scale bar, 4 μm. $*p < 0.05$; $**p < 0.005$; $***p < 0.001$. The data are expressed as mean values ± SEM. salNE, Saline-non-enriched animals; salE, saline-enriched animals; dex, dexefaroxan-non-enriched animals; labNE, labetalol-non-enriched animals; labE, labetalol-enriched animals.

enriched vs enriched, 24.8%; non-enriched vs dexefaroxan, 24.47%; non-enriched vs non-enriched labetalol-treated, 17.5%; non-enriched vs enriched labetalol-treated, 15.27%) (Fig. 2*E*). In other words, BrdU-positive cell maps of enriched and dexefaroxan-treated animals shared more similar features than with the three other maps, an observation which was in accordance with the behavioral performances.

Nevertheless, although sharing 51.56% of similarities, a more detailed analysis showed that the dexefaroxan-treated animals map was different from the enriched animals map. We measured the level of contrast (comparison of the distribution of high- vs low-density areas) in those two maps. For that purpose, we extracted from dexefaroxan and enriched animals maps the value of all bins and grouped them in two categories: the first category including values

comprised between the mean and the mean + 2SD ([mean; mean + 2SD]) which represent intervals of mean values. The other category included values above mean + 2 SD ([mean + 2SD; + ∞]) which represent intervals of more extreme values. We observed that the dexefaroxan-treated animals map presents more bins ranged in the interval of [mean; mean+2SD] compared with enriched-animals map ($p = 0.0112$) and less bins ranged in the interval of [mean + 2 SD; + ∞] ($p = 0.0328$) (Fig. 2*F*). These data indicated that adult-born cells are more broadly distributed after dexefaroxan treatment than after odor enrichment that could explain that dexefaroxan-treated animals are able to discriminate more odor pairs than the enriched animals.

In summary, we found that more surviving newborn cells was associated with better discrimination after learning. Furthermore, we showed that increases in the survival of adult-born cells with a broader distribution led to broad and nonspecific improvements in discrimination ability.

The noradrenergic system regulates the level of granule cell responsiveness to the learned-odorants

To determine whether the involvement of granule cell population in processing of the learned odors was related to the discrimination ability, we measured the expression of Zif268 as an index of cellular activation in animals from all five groups after odor stimulation. We first confirmed that the expression of Zif268 was modulated by the sensory activity, since sensory deprivation decreased its expression (Fig. 2*G*). We then assessed the responsiveness of granule cells, in response to +limonene (the odorant used for the enrichment regarding the groups of +/−limonene-enriched animals) or to an odorant that the animal had not previously been enriched with, decanal. We found that enriched and dexefaroxan-treated mice displayed increased granule cell Zif268 expression in response to +limonene (the learned odorant regarding the enriched animals) compared with all three other groups (group effect $F_{(4,12)} = 8.728$ $p = 0.002$; Fig. 2*H*). Indeed, levels of Zif268 expression were significantly higher in enriched animals ($p = 0.035$) or dexefaroxan-treated animals ($p < 0.0001$) compared with saline-non-enriched animals. In addition, Zif268 expression was similar between saline-non-enriched animals and both non-enriched ($p = 0.078$) and enriched ($p = 0.495$) labetalol-treated animals (Fig. 2*H*). When we assessed the responsiveness of granule cells in response to an odorant that the animal had not previously been enriched with, decanal, dexefaroxan-treated animals displayed an increase of Zif268-positive cells compared with the four other groups (group effect $F_{(4,14)} = 4.889$ $p = 0.011$; with saline-non-enriched similar to enriched ($p = 0.436$), to labetalol-non-enriched ($p = 0.542$) labetalol enriched ($p = 0.395$) but significantly different from dexefaroxan ($p = 0.001$; Fig. 2*Ii*,*Iii*), which is in accordance with the fact that only dexefaroxan-treated animals are able to discriminate the decanal/dodecanone pair. These data showed that improvement of discrimination after stimulation of noradrenergic transmission is accompanied by an increase of

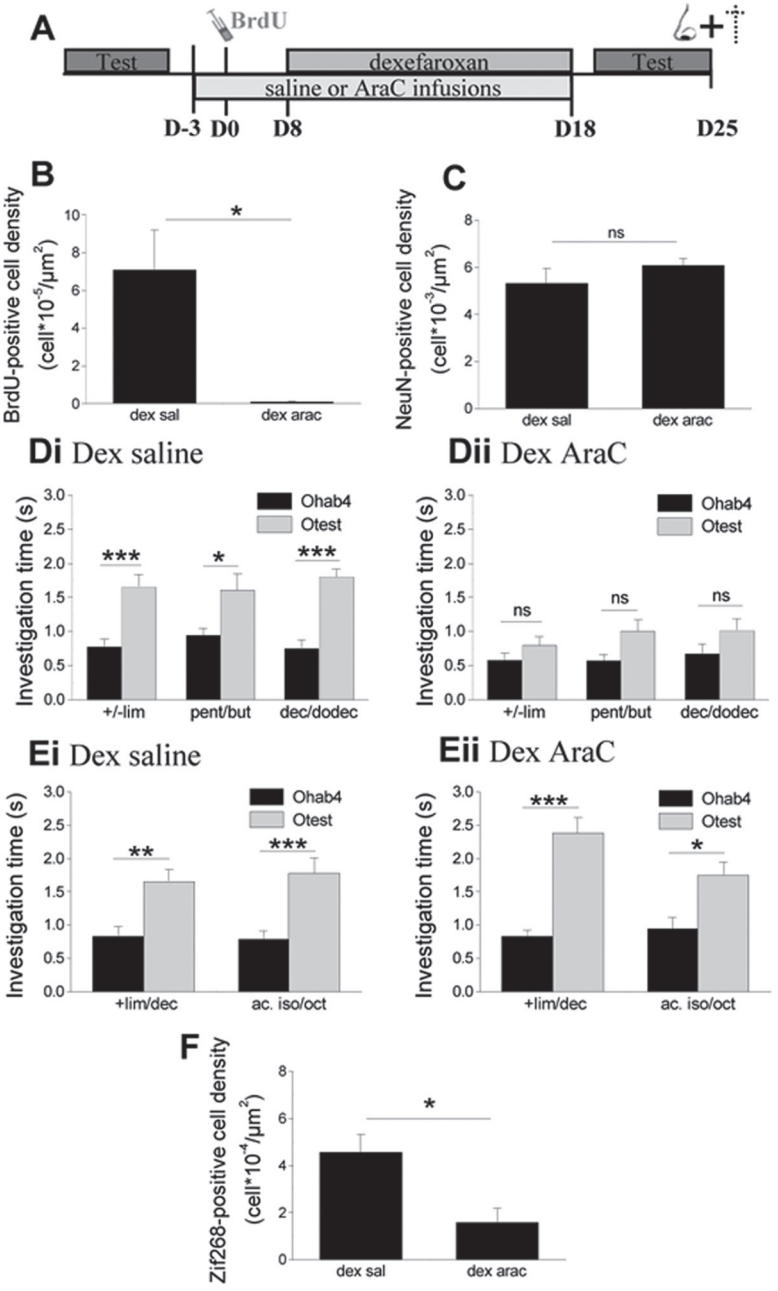

Figure 3. Olfactory perceptual learning requires the action of the noradrenergic system on adult-born neurons. ***A***, Experimental design. Saline or AraC was locally infused 3 d before the administration of BrdU and lasting for 21 d. Animals of both groups were treated with dexefaroxan once a day during 10 d. ***B***, Mice that received AraC had a significant reduction of BrdU-positive cell density in the granule cell layer of the OB compared with the saline-infused animals. ***C***, NeuN-positive cell density was similar between saline- and AraC-infused animals. ***Di***, In the saline-infused animals, treatment with dexefaroxan improved discrimination for the three odor pairs tested. ***Dii***, The strong reduction of bulbar neurogenesis in the AraC-infused group blocked the dexefaroxan treatment-induced improvement of discrimination. ***E***, Both groups of saline (***Ei***)- and AraC (***Eii***)-infused animals discriminate well a dissimilar pair of odorants (+limonene vs decanal and isoamyl acetate vs octanal). ***F***, Zif268-positive cell density is superior in the saline-infused dexefaroxan-treated animals compared with the AraC infused. *$p < 0.05$; **$p < 0.005$, ***$p < 0.001$. The data are expressed as mean values ± SEM.

granule cell responsiveness as in perceptual learning but with broader odor specificity.

Since it is known that after perceptual learning the functional activation of adult-born neurons is increased, we further examined whether this was also the case after modulation of the noradrenergic system. We assessed the percentage of adult-born granule cells expressing Zif268 in response to +limonene or decanal (Fig. 2*I*). Enriched and dexefaroxan-treated animals displayed more adult-born neurons expressing Zif268 in response to +limonene than non-enriched animals or both groups of labetalol-treated animals ($F_{(4,14)} = 12.509$, $p < 0.0001$; with saline non-enriched vs enriched $p < 0.0001$, vs dexefaroxan $p = 0.001$, vs labetalol non-enriched $p > 0.05$, vs labetalol enriched $p > 0.05$; Fig. 2*I*). After decanal stimulation, only dexefaroxan-treated animals displayed more BrdU-positive cells expressing Zif268 compared with the other groups ($F_{(4,13)} = 3.614$ $p = 0.034$; with saline non-enriched versus dexefaroxan $p = 0.007$; Fig. 2*I*).

In summary, we found that stimulating the noradrenergic system improved discrimination, newborn cell survival and functional responsiveness of newborn cells similarly to what it was observed in perceptual learning. Conversely, blocking noradrenergic system during perceptual learning cancelled the effect of enrichment on discrimination, survival and functional involvement of newborn cells.

Blocking neurogenesis prevents NA-induced improvement in olfactory discrimination ability

We have shown here that the noradrenergic system plays a significant role in regulating olfactory learning and neurogenesis. We have demonstrated that the presence and distribution of adult-born cells are correlated with improvements in discrimination. To test whether enhanced adult-born cell survival resulting from increased NA activity is necessary for behavioral improvements in discrimination ability, we injected mice with dexefaroxan once daily for 10 d, and in parallel, infused either the mitotic blocker AraC or saline into the subventricular zone of those same mice. This type of AraC treatment has previously been shown to block the division of constitutively proliferating cells (Enwere et al., 2004) in the subventricular zone and as a consequence inhibit neurogenesis (Moreno et al., 2009; Sultan et al., 2010) without affecting preexisting cells of the OB (Breton-Provencher et al., 2009). The AraC or saline infusion started 10 d before and was maintained during the 10 d period of noradrenergic stimulation (Fig. 3*A*).

We first confirmed that the AraC treatment induced a strong reduction in the density of BrdU-positive cells in the OB compared with saline-infused control mice ($F_{(1,5)} = 15.943$ $p = 0.01$) (Fig. 3*B*). We previously showed that treatment with AraC in the exact same conditions did not induce a significant decrease of hippocampal neurogenesis (Moreno et al., 2009). Moreover, this treatment did not alter the structure of the OB since no significant modification of NeuN-positive cell density was observed in the granule cell layer of the AraC-infused group compared with the saline-infused group ($F_{(1,6)} = 1.501$ $p > 0.05$; Fig. 3*C*).

Chronic infusion of AraC in the subventricular zone of dexefaroxan-treated animals had no effect on habituation memory, as evidenced by similar habituation curves obtained in treated and saline-infused animals (group effect $F_{(1,332)} = 0.089$ $p = 0.766$; see Table 4 for detailed statistics). Interestingly, in mice infused with AraC, dexefaroxan did not improve the ability of mice to discriminate any of the three odor pairs tested (Fig. 3*Dii*; Table 4) in contrast to saline-infused animals (Fig. 3*Di*; Table 4). As a control, and to insure that treatment did not simply disrupt olfactory function, we found that discrimination between dissimilar odorants was normal and well performed by both groups of mice (Fig. 3*Ei*,*Eii*). Thus, we found that the presence of adult-born cells is required for improvements in discrimination ability induced by increased noradrenaline release.

Table 4. Habituation and Discrimination performances after enrichment and/or drugs injections

		ANOVA		*Post hoc* Fisher test	
Groups	Odor pairs	*F* value	*p* value	Habituation	Discrimination
Saline infusion + dexefaroxan	+/− lim	$F_{(4,63)} = 9.365$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$, yes
	Pent/but	$F_{(4,57)} = 3.231$	$p = 0.019$	$p = 0.002$	$p = 0.013$, yes
	Dec/dodec	$F_{(4,67)} = 14.362$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$, yes
	+lim/dec	$F_{(4,40)} = 6.949$	$p < 0.0001$	$p < 0.0001$	$p = 0.002$, yes
	iso/oct	$F_{(4,44)} = 7.384$	$p < 0.0001$	$p < 0.0001$	$p = 0.000$, yes
AraC infusion + dexefaroxan	+/− lim	$F_{(4,69)} = 17.782$	$p < 0.0001$	$p < 0.0001$	$p = 0.383$, no
	Pent/but	$F_{(4,80)} = 13.530$	$p < 0.0001$	$p < 0.0001$	$p = 0.105$, no
	Dec/dodec	$F_{(4,76)} = 10.648$	$p < 0.0001$	$p < 0.0001$	$p = 0.213$, no
	+lim/dec	$F_{(4,57)} = 8.580$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$, yes
	iso/oct	$F_{(4,50)} = 9.222$	$p < 0.0001$	$p < 0.0001$	$p = 0.030$, yes

Pent, Pentanol; but, butanol; Dec, decanal; dodec, (2)-dodecanone; lim, limonene; iso, isoamyl acetate; oct, octanal.

Finally, treatment with dexefaroxan in saline-infused mice induced a significantly higher level Zif268 expression in the granule cell layer compared with the AraC-infused animals ($F_{(1,9)} = 9.093$ $p = 0.015$, Fig. 3*F*). This finding supports the implication of adult-born neurons in the increase in Zif268 expression observed in the granule cell layer after dexefaroxan treatment.

Since the effect of the noradrenergic system activation on the improvement of discrimination is abolished by neurogenesis blockade, it could be suggested that noradrenergic system controls perceptual learning through acting on newborn neurons within the OB.

To support these results we investigated whether noradrenergic fibers labeled with NET, projected directly onto newborn cells. To do so, we injected lentiviruses expressing GFP in the subventricular zone and 15 d later, analyzed both GFP and NET labeling in the OB. We found NET-positive fibers juxtaposed to newborn-GFP neurons (Fig. 4*A*).

Having established that newborn neurons receive noradrenergic innervations we investigate whether these cells are responsive to NA. In a recent publication Nai et al. (2010) reported that NA can either increases or decrease the excitability of adult granule cell depending on the applied concentration and the noradrenergic receptors subtype activated. Such effects were mediated by variation in the membrane resistance (R_m) through a modification of potassium conductance. To determine whether NA has similar effects on new born neurons, current-clamp recording were made from GFP-labeled cells in OB slices 12–18 d postinfection, i.e., the age at which new born neuron survival is affected by odor enrichment and the NA system. Newborn neurons did not spontaneously generate action potentials, presented an elevated membrane resistance (3.3 ± 0.4 GΩ), and fired few action potentials when depolarized by current injection (Fig. 4*B*), a characteristic of newborns granular cells (Carleton et al., 2003). Bath application of NA (10 μM) significantly modified R_m in seven of eight newborn neurons recorded (leading to increased R_m in five neurons and decreased R_m in two neurons; Fig. 4*C*,*D*).

Together, these data show that 12- to 18-d-old newborn neurons are already sensitive to the noradrenergic neuromodulation.

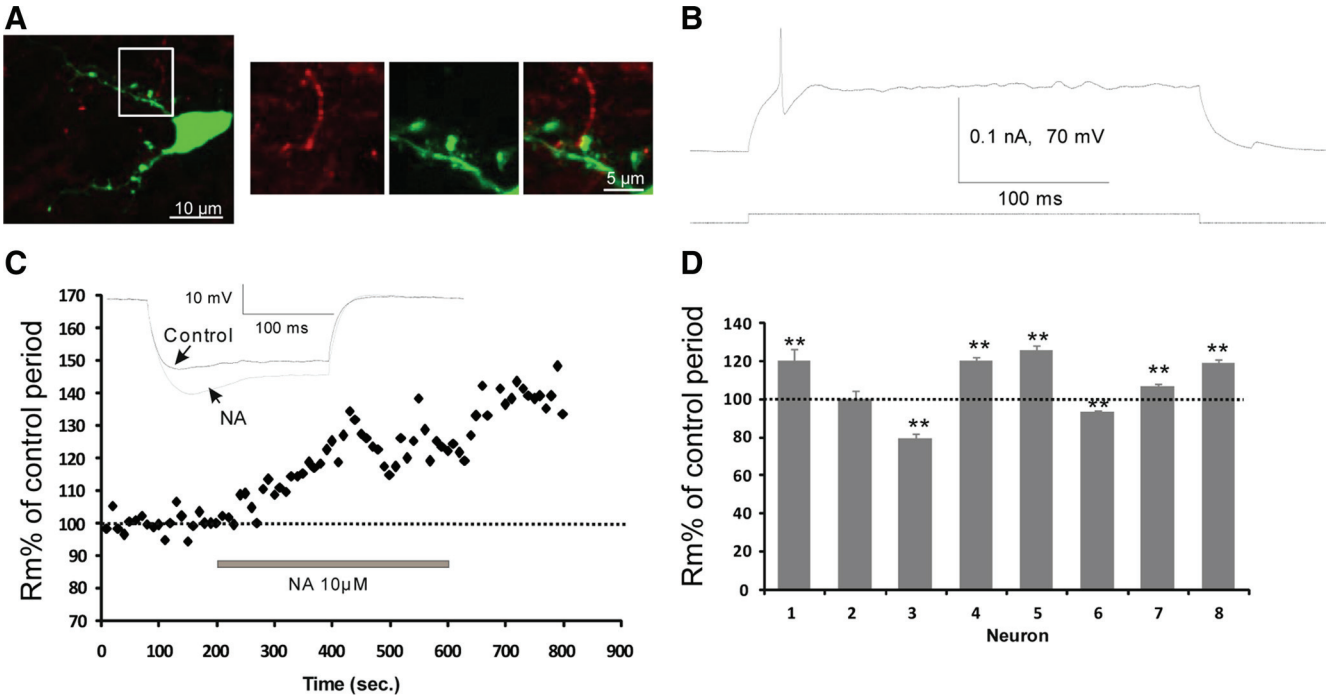

Figure 4. Adult-born neurons respond to noradrenaline. ***A***, Representative of NET labeling in the bulbar granule cell layer of animals injected with GFP lentivirus. We found that NET-positive fibers project onto newborn neurons. ***B***, Depolarization induced by current injection produced only few action potential in adult-born neuron. ***C***, Time-plot of noradrenaline effect on the membrane resistance of a newborn neuron. Inset, Membrane hyperpolarization produced by injected current (−0.005 nA) during control period (black trace) and in the presence of noradrenaline, 10 μM (gray trace). ***D***, Modifications induced by noradrenaline on the membrane resistance of eight different adult-born neurons. $^{**}p < 0.005$.

Discussion

Here, we show that noradrenaline is necessary for olfactory perceptual learning and that its efficacy requires the presence of adult-born neurons. Furthermore, we show for the first time that noradrenergic fibers impinge upon newly born cells and that newborn neurons are sensitive to application of noradrenaline. Based upon convergent lines of evidence we propose a model by which top-down release of noradrenaline supports the selective and region-specific incorporation of newly born neurons supporting enhanced discrimination abilities.

OB granule cells are continuously generated in adulthood (Lledo et al., 2006). The functional significance of adult-born neurons is a fundamental question in the field with broad implications. The survival of adult-granule cells has been shown to be modulated by associative learning (Alonso et al., 2006; Mandairon et al., 2006d; Sultan et al., 2010, 2011) required for remembering an operant olfactory learning (Mandairon et al., 2011) and necessary for perceptual learning (Moreno et al., 2009). However, the mechanisms underlying their integration into established neural networks are unknown. Here, we identify the noradrenergic system as a key signaling pathway regulating the selective survival of cells to support perceptual learning. Indeed, a 10 d treatment with α2-adrenoceptor antagonist dexefaroxan alone, which increased NA release in target areas (Mayer and Imbert, 2001), mimicked the effects of the odor enrichment by improving olfactory discrimination abilities. In contrast, a 10 d treatment by the mixed α1- and β-adrenergic receptor antagonist labetalol blocked the effect of olfactory enrichment on discrimination. We further found that in groups in which olfactory discrimination is improved (10 d enrichment or dexefaroxan treatment) the density of Zif268-positive neurons in the granule cell layer is increased whereas labetalol blocked that enhancement in animals receiving enrichment. These data indicate that perceptual learning requires activation of the noradrenergic system. These finding are further supported by the increase of DBH expression observed at the end of the perceptual learning which suggested an increased activity of the NA system during learning. These data are in accordance with previous studies showing activation of the noradrenergic system after enrichment (Veyrac et al., 2009; Rey et al., 2012) or leading to an increase in NA levels in the OB (Brennan et al., 1990; Veyrac et al., 2009).

NA modulation through α_2-adrenoceptor antagonist dexefaroxan treatment alone increased the survival of adult-born cells, an effect that is accompanied by an improvement in discrimination ability. We then asked whether the presence of adult-born cells versus preexisting cells was necessary for the improvement in discrimination ability following NA stimulation. To do this, we assessed olfactory perceptual learning in animals in which neurogenesis was blocked by local infusion of AraC along with a parallel stimulation of the noradrenergic system by dexefaroxan treatment. The intracerebral infusion of AraC had no observed side effects (the animals were able to detect and discriminate odorants that were perceptually very different and thus easy to discriminate). In addition, AraC-infused animals had normal locomotor or exploratory activity, motivation, and performance in the acquisition of an associative olfactory task and spontaneous discrimination (Breton-Provencher et al., 2009; Moreno et al., 2009; Sultan et al., 2010). However, dexefaroxan-treated animals with ablation of neurogenesis demonstrated no perceptual learning, suggesting that the beneficial effect of increased noradrenergic tone requires the presence of adult-born cells. Importantly, even though NA drug injections lead to systemic modulation of NA, the loss of efficacy following local infusion of AraC in the subventricular zone, strongly suggests that the efficacy of NA to support perceptual learning is through selective effects on bulbar adult-born cells. Such a hypothesis is

further bolstered by our confocal imaging and electrophysiological data showing that newborn cells are impinged upon by NA fibers and respond to NA.

Finally, in accordance with these findings, dexefaroxan-treated AraC-infused animals showed no increase in the density of Zif268-positive cells, indicating that in the absence of adult-born cells, dexefaroxan-induced augmentation of granule cell activity did not occur. Thus we conclude that NA and adult-born cells are responsible for the increased granule cell activation accompanying perceptual learning.

The careful analysis of the spatial distribution of adult-born cells indicated a high level of similarity between the enriched and dexefaroxan-treated groups in agreement with the improvements in discrimination in those two groups. Nevertheless, the survival of adult-born cells in the dexefaroxan group is more widely distributed within the OB than in the saline-enriched group which is consistent with the fact that noradrenergic fibers terminate broadly across the bulb (McLean et al., 1989) and that dexefaroxan-treated group discriminate more odor pairs than the saline-enriched group. In animals enriched with the +limonene/−limonene pair, the more clustered distribution of adult-born cells suggests that experience could lead to selective activation of noradrenergic afferents in those regions activated by the odorants and support selective cell survival. However, such a hypothesis would require more testing. In contrast, the mixed α1- and β-adrenergic receptor antagonist labetalol blocked the increase in neurogenesis in enriched mice. The injection of labetalol without any enrichment did not decrease the density of BrdU-positive cells compared with the control non-enriched animals, indicating that the NA blockade did not induce newborn cell death but rather prevented the increase of neurogenesis due to the enrichment in labetalol-enriched animals.

The role of NA in perceptual olfactory learning is reinforced by the fact that NA is important for the occurrence of long-term potentiation in the OB; indeed, NA antagonists disrupt induction of long-term potentiation (Wilson and Sullivan, 1994) and long-term potentiation in the OB seems to depend principally upon adult-born cells (Nissant et al., 2009). Here we found that, despite their immature electrical property, newborn cell are already sensitive to noradrenergic stimulation. NA is capable of triggering molecular mechanisms leading to long-term potentiation which could promote survival of newborn cells (Kermen et al., 2010) and increased excitability (Mouly et al., 1995). As a result, the neuromodulatory action of NA could alter receptive fields of the mitral cells to sharpen the contrast between odor neural representations and improve discrimination.

The present study examines the mechanisms underlying the survival of adult-born cells during perceptual learning. We found that the improvement of discrimination relied on the action of the noradrenergic system on the adult-born cells. Thus, our findings support a major role of the noradrenergic top-down control in perceptual learning, acting on inhibitory adult-born cells and refinement in odorant feature encoding, contributing to experience-dependent changes in odorant discrimination.

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Etude 3 :

Alteration of olfactory perceptual learning in aging mice and its cellular basis

Moreno MM, Richard M, Landrein B, Sacquet J, Didier A and Mandairon N, Soumis à Neurobiol Aging

Alteration of olfactory perceptual learning and its cellular basis in aged mice

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Abstract

Olfactory perceptual learning reflects an ongoing process by which animals learn to discriminate odorants thanks to repeated stimulations by these odorants. Adult neurogenesis is required for this learning to occur in young adults. The experiments reported here showed that (1) olfactory perceptual learning is impaired with aging and that this impairment is correlated (2) with a reduction of neurogenesis and (3) a decrease of granule cell responsiveness to the learned odorant in the olfactory bulb. Interestingly we showed (4) that the pharmacological stimulation of the noradrenergic system using dexefaroxan restores olfactory perceptual learning in old mice which is accompanied by an increase of granule cell responsiveness in response to the learned odorant without any improvement in neurogenesis. We provide the first evidence that, contrary to young adult mice, the improvement of olfactory performances in old mice is independent of the overall level of neurogenesis. In addition, restoring behavioral performances in old mice by stimulation of the noradrenergic system underlies the importance of this neuromodulatory system in regulating bulbar network plasticity.

Key words: olfactory bulb, behavior, discrimination, normal aging, Zif268, noradrenaline

1- Introduction

Among the various alterations in sensory processing that characterize normal aging, olfactory deficits are of special interest. Indeed, the olfactory system is important for animal's survival since it influences its perception of the environment and its behavior such as food intake, social interactions or reproduction. The alterations of olfactory functions during aging include deficits in olfactory perception (Nakayasu, et al. , 2000), discrimination (Enwere, et al. , 2004, Patel and Larson, 2009, Prediger, et al. , 2005), impairment in olfactory associative learning and memory (Guan and Dluzen, 1994, Prediger, et al. , 2005, Roman, et al. , 1996, Schoenbaum, et al. , 2002, Terranova, et al. , 1994) and reduction of the beneficial effect of olfactory enrichment (Rey, et al. , 2012). However, no data are available regarding the effect of aging on perceptual learning, which consists in the improvement of the discrimination of perceptually similar stimuli due to repeated presentation of these stimuli. This type of learning is crucial for basic olfactory functions because it sets the degree of discrimination between stimuli and thus reflects an ongoing process of sensorial environment assimilation (Gilbert, et al. , 2001, Mandairon, et al. , 2008a, Mandairon and Linster, 2009, Mandairon, et al. , 2006c, Mandairon, et al. , 2006d, Mandairon, et al. , 2006e, Moreno, et al. , 2009).

The olfactory bulb (OB), the first central relay of olfactory information transmission is involved in processing different aspects of olfactory perception including perceptual learning (Mandairon, et al. , 2008a, Mandairon and Linster, 2009, Mandairon, et al. , 2006c, Moreno, et al. , 2009). One of the OB specificity is that it is the target of an important adult neurogenesis. The newborn neurons originate from the subventricular zone of the lateral ventricles, migrate along the rostral migratory stream to reach the OB and differentiate into granule and periglomerular inhibitory interneurons (Alvarez-Buylla and Garcia-Verdugo, 2002, Lledo, et al. , 2006, Whitman and Greer, 2009). The adult-born neurons which regulate the mitral cell activity and per consequence the output message of the

OB (Shepherd, et al. , 2007) are necessary for olfactory perceptual learning by increasing the inhibition in the OB network (Moreno, et al. , 2009).

In addition, the OB is heavily innervated by the neuromodulatory noradrenergic system known to be important for olfactory learning (Fletcher and Chen, 2011), odor preferences (Moriceau and Sullivan, 2004, Sullivan and Wilson, 1994), discrimination performances (Doucette, et al. , 2007, Mandairon, et al. , 2008b), memory (Veyrac, et al. , 2007, Veyrac, et al. , 2009) and neural plasticity including neurogenesis modulation (Bauer, et al. , 2003).
Because the noradrenergic system and adult neurogenesis are both involved in perceptual learning (Moreno, et al. , 2012, Moreno, et al. , 2009) and altered during aging (Enwere, et al. , 2004, Luo, et al. , 2006, Rey, et al. , 2012, Tropepe, et al. , 1997), we expected perceptual learning to be affected in aged mice. We also studied whether and how this type of learning could be restored in aged mice and analyzed the underlying neural basis.

We report here that olfactory perceptual learning is altered in aged mice (18 months) whereas basic olfactory discrimination remains intact. We showed that the alteration of perceptual learning is correlated with a reduction of adult-born neuron survival and granule cell responsiveness to the learned odorant. Interestingly, we found that the pharmacological stimulation of the noradrenergic system (using dexefaroxan injection) mimics perceptual learning in old mice. This enhancement of performances is accompanied by an increase of granule cell responsiveness in old mice without any increase of overall neurogenesis. In conclusion, upon stimulation of the noradrenergic system, old mice are able to improve their discrimination performances through an activation of the granule cell network resembling that of young adult mice.

2- Material and methods

Eighty six male C57Bl/6J mice (Charles River, L'arbresles, France), aged respectively of 2 (n=40) and 18 months (n=46) were used in this experiment. All mice were housed under a 12h light/dark cycle in an environmentally controlled room and with an access to food and water *ad libitum*. All behavioral training was conducted in the afternoon (14:00-17:00). All efforts were made to minimize the number of animals used and their suffering during the experimental procedure in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC), and the French Ethical Committee.

2.1. Behavioral testing

Experimental Design

At the beginning of the experiment, all mice were tested on spontaneous discrimination between pairs of chemically and perceptually similar odorants (+Limonene/-Limonene, Butanol/Pentanol and Decanal/Dodecanone). Discrimination was tested using an olfactory habituation/dishabituation task. Mice were then enriched one hour daily during 10 days with + and - Limonene. After the enrichment period, mice were tested again on spontaneous discrimination between these three test odor pairs (Figure 2A). In the control non-enriched groups, mice were housed in the same condition except that the two tea balls contained 100 µL of mineral oil.

Enrichment

For the olfactory enrichment, swabs containing 100 µL of pure odorant (+ Limonene and - Limonene) were placed in two tea balls hanging from the cover of the standard breeding cages for one hour daily during 10 days. Young adult and old mice were submitted to the enrichment period.

Olfactory habituation/dishabituation

In this experiment, we assessed the spontaneous discrimination between three pairs of chemically and perceptually similar odorants: +Limonene/- Limonene, Butanol/Pentanol and Decanal/Dodecanone. These tested pairs of odorants exhibit various degree of response overlap (as measured by 2- deoxyglucose activation maps in the OB) with enrichment odors (the enantiomers of limonene). +Limonene and Pentanol are partially similar odorants since their activation patterns in the OB are overlapping, whereas decanal is a dissimilar odorant with no overlapping with +Limonene (Mandairon, et al. , 2006c, Moreno, et al. , 2009). The odorants were all diluted in mineral oil proportionally to their vapor pressure in order to reach a pressure of 1 Pa (see Table 1, (Cleland, et al. , 2002, Mandairon, et al. , 2006d)). Habituation experiments were performed in standard home cage and odorants were presented by placing 60 μL of odor stimulus onto a filter paper (Whatman). The filter paper was presented in a tea ball hanging from the cover of the cage. Each mouse was tested on the three odor pairs; the odor pairs were tested in a random order. A test session consisted of one 50-s presentation of mineral oil then four 50-s odor presentations of a first odorant (Ohab) at 5 min intervals, followed by one 50-s presentation of the second odorant of the pair (Otest). Investigation was defined as active sniffing within 1 cm of the tea ball.

Data analysis

Data analysis was performed using Systat statistical software (SSI, Richmond, CA, USA). Only mice that investigated at least 1 s during the first presentation of the Ohab were included in the analysis. Outlier trials that deviated from the mean by more than two standard deviations were also excluded from analysis in order to exclude trials during which mice may have been distracted by other stimuli (4.23% of trials). An ANOVA followed by *paired t-test* was performed in order to determine (1) whether mice exhibited habituation (ANOVA trial effect) and (2) the discrimination abilities by comparing Ohab4 and Otest. Discrimination was

indicated by a significant increase in investigation time during the test trial. The criterion for significance was set to $p = 0.05$.

2.2. Adult-born cells

Bromodeoxyuridine (BrdU) administration

To determine the fate of newborn cells in the OB, BrdU (Sigma) was injected intraperitonally 8 days before the enrichment period (25 days before the sacrifice). Three injections of BrdU at 2 h intervals (50 mg/kg in saline) were performed.

Histology

Five mice taken randomly from each experimental group were deeply anesthetized by injection of pentobarbital (2 g/kg), and killed with an intracardiac perfusion of 50 mL of cold fixative solution (paraformaldehyde 4% diluted in phosphate-buffered saline). Brains were then removed, cryoprotected in sucrose and frozen rapidly before being stored at -20 °C. OBs were then sectioned with a cryostat.

BrdU immunohistochemistry

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

2.3. Zif268 expression

To assess the expression of Zif268 in response to +Limonene, mice were put in a clean cage during one hour. Then, a tea ball containing 100 µL of pure +Limonene was introduced in the

cage during one hour. One hour after the end of odor exposure, mice were sacrificed (Moreno, et al. , 2009).

Immunohistochemistry

Every fifth section of the OB were transferred to 10% normal goat serum (Sigma) with 2% BSA and 0.1% Triton X-100 for 1 h to block non-specific binding and were then incubated overnight in a rabbit anti-Zif268 antibody (1:1000, Santa Cruz Biotechnology, Santa-Cruz, CA, USA) at 25°C. Sections were then incubated in a biotinylated anti-rabbit secondary antibody (1:200, Vector Laboratories) for 2 h. The remaining treatments were similar to those for the BrdU labeling.

2.4. Data analysis and positive cells mapping

The method used for positive cell counting and mapping has been previously described (Mandairon, et al. , 2006b)

Positive cells were counted on every fifth section (thickness = 14µm, sampling interval = 70µm) in the granule cell layer of the OB using a mapping software (Mercator, Explora Nova, La Rochelle, France) coupled to Zeiss microscope. Zif268-positive cells were automatically counted in the granule cell layer. The mean positive cell density of each array was calculated and averaged within each experimental group. Between-groups comparisons of the mean cell density were performed by ANOVA followed by bilateral unpaired t-test. The level of significance was set to 0.05.

Maps of positive cells were constructed as follows: the layer was divided into 36 sectors of 10° with a reference axis drawn parallel to the most ventral aspect of the subependymal layer of the OB (Mandairon, et al. , 2008a, Mandairon, et al. , 2006b, Sultan, et al. , 2011a). The cell density (number of labeled profiles/µm²) was calculated for each sector. Measurements were then merged into arrays of 10° x 70µm bins. The most rostral aspect of the accessory OB

served as an anatomical landmark to align the sections across animals (Matlab v.6). For visualization of the positive cell density maps, arrays were averaged across animals within each group, and colored image plot of the data was constructed in Matlab v.6. To further compare spatial patterns of labeled cells, a z-score (normalization of the matrix to mean = 0 and SD = 1) was calculated for each animal and z-scored maps were averaged within groups. Between-groups comparisons were achieved by Mann-Whitney tests performed on these z-scored arrays.

2.5. Double labeling

Immunohistochemistry

To determine the phenotype of BrdU-positive cells in the OB, double-labeling was performed using mouse anti-NeuN (1:500, Chemicon). For the Zif268/BrdU double labeling, a rabbit anti-Zif268 antibody (1:1000, Santa Cruz Biotechnology, Santa-Cruz, CA, USA) was used. The appropriate secondary antibodies, coupled to Alexa 633 (Molecular Probes) for revelation of BrdU and Alexa 488 (Molecular Probes) for revelation of the other markers, were used.

Analysis

On five animals of each experimental group, 30 BrdU-positive cells per animal were examined for co-labeling with NeuN- or Zif268. Double-labeling was analyzed by pseudo-confocal scanning microscopy using a Zeiss microscope equipped with an Apotome. Each labeled cell was examined along the z-axis to ensure proper identification of double labeled cells. A percentage of double labeled cells was calculated for each group and compared using ANOVA followed by bilateral unpaired t-test.

2.6. Pharmacological noradrenergic modulation

Twenty minutes prior to each day of the enrichment session, each mouse received a single i.p. injection of 100µl of freshly prepared drug or saline solution. The noradrenergic agent used was the α2 pre-synaptic receptor antagonist dexefaroxan hydrochloride (0.63mg/kg in saline) promoting noradrenaline release (Moreno, et al. , 2012, Veyrac, et al. , 2007).

3- Results

3.1.Olfactory perceptual learning is altered with aging

We used throughout this study an habituation/dishabituation test to assess spontaneous olfactory discrimination in young adult and old mice.

We first tested basic olfactory discrimination, by assessing the ability of old mice to discriminate between two dissimilar odorants, +Limonene and Decanal. Old mice exhibited normal habituation behavior ($F(4,65)=11.885$ $p<0.0001$, Figure 1) and were able to discriminate the two dissimilar odorants ($p<0.0001$ for difference between Hab4 and Otest), indicating that their basic discrimination ability was not impaired at the age of 18 months.

We then tested the spontaneous olfactory discrimination between three pairs of chemically similar odorants: +Limonene/-Limonene, Pentanol/Butanol, and Decanal/Dodecanone (Mandairon, et al. , 2006c), using the same habituation/dishabituation test. As we previously showed, young adult mice habituated correctly for the 3 odor pairs (see supp Table 1; supp Figure 1) but were not able to discriminate the two odors composing the pairs ($p>0.05$ for difference between Hab4 and Otest, Figure 2Bi). Similarly, old mice habituated correctly for the 3 odor pairs (see supp Table 1; supp Figure 1) and were not able to discriminate these odor pairs ($p>0.05$ for difference between Hab4 and Otest, Figure 2Ci).

Discrimination between perceptually similar odors can be improved in young adult mice by a 10-day period of odor enrichment with these odors, a process called perceptual learning (Mandairon, et al. , 2006c, Moreno, et al. , 2012, Moreno, et al. , 2009). We investigated

whether this type of learning was preserved in old mice. Therefore, we assessed spontaneous olfactory discrimination after a ten day enrichment period with +Limonene/- Limonene (Figure 2A) and compared control non-enriched animals to enriched animals. After the 10-day period, both young adult and old mice (enriched or not) exhibited a habituation behavior (supp Table 2, supp Figure 1). Focusing on the discrimination process itself (Ohab4 versus Otest), we observed a significant effect of age on discrimination performances ($F(1,312)=46.25$ $p<0.0001$) as well as an effect of enrichment ($F(2,322)=24.42$, $p<0.0001$)) and a significant age-enrichment interaction ($F(2,322)=5.98$, $p<0.003$ Figure 2Bii-Ciii). In young adult mice, we found a significant effect of enrichment ($F(1,190)=3.92$ $p<0.05$), trials ($F(1,190)=27.82$ $p<0.0001$) and an interaction between enrichment and trials ($F(1,190)=13.05$ $p<0.0001$) (Figure 2Bii-Biii). However, in old animals, neither the enrichment or the trials factors had an effect on the discrimination performances (enrichment effect: ($F(1,122)=0.00$ $p=0.99$); trials effect ($F(1,122)=1.45$ $p=0.2$); interaction ($F(1,122)=2.039$ $p=0.16$) (Figure 2Cii-Ciii). More specifically, among young adult mice, enriched animals were able to discriminate the two enantiomers of Limonene ($p<0.0001$ between Hab4 and Otest) (Figure 2Biii), whereas non-enriched animals could not discriminate these two odors (Figure 2Bii). In accordance with our previous data (Mandairon, et al. , 2006c, Moreno, et al. , 2012, Moreno, et al. , 2009), the improved abilities of adult enriched mice expanded to the discrimination of Butanol from Pentanol ($p=0.001$) (whose activation pattern overlap with +/-Limonene) but not the Decanal from Dodecanone ($p=0.31$; Figure 2Biii). On the contrary, enriched and non-enriched old mice both did not discriminate any of the three odor pairs tested ($p>0.05$ for the three odor pairs, Figure 2Cii and Ciii). These results show that enrichment with +Limonene/-Limonene did not improve the discrimination abilities of old mice.

In conclusion, we observed in 18 months old mice a normal habituation behavior (at 5 min inter trial interval) as well as a preservation of the discrimination ability between two dissimilar odors. However, perceptual learning (the improvement of discrimination between two similar odor after exposure to these odors), was inexistent in old mice.

3.2. The alteration of olfactory perceptual learning in aged mice is correlated with an impairment of neurogenesis

Since the basal rate of adult neurogenesis is impaired in old mice (Enwere, et al. , 2004, Rey, et al. , 2012) and an increased number of newborn neurons is necessary for perceptual learning to occur (Moreno, et al. , 2009), we compared the effect of a 10 day odor enrichment onto OB neurogenesis in young adult and old mice, by injecting BrdU 8 days before the enrichment period. Using that protocol, we labeled a cohort of newborn neurons arriving in the OB at the beginning of the enrichment period (Moreno, et al. , 2012, Moreno, et al. , 2009). BrdU-positive cells were counted in the granule cell layer of adult and old mice OBs (Figure 3). We did not assess neurogenesis in the glomerular cell layer of the OB because we previously found no modulation of periglomerular cells neurogenesis after perceptual learning in young adult mice (Moreno, et al. , 2009). An overall 2-way ANOVA with age (young adult and old) and enrichment (non-enriched and enriched), as main effects showed significant effect of age ($F(1, 11)= 305.117$ $p<0.0001$) and of enrichment ($F(1, 11)= 10.225$ $p=0.008$). An interaction between age and enrichment was also observed ($F(1, 11)= 10.599$ $p<0.0001$), indicating that the observed effect of enrichment depended on the age. More precisely, the BrdU-positive cell density was significantly lower in old mice compared to young adults ($p=0.0001$; Figure 3A). Interestingly, while odor enrichment lead to a significant increase of newborn cell density in young adult mice ($p=0.001$; Figure 3A) (Moreno, et al. , 2012,

Moreno, et al. , 2009), it did not affect the density of BrdU-positive cells in old mice ($p>0.05$; Figure 3A).

The analysis of BrdU-positive cell density on 2D maps representing the granule cell layer (Figure 3Bi) showed that the enrichment enhanced the density of BrdU-positive cells in specific regions only in young adult mice. We indeed observed the apparition of high BrdU-positive cell density areas mostly in the medial part of the OB (Figure 3Bii). In old mice, the density of BrdU-positive cells remained low before and after enrichment (Figure 3Bii). Then, using Mann Whitney tests, we counted the number of bins that showed significant differences between non-enriched and enriched maps (Figure 3D). Interestingly, we found that the modulation of adult-born cell distribution is stronger after enrichment in young adults compared to old mice, indicating a lower level of enrichment-induced neurogenic plasticity of the bulbar network in old animals.

Using BrdU/NeuN double-labeling, we analyzed the percentage of adult-born cells expressing the marker of mature neurons and found that old mice have a tendency toward a lower percentage of double labeled cells when compared to young mice (effect of age ($F(1,17)=3.22$ $p=0.09$)). We did not observe any effect of enrichment ($F(1, 17)= 0.014$ $p=0.908$) on the level of neuronal differentiation of newborn cells (Figure 3Ci and 3Cii).

All together these results showed that 18 months old mice displayed a reduced level of neurogenesis which cannot be increased by odor enrichment, contrary to the phenomenon existing during perceptual learning in young adult mice. This absence of increased neurogenesis in old mice parallels the absence of improvement of their discrimination abilities after odor enrichment.

3.3. The alteration of olfactory perceptual learning in aged mice is correlated with an alteration of granule cell responsiveness to the learned odorant

We previously showed in young adult mice that the improvement of sensory perception in perceptual learning was accompanied by an increase of granule cell responsiveness to the learned odorant (Moreno, et al. , 2009). We further examined here how the bulbar network activation was modulated by the enrichment in young adult and old mice.

For that purpose, we examined the granule cell responsiveness to the learned odorant using Zif268 as an index of cellular activation in response to odor stimulation (Inaki, et al. , 2002, Mandairon, et al. , 2008a, Moreno, et al. , 2012). More specifically, we assessed the density of Zif268-positive granule cells and mapped their distribution in young adult and old mice stimulated with +Limonene. An overall 2-way ANOVA with age (young adult and old) and enrichment (non-enriched and enriched) as main effects showed a significant effect of age ($F(1, 10)= 19.685$ $p=0.001$) and of enrichment ($F(1, 10)= 30.173$ $p<0.0001$) on the density of Zif268-positive cells. An interaction between age and enrichment was also observed ($F(1, 10)= 17.540$ $p=0.002$), indicating that the observed effect of enrichment depended on the age. More specifically, the density of Zif268-positive cells was similar in non-enriched mice whatever their age, suggesting a similar responsiveness of the network to +Limonene in young adult and old mice. However, the enrichment increases the density of Zif268-positive cells in young adult ($p=0.001$) but not old mice ($p>0.05$; Figure 4A), leading to a significantly higher density of Zif268-positive cells in young enriched mice when compared to old enriched mice ($p=0.0012$). To identify the bulbar regions specifically activated by the learned odorant, we mapped the distribution of Zif268-positive cells in the granule cell layer. In adult mice, we observed an important and mostly medial increase of Zif268-positive cell density after enrichment (Figure 4B). In old mice, the density of Zif268-positive cells remained low before and after enrichment (Figure 4B). Mann Whitney tests between non-enriched and enriched maps of Zif268-positive cells indicated a higher level of change in young adults compared to old mice regarding Zif268-positive cell density (Figure 4C). The changes in the

density of granule cells activated by the learned odorant are thus correlated with the behavioral performances.

Finally, we assessed the percentage of newborn granule cells activated by the learned odorant, *i.e.* expressing Zif268 in response to +Limonene (Figure 4D). We tested the age ($F(1,15)=4.23$ $p=0.057$) and enrichment effects ($F(1,15)=8.345$ $p=0.011$). Interestingly, we also found an interaction between age and enrichment ($F(1, 15)= 16.576$ $p=0.001$), indicating that the observed effect of enrichment depended on the age. Indeed, enrichment increased the percentage of BrdU/Zif268-positive granule cells in young adult mice ($p=0.0003$), while it had no effect in old mice ($p>0.05$). In non-enriched animals, the percentage of BrdU/Zif268-positive cells was similar whatever their age, suggesting an equal recruitment of adult-born neurons in young adult and old mice in basal conditions.

Altogether our data demonstrated an impaired ability of the aged bulbar network to increase its responsiveness to an odorant after a 10 day enrichment period, accompanying the absence of increased discrimination abilities. On the contrary, in young adult mice, a localized increase of inhibitory interneuron responsiveness (including newborn neurons) to the learned odorant occurred after perceptual learning.

3.4. Pharmacological noradrenergic stimulation induced olfactory perceptual learning in old mice

Since noradrenaline is necessary for perceptual learning in young adult mice (Moreno, et al. , 2012) and the noradrenergic system is altered in old mice (Rey, et al. , 2012), we hypothesized that noradrenergic deficits might be responsible for the alterations in perceptual learning that we reported above in old mice. Therefore, we tested whether restoring the noradrenergic system in old mice might allow an improvement in olfactory discrimination abilities.

We assessed the spontaneous olfactory discrimination after a 10-day period of saline or dexafaroxan treatment. Discrimination tests were done after a 2-3 day washout of the drug. We observed normal habituation curves in young adult and old mice injected with saline or dexefaroxan (Table 4; Supp Figure 2). In addition, focusing on the discrimination abilities, young adult and old mice behaved similarly after pharmacological treatment (no age effect $F(1,229)=0.12$ $p=0.72$). In both young adult and old mice, the overall ANOVA showed a significant effect of treatment (young adult: $F(1,91)=14.08$ $p<0.0001$; old: $F(1,138)=28.37$ $p<0.0001$)), of trials (young adult: $F(1,91)=12.3$ $p<0.001$; old: $F(1,138)=28.37$ $p<0.0001$), and an interaction between treatment and trials (young adult: $F(1,91)=6.92$ $p=0.01$; old: $F(1,138)=24.109$ $p<0.0001$). In details, dexefaroxan treatment improved the discrimination of the three odor pairs tested and mimicked perceptual learning in young adult mice, in accordance with our previous data (Moreno, et al. , 2012) (paired t-test, +/- limonene $p=0.03$, pentanol/butanol $p<0.0001$, decanal/dodecanone $p=0.01$ for difference between Ohab4 and Otest; Figure 5Ai and Aii). As expected, the performances of discrimination were not changed in saline non-enriched animals ($p>0.05$). Interestingly, we observed the same positive effect of dexefaroxan on discrimination performances in old mice (+/- limonene $p=0.003$, pentanol/butanol $p=0.0002$, decanal/dodecanone $p<0.0001$; Figure 5Bi and Bii), while saline non-enriched old mice did not discriminate ($p>0.05$ for all three odor pairs).

We then assessed the effect of noradrenergic stimulation on bulbar neurogenesis. The results showed an effect of age ($F(1, 11)=129.540$, $p<0.0001$), drug treatment ($F(1, 11)=7.022$, $p=0.023$) and an interaction between age and drug treatment ($F(1, 11)=6.537$ $p=0.027$) on the density of BrdU-positive cells. As reported in Figure 1, the BrdU-positive cell density was significantly reduced in old mice compared to young adults ($p<0.0001$). In addition, there was an increase of BrdU-positive cell density in young adult mice after dexefaroxan treatment

compared to saline treated mice (p=0.032; Figure 6A and C). This phenomenon was not observed in old mice (p>0.05 for difference between dexefaroxan and saline treated mice; Figure 6A and C). Finer analysis of the number of bins showing significant modulation of BrdU cell density between enriched and non-enriched animals revealed the same level of change in young adult and old mice indicating that in young adult group the increase of BrdU-positive cell density was homogeneously distributed across the granule cell layer (Figure 6D).

Finally, the neuronal phenotype of adult-born cells (percentage of BrdU/NeuN double labeled cells) was not modulated by the drug treatment ($F(1,13)=0.093$ $p=0.766$) (Figure 6B). We found a significant age effect ($F(1,13)=5.21$, $p=0.04$) but no interaction of age and treatment ($F(1,13)=1.61$ $p=0.2$). As presented as a trend in Figure 1, we found a significantly reduced level of neuronal differentiation in aged mice when compared to young adults (t-test; $p=0.019$).

Last, we assessed the granule cell responsiveness to the learned odorant after stimulation of the noradrenergic system. Interestingly, the density of Zif268-positive cells increased after dexefaroxan treatment compared to saline injected mice in both adult ($p=0.032$) and old mice ($p=0.0005$) (age effect $F(1, 10)=11.126$ $p=0.08$; enrichment effect $F(1, 10)=13.585$ $p=0.04$; interaction $F(1, 10)=4.774$ $p=0.0054$ Figure 7A). However, note that the density of Zif268-positive cells in dexefaroxan-treated old mice remained lower than in dexefaroxan-treated young adult mice ($p=0.04$). Averaged Zif268-positive cell density maps showed that the increase in granule cell responsiveness induced by dexefaroxan is widespread in young adult and old mice (Figure 7C) with old mice showing stronger dexefaroxan-induced modulation than young adult mice (t-test on proportion: $p=0.026$; Figure 7D).

In addition, treatment with dexefaroxan increased the percentage of newborn cells responding to the odorant (percentage of BrdU/Zif268 double labeled cells) in both young adult ($p=0.04$) and old mice ($p=0.0021$) (age effect $F(1,13)=2.81$ $p=0.11$, treatment effect $F(1,13)=4.820$

p=0.047; interaction $F(1,13=29.089$ $p<0.0001$; Figure 7B). This result showed that newborn neurons, although rare in the aged OB, can be recruited to process the learned odor.

Altogether, these data showed that increasing the level of noradrenaline was able to restore olfactory discrimination performances and increase the granule cell responsiveness in old mice (including the responsiveness of newborn neurons), but without increasing the overall level of neurogenesis.

In conclusion, our results showed that olfactory perceptual learning was altered in old mice and correlated with a low level of neurogenesis and granule cell responsiveness. However, the stimulation of the noradrenergic system was able to restore olfactory discrimination performances and increase the granule cell responsiveness including the responsiveness of newborn cells.

4- DISCUSSION

The present study was designed to investigate the effect of aging on olfactory perceptual learning, a fundamental type of learning belonging to the natural behavioral repertoire in mammals. We found that on contrary to young adult mice in which olfactory enrichment improved olfactory discrimination of similar odorants (Mandairon, et al. , 2006c, Mandairon, et al. , 2006d, Mandairon, et al. , 2006e, Moreno, et al. , 2012, Moreno, et al. , 2009), old mice did not show any improvement of discrimination after enrichment. Knowing that olfactory detection threshold is increased with aging (Mirich, et al. , 2002), we used relatively high concentrations of odorants (1 Pa) and ensured that all mice were able to detect the odorants using the habituation task. Indeed, 18 months old mice performed as well as young adult mice in that task indicating that the detection of such concentration was not altered at that age. Moreover, basic olfactory discrimination could be affected with aging (Enwere, et al. , 2004),

but the alteration of perceptual learning observed in our group of old mice was not due to a global defect of discrimination. Indeed, old mice discriminated well dissimilar odorants such as (+)-Limonene and Decanal (our study) or acetate esters or aldehydes which length differing from three carbons (Rey, et al. , 2012). Thus, these results indicated that the lack of improvement of performance after enrichment in 18 months old mice was due to a specific impairment of perceptual learning and not to alterations of detection threshold or basic discrimination abilities.

Our present study also sheds light on the neural basis of olfactory perceptual learning and more generally improved olfactory discrimination. An increase of neurogenesis has been correlated with an improvement of olfactory performances after olfactory associative learning (Mandairon, et al. , 2011, Mouret, et al. , 2008, Sultan, et al. , 2011a, Sultan, et al. , 2011b) and perceptual learning (Moreno, et al. , 2012, Moreno, et al. , 2009) in young adult mice. As expected, we observed in young adult mice an increase of newborn granule cell survival after enrichment. Such increase of neurogenesis did not occur in aging mice, which could explain the inefficiency of perceptual learning to induce discrimination improvement. This result is in accordance with our previous data showing that a blockade of neurogenesis, using infusion of an antimitotic drug directly in the subventricular zone, impaired olfactory perceptual learning in adult mice (Moreno, et al. , 2009). We now provide additional information using aging as a physiological model of neurogenesis' reduction. Old mice which presented a decrease of bulbar neurogenesis (and not a total ablation as did AraC-treated mice) were not able to perform olfactory perceptual learning. More precisely, the inability of neurogenesis to be increased by odor enrichment might be one of the reasons why old mice are not able to perform olfactory perceptual learning.

However, our data also interestingly revealed that increased newborn neuron survival is not always necessary for improved discrimination to occur. Indeed, noradrenergic stimulation in aged mice improved olfactory discrimination abilities without modifying the density of newborn granule cells, strongly suggesting that in old animals, adult neurogenesis is not the only neural basis of olfactory discrimination.

Noradrenergic neurons from the *Locus Coeruleus* have significant projections into the OB (McLean, et al. , 1989, Shipley, et al. , 1985), modify the excitability of multiple populations of OB neurons through diffusely expressed noradrenergic receptors (Holmberg, et al. , 2003, Jones, et al. , 1985, Palacios and Kuhar, 1982, Woo and Leon, 1995, Young and Kuhar, 1980), and are involved in numerous forms of learning (Mandairon and Linster, 2009, Mandairon, et al. , 2008b, Moriceau and Sullivan, 2004, Sullivan and Wilson, 1994) including perceptual learning (Moreno, et al. , 2012). A recent study pointed out a decrease of noradrenergic activity between 10 and 22 months in mice (Rey, et al. , 2012). Based on these results, we hypothesized that a stimulation of the noradrenergic system could restore perceptual learning in old mice. In line with the hypothesis, we reported an improvement of olfactory discrimination after dexefaroxan treatment in old mice compared to saline injected animals. Alteration of perceptual learning in aged mice is thus likely due to the deterioration of pre-synaptic noradrenergic mechanisms, as pharmacological stimulation of its release is sufficient to induce an improvement of the discrimination performances. These results imply that the noradrenergic signaling pathway (adrenergic receptors and intracellular signaling cascades) is still present and functional in the OB of aged mice. We cannot however exclude the involvement of other neuromodulatory systems such as the cholinergic system known to be also altered with aging (Mandairon, et al. , 2009) and involved in olfactory discrimination processes (Mandairon, et al. , 2006a). What would be the action of noradrenaline on bulbar neurons? The main difference between adult and old mice regarding the effect of

noradrenaline, is that newborn neuron survival is not increased by noradrenergic stimulation in aged animals. These data suggest that stimulating noradrenaline release can recruit neural mechanisms allowing improvement of discrimination and compensating for the lack of neurogenesis' response to enrichment in old mice.

Odor discrimination is thought to rely on a process of pattern separation mediated by increased lateral inhibition (Schoppa and Urban, 2003). We used the expression of Zif268, which is driven by neuronal activity (Inaki, et al. , 2002, Knapska and Kaczmarek, 2004, Moreno, et al. , 2012), to measure the level of inhibitory interneuron activation in response to odorant stimulation. In young adult mice, the density of Zif268-positive granule cells was increased after enrichment in accordance with our previous study (Moreno, et al. , 2009). Such increase of granule cell activation did not happen in old mice after enrichment. We also mapped the distribution of activated granule cells and quantified the extension of changes in activation occurring after enrichment. The amplitude of such changes was higher in young mice compared to old mice. In addition, we showed that in young adult mice but not in aged animals, adult-born cells responded preferentially to the learned odorant as seen by the increase of BrdU/Zif268 double-labeled cells after enrichment. The impairment of perceptual learning with aging is thus correlated with a loss of responsiveness of pre-existing and newborn granule cells to enrichment. Unlike odor enrichment, noradrenergic stimulation in aged mice was able to increase granule cell activation in response to odor stimulation, to recruit newborn neurons in the network and to improve olfactory discrimination. These results strongly support the hypothesis that improvement of the discrimination performances in old mice relies on the activation of the bulbar inhibitory network, composed of pre-existing and newborn granule cells. This suggest that newborn neurons despite their small number could be functionally recruited under dexefaroxan treatment and that these highly plastic newborn

neurons (Nissant, et al. , 2009) could contribute within the neural network to improvement in discrimination.

In conclusion, we have shown that perceptual learning is not effective in old animals but can be restored by increasing noradrenergic release. In this case, improving odor discrimination does not involve the modulation of newborn neuron survival but still is accompanied by the activation of the pre-existing inhibitory network and the recruitment of newborn neurons.

All together, our data indicate that decrease of noradrenergic activation could form the basis for the alteration of perceptual learning in aged mice. Importantly, they also indicate that the bulbar neural network in aged animals is still functional and positively respond to pharmacological stimulation of noradrenergic release to support perceptual learning.

Figures legends

Figure 1. Basic olfactory discrimination is not impaired in 18 months old mice

Discrimination between dissimilar odorants (limonene and decanal) was assessed in old mice using a habituation/dishabituation test. Old mice were able to habituate indicating that the animals detected and memorized the odorant. In addition, we observed an increase of investigation time when the second odorant of the pair is presented, compared to the last habituation trial, showing that the animals were also able to discriminate odorants. The data are expressed as mean values ± S.E.M (*** : $p<0.0001$, in response magnitude between Ohab4 and Otest).

Figure 2. Olfactory perceptual learning is impaired in old mice

A. Experimental design. To assess the performance of discrimination between three odor pairs, young adult and old mice were submitted to a habituation/dishabituation test before and after a 10-day enrichment period. **Bi. and Ci.** Before the enrichment period, none of the three odor pairs was discriminated by young adult and old mice. **Bii. and Cii.** The same result was observed in control non-enriched young adult and old mice. **Biii.** The enrichment with the enantiomers of limonene improved the capacities of young adult mice to discriminate +/- limonene and butanol/pentanol but not decanal/dodecanone. **Ciii** In contrary, old mice were not able to discriminate any odor pairs after the enrichment period. The data are expressed as mean values ± S.E.M (* : $p<0.05$; *** : $p<0.0001$; NS : non significant, in response magnitude between Ohab4 and Otest)

Figure 3. The decrease of olfactory perceptual learning performances was correlated with a low level of neurogenesis in aging mice

A. BrdU-positive cell density was increased in the granule cell layer after enrichment in young adult mice whereas no difference was observed in old mice. Old mice displayed a lower density of BrdU-positive cells when compared to young adult mice. The data are expressed as mean values ± S.E.M (** : $p<0.001$; *** : $p<0.0001$; NS : non significant). **B.** Averaged maps of BrdU-positive cell density in young adult and old mice before (left) and after (right) enrichment. A clustered distribution of BrdU-positive cells was observed after enrichment only in young adult mice. C**i.** Using BrdU/NeuN double-labeling in the granule cell layer, we did not observe any effect of enrichment on the neuronal phenotype of newborn cells in both young adult and old mice. **Cii.** Example of a BrdU/NeuN double labeled cell imaged through pseudo-confocal. (Scale bar, 5 μm). **D.** Numbers of bins with a p value inferior to 0.001 or superior to 1-0.001 in Mann-Whitney maps expressed as percentages of the total number of bins in the matrix. T-tests for comparison of proportions were applied; ***$P < 0.0001$.

Figure 4. The improvement of discrimination after perceptual learning is accompanied by an increase of granule cell responsiveness to the learned odorant in young adult but not in old mice.

A. In young adult mice, the density of Zif268-positive cells increased in the granule cell layer after enrichment whereas no such enhancement is observed in old mice. **B.** Averaged maps of Zif268-positive cell density in young adult and old mice in non-enriched (left) and enriched mice. Averaged maps showed a clustering of Zif268-positive cells preferentially located in the medial part of the granule cell layer only in young adult enriched mice. **C.** Numbers of bins with a p value inferior to 0.001or superior to 1-0.001 in Mann-Whitney maps expressed as percentages of the total number of bins in the matrix. T-tests for comparison of proportions were applied; *** $p < 0.05$. **Di.** Enrichment with +/-Limonene increased the percentage of

newborn neurons responding to +Limonene compared to control non enriched animals in young mice but not old animals. **Dii**. Example of a BrdU/Zif268 double labeled cell imaged through pseudo-confocal. (Scale bar, 10 μm) (*** : $p<0.0001$; NS : non significant).

Figure 5. Noradrenergic stimulation in old mice improved olfactory discrimination and mimicked olfactory perceptual learning.

Ai. and Bi. After ten days of saline injections, none of the three odor pairs is discriminated by young adult and old mice. **Aii. and Bii.** In contrary, after 10 days of Dexefaroxan injections, which increase the release of noradrenaline, the discrimination of the three odor pairs is improved in both young adult (**Aii**) and old (**Bii**) mice. The data are expressed as mean values ± S.E.M. (* : $p<0.05$; ** : $p<0.005$; *** : $p<0.0001$; NS : non significant, in response magnitude between Ohab4 and Otest)

Figure 6. Noradrenergic stimulation in old mice does not increase newborn neuron survival.

A. Dexefaroxan treatment increased the density of BrdU-positive cells compared to the saline injection in young adult but not old mice. **B.** We did not observe any effect of dexefaroxan injections on the neuronal phenotype of newborn cells in both young adult and old mice. **C**. Averaged BrdU-positive cells maps showed that dexefaroxan treatment induced a broad increase of neurogenesis only in young adult mice **D.** Graph showing the percentage of bins with a significant p value (bins with a p value inferior to 0.001 or superior to 1-0.001 in Mann-Whitney maps). T-tests for comparison of proportions were applied (N.S. non significant)

Figure 7. Noradrenergic stimulation in old mice increases the responsiveness of pre-existing and newborn granule cells.

A. In both young adult and old mice, the density of Zif268-positive cells is increased in the granule cell layer after dexefaroxan treatment compared to saline injected mice. **B**. Treatment with dexefaroxan increased the percentage of newborn neurons responding to +Limonene compared to saline injected animals in both young adult and old animals. **C.** Averaged Zif268-positive cell maps showed that dexefaroxan induced a widespread increase in Zif268-positive cells in young adult mice and at a lower level in old mice. D. Graph showing the percentage of bins with a significant p value (bins with a p value inferior to 0.001or superior to 1-0.001 in Mann-Whitney maps). T-tests for comparison of proportions were applied. The data are expressed as mean values ± S.E.M (* : $p<0.05$; *** : $p<0.0001$; NS : non significant).

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Disclosure statement

All authors declare to have no actual or potential conflicts of interest.

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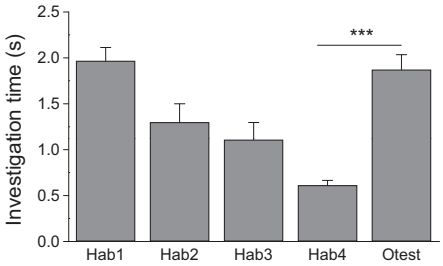

Investigation time (s)
2.5
2.0
1.5
1.0
0.5
0.0
***
Hab1
Hab2
Hab3
Hab4
Otest

FIGURE 1

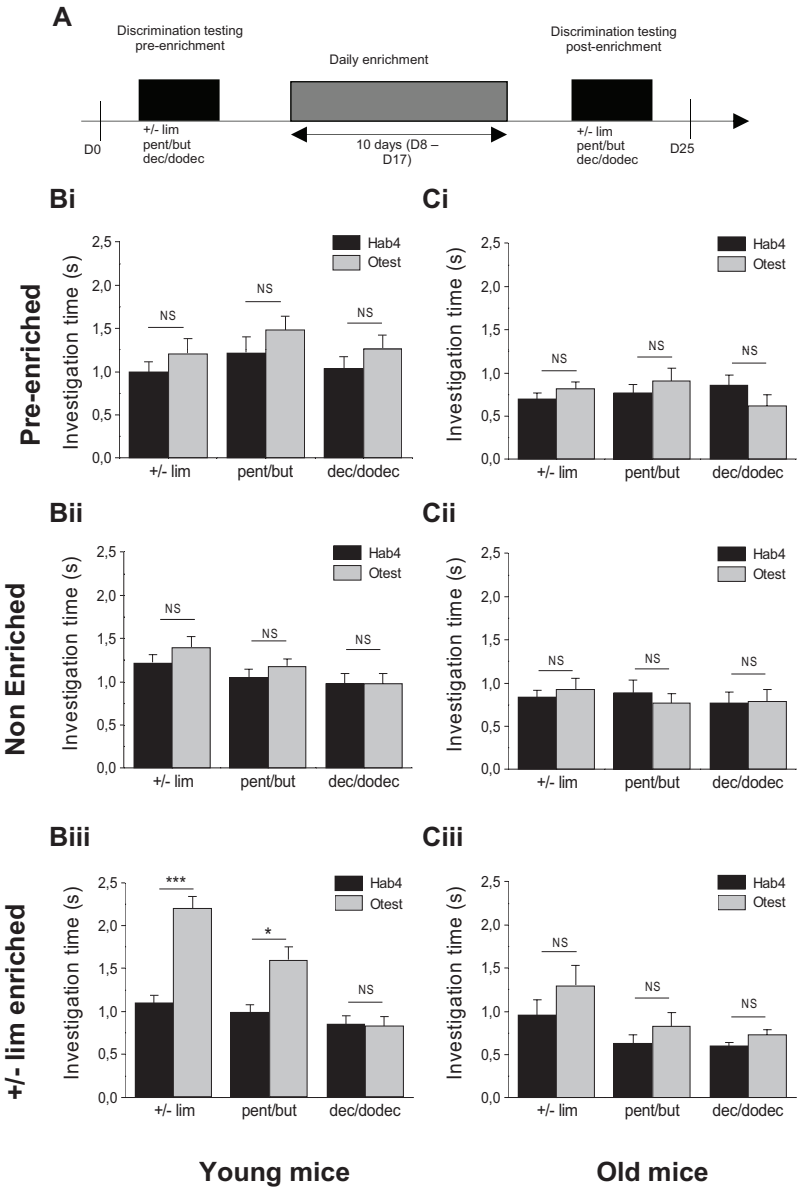

A
Discrimination testing pre-enrichment
Daily enrichment
Discrimination testing post-enrichment
D0
+/- lim pent/but dec/dodec
10 days (D8 – D17)
+/- lim pent/but dec/dodec
D25
Bi
Ci
Bii
Cii
Biii
Ciii
Pre-enriched
Non Enriched
+/- lim enriched
Investigation time (s)
Hab4
Otest
NS
***
*
+/- lim
pent/but
dec/dodec
Young mice
Old mice

FIGURE 2

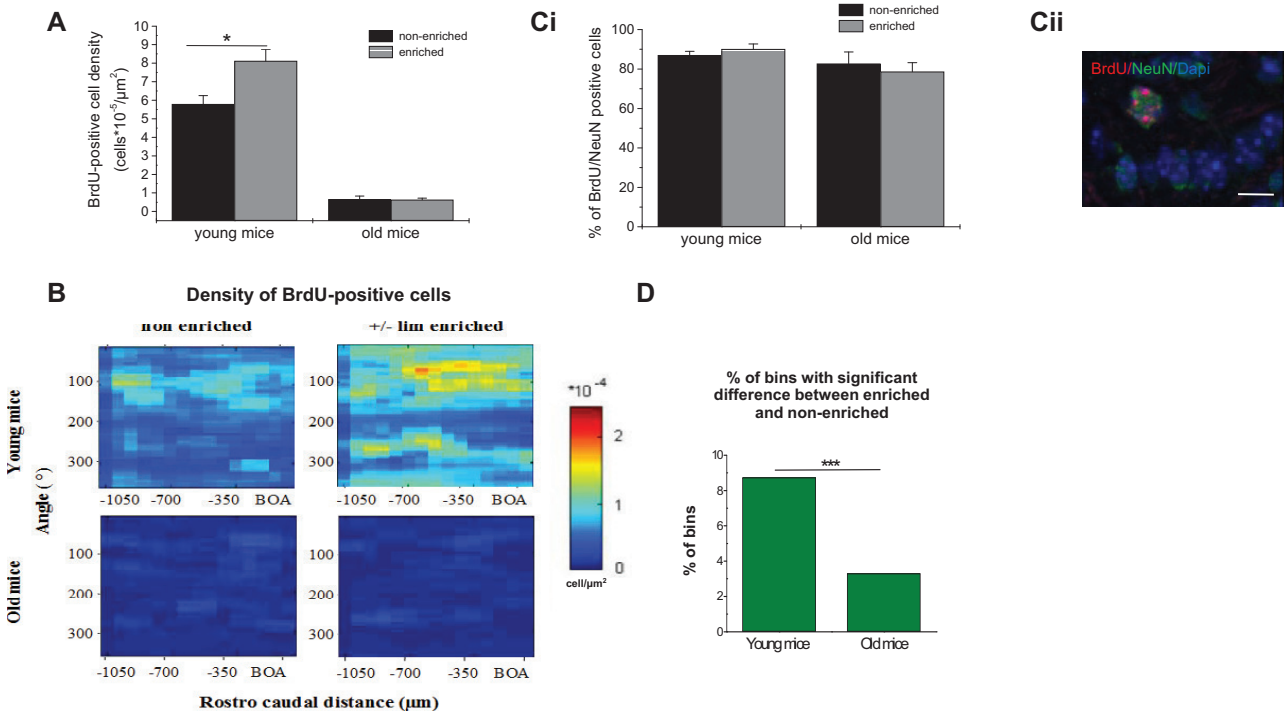

A
BrdU-positive cell density (cells*10^-5/µm^2)
non-enriched
enriched
young mice
old mice
Ci
% of BrdU/NeuN positive cells
non-enriched
enriched
young mice
old mice
Cii
BrdU/NeuN/Dapi
B
Density of BrdU-positive cells
non enriched
+/- lim enriched
Young mice
Old mice
Angle (°)
-1050 -700 -350 BOA
Rostro caudal distance (µm)
cell/µm²
D
% of bins with significant difference between enriched and non-enriched
% of bins
***
Young mice
Old mice

FIGURE 3

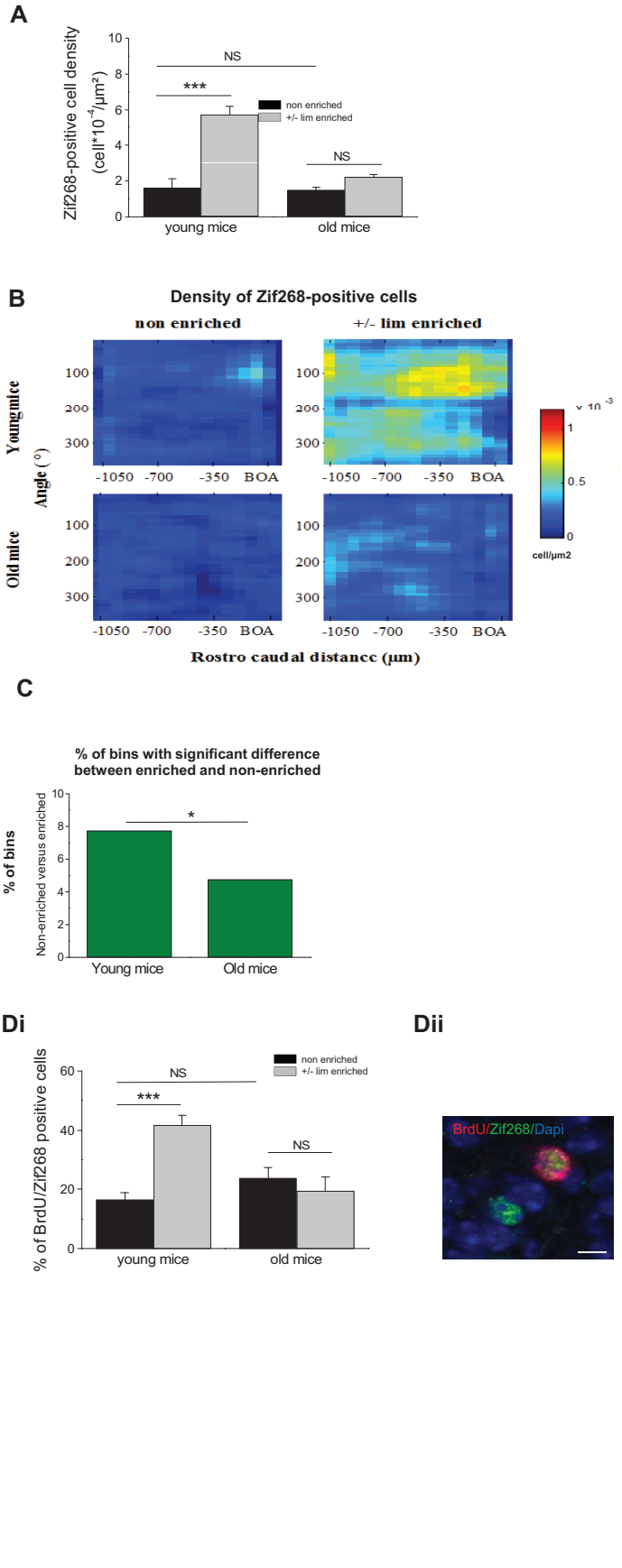

A
Zif268-positive cell density
(cell*10^-4/µm²)
NS
***
non enriched
+/- lim enriched
NS
young mice
old mice
B
Density of Zif268-positive cells
non enriched
+/- lim enriched
Young mice
Old mice
Angle (°)
-1050 -700 -350 BOA
cell/µm2
Rostro caudal distance (µm)
C
% of bins with significant difference
between enriched and non-enriched
% of bins
Non-enriched versus enriched
*
Young mice
Old mice
Di
% of BrdU/Zif268 positive cells
non enriched
+/- lim enriched
NS
***
NS
young mice
old mice
Dii
BrdU/Zif268/Dapi

FIGURE 4

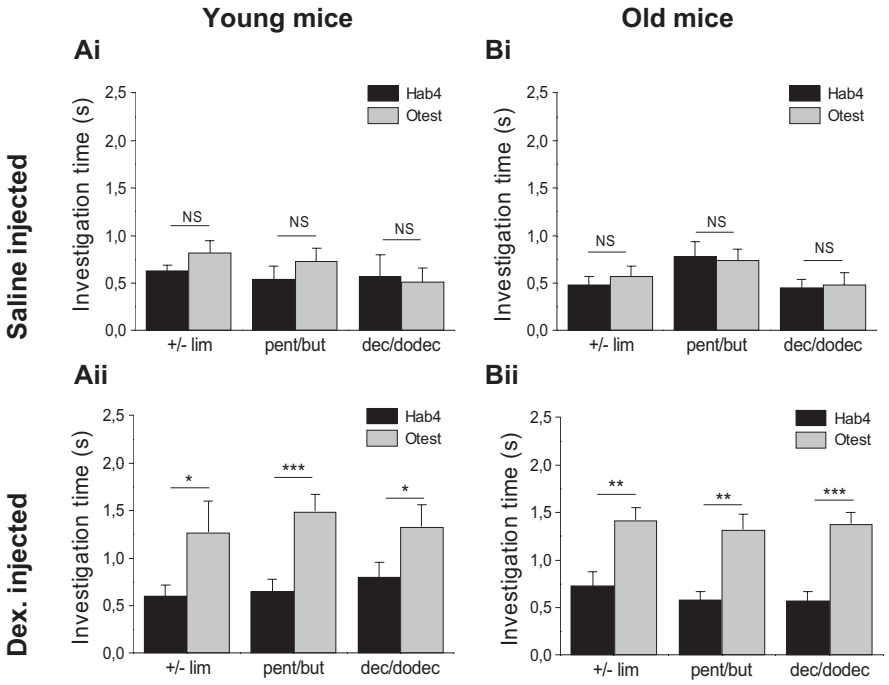

Young mice
Old mice
Ai
Bi
Aii
Bii
Saline injected
Dex. injected
Investigation time (s)
Hab4
Otest
NS
*
***
**
+/- lim
pent/but
dec/dodec
0,0
0,5
1,0
1,5
2,0
2,5

FIGURE 5

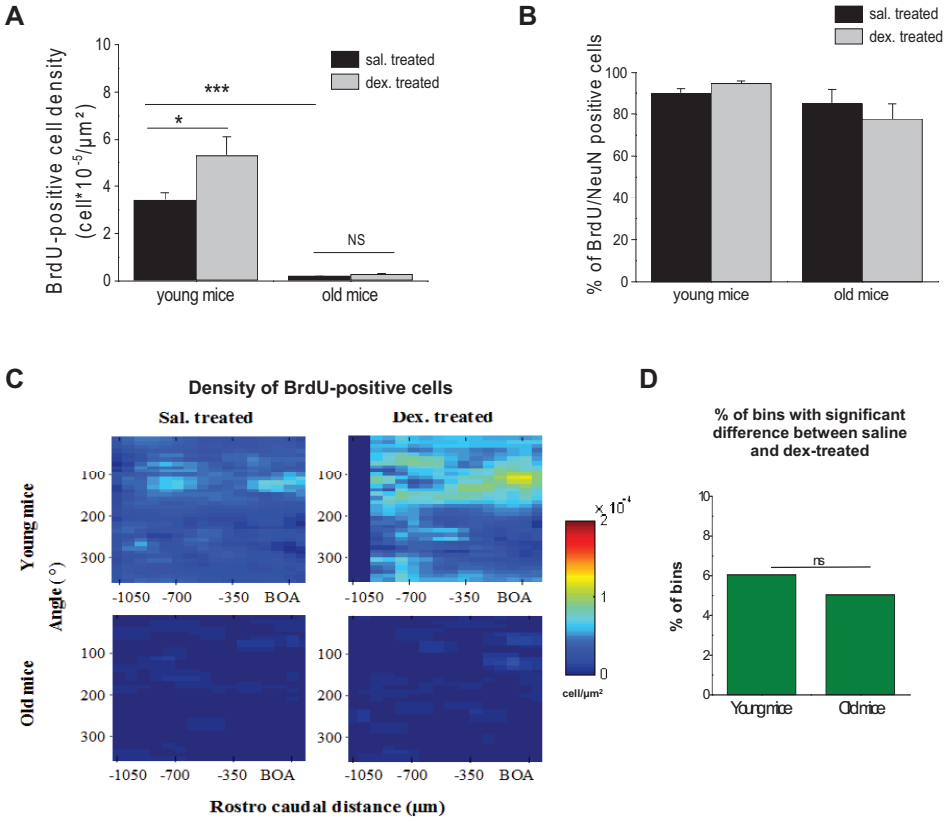

A
BrdU-positive cell density (cell*10-5/µm2)
sal. treated
dex. treated
***
*
NS
young mice
old mice
B
% of BrdU/NeuN positive cells
sal. treated
dex. treated
young mice
old mice
C
Density of BrdU-positive cells
Sal. treated
Dex. treated
Young mice
Old mice
Angle (°)
-1050 -700 -350 BOA
Rostro caudal distance (µm)
cell/µm²
D
% of bins with significant difference between saline and dex-treated
% of bins
ns
Youngmice
Oldmice

FIGURE 6

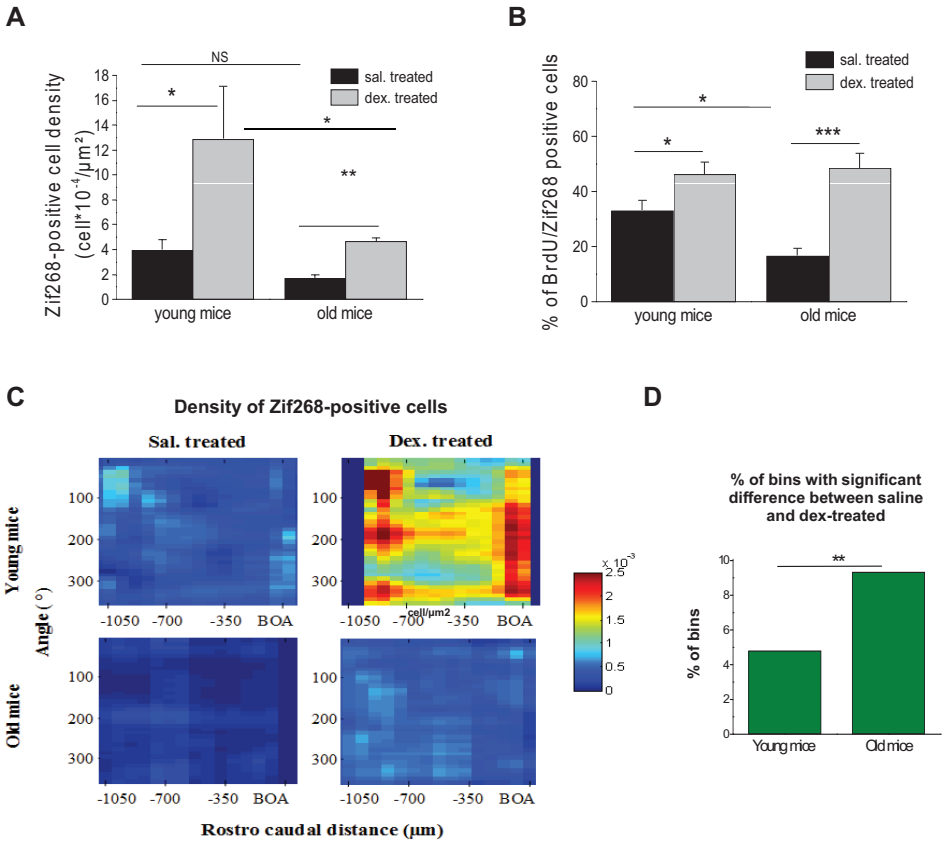
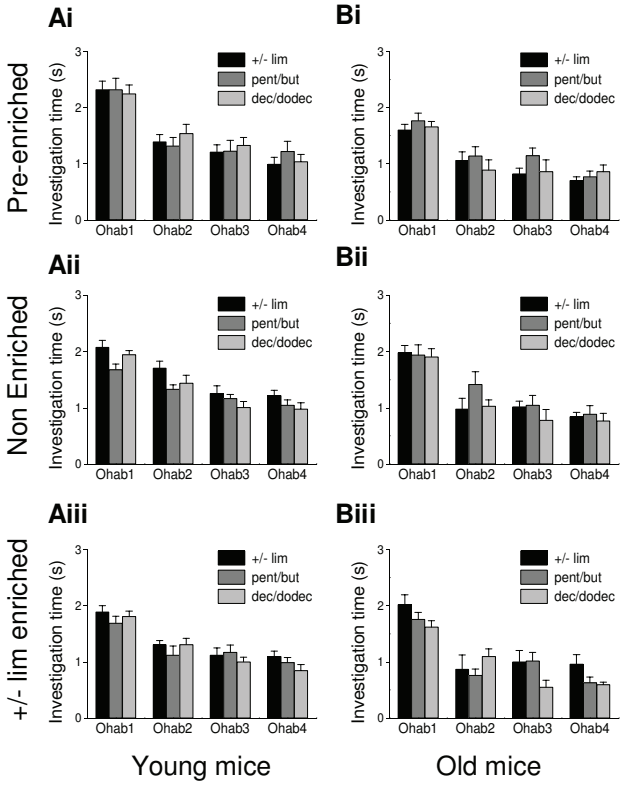

A
NS
*
*
**
sal. treated
dex. treated
Zif268-positive cell density
(cell*10⁻⁴/µm²)
young mice
old mice
B
sal. treated
dex. treated
*
*
***
% of BrdU/Zif268 positive cells
young mice
old mice
C
Density of Zif268-positive cells
Sal. treated
Dex. treated
Young mice
Old mice
Angle (°)
-1050 -700 -350 BOA
cell/µm2
Rostro caudal distance (µm)
D
% of bins with significant difference between saline and dex-treated
**
% of bins
Young mice
Old mice

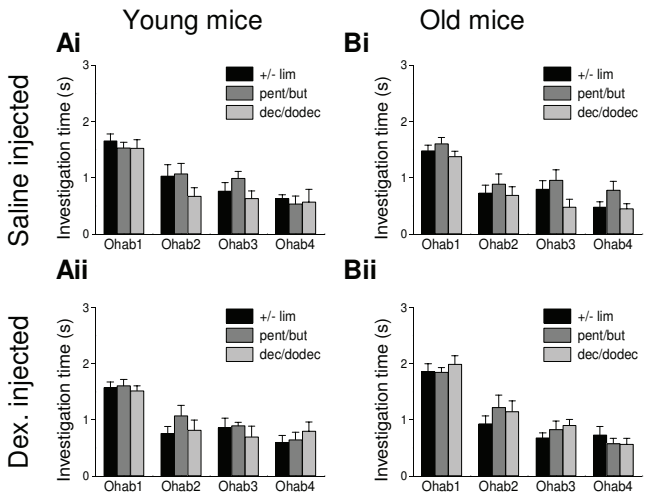
FIGURE 7

Odorants	Dilution
+ Limonene	0.204%
- Limonene	0.204%
Butanol	0.021%
Pentanol	0.074%
Decanal	1.776%
Dodecanone	12.498%

Table 1: Odorants used for discrimination task with corresponding v/v dilutions (1 Pa)



Supp Figure 1



Supp Figure 2

Groups	Odor pair	F value	p value
Adult mice	+/- lim	F(4,150)=13.761	<0.0001
	pent/but	F(4,123)=6.681	<0.0001
	dec/dodec	F(4,142)=10.124	<0.0001
Old mice	+/- lim	F(4,129)=11.895	<0.0001
	pent/but	F(4,96)=7.397	<0.0001
	dec/dodec	F(4,75)=6.828	<0.0001

Supplementary table 1 : Habituation performances before enrichment

Groups	Odor pair	F value	p value
Adult mice +/ + e+-icFed	(4, lim	150)=3.76<6p0	nb<bbb2
	8e+t4out	150)O9.7O<0=0	nb<bbb2
	dec4d/ dec	150)=3.72S<9O6	nb<bbb2
a ld mice +/ + e+-icFed	(4, lim	150)S3.722<S=b	nb<bbb2
	8e+t4out	150)3b.7=<099	nb<bbb2
	dec4d/ dec	150)39.722<0p=	nb<bbb2
Adult mice (4, lim e+-icFed	(4, lim	150)OS.726<6=O	nb<bbb2
	8e+t4out	150)O9.7S<0OO	nb<bbb2
	dec4d/ dec	150)=6.72O<bSO	nb<bbb2
a ld mice (4, lim e+-icFed	(4, lim	150)p=.70<9=O	b<bb3
	8e+t4out	150)Sp.722<2p6	nb<bbb2
	dec4d/ dec	150)p9.7O<90O	nb<bbb2

r u88leme+ty-: tyole 9 Hf yoituyti/ + 8e-s/ -my+ceh yste- e+-icFme+t

Groups	Odor pair	F value	p value
Adult mice saline	+/- lim	F(4,33)=7.472	<0.0001
	pent/but	F(4,39)=7.443	<0.0001
	dec/dodec	F(4,30)=6.540	0.001
Old mice saline	+/- lim	F(4,44)=10.539	<0.0001
	pent/but	F(4,44)=5.322	0.001
	dec/dodec	F(4,47)=10.773	<0.0001
Adult mice dexefaroxan	+/- lim	F(4,47)=4.574	0.003
	pent/but	F(4,42)=7.754	<0.0001
	dec/dodec	F(4,47)=4.545	0.003
Old mice dexefaroxan	+/- lim	F(4,76)=14.207	<0.0001
	pent/but	F(4,72)=10.055	<0.0001
	dec/dodec	F(4,67)=13.166	<0.0001

Supplementary table 3 : Habituation performances after treatment

DISCUSSION GENERALE

L'objectif de ce travail de thèse était d'étudier le rôle des nouveaux neurones qui arrivent chaque jour dans le bulbe olfactif. Comme le bulbe olfactif a été impliqué dans différents types d'apprentissage et de mémoires olfactives (Brennan and Keverne, 1997) et qu'il est le siège d'une neurogenèse importante, nous avons fait l'hypothèse d'un rôle central de la neurogenèse dans les processus d'apprentissage. Nous avons fait le choix d'étudier le rôle de la neurogenèse dans un modèle particulier d'apprentissage olfactif, l'apprentissage perceptif car cet apprentissage est très écologique pour l'animal. Il reflète un processus permanent d'assimilation de l'environnement sensoriel. L'expérience avec des odeurs de tous les jours influence la façon dont elles sont perçues. C'est un apprentissage de type implicite qui se définit comme une amélioration des performances de discrimination suite à une exposition répétée au stimulus.

I/ LES NOUVEAUX NEURONES DU BULBE OLFACTIF : SUPPORT DE L'APPRENTISSAGE PERCEPTIF

Des cellules souches présentes dans la zone sous-ventriculaire des ventricules latéraux donnent naissance à des neuroblastes qui migrent jusqu'au bulbe olfactif pour s'intégrer dans le réseau et donner des neurones matures (Alvarez-Buylla and Garcia-Verdugo, 2002). La moitié de ces neurones meurent dans les 30 jours après leur naissance dans les conditions contrôles (Mandairon et al., 2006a). Parmi ces différentes étapes de la neurogenèse : prolifération, migration et différenciation/survie, seule la dernière semble être modulée par l'entrée sensorielle (Rochefort et al., 2002; Mandairon et al., 2003; Alonso et al., 2006; Mandairon et al., 2006a) suggérant que l'entrée sensorielle jouerait un rôle clé dans le contrôle de la survie des nouveaux neurones dans le réseau bulbaire.
La signification fonctionnelle de la neurogenèse adulte reste cependant floue malgré le nombre croissant d'études effectuées.

Chez le rongeur, après 10 jours d'exposition à une paire d'odeurs très similaires d'un point de vue perceptif et physico-chimique, la discrimination de ces mêmes odeurs est améliorée. Les mécanismes cellulaires gouvernant cette plasticité fonctionnelle siègent dans le bulbe olfactif puisqu'elle est dépendante de l'activation des récepteurs NMDA présents dans le bulbe olfactif (Mandairon et al., 2006e). Nous avons tout d'abord établi que l'apprentissage olfactif perceptif était hautement dépendant de l'apport de nouveaux interneurones inhibiteurs dans le bulbe olfactif (Moreno et al., 2009). En effet, dans notre première étude, nous montrons tout d'abord une augmentation de la densité des néo-neurones dans la couche granulaire des animaux du groupe enrichi en comparaison au groupe contrôle non enrichi. Du fait du choix du protocole d'injections du BrdU, cette augmentation traduit une amélioration du taux de survie plutôt qu'une hausse de la

prolifération ou de la migration. Nous avons utilisé l'expression du gène précoce Zif268 comme marqueur d'activité (Inaki et al., 2002; Busto et al., 2009; Mandairon et al., 2008a) et nous avons observé que les nouveaux neurones répondent préférentiellement à l'odeur apprise. Ce résultat est en accord avec des études précédentes montrant que la réponse des cellules granulaires néoformées est modulée par l'expérience olfactive d'une façon spécifique du stimulus (Busto et al., 2009; Sultan et al., 2010). Dans un deuxième temps, grâce à l'utilisation d'un anti-mitotique, l'AraC, nous avons pu montrer le caractère nécessaire de la neurogenèse bulbaire dans la mise en place de l'apprentissage perceptif. En effet, la suppression de la prolifération neurogénique par des injections intra-cérébrales d'AraC durant une fenêtre temporelle courte (juste avant et pendant la période d'enrichissement) permet de bloquer l'arrivée de nouveaux neurones dans le bulbe olfactif. Pendant les tests de discrimination, la neurogenèse n'est plus bloquée. Ainsi, du fait de la courte période de blocage, la structure du bulbe olfactif n'est pas profondément modifiée puisque l'on n'observe pas de réduction significative du volume de la couche granulaire contrairement à ce qui est observé après blocage à long terme de la neurogenèse (Imayoshi et al., 2008).

Les nouveaux neurones sont donc indispensables à l'acquisition de l'apprentissage perceptif. Est-il possible de généraliser à d'autres formes d'apprentissages olfactifs ce rôle des néo-neurones ? Dans ce contexte, il est important de noter que le rôle de la neurogenèse dans l'apprentissage perceptif semble au moins en partie différent de celui rapporté dans l'apprentissage associatif. En effet, alors que les nouveaux neurones semblent indispensables dans la phase d'acquisition de l'apprentissage perceptif, ils ne le sont pas pour l'apprentissage associatif. Ils sont par contre support de la mémoire dans les deux apprentissages. Plus précisément, il a été montré que l'administration d'un agent antimitotique n'altère pas l'acquisition de l'apprentissage associatif (Sultan et al., 2010). Par contre, l'augmentation de la survie des nouveaux neurones induite par un apprentissage associatif, persiste tant que la tâche de discrimination est mémorisée et l'oubli de la tâche comportementale est corrélé à une élimination par apoptose des nouveaux neurones. Le rôle des nouveaux neurones comme support de la mémoire associative est montré puisque le blocage de la neurogenèse induite par l'administration d'un agent antimitotique empêche la rétention à long terme de la tâche (Sultan et al., 2011b, a).

Une partie de ces données a été confirmée par une autre étude dans laquelle le blocage de la neurogenèse a été pratiqué par irradiation de la zone sous-ventriculaire (Lazarini et al., 2009). Pourtant, ces deux études semblent en contradiction avec les données de Breton-Provencher et al., (2009) et d'Imayoshi et al., (2008). Dans Breton-Provencher et al., le blocage de la neurogenèse par l'AraC aurait un effet sur la mémoire non pas à long-terme mais à court-terme (Breton-Provencher et al., 2009) alors qu'Imayoshi et al., (2008) ne rapportent aucun effet d'une ablation génétique de la neurogenèse sur les performances olfactives en termes de mémoire à court ou à long-terme, de discrimination ou d'acquisition (Imayoshi et al., 2008). Ces deux dernières études ont utilisé un

apprentissage non-opérant pour tester la mémoire à long-terme alors que Sultan et al., (Sultan et al., 2011b) et Lazarini et al., (2009) ont utilisé un apprentissage opérant, ce qui pourrait expliquer les différences observées. Ainsi, en fonction de la nature du test comportemental (opérant/non-opérant ; associatif/perceptif), l'implication de la neurogenèse serait différente.

Nous avons fait l'hypothèse que la neurogenèse serait également support de la mémoire de l'apprentissage perceptif, puisque des études préliminaires que j'ai réalisées, montrent que les animaux discriminent aussi longtemps que les nouveaux neurones sont présents dans le bulbe olfactif (Figure 1).

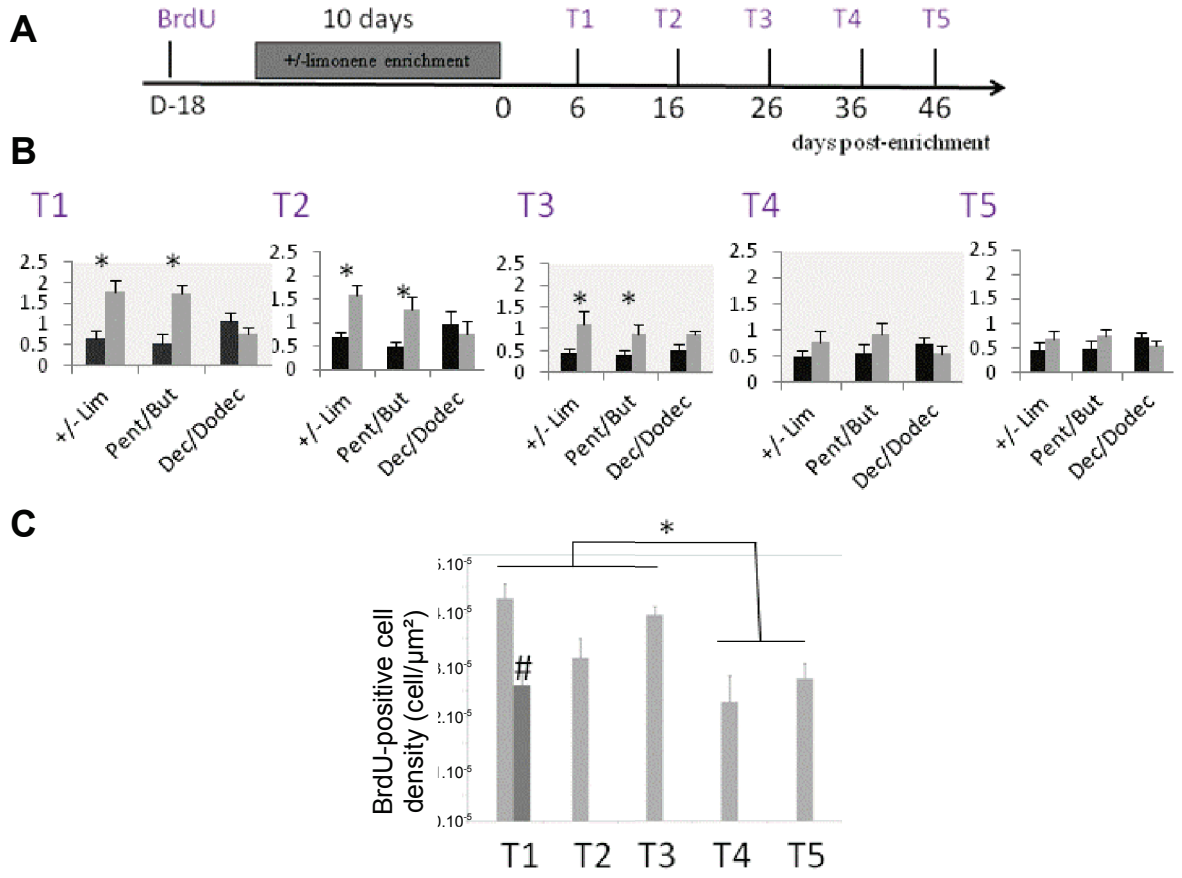


Figure 1 : **A.** Protocole expérimental : 6, 16, 26, 36 et 46 jours après la période d'enrichissement au +/- limonène, nous avons testé les performances de discrimination et évalué le taux de neurogenèse. **B.** L'amélioration des performances de discrimination perdurent jusqu'à 26 jours après enrichissement. **C.** La neurogenèse est augmentée jusqu'à 26 jours après enrichissement puis diminue significativement à partir de 36 jours. (*$p<0.05$; # $p<0.05$ pour différence entre groupes non-enrichi (gris foncé) et enrichi (gris clair))

Comme nos données préliminaires sur l'apprentissage perceptif suggèrent que les nouveaux neurones supportent la mémoire à long-terme, nous faisons l'hypothèse que l'effacement de la mémoire devrait altérer la survie des nouveaux neurones. Une façon écologique de tester cette hypothèse est de provoquer l'oubli de cet apprentissage perceptif et d'en regarder les effets sur la

discrimination.

C'est effectivement ce que nous observons en données préliminaires (Figure 2). L'apprentissage perceptif d'une deuxième paire d'odeurs placé juste quelques jours après la fin du premier apprentissage provoque l'oubli du premier apprentissage et la mort des nouveaux neurones. Ces données sont à rapprocher de celles obtenues lors de l'apprentissage associatif où après avoir créé une trace mnésique spécifique d'une association odeur-récompense, elle a été brisée de façon comportementale par une autre association pour voir les conséquences sur la survie des nouveaux neurones quand l'apprentissage est oublié. Dans ce contexte, les animaux ont subi un premier apprentissage odeur-récompense de 5 jours puis un deuxième apprentissage objet-récompense de 5 jours en présence de l'odeur présentée de façon aléatoire. On observe que le deuxième apprentissage provoque l'oubli du premier et qu'en parallèle il induit la mort par apoptose des nouveaux neurones impliqués dans le premier apprentissage (Sultan et al., 2011b).

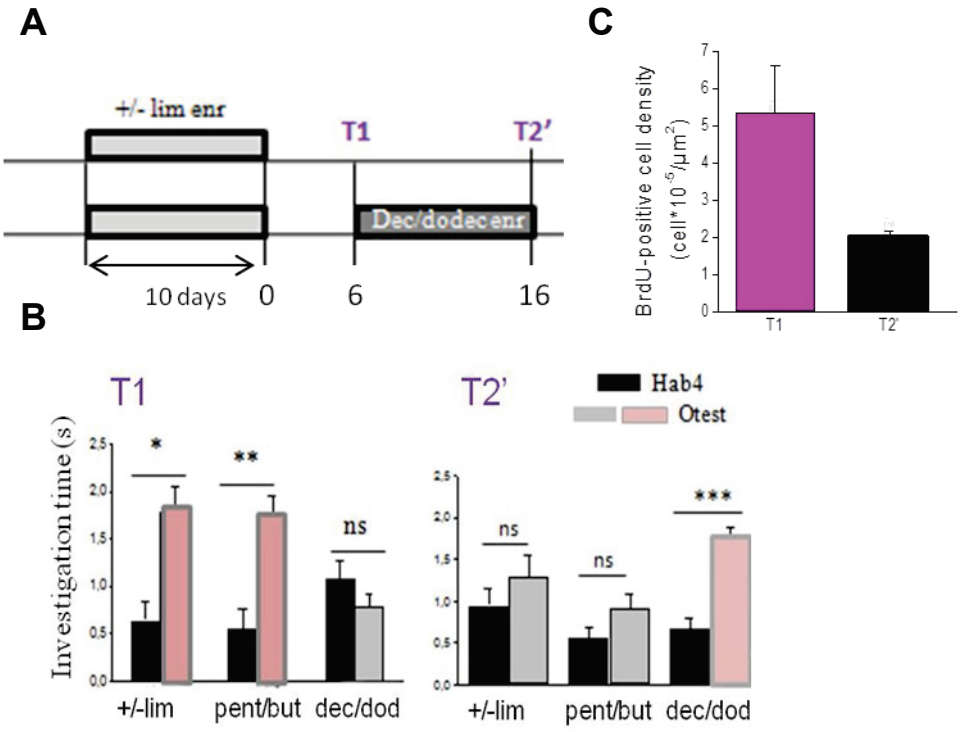


Figure 2 : A. Protocole expérimental : les animaux sont testés 6 jours après enrichissement au +/- limonene ou après un deuxième enrichissement au decanal/dodecanone. **B.** Les animaux discriminent le +/- limonène après enrichissement à cette paire d'odeurs. L'enrichissement successif au décanal/dodecanone améliore leur discrimination mais affecte l'amélioration de la discrimination au +/-limonène. **C.** L'enrichissement au décanal/dodecanone provoque la mort des néoneurones sauvés par le premier enrichissement (*p<0.05 ; ** p<0.001 ; ***p<0.0001).

Ainsi, malgré les différences entre les différents types d'apprentissage, un point commun émerge, il s'agit du rôle des nouveaux neurones comme support de la mémoire à long-terme. Les changements rapides de la survie des nouveaux neurones pourraient être d'une grande pertinence d'un point de vue biologique pour s'assurer que les informations importantes soient retenues et les informations obsolètes oubliées rapidement. Enfin ces données soulignent la flexibilité et réactivité du processus de sélection (survie/mort) des nouveaux neurones quand des modulations sont nécessaires pour ajuster le réseau bulbaire à l'environnement.

Mais quel est l'impact de l'apport ces nouveaux neurones sur le réseau bulbaire ? Nous avons montré que l'apprentissage perceptif était accompagné d'une augmentation de l'inhibition dans le bulbe olfactif, mise en évidence par une augmentation d'expression de la GAD65/67 et confirmée par une expérience d'électrophysiologie en double pulses. Ces deux techniques montrent une augmentation globale de l'inhibition dans la couche granulaire. Afin de déterminer si les nouveaux neurones sont responsables des modulations d'inhibition observées dans le bulbe olfactif, nous avons bloqué la neurogenèse pendant la phase d'enrichissement et évalué le taux d'expression de la GAD65/67. Dans ce cas, l'augmentation d'expression de la GAD65/67 n'est plus observée, ce qui indique que les nouveaux neurones sont responsables de l'augmentation de l'inhibition induite par l'apprentissage perceptif. Nous pouvons alors proposer l'hypothèse selon laquelle l'intégration sélective de nouveaux interneurones GABAergiques dans le bulbe olfactif permet l'apprentissage perceptif en augmentant l'activité inhibitrice dans le bulbe ajustant ainsi la représentation bulbaire des odeurs. La présence à long-terme de ces neurones dans le réseau permettrait la mémorisation de l'information.

En conclusion, des signaux sensoriels pertinents, comme la présence répétée d'odeurs dans l'environnement de l'animal, permettent un apprentissage de discrimination spécifique des odeurs grâce une augmentation ciblée de la survie des nouveaux neurones. La neurogenèse confère au réseau bulbaire adulte la capacité de s'adapter aux besoins éthologiques de l'animal.

II/ MÉCANISMES GOUVERNANT L'INTÉGRATION DES NOUVEAUX NEURONES LORS DE L'APPRENTISSAGE

Les mécanismes gouvernant l'intégration et la survie des neurones néoformés pendant l'apprentissage restent méconnus. Un candidat potentiel très intéressant est la noradrénaline. En effet, les cellules granulaires du bulbe olfactif, cible principale de la neurogenèse, sont largement innervées par le système noradrénergique en provenance du *Locus Coeruleus* (Shipley et al., 1985; McLean et al., 1989). De plus, la noradrénaline joue un rôle critique dans la perception olfactive et l'apprentis-

sage (Sullivan and Wilson, 1994; Sullivan et al., 2000; Fletcher and Chen, 2011), dans la discrimination spontanée (Mandairon et al., 2008b) et la mémoire à court-terme (Veyrac et al., 2007; Veyrac et al., 2009). Dans notre deuxième étude, nous nous sommes donc intéressés au rôle potentiel de la noradrénaline dans la médiation du signal d'intégration des nouveaux neurones pendant l'apprentissage perceptif. Chez la souris adulte, nous avons manipulé le système noradrénergique grâce à des moyens pharmacologiques et utilisé l'imagerie confocale et l'électrophysiologie pour rechercher les mécanismes potentiels par lesquels la noradrénaline pourrait directement influencer la survie des nouveaux neurones.

Nous avons trouvé que la manipulation directe de la transmission noradrénergique affectait significativement la survie des nouveaux neurones et les performances dans l'apprentissage perceptif. Plus précisément, nous avons trouvé que l'exposition aux odeurs ainsi que l'augmentation de libération de noradrénaline à l'aide du dexefaroxan (un antagoniste des récepteurs α2 présynaptiques noradrénergiques qui permet l'augmentation de la libération de noradrénaline) conduisaient à une amélioration des performances de discrimination chez la souris ainsi qu'à une augmentation de la survie des nouveaux neurones. Inversement, l'injection de labetalol, un antagoniste des récepteurs noradrénergiques, bloque l'amélioration des performances olfactives et n'augmente pas la neurogenèse.

Pour aller un peu plus loin, nous avons cherché à savoir si le système noradrénergique agissait sur les nouveaux neurones pour améliorer la discrimination. Pour cela, nous avons administré dans la zone sous-ventriculaire, de façon chronique l'AraC, conjointement à l'administration de dexefaroxan, et avons montré la nécessité de la présence des nouveaux neurones pour que la noradrénaline puisse améliorer les performances de discrimination.

Enfin, nous avons confirmé ces résultats grâce à la mise au point du marquage des nouveaux neurones par un lentivirus GFP injecté dans la zone sous ventriculaire. Nous avons pu observer en microscopie pseudo-confocale que ces neurones nouvellement formés GFP entraient en contact avec les fibres noradrénergiques et répondaient à la noradrénaline. Cette dernière partie a été permise grâce à une collaboration avec Nicola Kuczewski. Ces résultats mettent en évidence un lien important entre la transmission noradrénergique et la neurogenèse bulbaire comme mécanisme sous-tendant l'apprentissage perceptif.

Ces données supportent un rôle majeur du contrôle top-down noradrénergique dans l'apprentissage perceptif. En effet, la noradrénaline agirait sur les cellules nouvellement formées pour affiner le pattern de l'odeur, contribuant ainsi aux changements de discrimination dus à l'expérience. Ces données sont importantes car elles montrent pour la première fois le rôle d'une voie neuromodulatrice dans les mécanismes de sélection des nouveaux neurones et la modulation des performances olfactives.

Il serait intéressant d'étudier le rôle de la noradrénaline également dans l'apprentissage associatif. En effet, on a observé un rôle différent de la neurogenèse dans les deux types d'apprentissage (associatif et perceptif) et nous faisons l'hypothèse que les voies centrifuges ne sont pas sollicitées de la même façon en fonction de la tâche comportementale demandée. Une activation différentielle du système noradrénergique en fonction de l'apprentissage (perceptif/associatif, opérant/non-opérant) pourrait expliquer une action différente sur les nouveaux neurones, modulant ainsi leur intégration et fonction.

Le même type de démarche pourrait être appliqué au système cholinergique. En effet, on sait que le taux de neurogenèse est modulé par le système cholinergique (Lledo et al., 2006; Mandairon et al., 2006e) et que des fibres cholinergiques se projettent directement sur les nouveaux neurones (dès 10 jours après leur naissance) (Whitman and Greer, 2007). Ces données suggèrent que l'intégration des nouveaux neurones pourrait également dépendre de l'activation du système cholinergique sollicité lors d'un apprentissage.

Concernant la troisième voie neuromodulatrice se projetant sur le bulbe olfactif, le système sérotoninergique, il est très probable qu'il puisse aussi jouer un rôle important dans la régulation de la neurogenèse en condition d'apprentissage. C'est un champ qui reste à explorer.

En conclusion, les projections neuromodulatrices, permettant de moduler le traitement de l'information olfactive en fonction de l'expérience, du contexte d'apprentissage, ou de l'attention pourraient être des mécanismes clés de l'intégration des nouveaux neurones lors de l'apprentissage. Leurs activations spécifiques seraient capables d'induire des modifications à long terme du circuit bulbaire pour que l'animal puisse faire face à des situations comportementales spécifiques.

III/ L'EFFET DU VIEILLISSEMENT SUR L'APPRENTISSAGE PERCEPTIF

III. 1. Baisse de la neurogenèse et déclin olfactif

Le vieillissement est un processus physiologique qui va affecter l'ensemble d'un organisme, entraînant des modifications physiologiques et se traduisant généralement par des altérations des fonctions organiques mais aussi par des troubles cognitifs. Le système olfactif est également affecté par le vieillissement avec notamment un déclin des fonctions olfactives.

Grâce à une étude comportementale sur des souris âgées de 18 mois, nous avons trouvé que, contrairement aux observations faites sur des jeunes adultes, l'exposition répétée à deux odeurs similaires sur le plan perceptif ne permettait pas d'en améliorer leur discrimination. Comme l'olfaction est altérée chez la souris âgée avec notamment une diminution du seuil de détection (Mirich et al., 2002), nous nous sommes assurés que ces souris de 18 mois détectaient bien les odorants. En effet, nous avons utilisé, sur la base de la littérature, des concentrations largement

supraliminaires et nous avons testé leur détection. Nous avons également évalué la discrimination de base (discrimination entre odeurs très différentes) et nous avons montré qu'elle n'était pas altérée. En effet, les capacités de discrimination fine se dégradent à partir de 23 mois, alors que la discrimination d'odeurs distinctes est plus robuste et persiste chez la souris âgée (Enwere et al., 2004; Rey et al., 2012a; Wesson et al., 2010). Le troisième paramètre que nous avons contrôlé est la mémoire. En gardant un délai inter essais court dans le test d'habituation/deshabituation (5 min), nous avons réduit au minimum la composante 'mémoire' du test. Dans ces conditions, les courbes d'habituation obtenues chez les souris âgées montrent la même décroissance du temps d'investigation de l'odeur au fil des essais que chez les souris jeunes adultes indiquant que les souris habituent semblable aux plus jeunes. Ces données sont en accord avec une étude préalable menée dans l'équipe montrant que les altérations des performances de mémoire surviennent à l'âge de 23 mois chez la souris (Rey et al., 2012a). En conclusion, nous avons pu tester spécifiquement dans notre étude l'effet du vieillissement sur l'apprentissage perceptif et montrer son altération chez les souris âgées de 18 mois.

En parallèle aux évaluations comportementales, nous avons évalué le taux de neurogenèse chez les animaux âgés. Nous montrons que la neurogenèse est très fortement diminuée avec l'âge. Ces données sont en accord avec des études précédentes qui montrent une altération en deux temps de la neurogenèse lors du vieillissement: d'abord une forte diminution de prolifération, observable à âge moyen et qui serait due essentiellement à une diminution du nombre de précurseurs dans la zone sous-ventriculaire (Enwere et al., 2004, Rey et al 2012a), puis chez l'animal âgé, une diminution de la survie des néo-neurones et de leur taux de différenciation (Luo et al., 2006, Rey et al 2012a). De façon importante, nous montrons que chez la souris âgée, la neurogenèse, en plus d'avoir un taux de base faible, n'est pas augmentée après enrichissement indiquant une diminution drastique de la plasticité du processus neurogenique chez la souris âgée par rapport à la souris adulte jeune. Sur la base de ces éléments, nous pouvons suggérer que le déficit d'apprentissage perceptif chez la souris âgée serait dû à la diminution de la neurogenèse et de sa plasticité puisque dans notre première étude, nous montrons que chez la souris jeune adulte, l'apprentissage perceptif est hautement dépendant d'une neurogenèse fonctionnelle. Les altérations de la neurogenèse et de sa plasticité au cours du vieillissement pourraient découler de la réduction de la libération de certains facteurs trophiques et/ou d'une altération de l'expression de leurs récepteurs dans la zone sous-ventriculaire ou dans le bulbe olfactif. Par exemple, il a été montré que la voie du BDNF était importante dans la régulation de la neurogenèse bulbaire et dans la discrimination olfactive (Bath et al., 2008).

Notre troisième étude montre également que l'activité des cellules granulaires (mise en évidence par l'expression du Zif268) chez les souris âgées était similaire à celle observée chez les jeunes souris.

Cette donnée suggère que le niveau d'inhibition n'est pas altéré avec l'âge ce qui pourrait expliquer le maintien des fonctions olfactives de base chez la souris âgée comme la discrimination spontanée ou la mémoire à court terme. Par contre, il n'y a pas de hausse de l'activité des cellules granulaires préexistantes et néoformées après enrichissement chez la souris âgée. Ceci peut être relié au défaut d'apprentissage. Puisque chez la souris jeune, un blocage de la neurogenèse empêche l'augmentation générale de Zif268 dans la couche granulaire, l'altération de réponse neurogénique à l'enrichissement chez la souris âgée pourrait expliquer l'absence d'augmentation globale d'activité dans la couche granulaire ainsi que l'altération d'apprentissage.

Le maintien de la plupart des fonctions olfactives à 18 mois est en accord avec une étude récente indiquant que la structure du bulbe olfactif est très préservée avec l'âge, en termes de densité des différents types cellulaires, en dépit de la baisse drastique de neurogenèse, à l'exception de modifications synaptiques subtiles, essentiellement glomérulaires (Richard et al., 2010). De plus, la diminution progressive de la neurogenèse bulbaire précède souvent le déclin de performances olfactives (Rey et al., 2012a). Ceci suggère que le réseau bulbaire pourrait mettre en place des mécanismes compensatoires pour maintenir le fonctionnement basal et la perception olfactive, alors que les mécanismes de plasticité eux, seraient plus sensibles à l'âge.

III. 2. La noradrénaline : un mode d'action différent en fonction de l'âge

L'hypothèse proposée ci-dessus selon laquelle le bulbe olfactif âgé n'est pas simplement un bulbe dégradé mais de façon plus subtile et complexe un réseau différent qui s'est adapté à l'âge (Mandairon and Didier, 2010) est illustrée par les résultats que nous avons obtenus sur le mécanisme d'action de la noradrénaline chez la souris âgée Nous avons montré dans notre deuxième étude chez la souris jeune que la survie des nouveaux neurones permettant l'apprentissage perceptif était dépendante du système noradrénergique. Au cours du vieillissement, une diminution de l'expression de l'enzyme de synthèse de la noradrénaline (la DBH) dans le *Locus Coeruleus* survient à 23 mois ainsi qu'une diminution du nombre de fibres noradrénergiques dans le bulbe olfactif (Rey et al., 2012b). Nous avons donc supposé que la stimulation pharmacologique du système noradrénergique chez la souris âgée de 18 mois permettrait de restaurer un taux suffisant de noradrénaline pour permettre l'apprentissage perceptif.

En accord avec cette hypothèse, nous avons montré qu'une stimulation de la libération de noradrénaline par le dexefaroxan induit l'apprentissage perceptif chez la souris âgée, alors que l'enrichissement n'est plus capable de produire cet effet. Le système noradrénergique présente donc une réserve d'action disponible et accessible pharmacologiquement qui permet de restaurer l'apprentissage perceptif olfactif. Il a été montré que l'enrichissement augmentait l'expression de la DBH dans

le *Locus Coeruleus* chez les animaux de 10 mois mais plus à 23 mois (Rey et al., 2012b). Chez la souris âgée, la plasticité du système noradrénergique en réponse à l'enrichissement est perdue mais la stimulation directe du système noradrénergique semble court-circuiter ce mécanisme de plasticité et permettre ainsi l'apprentissage perceptif.

Un deuxième résultat important de cette étude est que la stimulation du système noradrénergique n'entraîne pas d'augmentation de la neurogenèse bulbaire comme c'est le cas chez la souris adulte jeune. Ce résultat suggère que la neurogenèse bulbaire à 18 mois ne possède pas de potentiel de restauration et est trop touchée par le vieillissement pour être stimulée par le système noradrénergique. Ainsi, de manière remarquable, chez la souris âgée, le système noradrénergique

seul permet de restaurer l'apprentissage perceptif olfactif et ce de manière indépendante de la neurogenèse bulbaire contrairement au mécanisme observé chez la souris jeune. Le réseau bulbaire vieillissant apparaît donc ici clairement comme un réseau se modifiant au cours du temps pour pallier aux déficits progressifs en neurogenèse au point que les effets de la noradrénaline, qui ont absolument besoin des nouveaux neurones pour s'exercer chez la souris jeune, semblent devenir indépendants de la neurogenèse chez la souris âgée. De plus, l'étude de l'activité des cellules granulaires via l'expression du Zif268 après stimulation noradrénergique, indique que l''inhibition est augmentée en lien avec l'amélioration des capacités de discrimination. La stimulation du système noradrénergique chez la souris âgée permettrait d'augmenter l'inhibition bulbaire de manière indépendante de la neurogenèse bulbaire, traduisant ainsi une modification progressive du réseau bulbaire au cours du vieillissement et la mise en place de mécanismes compensatoires complexes. Le système noradrénergique agirait vraisemblablement différemment chez la souris âgée et pourrait par exemple agir sur le développement de l'arbre dendritique et du nombre de contacts synaptiques des neurones plus anciens. Il ne peut pas non plus être exclu que les quelques néo-neurones qui persistent dans le bulbe olfactif âgé puissent aussi voir leur développement et intégration synaptique modulés par la noradrénaline. Ces paramètres morphologiques n'ont pas été mesurés dans notre travail. Des modifications de ce type pourraient intervenir et rendre compte au moins partiellement de l'amélioration des performances obtenue après stimulation noradrénergique, chez la souris âgée, sans que le taux de neurogenèse ne soit modifié. Ces modifications pourraient aussi bien entendu participer au processus d'apprentissage aussi chez la souris adulte jeune.

IV/ CONCLUSIONS

Le système olfactif apparaît comme un modèle pertinent pour étudier comment des représentations neurales peuvent expliquer le comportement et évoluer avec l'expérience. En effet, l'olfaction est un sens primordial chez le mammifère et le bulbe olfactif se prête particulièrement bien à ces études

compte tenu de son organisation anatomique et synaptique connue, de son accessibilité et des moyens expérimentaux permettant de contrôler efficacement l'entrée sensorielle. L'intérêt et la spécificité de ces études résident dans le caractère intégré de l'approche, qui consiste à travailler sur l'animal en comportement tout en décryptant les mécanismes cellulaires sous-jacents.

En résumé, l'ensemble de nos données a permis d'engager une voie de recherche essentielle pour la compréhension des mécanismes régulant la perception olfactive et plus largement pour celle des corrélats cellulaires du comportement. Enfin, les études sur les altérations de la plasticité perceptuelle avec l'âge contribuent à la compréhension des déclins sensoriels et cognitifs qui accompagnent le vieillissement.

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ANNEXE

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A computer-assisted odorized hole-board for testing olfactory perception in mice

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ABSTRACT

The present paper describes a behavioral setup, designed and built in our laboratory, allowing the systematic and automatic recording of performances in a large number of olfactory behavioral tests. This computerized monitoring system has the capability of measuring different aspects of olfactory function in mice using different paradigms including threshold evaluation, generalization tasks, habituation/dishabituation, olfactory associative learning, short-term olfactory memory with or without a spatial component, and olfactory preferences. In this paper, we first describe the hole-board apparatus and its software and then give the experimental results obtained using this system. We demonstrate that one single, easy-to-run experimental setup is a powerful tool for the study of olfactory behavior in mice that has many advantages and broad applications.

1. Introduction

The olfactory system is unique among brain structures in that it (1) is the target of an important adult neurogenesis (Lledo et al., 2006), (2) is heavily innervated by well-defined neuromodulatory systems known to be important for memory and neural plasticity (Luskin and Price, 1982; Shipley et al., 1996; Linster et al., 2003; Mandairon et al., 2006a, 2008a), and (3) is impaired in normal (Thomas-Danguin et al., 2003; Seiberling and Conley, 2004; Westenhoefer, 2005; Wilson et al., 2006; Hummel et al., 2007) and pathologic aging for example in Alzheimer's disease (Devanand et al., 2000; Kovacs et al., 2001; Wilson et al., 2007). In the field of olfaction, there has been explosive growth over the past decade in the understanding of the behavioral consequences of network modulation (Kaba and Keverne, 1988; Ravel et al., 1994; Sullivan and Wilson, 1994; Linster et al., 2001; Mandairon et al., 2006b,c, 2008b; Kiselycznyk et al., 2006; Doucette et al., 2007; Guerin et al., 2008) as well as the consequences of learning on the neural network (Gray et al., 1986; Kay et al., 1996; Kay and Laurent, 1999; Buonviso and Chaput, 2000; Rochefort et al., 2002; Spors and Grinvald, 2002; Fletcher and Wilson, 2003; Martin et al., 2004, 2006; Alonso et al., 2006; Mandairon et al., 2006a, 2008b; Woo et al., 2007; Beshel et al., 2007). This explains the growing interest for behavioral research into olfaction in mice. The research literature on olfactory system includes many behavioral studies that have used associative learning, positive or negative reinforcements, and observation of spontaneous behavior. However, because there exists no behavioral setup allowing a systematic and automatic recording of a large number of olfactory behavioral tests, the purpose of this paper is to describe a computerized monitoring system, designed and built in our laboratory, which has the capability of assessing olfactory detection, discrimination, memory and spontaneous preferences. This was done using olfactory threshold evaluation, habituation/dishabituation and generalization tasks, associative and non-associative learning (with or without a spatial component) and measurement of odor attractiveness. The detailed presentation of the experimental setup will be followed by the experimental results obtained using this system. The advantages and broader possible applications of this experimental setup will also be outlined.

2. Materials and methods

2.1. The apparatus

All behavioral experiments were conducted using a specially designed computer-assisted hole-board apparatus (Fig. 1A). This consisted of a flat, square board, 60 mm thick with 400 mm sides (Fig. 1B and C) with five holes (36 mm diameter) able to receive glass containers (30 mm inside diameter, 45 mm deep; Fig. 1D). Masks were used to hide or uncover the holes, meaning that the board could be configured with one to five holes, as required for each experiment (Fig. 1E). The masks were made of Teflon® *PTFE* (polytetrafluoroethylene) and were easy to remove and clean. When a hole

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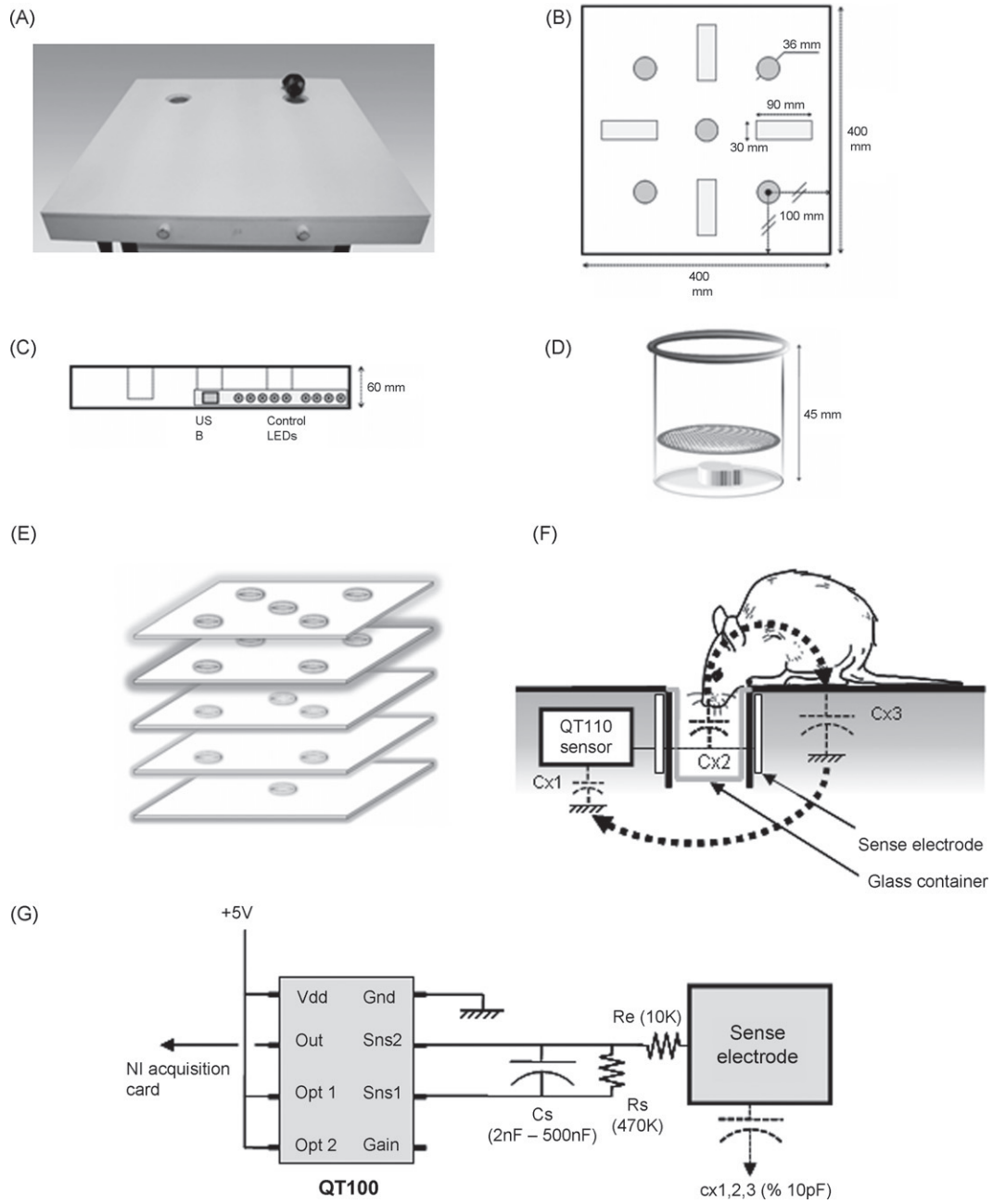

Fig. 1. Experimental setup. (A) The actual setup. (B and C) The computer-assisted hole-board apparatus consisted of a flat, square board, 60 mm thick with 400 mm sides in which five holes had been bored (36 mm diameter) to receive glass containers (30 mm inside diameter, 45 mm deep). The mouse could also be detected when traversing four rectangular areas located between the holes. (D) In each used hole, a polypropylene swab was placed under a metal grid at the bottom of the glass container and covered with bedding. (E) Several masks were used to hide or uncover the holes and allowed the hole-board to be configured for each experiment with from 1 to 5 holes. (F) The capacitance sensor uses Kirchoff's current law to detect a change of capacitance in an electrode. (G) Basic QT110 (Quantum Research Group®) circuit for the electronic cards used in the hole-board.

was in use, a polypropylene swab was placed under a metal grid at the bottom of the glass pot and covered with bedding (Fig. 1D). For trials involving odors, the swab was impregnated with the odorant at the appropriate concentration or with mineral oil for the controls. The bedding was replaced after each trial.

Five cylinder-shaped electrodes (positioned all around the hole) (Fig. 1F) were used to detect the presence of the mouse's nose in the holes and four rectangular electrodes, located between the holes were used to detect the position of the mouse on the board (Fig. 1B). The physical principle is based on charge-transfer. The presence of the mouse' nose in the hole induces a variation of capacitance detected by the electrode which is directly connected to an integrated circuit. This integrated circuit (QT110) uses Kirchoff's current law to detect a change of capacitance in the electrode (Fig. 1F) and generates an output transistor-transistor logic (TTL) signal (5 V) which is sent to an acquisition card (Fig. 1G).

Finally, the nine electrodes, five for the holes and four for the areas between the holes, were connected to nine small electronic cards placed under the upper surface of the board. Adjusting the sensitivity of each detector was possible by modifying the capacity (Cs) in the electronic circuit. The nine TTL signals were sent to an analog data acquisition card (National Instrument® NI USB-6000 series card) with the USB connector located on one edge of the hole-board. LEDs were also added to give visual confirmation that the system was functioning correctly. The electronic cards were powered via the USB connection.

2.2. The hole-board software

Any PC with a USB port could use the software we developed under LabView 8.5 (using DAQmx) (see: http://olfac.univ-lyon1.fr/unite/equipe-07/activite/holebox.html and for the software, contact the authors) after installing the appropriate NI acquisition card driver and the run-time program. Acquisition signal frequency was set at 100 Hz per channel which was sufficient to sample mouse detection and poking.

A user graphic interface was designed to perform several tasks and each experimenter could be logged and retrieve his global parameters. In testing mode, the hole-board is manually configured for each trial and the experimenter is free to test any configuration (selected holes and areas, odors, sensitivity of sensors). In protocol mode, a tool allows for the creation of protocols where every detail of the experiment is described. A text file is written containing the list of trials, details of the activated holes and areas, the odor stimulus and its distribution, and the maximal duration of the current trial.

During acquisition, all events (TTL signals) and their latency are monitored and stored in a results table. The different events that may occur were:

- The first TTL signal, coming from one of the four position electrodes, which was used to start the timer.
- The mouse enters or leaves an area.
- The mouse's nose pokes into a hole or leaves a hole.
- Additional keyboard event to log specific information (user-defined).

Results can be exported to a data-base for off-line analysis. In protocol mode, the trials are automatically sequenced but an inter-trial pause is proposed so that the experimenter can change the odorant swab if necessary.

2.3. Odorants

The odorants were presented pure or diluted in mineral oil proportionally to their vapor pressure. Briefly, the vapor pressures of the pure odorants were estimated with ACD Chemsketch software (Advanced Chemistry Development, Toronto, Ontario, Canada) and variously diluted in mineral oil to concentrations theoretically emitting the same vapor-phase partial pressure of each odorant (Cleland et al., 2002). All odorants used in this paper are reported in Table 1 with the corresponding dilutions. Because we wanted to demonstrate the maximum possibilities of the hole-board, we randomly used different odorants at different concentrations in the various behavioral tasks.

2.4. The olfactory tests

2.4.1. Animals

All the mice used in the experiments were housed upon arrival in the lab in groups of five in standard laboratory cages and were kept on a 12 h light/dark cycle (constant temperature) and with food and water *ad libitum*. Experiments were conducted in the afternoon (2–5 p.m.).

2.4.2. Olfactory detection

Odor set: We tested mice on threshold detection of propionic acid (PA) at different concentrations: 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} Pa (see Table 1).

Behavioral testing: Twenty mice were used in this experiment. The bedding was replaced after every trial. We used the hole-board in the one-hole configuration. The test consisted of two presentations of mineral oil, then one presentation of propionic acid at each concentration (2 min per trial; intertrial interval, ITI, 3 min). The duration of nose poking between odor presentation and no odor trials was used as a measure of threshold detection.

Table 1
Odors used for all experiments and corresponding dilutions.

Behavioral task	Odorants	Corresponding dilution (vol/vol)
Threshold detection	Propionic acid	0.0000033% 0.000033% 0.00033% 0.0033%
Generalization	Eugenol Guaiacol Heptanal	13.12% 2.08% 0.07%
Habituation/dishabituation	Pentanol Decanal	0.074% 1.78%
Discrimination learning Olfactory short-term memory	+limonene −carvone +limonene Citrate Cineol	pure pure pure pure pure
Olfactory and spatial memory	Carvone Geraniol Dodecanal Hexanol	2.36% 21.25% 2.74% 0.07%
Olfactory preferences	Allyl caproate Methylanthranilate Amyl butyrate Amyl phenylacetate Valeric acid Trimethyl amine	0.55% 12.65% 0.51% 59.13% 0.18% 0.0002%

Data analysis: All data analyses on time spent sniffing during odor presentation trials were performed with Systat software (SSI, Richmond, CA, USA). Outlier trials that deviated from the mean by more than two standard deviations were excluded from analysis. Data obtained for each concentration of the odor were averaged across animals and analyzed by ANOVA. The level of significance was set at 0.05.

2.4.3. Olfactory discrimination

2.4.3.1. Habituation–dishabituation paradigm. Odorants: All odorants were dissolved in mineral oil before the start of each experiment. Odors were presented by placing 60 μl of the odor stimulus (1 Pa) onto a polypropylene swab. We tested mice on a pair of odorants: pentanol and decanal (see Table 1).

Behavioral testing: This olfactory habituation task assessed the degree to which the mice spontaneously discriminated odorants by habituating them to an odorant (Ohab) and measured their investigation response to a second odorant (Otest). If the second odorant was not discriminated from the first, there would be no increased investigation response by the mouse. Food and water were continuously available during the course of the experiment. Because no reward was associated with either odorant in this task, and each test odor was presented only once, the task is probably a measure only of the basic similarities between odorants, unaltered by reinforcement. The mice ($n = 22$) were first familiarized with the experimental setup by a 3-min trial on the one-hole configuration of the board with no odorant. Then, habituation/dishabituation was assessed for the pair of odorants. A test session consisted of one 2-min presentation of plain mineral oil then four 2-min odor presentations of the Ohab at 5 min intervals, followed by one 2-min presentation of Otest.

Data analysis: All data analyses on time spent sniffing (nose poking) during odor presentation trials were performed with Systat software (SSI, Richmond, CA, USA). Outlier trials that deviated from the mean by more than two standard deviations were

excluded from analysis. Data obtained were averaged across animals and analyzed by unpaired *t*-tests to determine whether (1) the investigation time decreased between the first and the last Ohab trial and (2) the investigation time elicited by the test odor was significantly different from that elicited by the habituated odor during its fourth presentation. The level of significance was set at 0.05.

2.4.3.2. Generalization. Odor set: Three odorants were used, one conditioned odor (eugenol, odor C), and two test odorants: odorant 1 (guaiacol, O1) and odorant 2 (heptanal, O2) (see Table 1). The odors were diluted in mineral oil in such a way as to achieve an approximate vapor pressure of 1 Pa (Table 1).

Shaping: During shaping, the mice were exposed to the hole-board for 3 min over 2 days until they became accustomed to it. Then they were taught to retrieve a reward by digging in the holes. The hole-board was used in a two-hole configuration, one hole with the odor and a reward (a small bit of sweetened cereal, Kelloggs, Battle Creek, MI, USA) and the other hole with no reward and no odor. At the beginning of each trial, the mice were allowed to dig both holes until they retrieved the reward. During the first few trials, the reward was placed on the top of the odorized hole. After successful retrieval of the reward over several trials, the reward was buried deeper and deeper into the hole. Shaping was considered complete when a mouse would reliably identify the reward-containing hole and retrieve the deeply buried reward, and dig in the odor-containing hole even in the absence of a reward (thus controlling for the possibility of the mouse simply smelling the reward). Initial shaping was performed with odorants dissimilar to those used in other experiments (±limonene).

Behavioral testing: For this experiment, water was continuously available, but the mice were food-deprived for 8 h before the sessions. We used an odor generalization paradigm to measure the degree to which the mice generalized between odor C and the test odors. Each mouse was trained on odor C over six conditioning trials in which it had a choice between a scented hole containing the reward and an unscented hole containing no reward. Subsequently, three unrewarded test trials (in which the mouse was offered a choice between a hole scented with the test odorant and an unscented hole) were performed in a pseudorandom order; these test trials were separated by two rewarded conditioning trials to prevent extinction of the odor C-reward association. During the test trials, total digging times in the hole containing the test odor were recorded. The experimenter was unaware of the identity of the test odors during performance of these experiments. Trials were performed successively and only interrupted when it was necessary to change the odorant during which time the mice were put back in their cage.

Data analysis: The primary data consisted of digging times during the test trials. Outlier trials that deviated from the mean by more than two standard deviations were excluded from analysis. Data obtained for each odor test were averaged across animals. A paired *t*-test was performed in order to compare the digging time for the conditioned odor to the test odors (the level of significance was set at 0.05).

2.4.4. Olfactory memory

2.4.4.1. Associative learning. Odorants: We tested mice on a discrimination learning task between +limonene (20 μl of the pure odorant stimulus on a polypropylene swab) and mineral oil (Table 1) using the hole-board in its 2-hole configuration.

Shaping: Naive mice ($n = 15$) were first trained to retrieve a reward by digging through the bedding. The mouse was put in the start area and was allowed to dig for 2 min. During the first few trials the reward was placed on the top of the bedding of one of the two holes. After several successful retrievals, the reward was buried deeper into the bedding. Shaping was considered to be complete when a mouse could successfully retrieve a reward that was deeply buried in the bedding (8–12 trials).

Conditioning: During the discrimination learning experiments, water was continuously available, but the mice were food-deprived for 8 h before the sessions. In this experiment, we investigated how mice learn to discriminate +limonene from mineral oil. Each mouse was presented with a choice of two holes (one rewarded with the odorant, one unrewarded with no odorant) for 4 trials per day (2 min) for 5 days. During each trial, we recorded the hole in which the mouse dug first to retrieve the reward (correct response) and also the time needed by the mouse the find the reward (latency) as indicative of learning. In a previous experiment, we had verified that the animals were not guided by the odor of the reward by removing the reward for one trial and the percentage of correct responses was compared to the other trials. No difference was observed (Mandairon et al., 2006b).

Data analysis: ANOVA for repeated measures were applied to the latency values to assess the learning process (time effect). Data analyses were also performed using the number of correct responses as the dependent variable. The criterion for significance was set at 0.05.

2.4.4.2. Short-term memory. Odor set: Two odorants were used in this experiment: −carvone, +limonene. Odors were presented by placing 20 μl of the pure odor stimulus onto a polypropylene swab (Table 1).

Behavioral testing: 38 mice were first habituated to the experimental setup by a 2-min trial on the hole-board, with no odor. The mice then underwent the odor recognition task, which consisted of two trials each of 2-min duration. The hole-board was used in its two-hole configuration. During the first trial (acquisition) an unfamiliar odor (−carvone or +limonene) was presented in both holes. After 15, 30 and 60 min, the mice underwent the second trial (recall) in which one hole was odorized with the previously presented odor (−carvone or +limonene) and the other hole with a new odor (−carvone or +limonene). The odorants were randomized between the acquisition and recall trials. To avoid a place preference bias, odors were randomly placed in one or the other hole.

Data analysis: The time spent exploring the two holes during the recall was recorded. Results are expressed as the percentage of the time spent investigating the novel odor. Investigation of the novel odor above chance level indicated that it was recognized as novel and thus that the odor presented during acquisition had been recognized. Exploration of novel odor above chance level was evaluated using a one-sample *t*-test.

2.4.4.3. Spatial memory. Odor set: Four odorants were used: carvone, geraniol, dodecanal and hexanol. The odors were diluted in mineral oil in such a way as to achieve an approximate vapor pressure of 1 Pa (see Table 1).

Behavioral testing: Ten mice were individually habituated to the four-hole configuration for 2 min with no odorant. During the first two training sessions, four odorants were placed in the holes and the animal was allowed to explore for 6 min. During the last trial, two of the four odorants were switched and the animals were again allowed to explore freely for 6 min. During each trial, the number of visits was recorded for each hole. The animals were returned to their home cage for 3 min between each trial.

Data analysis: In order to measure olfactory spatial memory we compared, after habituation, the number of visits to the two control odorants versus the number of visits to the two odorants whose places on the board had been switched. Data obtained for each pair of odors (control and switched) were averaged across animals and analyzed by ANOVA followed by Fisher *post hoc* tests.

2.4.5. Olfactory preferences

Odor set: We tested the attractiveness of 6 odorants: methylanthranilate (MA), allyl caproate (AlC), amyl butyrate (AB), valeric acid (VA), trimethyl amine (TA), amyl phenylacetate (APA) (Table 1). A swab was impregnated with 60 μl of odorant (1 Pa).

Behavioral testing: The bedding was replaced after every trial. We used the hole-board in the one-hole configuration. One odorant was presented per day (random order for each animal). Each trial lasted 2 min. Duration of nose poking into the hole was used as a measure of odor preference. Ten mice were tested in this experiment.

Data analysis: All data analysis on time spent sniffing during odor presentation trials were performed with Systat software (SSI, Richmond, CA, USA). Outlier trials that deviated from the mean by more than two standard deviations were excluded from analysis. Data obtained for each odor were averaged across animals and analyzed by ANOVA. The level of significance was set at 0.05.

3. Results

3.1. Threshold detection

In order to test the detection threshold, the mice were first exposed to the mineral oil and then to propionic acid at each of four concentrations from 10^{-4} to 10^{-1} Pa (2 min per trial; ITI 3 min). We observe a significant effect of odor concentration on perception (ANOVA $F(5, 68) = 3.386$, $p < 0.009$). Below 10^{-1} Pa concentration the odor was not explored above that for mineral oil setting the detection threshold for propionic acid at 10^{-1} Pa (Bonferonni $p < 0.05$ difference from mineral oil; Fig. 2).

3.2. Olfactory discrimination

3.2.1. Habituation–dishabituation paradigm

In this experiment, we tested spontaneous discrimination between two odorants: pentanol and decanal. *t*-Test showed significant habituation for pentanol (decrease in odorant exploration time between the first and the fourth habituation trial; $p = 0.003$; Fig. 3). After habituation with pentanol, we observed an increase in the investigation time when the decanal was presented ($p = 0.038$) indicating that the mice spontaneously discriminated decanal from pentanol.

3.2.2. Generalization

After conditioning to odor C (EUG), the mice responded strongly (long digging time) to that odorant in a choice test procedure, in which they were presented with a hole scented with odor C and an unscented hole (Fig. 3). On average, the longest digging times were observed in response to odor C compared to the test odors (O1: GUA or O2: HEP; *t*-test, $^{**}p < 0.01$; Fig. 4), suggesting no generalization between odor C and the test odors.

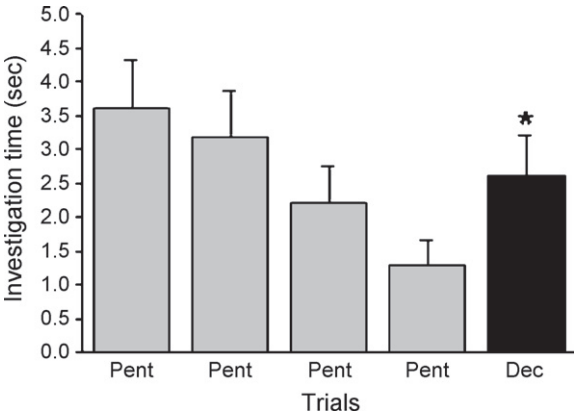

Fig. 3. Habituation/dishabituation task. Responses during the four habituation trials for pentanol declined with increasing numbers of trials. Conversely, the response times for decanal were high, indicating that mice discriminated pentanol from decanal. The asterisks indicate a significant difference ($^{*}p < 0.05$) in response magnitude between Trials 4 and 5.

3.3. Olfactory memory

3.3.1. Olfactory discrimination learning

15 mice underwent a discrimination learning task using +limonene and mineral oil. +limonene was positively reinforced by a food reward. The first visit during a trial to the reinforced hole was recorded as a successful trial (correct response, Fig. 5A). The percentage of correct responses improved significantly over time (ANOVA for repeated measures, $F(4, 70) = 6.776$, $p < 0.0001$). The learning process was also reflected in the time spent to find the reward (latency). The evolution of latency across the learning sessions is presented in Fig. 5B. The latency decreased with learning (day effect ($F(4, 70) = 19.114$, $p < 0.0001$). Taken together, these data indicate that the learning process was successful.

3.3.2. Olfactory short-term memory

15 min after first presentation of the novel odor, the mice explored it above chance level, in terms of number of visits, (one-sample *t*-test, $p = 0.002$). This was not so after 30 or 60 min ($p > 0.05$),

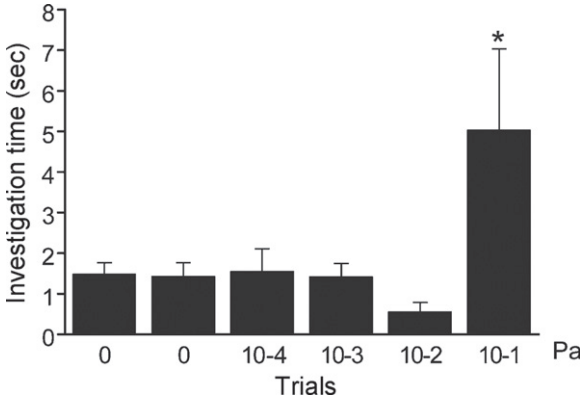

Fig. 2. Threshold detection. Mean investigation time of the odorized hole s ± s.e.m. is shown for propionic acid at 5 different concentrations during each 3-min trial. We observed significant detection of the propionic acid at a concentration of 10^{-1} Pa. The asterisks denote a significant difference in investigation time compared with the mineral oil trial ($^{*}p < 0.05$).

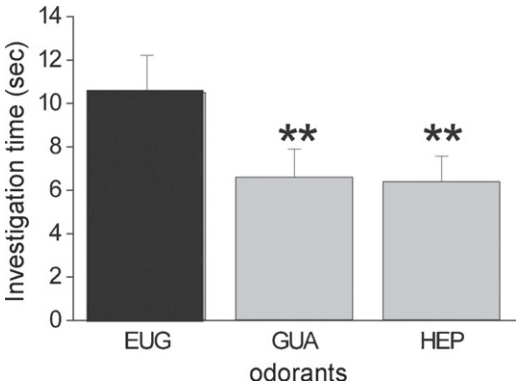

Fig. 4. Generalization task. Mean (± s.e.m.) digging times of 20 mice during non-rewarded test trials in response to the conditioned odor (eugenol, EUG) and the test odors guaiacol (GUA) and heptanol (HEP). The asterisks denote a significant difference in investigation time compared with Eugenol ($^{**}p < 0.01$).

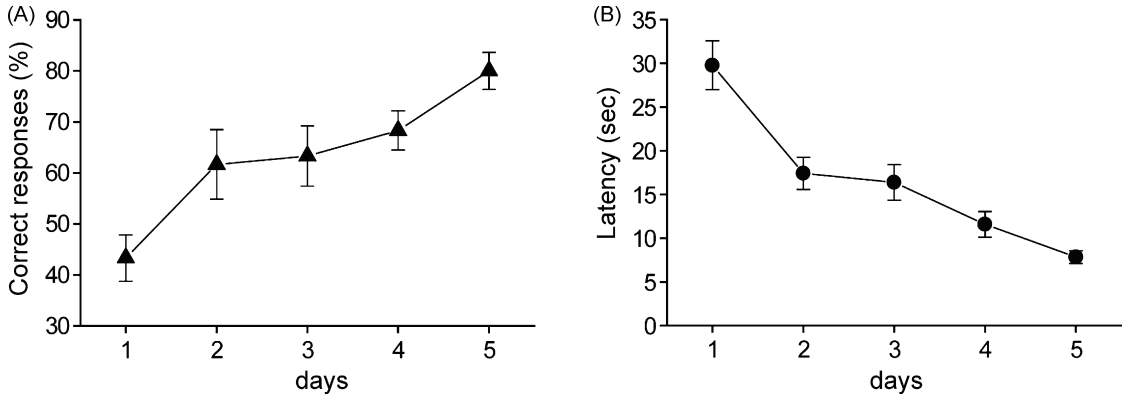

Fig. 5. Associative learning. (A) For conditioning, +limonene was systematically reinforced compared to unrewarded non-odorized hole. The first visit of a trial to the reinforced hole was recorded as a successful trial (correct response). Performance in the discrimination task increased significantly over time. (B) Latency is the time spent on the hole-board before the reward is found and also reflects the learning process. The latency decreased significantly with learning.

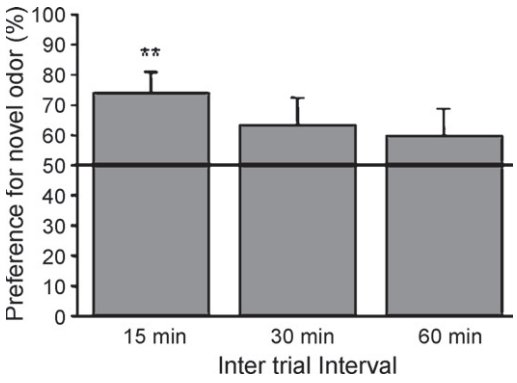

Fig. 6. Short-term memory. Effect of different inter-trial intervals on odor recognition. An investigation time for the novel odorant during recall above 50% (preference for novel odor) is indicative of a memory of the odor presented during acquisition. The preference for the novel odor was significant 15 min after the first exposure (one sample *t*-test for difference from 50%, $^{**}p < 0.001$) but not at 30 and 60 min.

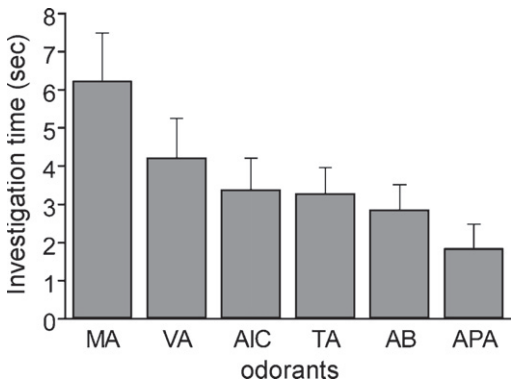

Fig. 8. Olfactory preferences. The duration during which the mouse investigated the hole was measured for various odorants. Mean investigation times (s) ± s.e.m. are shown for each odorant during the 2-min test period. We observed significant differences in investigation time reflecting preferences for certain odors.

indicating that the mice remembered the odorant only 15 min after the first presentation (Fig. 6).

3.3.3. Spatial memory

To assess spatial memory, we used a two-trial 6-min training protocol (see Section 2). During training, mice were allowed to explore odorants and the number of visits to these odorants was recorded. In a third recall trial, two of the four odorants were spatially switched and the animals were again allowed to explore for a further 6 min. We observed a significant difference in the number of visits to the switched odorants compared to the others (Unilateral *t*-test $p < 0.05$; Fig. 7).

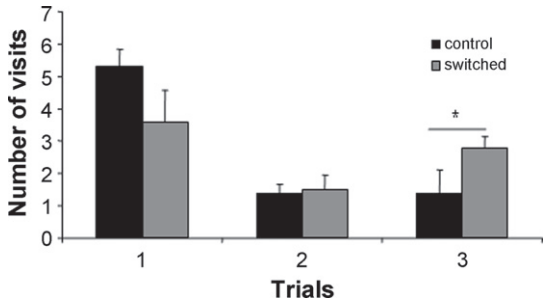

Fig. 7. Spatial odorant recognition memory. The number of visits to the four odorants was measured during two 6-min acquisition trials (trials 1 and 2). Then, two of the four odorants were spatially switched from one hole to another and the number of visits was assessed for a further 6-min (trial 3). The figure shows an increased number of visits to the two odorants which had been spatially switched. Data is expressed as mean ± s.e.m. ($^{*}p < 0.05$).

3.3.4. Olfactory preferences

In the preference test (Fig. 8), the hole containing the odorant was presented to the mice. The length of time the mouse spent investigating each odorant was automatically recorded. In this experiment, six odorants were selected (AlC, MA, AB, APA, TA, VA) and it was found that the mice demonstrated significant differences in their exploration time of these odorants ($F[5, 50] = 2.689$, $p = 0.031$) indicative of the differential attractiveness of the odorants (odor preferences).

4. Conclusions

The results of the present series of studies suggest that the olfactory hole-board provides a reliable method for measuring olfactory performance. The hole-board, which does not require complex and expensive olfactometric equipment, allows assessment of the main aspects of olfactory function such as olfactory threshold, discrimination or memory. For olfactory threshold evaluation, although the precise concentration of an odorant at the top of a hole is unknown it is still possible to compare behavior in response to different concentrations and establish a useful scale of detection abilities. Regarding discrimination and memory, each of the tasks described in this study aimed to measure one aspect of olfactory perception but one should be aware that none of them are totally specific. For instance, the habituation/dishabituation task depends on short-term mem-

orization of an odor between successive trials. Consequently, the shorter the interval between trials the more the memory component is reduced. Conversely, the associative memory task presented here requires discrimination between odor and mineral oil. However, since discrimination problem is very simple, the test mostly assesses associative memory.

Other published studies have suggested that with a very similar experimental design, the system is capable of assessing the affect of pathological aging on olfactory performance (Guerin et al., 2009). In this paper, we were able to characterize some discrete olfactory memory deficits in young Tg2576 mice, a model for Alzheimer's disease. The main deficit was found in the habituation task when the inter-trial interval was set at 15 min whereas when it was set at 5 min, no alteration was found.

The computerized system was also effective in demonstrating slight changes in behavior due to the difficulty of the task (Mandairon et al., 2006b). In this paper, the animals had to learn to discriminate between a pair of similar odorants (the two enantiomers of limonene) and a pair of dissimilar odorants (+limonene and propionic acid). Using a habituation/dishabituation task, we first showed that there was no spontaneous discrimination between ±limonene. Despite this, adult mice learnt to discriminate them as shown by their ability to perform the associative task when one enantiomer of the pair was positively reinforced. The learning curve obtained in this group exhibited a 4-day lag compared with the learning curve obtained when two distinct odorants (+limonene and propionic acid) were used indicating that the more difficult the task, the more time is needed to perform it. Taken together, these data indicate that this set up is highly sensitive and is suitable for assessing small differences in olfactory performance.

In a previous study, we examined several combinations of drug treatments on olfactory memory performance and revealed the usefulness of the apparatus for pharmacological approaches to olfactory function. More specifically, a study examined the influence of pharmacological modulations of the *Locus Coeruleus* noradrenergic system on odor recognition in the mouse. Treatment with the alpha(2)-adrenoceptor antagonist dexefaroxan was able to increase the odor memory from 15 to 30 min post-training. In contrast, both the alpha(2)-adrenoceptor agonist UK 14304 and the noradrenergic neurotoxin DSP-4 prevented the recognition of the familiar odor 15 min after the first exposure (Veyrac et al., 2007).

Finally, using the experimental setup, we are able to assess olfactory preferences in mice. In a recent study, we compared the olfactory preferences of mice and humans to 19 odorants. We showed that odorants rated as pleasant by humans were also those which, behaviorally, mice investigated longer, thereby revealing for the first time a hedonic component of olfactory perception conserved across species. Consistent with this, we further showed that odor pleasantness ratings in humans and investigation time in mice were both correlated with the physicochemical properties of the molecules (Mandairon et al., 2009).

Therefore, this new olfactory setup may provide a valuable tool for the study of olfactory behavior in mice and more particularly to studying the link between the olfactory network and olfactory behavior. Moreover, the hole-board can be an interesting set up for screening olfaction in transgenic mice and drugs with therapeutic potential for use on memory.

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