
UNIVERSITY OF TURIN

DEPARTMENT OF LIFE SCIENCES AND SYSTEMS BIOLOGY


PhD IN NEUROSCIENCE

XXIX CYCLE

# Modulation of Olfactory Bulb Plasticity Underlying Reproductive Behaviour at Puberty


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# Outline of the thesis

**Chapter 1** is the introduction, which is made up of four different subchapters. The first part provides a general introduction on the neuroanatomy, connectivity and function of the olfactory system. The second part gives an overview of the neuroendocrine system controlling reproduction and fertility: the gonadotropin-releasing hormone (GnRH) neurons. The third part deals with the constitutive adult neurogenic areas, the subventricular zone (SVZ)-olfactory bulb (OB) system and the dentate gyrus (DG) of the hippocampus. In this part, both the precise cellular composition and multiple regulatory mechanisms underlying the function of the adult neurogenic niches are deeply described. Finally, the last part includes a detailed overview of the present knowledge about the mutual link existing between adult neural plasticity and the endocrine system. This part includes also an overview of a critical period for this crosstalk, that is puberty.

**Chapter 2** reports a paper published in Frontiers in Neuroanatomy, 2017, concerning the modulation of olfactory bulb neurogenesis at puberty (Oboti, Trova *et al*., 2017). In this study I am co-first author, and I personally contributed to the vast majority of the experimental procedures, data analysis and preparation of the manuscript.

**Chapter 3** consists of unpusblished data of a still ongoing work regarding the influence of the GnRH system in the olfactory bulb function and in the modulation of adult neurogenesis. This work is based on the exploitation of a mouse model recently developed in the laboratory of Development & Plasticity of Neuroendocrine Brain in Lille, France, directed by Dr. Paolo Giacobini, in which I spent 5 months during the second year of my PhD. These mice are featured by impaired GnRH secretion during the peripubertal period, and thus offer a unique opportunity to investigate the effects of a loss of GnRH secretion during adulthood. This part has been already organized as a manuscript that we intend to conclude it in the near future. In this study, I have directly carried out all the experiments, analyzed the data and wrote the manuscript with the supervision and contribution of Prof. P. Peretto and P. Giacobini.

**Chapter 4** provides a general discussion with an overview of the main findings of my studies and future perspectives.

**Chapter 5** consists of references regarding chapter 1 and appendix, whereas the citations for both chapters 2 and 3 are at the end of each chapter for simplicity.

**Chapter 6** reports acknowledgments and personal information

The **appendix** at the end of the thesis consists of additional information mostly regarding chemical cues, GnRH system and anatomy of the hippocampus. Moreover, I added two studies not included in the core of this thesis because referring to different research projects. The first one has been published in Scientific Reports, 2016, in which I contributed during my PhD thesis by performing some olfactory tests and molecular experiments. The other one has been published in Development, 2015, and my contribute was related to quantitative analyses, and performed mainly during my master's degree.

## Summary

Aim of my PhD thesis was to investigate mechanisms underlying neural plasticity in the adult mammalian brain. In particular, I have addressed how adult neurogenesis (AN), a striking form of neural plasticity, is involved in the modulation of social-reproductive behaviors. Indeed, several data suggest that in rodents salient chemosensory cues (i.e., pheromones) and internal factors (i.e., hormones) cooperate to modulate the process of AN, and in turn newborn neurons integrated in the olfactory bulb (OB) seem essential to optimize olfactory-dependent reproductive behaviors. How this complex integration among social cues, hormones and AN occurs is still largely unknown. Unraveling this mechanism is key not only to identify the specific role of the OB neurogenesis in mediating social cues, but also to understand how the brain integrates external and internal cues to produce appropriate behaviors. In order to achieve these goals, I focussed mostly on puberty, as a major life transition during which an individual's physiology undergoes a profound reorganization, especially for what concerns the neuroendocrine system and the reproductive behavior. Importantly, several data have shown that AN undergoes a sharp decline during puberty, supporting this is an optimal period to investigate processes modulating AN. Another related key question of my thesis, was to investigate the possible role of the gonadotropin releasing hormone (GnRH) system in the OB plasticity. The GnRH system, starting from puberty, orchestrates reproduction through its activity on the hypothalamic-pituitary-gonadal axis (HPG), and by integrating multiple sensory stimuli, including the olfactory ones. In addition, recent data have shown GnRH-ir cells in the OB region of both mice and humans. I took advantage of mice deprived of gonadal hormones through gonadectomy and of a recently developed mouse model in which GnRH secretion is abolished just before puberty. Overall the results obtained indicate that: i) the pheromonal-dependent modulation of neurogenesis in the accessory OB of females starts only after puberty, and is induced only by urine compounds produced by mature males; ii) the granule cell increase induced by mature male odor exposure is not prevented by pre-pubertal ovariectomy, indicating a lesser role of circulating estrogens in this plasticity; iii) the intake of adult male urine-derived cues by the female

vomeronasal organ increases during puberty, suggesting a direct correlation between sensory activity and accessory OB neuronal plasticity. For what concerns the role of GnRH in modulating the OB plasticity, we found that GnRH immunoreactivity in the OB region is due to a relevant system of fibers and cells surrounding the bulb and in close contact with both the main and accessory OB nerves, as well as to the dopaminergic OB periglomerular cells. This supports that this extra hypothalamic GnRH population can be directly involved in modulating olfactory sensory perception. In addition, in this model we found alteration of the process of adult neurogenesis in both the OB and DG of hippocampus. Finally, we also found abnormal olfactory-dependent behaviors. Although further investigation is required (several experiments are already ongoing) to evaluate whether the activity of GnRH is direct or mediated by the HPG axis, we can state it is clearly involved in modulating neural plasticity in the adult brain.

# CHAPTER I

# Introduction

## The Sense Of Smell

Since the late eighteenth century, we know that the smell of an object is due to volatile molecules that it releases. In 1821 the French anatomist Hippolyte Cloquet (1787–1840) noted the importance of smell for animal survival and reproduction, and the Spanish neuroanatomist Santiago Ramòn y Cajal (1852–1934) traced the architecture of the nerves leading from the nose to and through the brain. Nevertheless, a real progress in understanding the molecular and functional bases of the sense of smell has been achieved only starting from 1980–1990s decades, especially with the seminal studies of Linda Buck and Richard Axel (1991). Since then, giant leaps have been made for the comprehension of olfactory transduction mechanisms and the organization and function of the olfactory system (Bakker *and* Leinders-Zufall, 2016).

Olfaction is a primary sense for most mammals, except for some primates, such as humans, in which the sense of vision takes this place. Olfactory detection is crucial for finding food, identification of predators, recognition of conspecifics, reproductive behaviors (e.g. opposite-sex attraction, sexual behaviour, maternal care) and many other aspects of animal physiology. A vaste range of both volatile and non-volatile molecules carrying information on the animal's environment are perceived through the sense of smell. Due to the huge complexity of these environmental cues, the olfactory system has evolved into multiple olfactory sub-systems/pathways, including the main and the accessory olfactory systems (MOS and AOS).

The first appearance of a vertebrate-like olfaction was reported in early chordates, more than 500 million of years ago: genomic analyses revealed the presence of intact olfactory receptor genes in amphioxus (Churcher *and* Taylor, 2009; Grus *and* Zhang, 2009; Niimura, 2009). The MOS is present in all vertebrates (including teleost fishes), and its structure is highly conserved throughout evolution. The presence of a distinctive accessory olfactory pathway appeared firstly in lungfish (*Protopterus*), which possess the most ancient form

of distinctive segregation between olfactory and accessory pathways from the nose to the brain (González *et al*., 2010; Nakamuta *et al*., 2012; Northcutt *and* Rink, 2012). A distinctive and concerted action of these two systems is displayed also by amphibians, reptiles, many mammals, but not by birds and humans (Suárez *et al*., 2012). At present, there are no evidences for a functional accessory olfactory system in humans (Boehm *and* Gasser, 1993; Boehm *et al*., 1994; Meisami *and* Bhatnagar, 1998).

The MOS essentially perceives a large variety of volatile odorants, while the AOS is thought to mediate the detection also of non-volatile odorants, e.i. most gender and species-specific cues involved in the control of mating and aggressive behaviours (Dulac *and* Torello, 2003). Among non-volatile odorants, pheromones have aroused particularly interest since they are involved in several aspects of animal's physiology, e.g. in reproductive behaviors. In Appendix I provide a full description of the different kinds of semiochemicals present in nature: pheromones, kairormones, allomones and synomones .

## Organization of the Olfactory System

As introduced before, in vertebrates the concept of a unique/single olfactory system is oversimplified. For example, in rodents, the olfactory system is actually composed by the main olfactory system (MOS) and the accessory olfactory system (AOS), and a number of subsystems, in particular the Septal Organ of Masera (SOM) and the Grueneberg ganglion (GG). All these systems are segregated into the nasal cavities, and make distinct neural connections to the olfactory forebrain (Fig. 1; Munger *et al*., 2009). These two systems could be distinguished also for the receptors they express. Generally, distinct plethora of molecules activate these distinct olfactory pathways (Munger *et al*., 2009).

In this thesis, I will specifically refer on the main and accessory olfactory systems, focussing on the mutual influence and plasticity of these two systems with the internal and external environment.

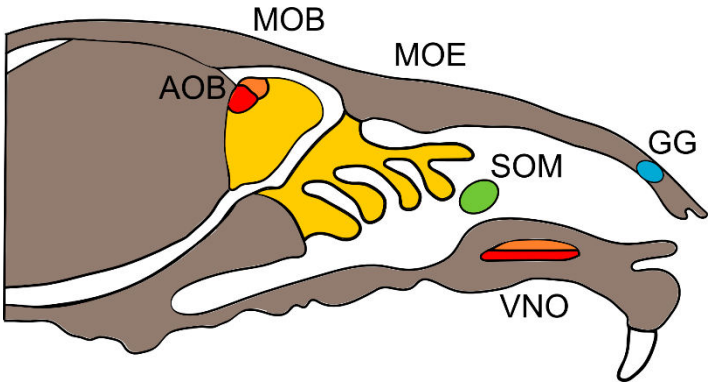


**Figure 1. The mouse dual olfactory system.** Sagittal section through the nasal region of the head. The main olfactory epithelium (MOE) and the main olfactory bulb (MOB) are shown in yellow. The two layers of the vomeronasal organ (VNO) and corresponding connection regions in the accessory olfactory bulb (AOB) are shown in orange and red. The septal organ of Masera (SOM) is shown in green and the Grueneberg Ganglion (GG) is shown in blue. *[Modified from Brennan and Zufall, 2006; and from Tirindelli et al., 2009]*

*Anatomical organization of the main and the accessory olfactory system*

The MOS and the AOS share a similar anatomical organization, consisting of three main parts.

1) The sensory epithelium: the olfactory sensory neurons (OSNs) of the main olfactory epithelium (MOE) and the vomeronasal sensory neurons (VSNs) of the vomeronasal organ (VNO). These cells represent the detection unit of the MOS and AOS, and are the predominant cell type within the epithelium of the sensory organs. Other cells present in the two sensory units are sustentacular cells, that may serve a glia-like role, and progenitor cells, that replenish this regenerating tissue. Progenitor cells in the VNO also occur at the edge between the sensory and non-sensory epithelium (Suárez *et al.*, 2012).

2) The forebrain center: the first processing center of olfactory information of the MOS and the AOS (that are innervated by their specific sensory neurons) are the main olfactory bulb (MOB) and the accessory olfactory bulb (AOB), respectively.

3) The higher olfactory centers: these regions reside in cortical and sub-cortical areas of the brain and receive direct or indirect information from the MOB and the AOB, respectively (Firestein, 2001).

*Functions of the main and the accessory olfactory system*

The MOS is a broadly tuned odor sensor; it responds to thousands of volatile chemicals carrying information about the quality of food and the presence of pathogens, prey, predators, or potential mates (Firestein, 2001). There is a growing recognition that the MOS is also responsive to semiochemicals that can elicit specific behaviours or hormonal responses (Zufall *and* Leinders-Zufall, 2007). On the other hand, the essential role of the AOS is in chemical communication and the regulation of social behaviours (Brennan *and* Zufall, 2006; Dulac *and* Torello, 2003; Halpern *and* Martinez-Marcos, 2003; Luo *and* Katz, 2004; Broad *and* Keverne, 2008).

In the following part, I will give a more detailed description of the two subsystems.

## The Main Olfactory System (MOS)

The olfactory sensory neurons (OSNs) are bipolar ciliated neurons located in the olfactory epithelium (OE, which lies the roof of the nasal cavity) with axons projecting to the glomerular neuropil in the olfactory bulb (OB – Figure 2). The OE consists of multiple laminar folds of neuroepithelium, where volatile molecules come into contact with the mucosa during either passive respiration or active sniffing. The OE contains millions of OSNs extending multiple cilia into the mucous lining of the neuroepithelium. In the mouse, each OSNs expresses only one of ~1000 olfactory receptor genes (ORs). ORs themselves are broadly tuned (Mombaerts, 2004; Gold, 1999), and multiple ORs can respond to the same odorant, although usually with different efficacies (Mombaerts, 2004). Thus, odors are encoded through use of a combinatorial strategy (Buck, 2000; Malnic *et al*., 1999). Those OSNs expressing a particular OR are restricted to only part of the MOE, although they are randomly distributed throughout that zone (Mombaerts *et al*., 2004). Axons of OSNs expressing the same OR converge on a few glomeruli (usually two) within the MOB. The convergent innervation of individual glomeruli by OSNs expressing the same OR argues that the glomerulus, not the receptor, is the fundamental unit of odor coding. The volatile-olfactory receptor binding leads the activation of cAMP

cascade and the activation of cyclic nucleotide-gated and calcium-activated chloride channels (Suárez *et al.*, 2012; Mombaerts *et al.*, 2004; Munger *et al.*, 2009; Ressler *et al.*, 1994; Vassar *et al.*, 1994; Strotmann, 2001).

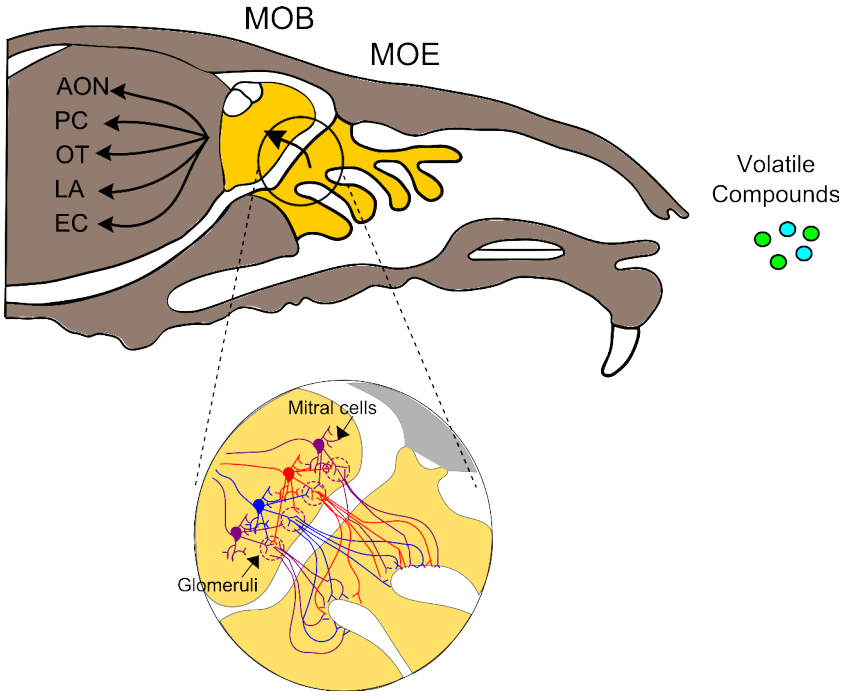


**Figure 2. The Main Olfactory system in rodents.** Olfactory sensory neurons in the main olfactory epithelium (MOE) are specialized for detecting small volatile odorants. The olfactory information is transmitted from the MOE to the main olfactory bulb (MOB), then to distinct brain nuclei that form the primary olfactory cortex and that include the anterior olfactory nucleus (AON), the piriform cortex (PC), the olfactory tubercle (OT), the lateral part of the cortical amygdala (LA) and the entorhinal cortex (EC). Inset shows synapses between olfactory sensory neurons and mitral cells. Each glomerulus in the MOB receives axons only from cells expressing the same OR receptor type, indicated by different color, and connects to dedicated mitral cells. [Modified from Dulac *and* Torello, 2003].

The axonal projections of the OSNs form the first layer of the MOB, the olfactory nerve layer (ONL). The olfactory glomeruli, about 2000 in the rodents OB, are spherical structures, located in glomerular layer (GL), receiving axons from OSNs. Each glomerulus is innervated by up to 20-40 mitral /tufted cells [Fig. 3A, B; M/T - which reside in the mitral cell layer (MCL) and external plexiform layer (EPL), respectively]. The synaptic contacts between glomerular cells and mitral/tufted cells are glutamatergic synapses, which represent the first-order synaptic region between sensory region and the forebrain (Fig. 3; Mombaerts *et al.*, 2006; Nagayama *et al.*, 2014). Before traveling to the olfactory

cortex, odor information is further processed by the activity of inhibitory interneurons: granule cells, which cell body is located in the granule cell layer - GCL), and periglomerular cells, located in the glomerular layer. Moreover, interneurons receive depolarizing input from M/T dendrites and in turn inhibit current spread in M/T dendrites via hyperpolarizing reciprocal dendro-dendritic synapses. Therefore, the interaction between stimulatory and inhibitory inputs induces a fine-tuning of the signal. Odor Information is finally sent to telencephalic structures collectively known as the primary olfactory cortex (OC). The OC is considered as the assembly of brain regions that receive direct input from the olfactory bulbs and it is composed of several anatomically distinct areas: the piriform cortex, the olfactory tubercle, the anterior olfactory nucleus, the cortical amygdala, and the enthorinal cortex (Fig. 2). In turn, most of these cortical regions project back to the main olfactory bulb and the other areas of the brain, including the thalamus, the hypothalamus, the hippocampus, the frontal cortex, and the orbitofrontal cortex. This anatomical feedback of the cortical brain areas on the olfactory bulb implies a mutual control in the odor information processing, from the center to the periphery (Bakker *and* Zufall, 2015).

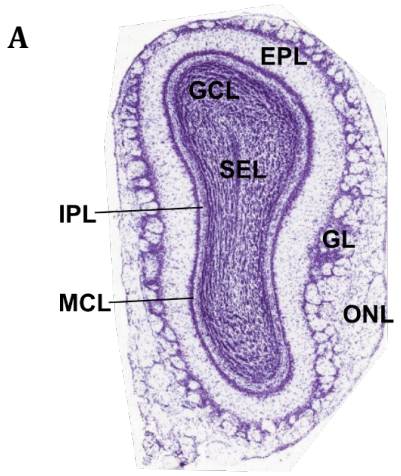


**Figure 3A. Coronal section of the mouse adult olfactory bulb stained with Nissl staining.** Laminar organization is well visible. From periphery to center: olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), granule cell layer (GCL), subependymal layer (SEL).

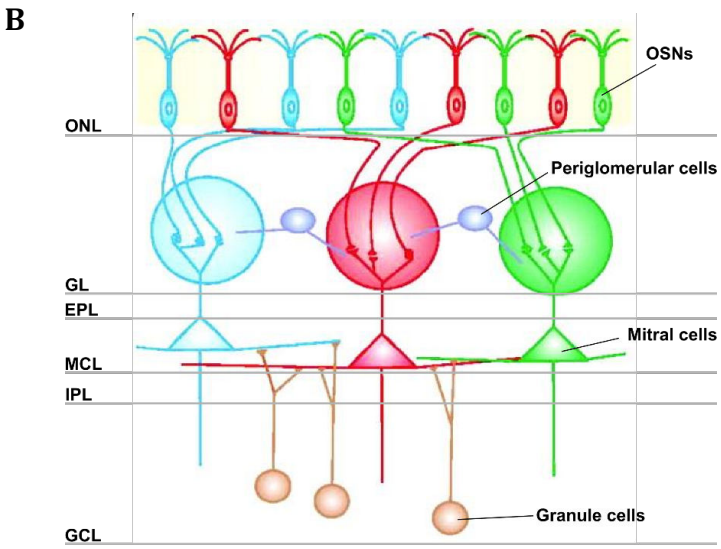


**Figure 3B. Basic model of the olfactory bulb network.** The axons of olfactory sensory neurons (OSNs) make synapses in the glomerular layer (GL), consisting of spherical structures called glomeruli. Each glomerulus represents a single odorant receptor (OR). Neurons surrounding glomeruli in the GL are mainly periglomerular (PG) cells. The somata of the mitral cells are located in the mitral cell layer (MCL). Mitral cells project a single primary dendrite into a single glomerulus, where they receive synaptic inputs from the axons of olfactory sensory neurons and make reciprocal synapses with the dendrites of PG cells. Secondary dendrites of mitral cells are elongated in the external plexiform layer (EPL), where reciprocal synapses are formed with granule cell dendrites. The internal plexiform layer (IPL), in which axons from mitral cells run, and the granule cell layer (GCL), which is largely composed of granule cells, both lie beneath the MCL. Granule cells are axon-less interneurons extending dendrites apically into the EPL. Abbreviation: ONL, olfactory nerve layer [*Modified from Tirindelli et al., 2009*].

## Accessory Olfactory System (AOS)

In addition to the MOS, many mammals possess an accessory olfactory system (AOS). The AOS has attracted a great deal of attention over the past ten years because of a growing recognition of this system's essential role in chemical communication and the regulation of social/reproductive behaviors (Brennan *and* Zufall, 2006; Brus *et al*., 2016; Schellino *et al*., 2016). The sensory epithelium of the AOS is the vomeronasal organ (VNO), a blinded-ended tube located bilaterally at the base of the nasal cavity (Fig. 4; Meredith *et al*., 1980). This system is primary (but not exclusively) involved in the detection of non-volatile compounds, due to both location and structure of the VNO. The VNO opens via a narrow duct into the nasal cavity or into the mouth depending on the species and therefore the volatile molecules cannot reach directly the microvillar vomeronasal sensory neurons (VSNs) located into the VNO. Only after the investigation of the olfactory source, often through a direct physical contact, animals activate a vascular pumping mechanism able to move chemosensory cues into the lumen of the VNO (Meredith *et al*., 1994).

The VNO contains not only VSNs, but also sustentacular cells, basal (progenitor cells), and gland cells. The VSNs are bipolar neurons having a dendrite extending to the lumen in which they emanate microvilli. Axons of the VSNs form fascicles (the vomeronasal nerves) that travel along the septum, cross the cribriform plate, and end in the accessory olfactory bulb (AOB). VSNs are divided into two major subgroups, based on their location into the VNO, the expression of a particular G-protein coupled receptor and the portion of the AOB they synapse with. In particular, the apically located VSNs express the Vomeronasal Receptor family Type 1 (V1R), coupled with $G_{\alpha i2}$-protein. These neurons innervate the anterior region of the AOB (aAOB; Fig 4). The basal located VSNs express the Vomeronasal Receptor family Type 2 (V2R) associated with the G-protein $G\alpha_o$ and innervate the posterior region of the AOB (pAOB; Fig 4; Dulac *and* Axel, 1995; Berghard *and* Buck, 1996; Herrada *and* Dulac 1997; Matsunami *and* Buck, 1997; Ryba *and* Tirindelli, 1997; Buck, 2000; Dulac *and* Wagner, 2006). Sequence analyses have indicated that vomeronasal receptors genes comprise a completely independent family of seven transmembrane

domain proteins unrelated to olfactory receptors expressed in the MOE (Dulac *and* Axel, 1995). In addition, the olfactory cues-vomeronasal receptor binding leads a different transduction pathway compared to the MOS. The ligand-receptor binding in the VSNs activates the IP3/DAG cascade and TRPC2 channels (Zufall et al., 2005). The importance of TRPC2 channels in the AOS activity have been elucidated through experiments of genetic ablation of the TRPC2 –channel (TRPC2 deficient mice). Genetic inactivation of TRPC2 channels results in a number of alterations in female reproductive behaviors, such as the absence of puberty acceleration, maternal aggression, lordosis and also defects in the female AOB neurogenesis typically enhanced after male bedding exposure (Haga *et al*., 2010; Leypold *et al*., 2002; Flanagan *et al*., 2011; Kimchi *et al*., 2007; Hasen *et al*., 2009; Oboti *et al*., 2011). VSNs express one receptor protein out of ~200 V1R or ~80V2R genes and each VSN innervates 3-9 glomeruli from neurons expressing the same genetically related receptors (Belluscio *et al*., 1999; Rodríguez *et al*., 1999; Del Punta *et al*., 2002; Wagner *et al*., 2006; Larriva-Sahd *et al*., 2008).

The first processing center of the VNS is the AOB. The AOB is located in the posterior dorsal portion of the MOB, and it has a laminar organization as the MOB with some substantial differences (reported by Larriva-Sahd, 2008; Munger *et al*., 2009). It has an extra subdivision into the anterior AOB (aAOB) and posterior AOB (pAOB) based on the anatomical location, the expression of G-proteins and the innervation from VSNs. This topographical segregation is thought to sustain functional differences, as the anterior and the posterior part of the AOB seem to respond differentially to several pheromonal stimuli in mice (Nunez-Parra *et al*., 2011). This segregation is further maintained in higher levels of the CNS with the anterior and posterior divisions of the AOB projecting to different areas of the amygdala (Mohedano-Moriano *et al*., 2007).

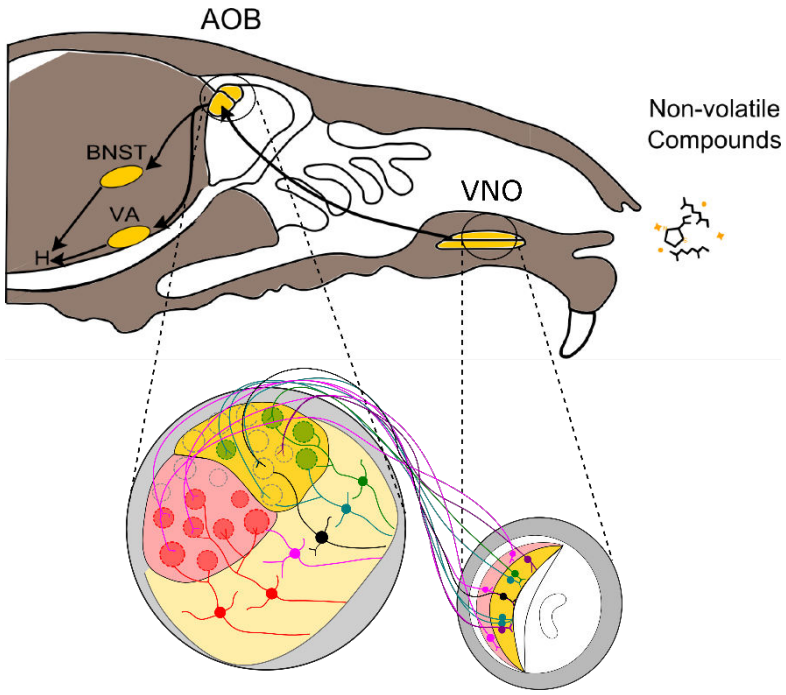


**Figure 4. The Accessory Olfactory system in rodents.** Information provided by non-volatile compounds (e.g. pheromones) is primarily processed by distinct neural circuits. Pheromones are mostly detected by sensory neurons in the vomeronasal organ (VNO), a bilateral tubular structure in the anterior region of the nasal cavity. VNO axons project to the accessory olfactory bulb (AOB), which in turn transmits sensory information to the vomeronasal amygdala (VA) or to the bed nucleus of the stria terminalis (BNST) and then to specific nuclei of the hypothalamus (H), which are involved in regulating genetically pre-programmed physiological and reproductive responses. Insets show synapses between vomeronasal sensory neurons and mitral cells in the AOB. Vomeronasal sensory neurons that express the same V1R or V2R project to multiple, small glomeruli in the AOB. The apical layer of the epithelium projects to the rostral half of the AOB (yellow); the basal layer projects to the posterior half (red). [*Modified from Dulac and Torello, 2003; Mombaerts, 2004*].

Similar to the MOB, the AOB is composed by a glomerular layer (GL, the first relay station of the vomeronasal chemosensory cues), an external plexiform layer (EPL), containing mitral/tufted cells, and a Granular Layer (GrL), containing mainly inhibitory interneurons. The lateral olfactory tract (lot), which is a bundle of afferent nerve fibers from the mitral and tufted cells, separates the EPL from the GrL of the AOB (Fig. 5A, B).

In contrast with the MOB, in which olfactory information directly passes through cortical structures, olfactory information processed by the AOB is sent to subcortical areas involved in reproductive physiology and behavior, but also to areas involved in those behaviors influencing feeding, fight and flight. In particular, AOS has direct connections to third-order limbic regions, including

the medial amygdala and the posteromedial cortical nucleus (together representing the vomeronasal amygdala-VA, Fig. 4), which in turn are connected with the hypothalamus. The medial amygdala is the major hub in pheromone-processing circuitry (Nodari *et al*., 2008). Given the direct connection of the AOS to central brain areas involved in reproduction, I will discuss with major attention on how the social/reproductive olfactory information is processed within the AOS and how in turn could affect reproductive physiology and behavior.

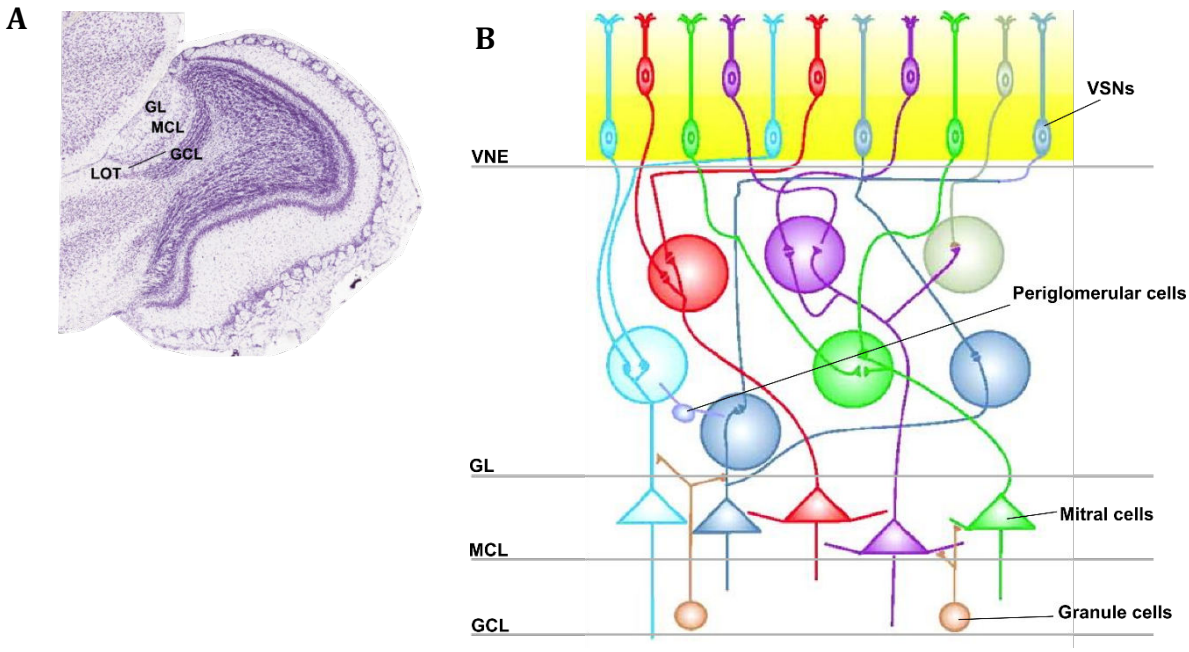


**Figure 5A. Sagittal section of the adult mouse olfactory bulb stained with Nissl staining.** Laminar organization is well visible. From periphery to centre: glomerula layer (GL), mitral cell layer (MCL), lateral olfactory tract (LOT), granule cell layer (GCL). **B. Basic model of the accessory olfactory bulb network.** Vomeronasal sensory neurons (VSNs) that express the same vomeronasal receptor project to multiple small glomeruli. Six populations of VSNs, each expressing one different type of vomeronasal receptor, are represented in different colors. They are located in the vomeronasal epithelium (VNE). The axons of VNSs make synapses in the glomerular layer (GL). Neurons surrounding glomeruli are periglomerular (PG) cells. The somata of the mitral cells are located in the mitral cell layer (MCL). Secondary dendrites of mitral cells reciprocal synapse with granule cells dendrites. The granule cell layer (GCL) is largely composed of granule cells. [*Modified from Tirindelli et al., 2009*].

## Synergistic roles of the MOS and the AOS in the regulation of social behaviors

Conventionally, the MOS and the AOS have been considered as two separated and non-overlapping systems, from both anatomical and functional points of view. The first one involved primarily in the detection of a large variety of volatile odorants, while the second specifically related to the detection of gender and species-specific cues (i.e., pheromones) and thus involved in the control of mating and aggressive behaviour (Baum, 2012). Recently, the idea of two separated subsystems has been largely outdated thanks to findings strongly supporting that the MOS and AOS play synergistic roles in the regulation of a range of olfactory-guided behaviors, from reproductive and social interactions to foraging and defensive contexts. This functional integration takes place thanks to the existence of anatomical convergence points between the two systems (Mucignat-Caretta *et al*., 2012; Baum, 2012). Firstly, the AOB receives direct projections from the MOB (Fig. 6; Larriva-Sahd, 2008), while no intrabulbar connections have been identified from the AOB to the MOB. In addition, the anterior part of the Medial Amygdala (MA) receives direct inputs from both the MOB and the AOB main neurons (Fan *and* Luo, 2009; Kang *et al*., 2009; Mohedano-Moriano, 2012). Consistently, individual MA and cortical amygdala neurons can be activated by both the MOB and AOB inputs (Lich *and* Meredith, 1987), thus supporting functional convergence of AOS and MOS at the level of cortico-medial amygdala. Moreover, the anterior (ACo) and postero-lateral (PLCo) cortical amygdala that receive MOB input project, in turn, to MA and other downstream structures, such as the posterior bed nucleus of the stria terminalis (BNST), that also receive direct AOB inputs (Maras and Petrulis, 2010; Martinez-Marcos, 2009). The MOB target region in the MA sends feedback projections to the AOB influencing its responses to volatile chemosignals (Martel *and* Baum, 2009). Lastly, detection of volatiles chemosignals by the MOS can stimulate activity of the VNO pumping mechanism, leading to increased AOS sampling of chemochemicals (Meredith, 1998). Points of anatomical contacts are depicted with red crosses in figure 6.

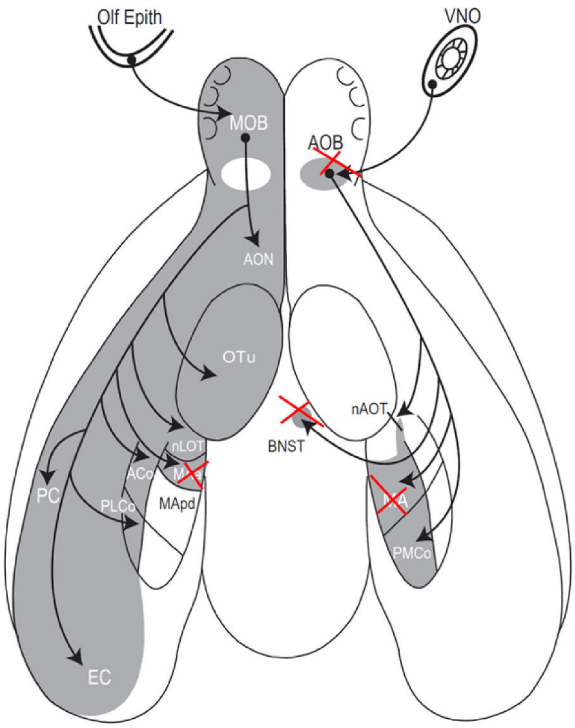


**Figure 6. Schematic of the main (left) and accessory or vomeronasal (right) olfactory systems highlighting projections from the MOB and the AOB to telencephalic brain regions.** Red crosses indicate points of anatomical contacts between the two systems. For clarity, only unidirectional MOB/AOB connections are presented. Abbreviations: AOB, accessory olfactory bulb; AON, anterior olfactory nucleus; ACo, anterior cortical amygdala; BNST, bed nucleus of stria terminalis (posterior); EC, entorhinal cortex; MA, medial amygdala; MAa, anterior medial amygdala; MApd, posterodorsal medial amygdala; MOB, main olfactory bulb; MOE, main olfactory epithelium; nAOT, nucleus of the accessory olfactory tract; nLOT, nucleus of the lateral olfactory tract; OTu, olfactory tubercle; PC, piriform cortex; PLCo, posterolateral cortical amygdala; PMCo, posteromedial cortical amygdala; VNO, vomeronasal organ [*Modified from Petrulis et al., 2013*] .

The anatomical convergences between the MOS and the AOS presuppose integration also from a functional point of view. Indeed, both the OSNs and VSNs can respond to the same sensory stimuli (Brennan *and* Zufall, 2006). Moreover, both the main and the accessory olfactory bulb interact in the control of mate recognition and sexual behaviour (Keller *et al*., 2009). Female recognize airborne volatiles from males detected through the MOE by association with involatile scent information gained through the VNO during nasal contact with scents (Ramm *et al*., 2008). Without this previous contact with the VNO, females show an inherent attraction to adult male compared to either female scents (Moncho-Bogani *et al*., 2002; 2005) or to castrated male scents (Martinez-Ricos *et al*., 2008). These evidences denote a mutual interaction between the MOS and the AOS.

In agreement with these data, the anatomical and functional overlapping of these two systems concerns mainly those pathways underlying social/reproductive physiology and behaviour. Indeed, volatile compounds processed by the MOS impact largely the AOS downstream pathways, suggesting that the reproductive system needs a greater fine tuning regulation upon

olfactory stimulation (coming from both volatile and non-volatile cues), that implies a mutual interaction of the two olfactory systems.

The AOS is prevalently considered the main system involved in the processing of pheromonal information, due to its connection with the reproductive hypothalamus and therefore it has always been conceived to mediate the physiological and neuroendocrine changes induced by pheromones. Accordingly, through the AOS, male pheromones can accelerate puberty in females, a phenomenon called Vandenbergh effect (Lombardi *et al.*, 1976; Vandenbergh, 1974). Pheromones can also induce the pregnancy block (or the Bruce effect; Bruce, 1959), that is the failure of embryo implantation, after the exposure of females to unfamiliar male pheromones soon after mating. The importance of AOS in reproductive physiology has been also elucidated through VNO removal experiments. For instance, the VNO is essential for the expression of lordosis behaviour in female mice, but not for male-female odor discrimination, for which the MOS plays a major role (Keller *et al.*, 2006). Nevertheless, the VNO is essential to drive the motivation of individuals to investigate opposite-sex cues, thus enhancing the interest of mice to investigate opposite-sex urine (Keller *et al.*, 2006; Pankevich *et al.*, 2004). Besides the detection of non-volatile compounds, the VSNs can in part detect volatile pheromones, and this was shown through in vitro electrophysiological and imaging methods (Kang *and* Baum, 2009). However, it is still debated whether these volatiles can have free-access to the lumen of the VNO, or if they need a carrier protein such as members of lipocalin family, like Major Urinary Proteins (MUPs).

Pheromonal cues are a large class of molecules including proteins and peptides and also small volatile molecules. Volatile pheromones are perceived by the MOS. Therefore, also the MOS has the ability to detect and process pheromones related to social attraction and individual recognition. In particular, accumulating evidences support an involvement of MOE in setting up the preference for the opposite-sex and mating initiation. For example, the volatile compound (methylthio)methanethiol (MTMT) present in male mouse urine activates a subset of mitral cells in the female MOB and can enhance the attractiveness of castrated male urine supplemented with MTMT to female mice

(Lin *et al*., 2005). Moreover, the detection of opposite-sex volatile compounds, essential for pre-copulatory behaviors, is mediated by the MOS, which is necessary for the detection of volatile cues at a distance and therefore mediate the attraction toward them. Indeed, the chemical destruction of the MOE with zinc sulfate abrogates urinary odor preference in female mice (Keller *et al*., 2006). As previously reported, a functional cooperation between the MOS and the AOS is also required to drive female attractiveness toward male scents (Keller *et al*., 2009; Ramm *et al*., 2008)

Overall, these data support an important link between the MOS and the AOS at different levels of the odor information processing, from more peripheral (olfactory sensory epithelium) to more central areas (cortical and sub-cortical areas), functionally related to optimize social/reproductive behaviors.

## The Gonadotropin-Releasing Hormone (GnRH) System

A link between the olfactory system and reproduction was firstly recognized almost 70 years ago by de Morsier, who described several cases of hypogonadism associated to anosmia, using the term “olfactogenital dysplasia”. These cases were often in association with a number of median cranioencephalic dysraphias (de Morsier, 1954). In 1944 Kallman and collegues described the genetic nature of these conditions, and since then the eponym Kallman syndrome (KS) has labelled any human syndrome characterized by the association of hypogonadotropic hypogonadism and anosmia (Kallman, 1944). This condition is genetically heterogeneous, with both autosomal and X-linked forms, with only the latter is now understood at the molecular level.

The hint of the biological explanation behind the association of hypogonadism and anosmia is now evident after the disclosure of the common origin of olfactory neurons and neurons controlling reproduction and fertility, the Gonadotropin-Releasing Hormone (GnRH) neurons. Indeed, both olfactory sensory neurons and GnRH neurons derive from progenitor cells in the epithelium of the olfactory placode. GnRH neurons then travel inside the central

nervous system (CNS), by crossing the cribriform plate. This migration is supported by olfactory and vomeronasal nerves scaffolds (Wray *et al*., 1989; Schwanzel-Fukuda et al., 1989; see appendix for more details on embriological origin of GnRH neurons). Therefore, defects in the development and migration of olfactory nerves and GnRH neurons could result in both hypogonadism and anosmia (KS). Confirmation came from anatomo-pathological examination of a 19-week-old human fetus affected by KS (Schwanzel-Fukuda *et al*., 1989). In this fetus, the olfactory, vomeronasal, and terminalis nerves were not in contact with the brain, but terminated their path within the meninges forming an abnormal neural tangle. Moreover, GnRH neurons failed to enter the brain and were found in the nasal cavities and on the dorsal surface of the cribriform plate in the above-mentioned tangle. Olfactory bulb and tract aplasia or hypoplasia is a consistent neuroradiological finding in patients with KS. Moreover, now we know that several genes mutated in KS affect the fate and migration of GnRH neurons. Among them, the *KAL* gene (Franco *et al*., 1991; Legouis *et al*., 1991) was the first gene associated with KS. The *KAL* gene, now renamed ANOS1, is located in the X chromosome at Xp22.3, and codes for an extracellular matrix protein called anosmin1, which is involved in the migration of GnRH cells during embryogenesis (Soussi-Yanicostas *et al*., 1998).

The GnRH neurons represent the key elements of the neuroendocrine gonadotropin releasing hormone (GnRH) system (also known as the luteinizing hormone-releasing hormone system- LHRH), an evolutionary innovation specific to vertebrates (Sower *et al*., 2009; Campbell *et al*., 2004). Cells within this system express the ‘mammalian form' of the GnRH transcript (GnRH-I) and are integral members of the hypothalamic-pituitary-gonadal (HPG) axis. There are between 800 and 2000 neuroendocrine GnRH-1 cells in mammals (including humans). Within the forebrain of mammals, birds, reptils and amphibians, the ~70% of hypophysiotropic GnRH-1 neurons have their perikarya in the Preoptic Area (POA); the other ~30% are localized in extrahypothalamic brain regions in addition to the hypothalamus. Thus, GnRH-1 cells can be found distributed in a bilateral continuum from the olfactory bulbs to the anterior pituitary (Fig. 7; Silverman, 1988).

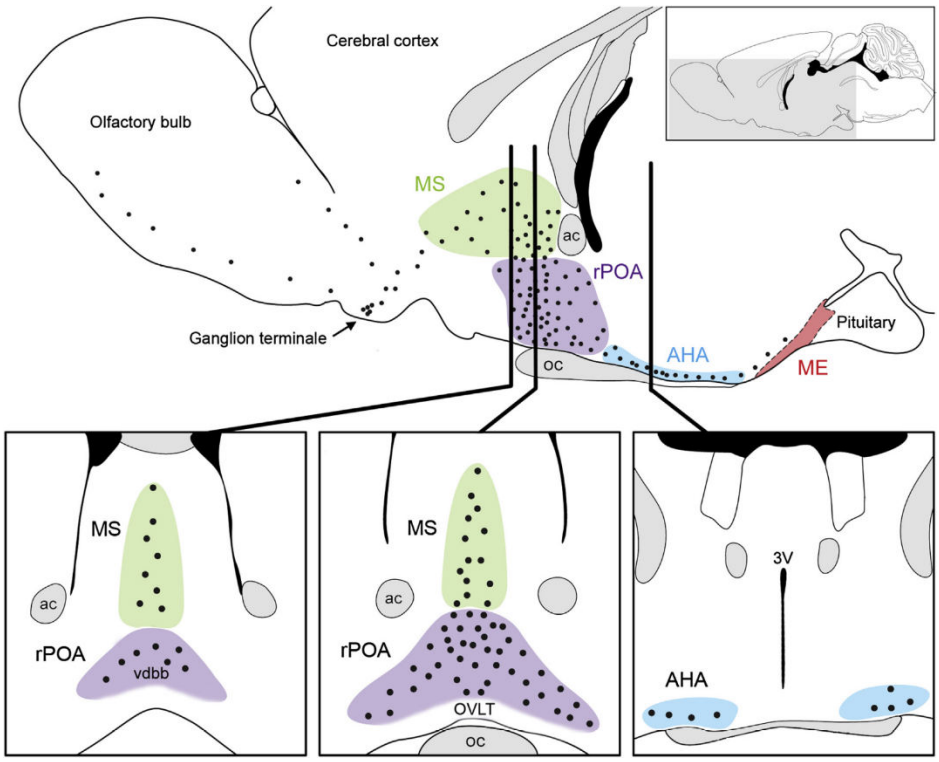


**Figure 7. Location and distribution of GnRH cells in the adult mouse brain.** Sagittal section of a mouse brain indicating the distribution and location of GnRH cells (black dots). The majority of them are located in hypothalamic regions (represented in coronal view on the bottom: MS, rPOA and AHA). Abbreviations: MS, medial septal nucleus; rPOA, rostral preoptica area; AHA, anterior hypothalamic area; Ac, anterior commissure; Oc, optic chiasm; ME, median eminence; OVLT, organ vasculosum of the lamina terminalis; Vdbb, diagonal band of Broca [*Schematic taken Knobil and Neill, 2014*].

Independent from their location, the majority of GnRH-1 cells send their axons to the median eminence where they access (via fenestrated capillaries) the pituitary portal capillary system. The release of GnRH-1 in this site is synchronized and pulsatile and affects the synthesis and secretion of gonadotropins, the luteinizing (LH) and follicle stimulating (FSH) hormones from cells in the anterior pituitary (Fig. 8; Christian *and* Moenter, 2010). The pattern of pulsatile release regulates the activity of the ovary and testis and is responsible for the secretion of the reproductive hormones. In the ovary, LH acts upon theca cells that produce the androgen substrate required for ovarian estrogen biosynthesis. In the testis, LH induces Leydig cells to produce testosterone. In both males and females, FSH induces maturation of germ cells.

A pulsatile secretion of GnRH characterizes the early neonatal period ('mini-puberty of infancy') whereas during childhood there is a phase of quiescence. A subsequent GnRH secretion is evident starting from adolescence.

Reactivation of the GnRH pulse generator during adolescence indicates the onset of puberty. The GnRH pulse generator is guarantee by complex and coordinated activities of excitatory (kisspeptin, serotonin, glutamate, NPY, galanin) and inhibitory signals (GABA, opioids) that act upon GnRH neurons (Radovick *et al*. 2012). The adult homeostasis of the system is accomplished by positive and negative feedback coming from gonadal steroids that act at both the level of the pituitary and the hypothalamus (Fig. 8; Radovick *et al*. 2012).

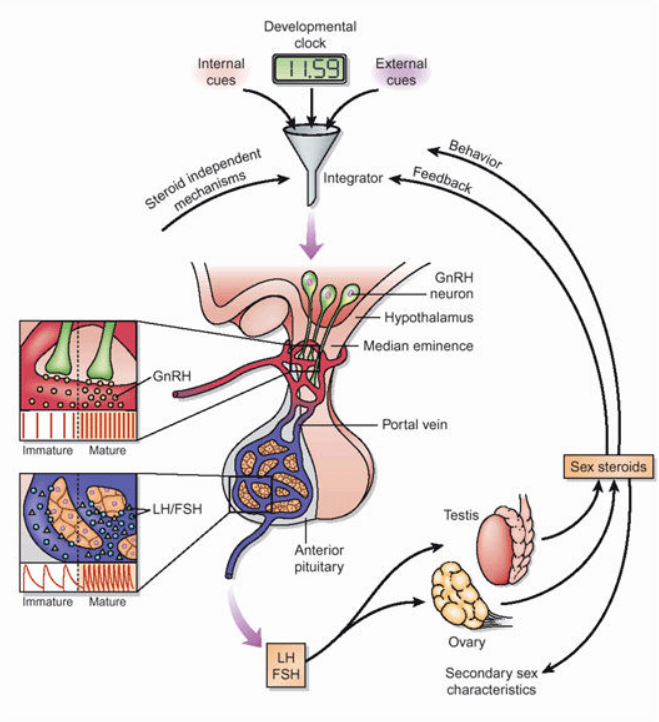


**Figure 8. GnRH neurons projecting to the median eminence direct pituitary gonadotropin and gonadal steroid hormone secretion.** Sex steroids promote secondary sex characteristics in peripheral tissues, regulate GnRH neurons via neuroendocrine feedback loop and facilitate social behaviors by acting on central neural circuits. The pubertal increase in GnRH neuronal activity and episodic gonadotropin secretion is grossly timed by a developmental clock fine-tuned by the neural integration of multiple permissive internal and external signals. At the onset of puberty, steroid feedback and steroid-independent neural mechanisms are engaged to disinhibit and excite GnRH neurons *[From Sisk and Foster, 2004]*.

The GnRH-I function in controlling HPG axis was first documented by Mason and coworkers in 1986 who carried out gene therapy/rescue experiments in mice having a mutation in the GnRH-I gene: the hypogonadal (*hpg*) mice (mice deficient in the pituitary gonadotropin secretion leading to a failure in gonadal postnatal development; Mason *et al*., 1986a, 1986b). By introducing an intact GnRH gene, *hpg* mice completely restored their normal phenotype. The same role of GnRH-I neurons in humans was first shown in a family carrying homozygous loss of function mutations in the GnRH1 gene (Bouligand *et al*., 2009). Hence, failure in the development of GnRH system (Schwanzel-Fukuda *et al*., 1989; Quinton *et al*., 1997; Wray, 2002) or defects in

GnRH synthesis (Cattanach *et al*., 1997), release (de Roux *et al*., 2003; Giacobini *et al*., 2014) or ligand/receptor (GnRH/GnRHR) pairing (Quinton *et al*., 1997; Chevrier *et al*., 2011) are associated to clinical syndromes of reduced sexual competence or infertility (Bohem *et al*., 2015; see appendix for a more detailed description of GnRH isoforms).

Besides the primary function of GnRH neurons in governing the HPG axis (that is linked to their location into hypothalamic regions), the presence of these neurons into extrahypothalamic regions could sustain additional properties and functions. Indeed, GnRH neurons are widely distributed throughout the brain: a study in rats showed GnRH immunoreactive cells in olfactory bulb, tubercle, diagonal band of Broca, medial septum, medial preoptic and suprachiasmatic areas, anterior and lateral hypothalamus and several regions of the hippocampus (Fig.7; Shinoda *et al*., 1989; Lin *et al*., 2004). Moreover, GnRH cells have been found in the amygdala, stria terminalis, periventricular plexus, periaqueductal gray of the mesencephalon and extra-cerebral regions, such as the nervus terminalis and its associated ganglion (Merchentaler *et al*., 1989). This broad distribution through the entire brain suggests that GnRH neurons could be involved in monitoring and regulating a wide variety of sensory systems, in addition to their classical function in governing the HPG axis. In turn, extrahypothalamic GnRH neurons could be modulated by multiple sensory inputs. It is to note that, besides the control of reproduction, GnRH neurons have been already demonstrated to play a role in governing body weight and nutritional status, circadian and light/dark cycles; stress and adrenal function and the perception of other environmental variables that might be threaten the species (Balasubramanian *et al*., 2010). Hence, the role of GnRH neurons could be more complex than expected, since they have to harmonize multiple sensory signals to set up the right moment and environment to support reproduction and fertility.

**Adult neurogenesis in the Olfactory System**

The olfactory system is characterized by continuous morphological and physiological changes at the level of its neural circuits. This quality ensures flexibility in the face of a varying internal (hormonal) and external (olfactory cues) environment. Brain and spinal cord remodeling during and after the developmental period are known with the term of plasticity**.** Neural plasticity is a characteristic of the CNS implying changes in the structure and function of neural circuits throughout life, allowing the nervous system to be adaptable during maturation, learning, environmental changes and pathology**.**

Adult Neurogenesis (AN) is a striking form of structural plasticity implying genesis and integration of new neurons into pre-existing circuits. It occurs mainly into two brain areas: the Subventricular Zone - Olfactory Bulb system (SVZ-OB) and the Dentate Gyrus of the Hippocampus (DG; Lledo *et al*., 2006; Ming *et al*., 2005). AN refers to the production of new neurons in the postnatal period, considering both those produced in the juvenile period (from birth to puberty) as well as those produced in the adult life, by keeping in mind that this phenomenon varies and is differently modulated during critical life stages (e.g. early postnatal development and puberty; Díaz *et al*., 2017).

The first anatomical evidences for the presence of newly generated granule cells in the postnatal olfactory bulb and hippocampus of mammals were provided in the 1960s by Joseph Altman's pioneer studies in rats, involving the use of tritiated thymidine, which is incorporated in proliferating cells and can be detected through autoradiography (Altman *and* Das, 1965). In the 1980s, studies from Fernando Nottebohm's group showed neurogenesis in the vocal control centers of adult songbirds. Songbird neurogenesis were found to be seasonal, functional, and related to song learning (Alvarez-Buylla *and* Nttebohm, 1988; Goldman *and* Nottebohm, 1983; Paton *and* Nottebohm, 1983), nevertheless adult neurogenesis was initially dismissed as occurring only in birds, and likely because in the early studies authors were not able to clearly demonstrate the neuronal phenotype of newly born cells. It was not until the 1990s, with the isolation of multipotent NSCs from adult mammalian brain (Reynold *and* Weiss, 1992), and the demonstration that dividing cells in the lateral ventricles could

migrate and become functional neurons (Lois *and* Alvarez-Buylla, 1993; Luskin, 1993) that adult neurogenesis in mammals became widely accepted.

Significant progress in the field of AN has been made after the introduction of bromodeoxyuridine (BrdU), a nucleotide analog that can be used as a lineage tracer (Kuhn *et al*., 1996), and after multiple demonstrations of life-long continuous neurogenesis in almost all mammals examined, including humans (Erikkson *et al*., 1998).

## AN in the Subventricular Zone – Olfactory Bulb system (SVZ – OB)

In the olfactory system, newly-formed neurons are generated in the subventricular zone (SVZ) lining the lateral ventricles and then migrate along a well-defined pathway, the rostral migratory stream (RMS) into the olfactory bulb (including both the MOB and the AOB), where they differentiate into granule cells and periglomerular cells (Luskin, 1993; Lois *and* Alvarez-Buylla, 1993; Alvarez-Buylla *and* Garcìa-Verdugo, 2002; Bonfanti *et al*., 1997; Martinez-Marcos *et al*., 2001; Huang *and* Bittman, 2002). Olfactory enrichment or deprivation studies have shown that the maturation and survival of these newborn neurons depends on sensory inputs (Cummings *et al*., 1997; Rochefort *et al*., 2002; Mandairon *et al*., 2006). Importantly, loss or ablation experiments of newborn neurons in the MOB can impair olfactory function (Breton-Provencher *et al*., 2009; Mandairon *et al*., 2011).

In rodents, estimates suggest that around 10000/30000 adult-born neurons reach the olfactory bulb every day (Lledo *et al*., 2006). After just a few days/weeks they integrate into existing neural circuitry and they play essential roles in the olfactory processing and function (Alvarez-Buylla *and* Garcia-Verdugo, 2002; Lazarini *and* Lledo, 2011). Several studies, including loss-of-function and gain-of-function experiments, have clearly explained the key role of adult-born OB neurons in different olfactory-related behaviours. For instance, they are implicated in olfactory perceptual learning, olfactory discrimination, olfactory short- and long- term memories (functions more related to the MOS; Moreno *et al*., 2009; Breton-Provencher *et al*., 2009; Lazarini *et al*., 2009; Sultan

*et al*., 2010; Lepousez *et al*., 2013; 2015; Gheusi *et al*., 2013). Moreover, they are crucial for predator avoidance and opposite-sex odor learning, vital activities required for pheromonal imprinting and mate recognition (functions more related to the AOS; Oboti *et al*., 2011; Sakamoto *et al*., 2011; Peretto *et al*., 2014).

## The multistep process of AN in the SVZ-OB region

The production of new neurons in adulthood is similar to that arising during development: same mechanisms and factors regulate neurogenesis before and after birth. In the SVZ – OB system, the process of AN is characterized by three major steps: proliferation, migration and survival/integration. In the following paragraphs, I will provide a summary of these steps and I will focus only on those aspects necessary for a full comprehension of the present thesis. I refer the reader to the literature of specific points of the AN process not directly addressed in this thesis.

### *Proliferation*

The first step in the production of new neurons occurs in the SVZ. The SVZ is located in close proximity to the lateral telencephalic ventricles, and specifically it lines the wall of the lateral ventricles. The SVZ is a neurogenic niche consisting of different kinds of cells: adult neural stem cells (NSCs) and immature progeny, ependymal cells, mature vasculature and a multitude of different axonal terminals (Fig. 9). Adult NSCs within the SVZ are slowly dividing primary progenitors and are usually called B1 cells. These cells display astroglial properties in terms of morphology, ultra-structure and marker expression. In fact, as mature astrocytes, they express the glial-fibrillary acidic protein (GFAP) and other cellular markers, like the glutamate aspartate transporter (GLAST), the brain lipid-binding protein (BLBP) and SRY-related high-mobility group (HMG) box (Sox) family member Sox2 (Lim *and* Alvarez-Buylla, 2014). SVZ NSCs could be in either a quiescent or an activated state (Codega *et al*., 2014; Mich *et al*., 2014) and the two states can be discriminated by the expression of Nestin, which

characterizes the activated state. Activated B1 cells give rise to intermediate progenitor cells (IPCs), or type C cells, which lose GFAP immunoreactivity and acquire the expression of the achaete-scute homolog 1/mammalian achaete-scute homolog-1 (Ascl1/Mash1) and the distal-less homeobox 2 (Dlx2). Type C cells are often found in clusters near blood vessels and they do express Ascl1/Mash1, Dlx2, and the paired box protein Pax6 (Kriegstein *and* Alvarez-Buylla, 2009; Brill *et al*., 2008). Through several rounds of symmetric division, IPCs give origin to a pool of neuroblasts, named type A cells, expressing the polysialylated form of neural cell adhesion molecule (PSA-NCAM) and the early neuronal marker doublecortin (DCX; Alvarez-Buylla *and* Garcìa Verdugo, 2002; Ponti *et al*., 2013).

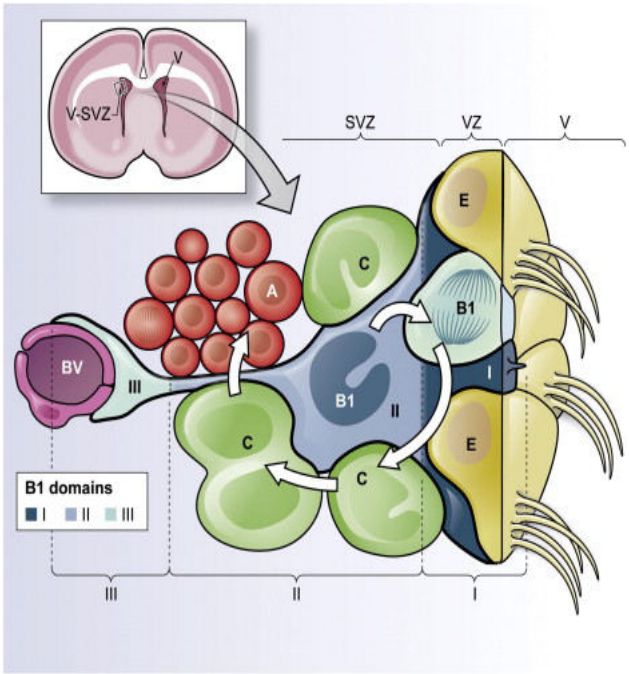


**Figure 9. Schematic representation of the adult SVZ.**
The upper left panel shows a coronal section of the adult mouse brain and the location of the SVZ is indicated within a box. The lower panel illustrates the cellular composition of the adult SVZ niche and the different subdomains of a quiescent B1 NSC (qB1; blue). B1 cells are surrounded by ependymal cells (E cells, yellow). Active B1 cells (aB1; light green) give rise to IPCs (or C cells; green), which divide to generate neuroblasts (type A cells; red). qB1 cells show a thin apical process (with a primary cilium) that contacts the lateral ventricle (V) and a long basal process ending on blood vessels (BV; light purple). B1 cells can be subdivided into three domains: Domain I (proximal or apical, dark blue); Domain II (intermediate, medium blue); Domain III (distal, light blue). *[Modified from Fuentealba et al., 2012].*

After birth in the SVZ, neuroblasts organize themselves into chains as they join the RMS (Fig. 10; Peretto *et al*., 1999). Neuroblasts migration in the SVZ occurs in various directions, but within the RMS they migrate in chains and at around the 80% move rostrally, towards the olfactory bulb (Bolteus *and* Bordey, 2004). In adults, neuroblasts migrate tangentially through the RMS in chains of cells, associated closely with astrocytes (Fig. 10; Peretto *et al*., 1999; Lois *and* Alvarez-Buylla, 1994). When they reach the olfactory bulb, neuroblasts detach from the RMS chains and migrate radially to their final position in the GCL or glomerular layer of the MOB or in GCL of the AOB. This type of migration differs from that during embryonic development: RMS neuroblasts do not have a scaffolding of radial glia that mediates migration, either during tangential or radial migration. Indeed, a scaffold for migrating neuroblasts is sustained by blood vessels into the RMS (Whitman *et al*., 2009) and by laminae within the olfactory bulb (Bovetti *et a*l., 2007). Petreanu and Alvarez-Buylla (2002) have defined five stages in the differentiation of adult-born granule cells. Stage-1 (days 2-7 after their birth) cells are migrating tangentially through the RMS, and have a very simple morphology with a prominent leading process and a small trailing process. Stage-2 cells (days 5-7) are migrating radially through the GCL, also with a simple morphology. Stage-3 cells (days 9-13) appear to have stopped migrating and reached their final position in the GCL. They have a simple dendrite extending toward, but not past, the mitral cell layer. Stage-4 cells (days 11-22) have an extensive dendritic arbor, but no spines, and at stage-5 (days 15-30) cells have a mature granule cell morphology, with spiny dendrites. The morphological development of these cells could be influenced by environmental signals.

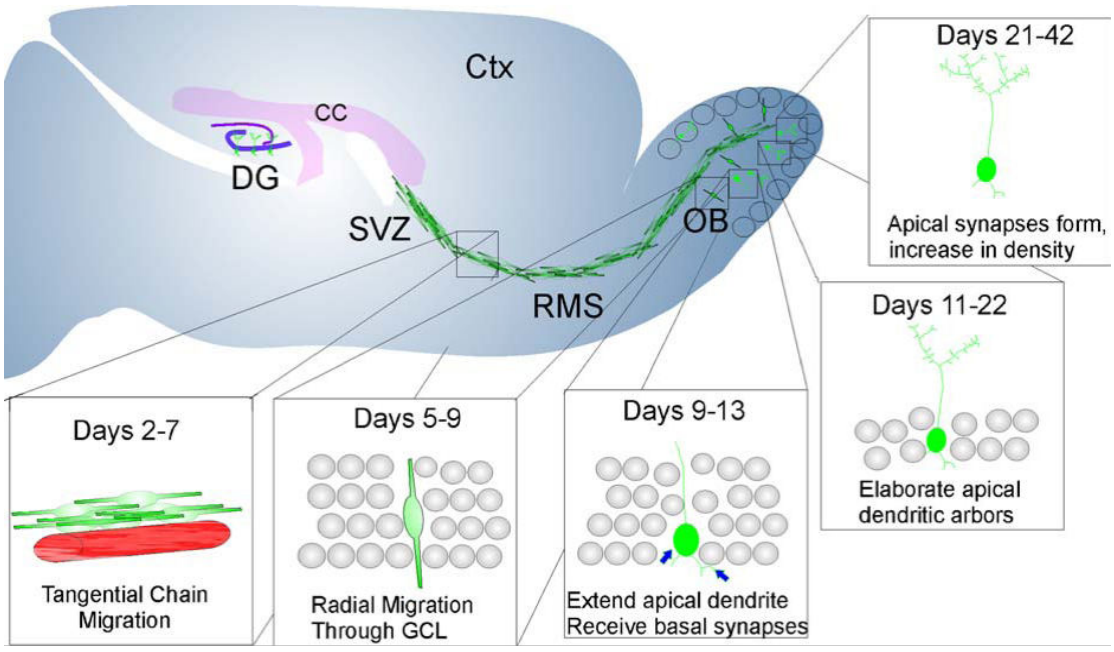


**Figure 10. Schematic of neurogenic regions of the mouse brain and developmental stages of new interneurons in the olfactory bulb.** Neuroblasts born in the SVZ migrate through the RMS using a unique form of migration, tangential chain migration. In the olfactory bulb, neurobasts migrate radially into the granule cell layer and glomerular layer and differentiate into granule cells and periglomerular cells. Granule cells first receive synapses on their basal dendrites, as their apical dendrites grow into the external plexiform layer. They then elaborate and extensive apical dendritic arbor and form spines and reciprocal synapses with the dendrites of mitral and tufted cells. Abbreviations: DG, dentate gyrus; CC, corpus callosum; Ctx, cortex; SVZ, subventricular zone; RMS, rostral migratory stream; OB, olfactory bulb [*from Withman and Greer, 2009*]

Many factors have been shown to influence the migration of newborn neurons. Within the SVZ, the flow of cerebral fluid (CSF) may influence neuroblast migration (Sawamoto *et al*., 2006). After removal of the olfactory bulb, precursors continue to divide in the SVZ and migrate through the RMS. Without their target, however, neuroblasts accumulate in the RMS, and after several months cell death increases (Kirschenbaum *et al*., 1999; Mucignat-Caretta *et al*., 2006). Activity from the olfactory bulb can influence proliferation and survival along the pathway: olfactory enrichment (with both non-social odors or pheromones) causes increases in proliferation of putative stem cells in both SVZ and RMS (Alonso *et al*., 2008; Mak *et al*., 2007).

In addition, non-physiological conditions lead neuroblasts to change their canonical migratory route. After a lesion, neural progenitors in both the DG and

SVZ increase their proliferation, and SVZ neuroblasts migrate toward injured areas (Kernie *et al*., 2010). For example, striatal stroke or progressive striatal degeneration induce SVZ neuroblasts to migrate as chains through the callosal striatal border and then enter into the striatal parenchyma as individual cells (Yamashita *et al*., 2006; Luzzati *et al*., 2011). Nonetheless, different brain lesions paradigms can further induce neurogenic events in normally non-neurogenic sites, such as the striatum, the neocortex or the CA1 of the hippocampus (Nato *et al*., 2015; Luzzati *et al*., 2011; Arvidsson *et al*., 2001; Parent *et al*., 2002; Nakatomi *et al*., 2002; Magavi *et al*., 2000; Collin *et al*., 2005; Kernie *et al*., 2010). However, newborn cells in injured regions generally show low survival rate, and although replacement of a few neuronal cells has been reported, the fate/functional meaning of lesion-induced neuroblasts remain unclear (Arvidsson *et al*., 2002; Parent *et al*., 2002; Magavi *et al*., 2000; Collin *et al*., 2015).

*Functional integration*

Between 15 and 45 days after generation, approximately 50% of the newly generate granule cells die; the remaining cells can survive up to 1 year (Petreanu *and* Alvarez-Buylla, 2002; Winner *et al*., 2002). In the olfactory bulb, these cells turn and migrate radially, becoming periglomerular and granule cells (Peretto *et al*., 1999). In the AOB, addition of new cells was previously thought to be restricted to the early postnatal period (Bayer, 1983), nevertheless, during the last two decades arrival of new neurons in the AOB has been clearly demonstrated also during adulthood (Peretto *et al*., 1999; Bonfanti *et al*., 1997; Martinez-Marcos *et al*., 2001).

Overall, adult-born olfactory neurons are interneurons that modulate the activity of neurons that project to olfactory cortex (those integrating in the MOB) and subcortical areas (those integrating in the AOB). In both the MOB and the AOB, after 1 month many newly-formed neurons acquire phenotypes of mature neurons indicating their integration in pre-existing circuits. In particular, in the MOB periglomerular cells can be subdivided into three non overlapping populations of cells: calretinin- (CalR+) and calbindin-expressing (CalB+) cells,

and tyrosine hydroxylase-expressing (TH+) dopaminergic cells (Kosaka *et al*., 1995). Granule cells include deep, superficial, and CalR+ cells (Price *et al*., 1970; Jacobowitz *et al*., 1991). In the AOB, similar to the MOB, newborn neurons are inhibitory interneurons, since they express GABA+ and GAD67+, the typical interneuronal inhibitor neurotransmitter and its synthesizing enzyme (Mugnaini *et al*., 1984). In contrast with the MOB, only few newborn interneurons in the AOB express CR and TH expression has not been identified in AOB newborn neurons, that it is consistent with the relative low number of TH-positive cells in the GL of the AOB (Oboti *et al*., 2009). Interestingly, about 30% of newly-formed neurons in the AOB are positive for nNos (Oboti *et al*., 2009), which is strongly expressed in the AOB Gr-L (Bredt *et al*., 1991; Porteros *et al*., 1994; Nelson *et al*., 1997) and functionally involved in the modulation of synaptic activity between granule and mitral cells (Okere *et al*., 1995; Kendrick *et al*., 1997). Moreover, neither ChAT nor NPY, two molecules described in a restricted neuronal population of the AOB GrL of goats and dogs (Nakajima *et al*., 1998; Mogi *et al*., 2007), are expressed by newly-formed cells of the AOB (Oboti *et al*., 2009).

It is now clear that different types of interneurons arise from progenitors in specific areas of the SVZ (Merkle *et al*., 2007; 2014). Neuroblasts are heterogeneous before reaching the olfactory bulb (Hack *et al*., 2005; Kowhi *et al*., 2005). This was demonstrated by specifically targeting stem cells in different rostro-caudal and dorso-ventral regions of the SVZ, and by following their progeny in vivo. Specifically, periglomerular cells are produced in anterior and dorsal regions of the SVZ: TH+ cells are produced mainly in dorsal regions, CB+ cells are produced in more ventral regions and CR+ cells are produced especially in medial regions. Granule cells are also produced through the dorsal-ventral extent of the SVZ: dorsal regions tended to produce superficial granule cells, whereas ventral regions produced mostly deep granule cells (Fig. 11; Merkle *et al*., 2007). In addition, a recent work from the same group (Merkley *et al*., 2014), reported the generation of four previously unknown olfactory bulb interneurons subtypes that are produced in finely patterned progenitor domains in the anterior ventral V-SVZ of both the neonatal and adult mouse brain (Fig. 11). Progenitors of these interneurons are organized into micro-domains that correlate with the expression domains of two specific transcription factors,

Nkx6.2 and Zic family. These works reveal unexpected degree of complexity in the specification and patterning of NSCs in the postnatal mouse brain. Nevertheless, they are restricted to the study of MOB newborn interneurons. Thus, the specification of AOB interneurons from distinct SVZ progenitors needs to be still investigated.

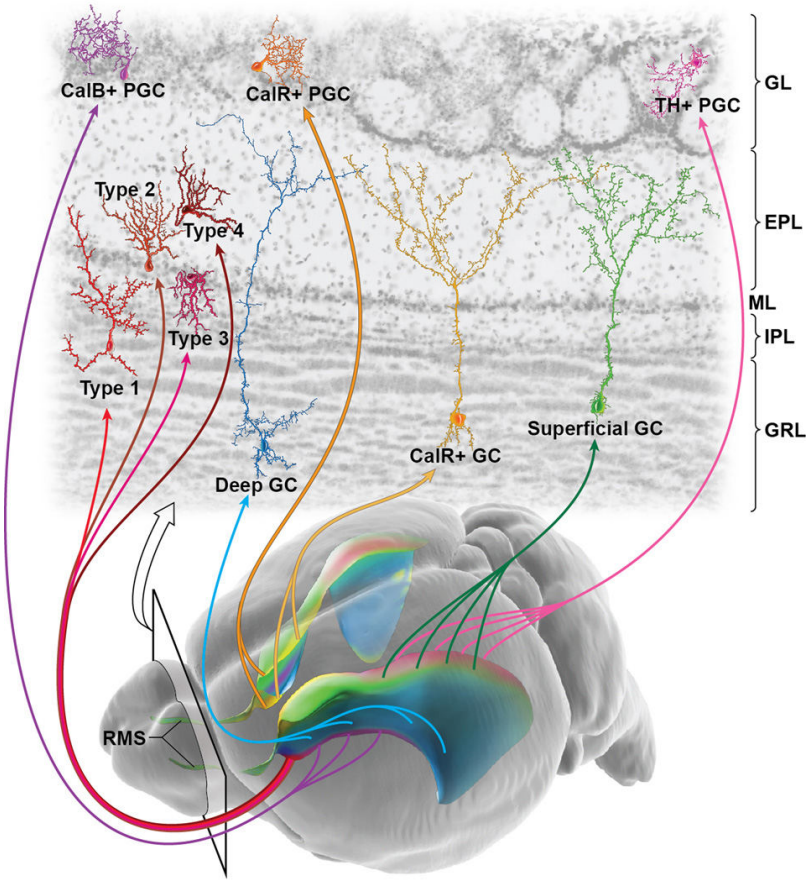


**Figura 11. Regional Organization of the adult NSCs within the SVZ.** Neural cells born in different subregions of the adult SVZ, migrate along the RMS into the OB to give rise to unique types of interneurons. Examples of three types of granular cells (GC) [green: superficial; yellow: calretinin (CalR) superficial; blue: deep], three subtypes of cells in the GL [pink: tyrosine hydroxylase (TH); orange: calretinin (CalR); purple: calbindin (CalB)] and four novel subtypes (type 1-4) of interneurons derived from the most anterior SVZ. Abbreviations: CalB, calbindin; CalR, calretinin; TH, tyrosine hydroxylase; PGC, periglomerular cell; GC, granule cell; GL, glomerular layer; EPL, external plexiform layer; ML, mitral cell layer; IPL, internal plexiform layer; GRL, granular layer. [*From Lim and Alvarez-Buylla, 2014*].

## AN in the Hippocampal Dentate Gyrus

The Dentate Gyrus (DG) of the Hippocampus represents the other brain area of persistent adult neurogenesis in mammals. Adult neurogenesis in the hippocampus is present in almost all the mammalian species in which it has been investigated, including humans (Spalding *et al*., 2013; Kempermann, 2012). The hippocampus is a region in the brain that has been shown to be involved in different kinds of cognitive functions, including learning and memory (Kempermann, 2011; Ming *and* Song, 2011; see appendix for the anatomy of the hippocampus).

The subgranular zone (SGZ), the stem cell niche, lines the hilar side of the granule cell layer of the dentate gyrus (Alvarez-Buylla *and* Lim, 2004; Lledo *et al*., 2006). Although the process of AN in this region share several similarities with that occurring in the SVZ-OB system, there are some substantials differences.

Similar to the SVZ progenitors, the hippocampal stem cells are in a quiescent state, they next exit form quiescence, start to proliferate and give rise to a population of highly proliferating intermediate progenitors cells (IPCs) that finally generate new granule cells (DGCs). Adult NSCs of the SGZ are referred as type-I cells, express astroglial markers and have characteristics of astrocytes (like in the SVZ; Seri *et al*., 2011; Kempermann *et al*., 2004). Type-1 cells are also known as radial glial like cells (RGLs), since they have a single radial process going through the granule cell layer (GCL) and branching into the molecular cell layer (MCL; Kriegstein *and* Alvarez-Buylla, 2009; Ming *and* Song, 2005). A second class of type-1 cells, recently identified, have short tangential processes extending along the border of the granule cell layer and hilus (Lugert *et al*., 2010). Thanks to these processes progenitor cells can interact with the vasculature, directly with their progeny and are exposed to neuronal networks (Fuentenalba *et al*., 2012). As the SVZ, the SGZ is located next to an extensive vascular niche, suggesting that factors derived from blood vessels influence the behaviour of NSCs in the SGZ (Alvarez-Buylla *and* Lim, 2004; Lledo *et al*., 2006). Once activated, type-1 cells give rise to type-2 cells, also known as intermediate progenitor cells (IPCs). In contrast with type-1 cells, IPCs show a high proliferative activity, as they rapidly divide and quickly re-enter in cell cycle

multiple times (Kronenberg *et al*., 2003; Farioli-Vecchioli *et al*., 2014). Morphologically, type-2 cells are small cells with short horizontal processes and are often found in clusters within the SGZ. Two types of IPCs cells could be distinguished based on different expression markers. Type-2a (also called early IPCs) still express typical glial markers, such as Sox2 and BLBP, but have distinct morphologies compared to type-1 cells. Type-2b cells (also called late IPCs) start to express neuronal genes, such as the pro-neuronal bHLH factors NeuorD1, DCX and the homeobox factor Prox1 (Fig. 12; Steiner *et al*., 2006; Kempermann *et al*., 2015). This high proliferative step (i.e. expansion phase) allows the expansion of the pool of precursor cells that differentiate into neurons. Type-2 cells generate in turn type-3 cells, also referred as neuroblasts, which display medium/long horizontally oriented processes, and sometimes vertically growing neuritis. Many of them are still in the cell cycle, even if they show low proliferative activity compared to IPCs cells (Kroenenber *et al*., 2003; Kemperman *et al.*, 2004). When neuroblasts exit the cell cycle, they become post-mitotic immature neurons transiently expressing CR (Fig. 12; Brandt *et al*., 2003). During this stage a massive negative selection of newborn neurons occurs. Unlike to the SVZ, the migration of newborn cells in the hippocampus is shorter in both space and time. Recent studies have underlined that neuroblasts migration is tangential to the SGZ, followed by a limited radial migration towards the inner GCL (Sun *et al*., 2015). At around 4 – 8 weeks, newborn cells undergo fine-tuning and late maturation (Ge *et al*., 2007). Newborn neurons establish glutamatergic synaptic connections and show an initial phase of enhanced synaptic plasticity, in which the threshold to induce the long-term potentiation (LTP) is lower than in mature granule cells (Wang *et al*., 2000; Schmidt-Hieber *et al*., 2004). After several weeks newborn granule cells will become electrophysiologically indistinguishable from the fully mature or embryonic-generated granule neurons (van Praag *et al*., 2002; Ambrogini *et al*., 2004; Yang *et al*., 2015).

As in the SVZ, the activity of the hippocampal neurogenic niche is highly regulated and fine-tuned by both intrinsic and extrinsic factors. Indeed, there are molecules and experiences able to deviate this process from the baseline (Kempermann, 2011). Different molecules and several stimuli can modify the different developmental steps of adult hippocampal neurogenic process,

including proliferation, differentiation and maturation stages (Zhao *et al*., 2008; Ming *and* Song, 2011; Hsieh, 2012).

The major intrinsic factors that regulates adult hippocampal neurogenesis are transcription factors (e.g., Sox2, COUP-TFI, FoxO3), cell cycle regulators and epigenetic mechanisms (Zhao *et al*., 2008). Among extrinsic factors, morphogens (e.g., Notch, Shh, Wnts and BMPs) serve as niche signals to regulate multiple aspects of adult hippocampal neurogenesis. In addition, hormones, growth factors, neurotransmitters and cytokines are important regulators in this process (Zhao *et al*., 2008). For example, BDNF and VEGF, two neurotrophic factors, are mediators of antidepressants and environmental stimuli that modulate the neurogenic mechanisms (Rossi *et al*., 2006; Castren *et al*., 2007; Warner-Schmidt *and* Duman, 2007). In agreement with the existence of several differences between the two adult neurogenic niches, the same factor (intrinsic or extrinsic) could modulate in a different manner the SVZ or the SGZ niche (see the next paragraph for more details on this topic).

Environmental stimuli are also important regulators of adult neurogenesis in the hippocampus, at distinct levels of the neurogenic process. In general, stimuli that control the expansion phase (i.e. expansion of intermediate progenitors) tend to be rather unspecific (e.g. physical activity, seizures), whereas stimuli that are more specific and dependent to hippocampal functions (such as learning and memory tasks) affect much more the survival phase (Gould *et al*., 1999; Kempermann *et al*., 2015). Interestingly, the increased survival occurs only when learning happens at a specific critical period, approximately 1 week after cell division (Epp *et al*., 2007). Ablating neurogenesis impairs spatial memory and contextual fear conditioning (Saxe *et al*., 2006; Imayoshi *et al*., 2008), but actually improves performance on some working memory tasks (Saxe *et al*., 2007). Reduced neurogenesis also impairs long-term retention of spatial memory as well as object recognition tasks (Jessberger *et al*., 2009). In addition to effects on learning and memory, hippocampal neurogenesis is important in mood regulation. Chronic stress leads to decreases in hippocampal neurogenesis, an effect that can be reversed by antidepressant treatment (Duman, 2004).

Neurogenesis in the SGZ is also enhanced by physical exercise, such as voluntary wheel running, which increases in particular proliferation (Brown *et*

*al*., 2003), or an enriched environment, which increases survival (van Praag, 1999). Moreover, running could also induce the activation of quiescent radial NSCs, but a lower extent, compared to the expansion in late progenitors (Lugert *et al*., 2010).

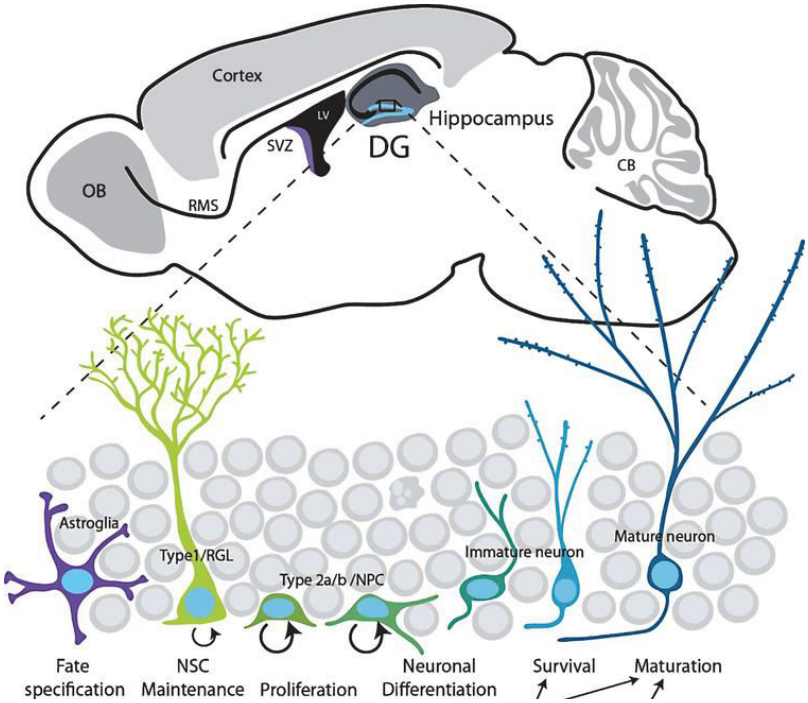


**Figure 12. Adult neurogenesis in the DG.** Schematic drawing illustrates the two neurogenic regions in the adult rodent brains.(top). Adult neurogenic niches in the brain include the dentate gyrus (DG, blue) and the subventricular zone (SVZ, purple). On the bottom, the stages of DG neurogenesis indicating different NSCs phases [*Modified from Jobe and Zhao, 2016*].

**The Link Between Adult Neurogenesis and Reproduction**

It is now clear that intense events of environmental-elicited brain remodeling besides the fetal and early postnatal development, also occur during puberty and adulthood. This brain ability called neural plasticity is finalized at optimizing the brain circuits to the changing environmental conditions (internal and external), and among the mechanisms of brain plasticity adult neurogenesis seems to play a key role during the whole postnatal and adult life (Ming *and* Song, 2011). A large amount of data from the last 50 years of research in the field of neuroendocrinology have progressively shown that during early postnatal life and puberty some events of brain remodeling and hormonal readjustment are specifically aimed at reaching the reproductive function (Nowicki *et al*., 2002; Ramm *et al*., 2008; Bouret, 2012; Saleem *et al*., 2014). In addition, also during adulthood, a large variety of life events, including, circadian and seasonal cycles; pregnancy; motherhood; lactation; feeding; social interaction and behavioural performance are characterized by both brain remodeling and hormonal modifications mostly aimed at driving reproduction (Galea *et al*., 2008; Roa *et al*., 2013). Importantly, a cross talk between the brain and the endocrine system appears mandatory in this function, as well as to reach and maintain a general physiological equilibrium of the entire organism. The interaction between the endocrine and the nervous system impacts on higher level brain circuits functions involved in driving emotions, feelings, and motivations (Damasio *et al*., 2003). Interestingly, recent data have clearly shown that adult neurogenesis is modulated in multiple reproductive social behaviors (Feierstein *et al*., 2012; Peretto *et al*., 2014; Oboti *et al*., 2009; Brennan *et al*., 2006; Gheusi *et al*., 2009) and that hormones play a major role in this regulation. In the following paragraphs, I will discuss in more detail the impact of hormones on AN in both neurogenic niches, with major attention on the SVZ-OB system, and how social/reproductive behaviors (in particular pheromonal cues) modulate AN.

## Hormonal modulation of AN

Different types of hormones can affect the process of AN in both neurogenic niches and at different steps of the neurogenic process (proliferation, migration and functional integration). Sex hormones are major regulators of this process and they can affect AN in a dose- and time- dependent manner. Depending on the experimental design, estradiol could either enhance or reduce progenitor cell proliferation and survival of newborn cells in female rodents (Brock *et al*., 2010; Veyrac *and* Bakker 2011; Galea *et al*., 2013; McClure *et al*., 2010; Chan *et al*., 2014; Farinetti *et al*., 2015). For instance, in 2006, Hoyk and collaborators analyzed the effects of estradiol on the survival of newborn interneurons in the accessory olfactory bulb of rats. Specifically, they found that administration of estradiol for 6 consecutive days on ovariectomized rats before BrdU treatment, reduces the number of BrdU-labeled neurons in the AOB granule cell layer at 21 days survival time. Interestingly, they didn't found any difference in the number of BrdU-labeled neurons in the main olfactory bulb (Hoyk *et al*. 2006). The effect of estradiol could affect the proliferation, the migration and/or the survival and integration of newborn neurons. In this case, by administrating estradiol prior to BrdU injection, the effect of estradiol was on the proliferation of newborn neurons. Another study showed that estradiol down-regulates cell proliferation in the SVZ leading to a decreased number of newborn cells in the main olfactory bulb of adult female mice (Brock *et al*., 2010). In a more recent work, a different role of estradiol was indicated. In particular, by using a model the ArKO mice, characterized by the absence of aromatase (the enzyme responsible for the synthesis of estrogens through testosterone aromatization – Carolyn *et al*., 1998), that leads to the inability to produce estradiol across life span, they found that the survival of newborn neurons in the AOB of female mice is reduced, with no alterations in the survival of newborn neurons in the MOB and in the DG of hippocampus (Brus *et al*., 2016; Veyrac *and* Bakker, 2011). Moreover, cell proliferation was not altered in the SVZ, although a reduction in this process has been found in the DG (Brus *et al*., 2016). In addition, it was found that the absence of estradiol altered the responses (c-fos expression) elicited by male urine cues in newborn neurons of

both the main and accessory olfactory bulb (Brus *et al*., 2016; Veyrac *and* Bakker, 2011). From a functional point of view, although these results need further investigation, they support a role of estradiol as a key factor in modulating adult SVZ neurogenesis in the context of social interaction.

As above mentioned, in addition to the regulation of olfactory neurogenesis, estradiol regulates also hippocampal neurogenesis. In 1999 two papers demonstrated that cell proliferation in the adult dentate gyrus is affected by gonadal hormones. In particular, the work of Galea and collaborators (1999) demonstrated that the proliferation of newborn neurons in the DG of females wild meadow voles was different in the breeding season compared to the nonbreeding season. The proliferation was higher during the nonbreeding season. These effects were correlated to the fluctuation in the levels of adrenal steroids and gonadal steroids occurring in the female meadow voles. In particular, high levels of corticosterone and estradiol were correlated with a decreased cell proliferation (Galea *and* McEwen, 1999). The same year, Tanapat and co-workers reported that ovariectomy decreases the proliferation of newborn cells in the DG of rats, and that estradiol administration after ovariectomy restores the number of proliferating cells. Moreover, they also reported that the level of proliferation varies during the estrus cycle, by having more cells proliferating during the proestrus compared to estrus and diestrus (Tanapat *et al*., 1999). These two works reported different regulations of estradiol on DG proliferation in different species: in meadow voles estradiol seems to decrease cell proliferation, whereas in rats it seems to increase cell proliferation. This contradiction could reflect besides specie-specific differences, differences in the time, duration and the dose of estradiol action. Subsequent works demonstrated that estradiol have a biphasic effect on hippocampal cell proliferation: it first enhances cell proliferation after an acute administration (Banasr *et al*., 2001; Ormerod *and* Galea, 2001; Tanapat *et al*., 2005), but chronic administration of the hormone does not affect cell proliferation in the DG (Perez-Martin *et al*., 2003; Tanapat *et al*., 2005). These data suggest that the effect of estradiol on AN in both neurogenic systems is much more complex than expected, and should regard major attention.

A direct action of estradiol on the production and survival of new neurons is supported by the expression of estradiol receptors (ERs) in both neurogenic systems. The mapping of ERs in the mouse central nervous system showed Erα-immunoreactive (ir) cells in the granular layer of the olfactory bulb, and depending on the study, none to moderate/strong numbers of ERb-ir cells were reported in this layer (Mitra *et al*., 2003; Merchenthaler *et al*., 2004). In the hippocampus, only a few weakly stained ERα and ERβ-ir cell nuclei were observed in the most ventral regions, including the CA2-3, the subiculum and the paraventriculum. Intriguingly, the deep layer of the DG, where proliferating cells and neuronal progenitors are present (Merchenthaler *et al*., 2004), contain some Erα-ir cells that were not positive for ERβ.

Another gonadal hormone modulating AN is testosterone (TST). TST increases proliferation in the SVZ of adult male rats (Farinetti *et al*., 2015). In addition, TST is involved in the modulation of newborn cell survival elicited by exposure to male pheromones. In the Semaphorin 7A knock out males (Sema7A ko - Pasterkamp *et al*., 2003; Messina *et al*., 2011), that similar to females have low levels of TST, an increase of neurogenesis in the AOB occurs after male bedding exposure (Oboti *et al*., 2009-11; Schellino *et al*., 2016). This TST-dependent modulation of AOB AN was also confirmed by using wild-type castrated males, and by treating Sema7A ko and castrated males with chronic administration of TST, a condition that fully reversed the neurogenic response to male pheromonal exposure (Schellino *et al*., 2016). TST acts either directly, via binding to androgen receptor (AR), or through its aromatization, by activating ERα and ERβ (Ogawa *et al*., 2000; Sato *et al*., 2004). In Sema7A ko mice, AR and not ERs levels are reduced in the olfactory bulbs and TST administration does not restores the normal AR levels. Hence, the action of TST on the sex-specific control of AOB neurogenesis could be not mediated by its specific receptor, but probably through ERs, after its aromatization (Schellino *et al*., 2016).

Besides sex hormones, other hormones have been described to directly modulate the process of AN. The anterior pituitary hormone prolactin (PRL) has been identified as one of the main endogenous cues promoting proliferation of SVZ progenitors in the reproductive context (Larsen *et al*., 2008; Mak *et al*., 2007, 2010; Shingo *et al*., 2003). PRL mediates several reproductive/social behaviors.

Indeed, PRL rises in female rodents during mating (Erskine *et al*., 1995), pregnancy and lactation (Grattan *et al*., 2008) and after prolonged exposure to male pheromones (Larsen *et al*., 2008). Socially induced release of PRL increases proliferation in the SVZ progenitor cells, increasing the number of newborn neurons integrating into the OB circuits in female mice 15-20 days thereafter (Mak *et al*., 2007; Larsen *et al*., 2008; Shingo *et al*., 2003). Thus, this increase in the proliferation in early gestation mediated by PRL is possible required later on for the expression of normal maternal behaviors during the postpartum period (e.g. pup recognition). Interestingly, postpartum period is characterized by fluctuation on SVZ proliferation (Shingo *et al*., 2003) and by a reduced proliferation in the DG of rats (Leuner *et al*., 2007). It is to note that proestrus increase in estrogen induces a surge in PRL and luteinizing hormone (LH; Becker *et al*., 2005; Brennan *et al*., 2004; Zhang *et al*., 2001). Thus, the increase in cell proliferation seen in proestrus (aforementioned) could be mediated directly by PRL, and not exclusively by estradiol. Accordingly, PRL receptor (PRLR) is expressed in the dorsolateral corner of the SVZ and in the choroid plexus in the adult female forebrain, but not present in the DG (Shingo *et al*., 2003; Mak *et al*., 2007). Another hypothalamic-pituitary axis hormone, the luteizing hormone (LH), is a well-known mediator of social and reproductive functions (Rao *et al*., 2002). The receptor of this hormone, the luteizing hormone receptor (LHR) is expressed in the SVZ and also in the DG (Mak *et al*., 2007). Exposure to dominant male semiochemicals, besides increasing SVZ neurogenesis through PRL release, enhances cell proliferation in the DG of female via LH. This hormone-mediated neurogenic effect is correlated with the mate-choice behavior, since only exposure to male dominant chemosensory cues was able to enhance neurogenesis. Moreover, mate preference was lost in females with impaired PRL and/or LH function (Mak *et al*., 2007).

The link between hormones and AN is also evident in endocrine diseases. Indeed, there are substantial evidences of neuronal defects as a consequence of thyroid hormone alteration in adults, that could result in cognitive deficits and depressive symptoms. For instance, we now know that the effects of hypothyroidism in adult life include an impairment of hippocampal neurogenesis and hippocampal morphological and functional synaptic plasticity (Garcìa-

Segura, 2009). Moreover, impairment on AN in the hippocampus may be involved in the cognitive effects of diabetes. Also the growth hormone and/or IGF-1 have been shown to increase adult hippocampal neurogenesis (Garcìa-Segura, 2009).

Adenohypophyseal and gonadal hormones *per se* could not be the only mediators in the modulation of AN. On the basis of the known role of GnRH in regulating these hormones, it is possible to hypothesize that hypotalamic regulation could also directly affects AN. Accordingly, GnRH could work as primary mediator independently on a specific sex hormone. In 2011, Zhang and collaborators found that, by delivering GnRH into the hypothalamic third-ventricle of old mice and by analyzing cell proliferation after BrdU administration, GnRH promoted adult neurogenesis despite aging. In particular, this effect was evident not only into the hypothalamus, but also in the hippocampus and other brain regions. This result reflects the fact that GnRH travels within the brain to promote neurogenesis (Zhang *et al.*, 2013). Nevertheless, data are still lacking regarding a direct involvement of GnRH action on the process of AN.

Overall, these findings indicate that AN is regulated in a complex manner by hormones, which in turn could modulate different steps of the neurogenic process, depending on the time, dose and duration of hormonal action. Moreover, the two neurogenic niches are regulated by different hormones or by the same hormone but in separate and well-defined ways. This could suggest that the molecular mechanism underlying the SVZ and SGZ fine-tuning could reflect different roles of these niches on brain physiology.

## The Role of Olfactory Neurogenesis in Reproductive/Social Behavior

The data presented in the previous paragraph clearly show that AN is modulated by hormones that are involved in several aspects of reproduction, thus supporting that a direct and complex link exists between AN and reproduction. In addition to these data, other experimental evidences further support this hypothesis. Indeed, in the last decade, several studies have depicted

the important role of olfactory neurogenesis in multiple social/reproductive behaviours (Feierstein *et al*., 2012; Gheusi *et al*., 2009). Mate selection, pregnancy, maternal recognition and paternal recognition are all social/reproductive behaviours in which a role of olfactory neurogenesis have been suggested (Mak *et al*., 2007; Shingo *et al*., 2003; Larsen *and* Grattan, 2010; Mak *and* Weiss, 2010).

Pheromonal cues, which convey information about species-specificity, gender, social status, health, genetic advantage and individual recognition (Ma *et al*., 2009) are considered as the major external stimuli able to enhance neurogenesis in the adult brain (Brus *et al.*, 2016a; Mak *et al*., 2007; Oboti *et al*., 2009; 2011; Sakamoto *et al*., 2011). In order to significantly regulate AN, pheromonal cues need to be perceived by both the main and the accessory olfactory systems, through a direct sensory stimulation that implies activation of both the vomeronasal and main olfactory sensory epithelium (fully exploration of pheromonal cues and/or coupling). Thus, the cooperation between the two olfactory systems is necessary in this social/reproductive behavior. However, the relative contribution of the two olfactory pathways on the neurogenic process triggered by olfactory social stimuli remains poorly understood.

In general, the exposure to social olfactory cues, such as those released during sex and parent-offspring interactions, elicits neurogenesis in both SVZ and SGZ (Mak *et al*., 2007; Larsen *et al*., 2008; Mak *et al*., 2010). On the contrary, the exposure to "generic odorants", or physical activity and environmental enrichment, seems to selectively regulate AN in the SVZ or in the SGZ, respectively (Rochefort *et al*., 2002; Brown *et al*., 2003).

Several studies have shown that the exposure to male soiled bedding, which contains semiochemicals present in urine and exocrine gland secretion (Brennan *et al*., 2004; Dey *et al*., 2015) significantly increases the integration/survival of newborn neurons in the accessory olfactory bulb of adult females (Nunez-Parra *et al*., 2011; Oboti *et al*., 2009, 2011; Schellino *et al*., 2016). In the main OB, the sensory activity enhances the survival/integration of newborn neurons during a critical time window of neuroblast maturation (Petreanu *et al*., 2002; Winner *et al*., 2002; Yamaguchi *and* Mori, 2005). As in the MOB, the AOB pro-survival effect elicited by male bedding exposure is achieved

favoring the integration of newborn cells aged between 7 and 14 days (Oboti *et al*., 2011; Wu *et al*., 2013), the critical time window for survival of AOB neurons (Oboti *et al*., 2009). Enhanced survival in the AOB can also occur after an increase in cell proliferation in the SVZ. Indeed, female mice, exposed for 7 days to male odors prior to BrdU injections, and thus affecting cell proliferation rather then survival (Larsen *and* Grattan, 2012; Mak *et al*., 2007), show an increase in the number of BrdU+ cells in the SVZ, in the MOB and in the AOB (Nunez-Parra *et al*., 2011). Moreover, estrous induction in females prairie voles by exposure to males increases cell proliferation in the SVZ (Smith *et al*., 2001). This phenomenon is gender-specific: neither females exposed to females odors, nor males exposed to females/males odors, show a similar effect; and definite: it occurs only in post-pubertal females (Nunez-Parra *et al*., 2011; Oboti *et al*., 2011).

## Functional involvement of AOB newborn neurons in reproduction

In this paragraph I will describe a series of experimental evidences supporting that adult generated OB neurons, particularly those of the AOB, are functionally involved in the modulation of olfactory-dependent reproductive behaviors in mice. One first evidence of this functional role has been achieved through analyses of c-fos expression (as a marker of cellular activity, Morgan *et al*., 1987), in the AOB of female mice after male pheromonal stimulation. Indeed, AOB newborn neurons show preferential responsiveness to male familiar (1-week experienced) stimuli when compared with those from unfamiliar (never experienced) males (Oboti *et al*., 2011). Interestingly, this preferential responsiveness of newborn cells is transient (disappears after 7 days, Oboti *et al*., 2011) and estradiol-dependent (Brus *et al*., 2016). Moreover, analysis of the activation in the AOB downstream vomeronasal nuclei involved in the control of estrus induction showed that exposure to the same familiar cues induces diverse responses (low c-fos expression) when compared to male unfamiliar stimuli (Oboti *et al*., 2011). These data supported the hypothesis that in mice AOB newborn neurons in females were linked to behaviors elicited by opposite sex-

pheromones. This idea was confirmed by a study in our Laboratory (Oboti *et al*., 2011) by chronic administration of the anti-mitotic drug Ara-C (four-weeks) to block the genesis of newborn interneurons on female mice during the post-mating critical period. It is known that soon after mating the exposure to male unfamiliar cues drives to a pregnancy block (known as the bruce effect, Bruce 1966) that does not occur if the female is exposed to male familiar cues. This sort of "protection" from male pheromones is assured by the AOB circuits in which a transitory memory for the mating partner occurs (Oboti *et al*., 2011; Portillo *et al*., 2012). Strikingly, the ablation of AN resulted in females inability to recognize their mating partners (Oboti *et al*., 2011), indicating the AOB newborn neurons as the preferential cellular substrate to form this kind of memory. Importantly, other studies based on the depletion of AN obtained through genetic targeting of newborn neurons in the forebrain (Sakamoto *et al*., 2011), and/or focal irradiation of the SVZ (Feierstein *et al*., 2010) further support that continuous OB neurogenesis is required to sustain appropriate sex-specific behaviors.

A functional modulation/involvement of AOB neurogenesis during social behaviors has been observed also in males. Nunez-Parra and co-workers have found that male mice exposed to aggression exhibited a region-specific increase in neurogenesis that was limited to the anterior AOB. Male intruders had a significant increase in the number of new neurons in the aGCL compared to individually-housed controls (Nunez-Parra *et al*., 2011). In addition, in another study (Mak *et al*., 2010) it has been suggested that in adult male mice the exposure to pup cues can modulate AN in the OB and that this modulation is functional in order to recognize the pups and probably avoid infanticide.

Beside olfactory neurogenesis, also adult hippocampal neurogenesis seems influenced by sexual stimuli/behavior, and this is dependent on the familiarity of the sexual interaction. In fact, after sexual interaction with unfamiliar females, adult males rats show a reduction in neurogenesis (in particular in the proliferation) in the dentate gyrus of the hippocampus compared to males that sexually interact with familiar females. Notably, males that interact with familiar females display a greater sexual behaviors (in terms of number of ejaculations and intromissions) compared to males interacting with

unfamiliar females, suggesting that probably the level of sexual activity rather than the familiarity with females, influences neurogenesis (Spritzer *et al*., 2016).

Overall, these data clearly show a direct involvement of newborn neurons of both the OB and the DG in social/reproductive behaviors.

## Modulation of AN at Puberty

Puberty is one of the most important critical periods in vertebrates. This period of life is featured by drastic changes in both the physiology and behavior that imply readjustment in the activity of endocrine glands and structural and functional transformations of the central nervous system (Romeo *et al*., 2003; Sisk *and* Zehr, 2005; Blakemore *et al*., 2010; Piekarski *et al*., 2017). For example, during puberty a fully functional maturation of the hypothalamic-pituitary-adrenal axis occurs (Di Luigi *et al*., 2006; Goel *and* Bale, 2007; Meaney *et al*., 1985; Romeo *et al*., 2004). Interestingly, several studies have suggested a pubertal-related decline in AN (Ho *et al*., 2012). Accordingly, the production of new neurons appears much higher in the prepubertal period compared to the postpubertal period (Rankin *et al*., 2003; Crews *et al*., 2007; Ho *et al*., 2012). He and Crews (2007) reported higher level of neurogenesis in both the SGZ and SVZ of prepubertal mice than in adult mice. The decline in hippocampal neurogenesis after puberty of rodents seems to be independent of the pubertal rise in gonadal hormones (Ho *et al*., 2012) and a similar mechanism has been proposed also for primates (Eckenhoff *and* Rakic, 1988). In humans, this decline is moderate throughout adulthood, without a massive decrease around puberty (Spalding *et al*., 2005).

The decline in olfactory neurogenesis could be the cause or the consequence of the olfactory dysfunction reported by the elderly population (Kovács, 2004). This is important because olfactory dysfunction represents one of the first symptoms manifested by people suffering from neurodegenerative diseases. Similarly, a decrease during puberty in the survival of newborn

neurons has been demonstrated also in both the MOB and the AOB neurogenic niches (Enwere, 2004; Nunez-Parra *et al*., 2011).

Considering the key role played by olfactory neurogenesis in: I) neural plasticity, ii) its modulation by opposite-sex stimuli, iii) its role in the socio/sexual context (see paragraphs above), and iv) that reproductive maturity is achieved only after puberty, we can argue that the possible pubertal-related modulation of olfactory neurogenesis could be functionally related to the accomplishment of "new" reproductive/social functions, unnecessary before. According to this hypothesis, in mice, at puberty chemical signaling begins to be used to advertise sexual receptivity and to assess both the quality and the readiness of mating partners (Moncho-Bogani *et al*., 2002; Ramm *et al*., 2008). Nevertheless, important gaps need to be filled to sustain this hypothesis. Among them further studies are necessary to identify the factors/mechanisms involved in the modulation of AN during the pubertal period, and the role played by hormones in modulating AN at puberty. Moreover, open questions remain, such as how AN achieves different functions before and after puberty and how pheromonal cues are processed during puberty. Some of these question were addressed in this PhD thesis.

# Aim of the Study

Over the past years, many researchers have made several efforts to figure out the complex regulation of adult neurogenesis (AN), a striking form of neural plasticity that implies genesis and integration of new neurons into pre-existing mature brain circuits.

At present, we know that under physiologic condition AN occurs mainly into two specific sites of the adult brain (the SVZ-OB system and the DG of hippocampus). Its role in these regions has been linked to important cognitive functions, such as learning and memory. Nevertheless, several gaps of knowledge still remain for what concerns the mechanisms by which adult generated neurons contribute to these functions. Recently, many studies have also indicated that the OB neurogenesis in rodents is involved in the modulation of social-reproductive stimuli. Accordingly, pheromonal cues and gonadal hormones, which cooperate to tune the reproductive behavior, have been shown to be key factors to regulate the process of AN in the OB. In turn, bulbar newborn neurons are essential to optimize intersex and parental social behaviors. How this complex integration among social cues, hormones and newborn neurons occurs is still largely unknown. To unravel this mechanism appears essential not only to identify the specific role of the OB neurogenesis in mediating social cues, but it gives a unique opportunity to investigate how the brain integrates external and internal cues to produce appropriate behaviors.

To address this issue, I focussed firstly on a critical stage of life, that is puberty. Puberty is a major life transition during which an individual's physiology undergoes a profound reorganization. This is particularly true for what concerns the neuroendocrine system and behavior, especially the reproductive one. Interestingly, several data have shown that AN undergoes a sharp decline during puberty, indicating this is an optimal period to investigate processes modulating AN. Another related key question of my thesis, is to investigate the possible role of the gonadotropin releasing hormone (GnRH) system in the OB plasticity. Accordingly, this system, starting from puberty, orchestrates reproduction through its activity on the hypothalamic-pituitary-

gonadal axis, and by integrating multiple sensory stimuli, including the olfactory cues. In addition, GnRH-ir cells have been recently described in the olfactory bulb region of both mice and humans.

Based on these considerations, specific questions of this thesis were:

1) How adult OB neurogenesis is regulated during puberty?
2) How the neuroendocrine system is involved in modulating adult neural plasticity in the olfactory bulb region?

To address these main issues I took advantage of mice deprived of gonadal hormones through gonadectomy and of a recently developed mouse model in which GnRH secretion is abolished just before puberty.

# CHAPTER II

# Activity Dependent Modulation of Granule Cell Survival in the Accessory Olfactory Bulb at Puberty

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## Abstract

The vomeronasal system (VNS) is specialized in the detection of salient chemical cues triggering social and neuroendocrine responses. Such responses are not always stereotyped, instead, they vary depending on age, sex and reproductive state, yet the mechanisms underlying this variability are unclear. Here, by analyzing neuronal survival in the first processing nucleus of the VNS, namely the accessory olfactory bulb (AOB), through multiple bromodeoxyuridine (BrdU) birthdating protocols, we show that exposure of female mice to male soiled bedding material affects the integration of newborn granule interneurons mainly after puberty. This effect is induced by urine compounds produced by mature males, as bedding soiled by younger males was ineffective. The granule cell increase induced by mature male odor exposure is

not prevented by pre-pubertal ovariectomy, indicating a lesser role of circulating estrogens in this plasticity. Interestingly, the intake of adult male urine-derived cues by the female vomeronasal organ (VNO) increases during puberty, suggesting a direct correlation between sensory activity and AOB neuronal plasticity. Thus, as odor exposure increases the responses of newly born cells to the experienced stimuli, the addition of new GABAergic inhibitory cells to the AOB might contribute to the shaping of vomeronasal processing of male cues after puberty. Consistently, only after puberty, female mice are capable to discriminate individual male odors through the VNS.

## Introduction

Postnatal development is a critical period for the maturation of brain circuits and implies extensive interactions between external and internal factors affecting both their wiring and tuning. This is particularly important for the brain systems implied in goal oriented behaviors (such as mating or foraging) as they need to adaptively match external stimuli to internal states and drives, in order to limit non adaptive decisions that could be detrimental for survival (Bouret *et al*., 2012; Saleem *et al*., 2014; Ramm *et al.,* 2008; Nowicki *et al*., 2002). Because mating and feeding circuits have been mainly considered hard-wired or innate (Kohl *et al*., 2017; Anderson, 2016), the neural mechanisms involved in this tuning remain poorly investigated. Here, we have analyzed the role of sensory activity in the peripubertal activity-dependent maturation of inhibitory interneurons in the accessory olfactory bulb (AOB), a bulbar circuit specialized in the elaboration of salient signals for reproductive and mating behaviors (Brennan, 2009; Ferrero *et al*., 2013; Flanagan *et al*., 2011; Haga *et al*., 2010; Hendrickson *et al*., 2008; Keller *et al*., 2006; Mucignat-Caretta *et al*., 1995). The onset of female puberty represents a critical period for the development of both reproductive organs and brain circuits regulating the neuroendocrine aspects of sexual behavior (Romeo, 2003). Also, puberty represents a stage for important behavioral changes (Blakemore *et al*., 2010; Sisk *and* Zehr, 2005) and a critical period for the activity dependent remodeling of cortical circutis (Piekarski *et al*.,

2017). In mice, at puberty chemical signaling begins to be used to advertise sexual receptivity and to assess both the quality and readiness of mating partners (Ramm *et al.,* 2008; Moncho-Bogani *et al*., 2002). These two aspects of sexual maturation - the internal physiology and the sensitivity to environmental cues - obviously interact, and, through the vomeronasal system (VNS), chemical stimuli can affect both the development (Vandenbergh, 1974; Flanagan *et al*., 2011; Oboti *et al*., 2014) and the adult display (Oboti *et al*., 2014; Ferrero *et al*., 2013; Haga S. *et al*., 2010; Roberts *et al*., 2010) of female reproductive behaviors.

A tight interaction between neuroendocrine responses and the molecular nature of specific triggering signals has long been considered the main mechanism by which both aspects of sexual maturation are matched. However, chemical signaling is flexible and subjected to changes depending on both external and internal factors (Garratt *et al*., 2011; Ferrero *et al*., 2013; Drickamer *et al*., 1992; Lemmen *and* Evenden, 2009; Villar *et al*., 2015). In fact, the same compounds might acquire different signaling values depending on behavioral and physiological needs (Mucignat-Caretta *et al*., 1998; Brennan, 2009; Martín-Sánchez *et al*., 2015). Therefore, the question of how the VNS determines the onset of social behavioral responses by detecting such variable signals is unclear. Clarifying this issue is further complicated by the presence of ongoing neurogenesis in both the vomeronasal epithelia (Weiler, 2005) and the AOB (Oboti *et al.*, 2009; see for a review Oboti *and* Peretto, 2014). Specifically, how this plasticity relates to the onset of puberty and to more mature behavioral displays is still unclear.

In our previous works we have extensively characterized the process of adult neurogenesis in the AOB, showing that, as in the main olfactory bulb (MOB), adult neurogenesis in this region involves a process of continuos addition of GABAergic interneurons originating in the subventricular zone (SVZ; Bonfanti *et al*., 1997; Peretto *et al*., 2001; Oboti *et al*., 2009; 2011). In addition, we have also shown that the integration and survival of AOB immature neurons is modulated by sensory activity (Oboti *et al*., 2009; 2011). In particular, exposure to vomeronasal organ (VNO)-detected stimuli contained in male urine, during a critical stage of newborn cell maturation, increases neuronal survival in the AOB of female mice (Oboti *et al*., 2011).

Here, by incorporating bromodeoxyuridine (BrdU) in immature SVZ-derived neurons reaching the AOB, we show that granule cell (GC) survival in the female AOB begins to be particularly sensitive to opposite-sex olfactory stimuli at the onset of puberty. We further show that the number of GCs integrating in the AOB is modulated by olfactory stimuli derived from adult but not juvenile male urine. Moreover, short term post-pubertal exposure of females to adult male derived cues promotes the integration of immature GCs in the bulbar circuits implied in male odor processing. This further correlates with the onset of individual specific responses to male odors detected through the vomeronasal system, normally displayed by mature females (Moncho-Bogani *et al*., 2002; Ramm *et al*., 2008). By combining ovariectomy with male odor exposure in BrdU-treated animals, we next provide evidence supporting that the effect of male derived cues on AOB GCs does not depend on ovarian hormones. Rather, increased VNO activity levels shown by post-pubertal females in response to these stimulations suggest a major role of sensory activity in this process. Overall, our results indicate that through activity dependent regulation of AOB GC development, VNO- detected stimuli rearrange the AOB inhibitory network involved in their processing, when these cues acquire the meaning of mating signals after puberty.

## Materials and Methods

### Animals

All experiments were done using CD-1 mice (Charles River, Italy): females and males, pre-pubertal (postnatal day P21-P28), peri-pubertal (P35-P42), and post-pubertal (P52-P90). Animals were housed under a 12-hour light-dark cycle in an environmentally controlled room. Female subjects of the same group were maintained 4-6 per cage, whereas male subjects were kept or in isolation for the duration of all experiments or in cages with other male subjects of the same group. Experimental procedures were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), Recommendation 18/06/2007, Dir. 2010/63/UE, and the Italian law for care

and use of experimental animals (DL116/92) and were approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin (Protocol Number DGSAF0007085-A05/04/2013). All animal care and procedures conducted at the University of Maryland, Baltimore County were in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals (2006) and approved by the institutional Animal Care and Use Committee. All experiments were designed to minimize the number of animals used.

### Assessment of puberty onset

CD1 female mice were weaned at P21 and checked daily for vaginal opening. After vaginal opening, vaginal smears were performed daily and analyzed under an inverted microscope to identify the specific day of first estrus.

### BrdU treatment and bedding exposure

To identify newly generated cells in the AOB, female mice were intraperitoneally injected with bromodeoxyuridine (BrdU) in 0.1M Tris (pH 7.4) twice in one day (delay = 8h, 100mg/kg body weight), and sacrificed 28 days later for the evaluation of neuronal survival. For all experimental groups, bedding stimulations (consisting of daily renewed male bedding material added with male urine) were timed during the second week after BrdU injection. Males were caged individually or in groups of 3-4 animals and the bedding was left untouched for at least one week before being transferred to female cages. Exposures were performed at different stages of peripubertal maturation: from P28 to P35 (pubertal); from P35 to P42 (postpubertal) and from P52 to P59 (mature). Different sources of male soiled-bedding material were used for each experimental group. Females exposed to sexually mature males, were given a mixture of bedding from 3 different adult reproductive males not kin-related with females (MB); females exposed to coetaneous males bedding, were given a mixture of bedding from 3/4 kin-unrelated males of the same age of female subjects (cMB; P28-P35 and P35-P42 for the peripubertal exposure; P52-P59 for the adult exposure); females exposed to kin or foster males (of the same age as females) bedding, were given a mixture from 3/4 kin (kMB) or foster (foMB)

(cross-foster with females at the age of P2 until P21) males, respectively. The "control" groups were treated similarly but with clean bedding.

## Ovariectomy

Juvenile (P21, n=7) CD1 female mice were deeply anaesthetized with a 3:1 solution of ketamine (Ketavet; Gellini, Italy) and xylazine (Rompun; Bayer, Germany) and using aseptic procedures both ovaries were removed by two small incisions on each side in the abdominal area, one through the skin and then another through the muscle wall. Each ovary was tied off with absorbable surgical thread and removed; after that the muscle incision and skin incision were closed using sutures. Sham-operated juvenile (P21, n=6) CD1 female mice have been subjected to same surgical manipulations without removal of the ovaries. Mice were then allowed to recover for 1 week before further treatments.

## VNO dye-access assay

Juvenile (3 weeks old) sexually naïve CD1 female mice were purchased from the Jackson Laboratory and grown to designed ages of either 28, 35, or 52 days old, respectively (n=5-7 per group).

*Urine Collection and urine-dye mixture*

Male urine was freshly collected on the same day of the experiment from the same males that were sexually experienced. To induce urination from the males, we placed two female CD1 mice into two clean separate cages without bedding for two minutes. We then removed the female mice and transferred the male mice into the scented cages. Care was taken to remove female urine or feces, if any, before introducing the males into the cages. If no urine appeared after two minutes, the procedure was repeated or the animal was held by hand and the bladder region gently pressed to induce urination. Male urine was immediately collected after urination using a pipette. Urine was pooled into an Eppendorf tube, and placed on ice before use. For the stimulus-dye VNO access assay, a 100µl of urine was mixed with Rhodamine 6G fluorescent dye (Sigma) to yield a final concentration of urine in 1:10 dilution and 0.002% dye.

*Delivery of urine-dye mixture*

The VNO dye access assay was performed following a previously developed method from Ogura *et al.* (2010). Briefly, an individual mouse was transferred and acclimated in a chamber made from clean empty, plastic, pipette tip boxes with clear lids 12.7 x 7.6 x 5.1cm (width x depth x height) with a 1.27cm square hole on a side wall for ten minutes. Upon acclimation, a pipette tip containing 5µl of urine-dye solution was presented to the nostrils in small drops, when the mouse protruded its nose from the small hole. The process was repeated until all 5µl was delivered. The process usually lasted 3-10 minutes. For each post-developmental stage, five to seven mice were examined.

*VNO dissection and fluorescence image acquisition*

Immediately after the assay, mice were euthanized by $CO_2$ inhalation followed by cervical dislocation. The head was then removed, split roughly along the midline to expose the VNO. The half head containing the VNO was placed on a premade mold and epi-fluorescence images were taken for both lateral and ventral VNO using a 4X lens and an Olympus BX 41 epi-fluorescence compound microscope equipped with a Retiga 4000R camera and Image-Qcapture Pro 7 (QImaging, British Columbia, Canada). Fluorescence dye intensity of the VNO proper and entrance duct, which is narrow, approximately 0.4mm in length, tubular structure anterior to the VNO proper (Ogura *et al*. 2010), were measured using NIH ImageJ. Background intensity was measured from the septal respiratory epithelium dorsal to the VNO proper.

## Tissue preparation for immunohistochemistry

Mice were deeply anesthetized with an intraperitoneal injection of ketamine (Ketavet; Gellini, Italy) and xylazine (Rompun; Bayer, Germany) 3:1 solution. All the animals were transcardially perfused with 0.9% saline solution followed by cold 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB), pH7.4. Brains were removed from skull and post-fixed for 4h in 4% PFA at 4°C, followed by a cryopreservation step using a 30% sucrose solution in 0.1M PB pH

7.4 at 4°C. The two hemispheres were separated and embedded in OCT (Bio-Optica), and then frozen and cryostat-sectioned. Free floating parasagittal sections (25µm) were collected in multi-well dishes in representative series of the AOB. Sections were stored at -20°C in an antifreeze solution (30% ethylene glycol, 30% glycerol, 10% phosphate buffer: 189mM $NaH_2PO_4$, 192.5 mM NaOH; pH 7.4) until use.

**Immunohistochemistry**

After rinsing in phosphate-buffered saline (PBS) to remove the antifreeze solution, sections were incubated for 24h at 4°C in primary antibodies diluted in 0.01 M PBS, pH 7.4, 0.5% TritonX-100, and 1% normal sera, made in the same host species of the secondary antibodies. As primary antibodies we used: anti-BrdU, rat IgG monoclonal, dilution 1:5000 (ABC), AbD serotec, Bio-Rad Laboratories, code number OBT0030CX (Liu *et al*., 2009); anti-c-Fos, rabbit IgG polyclonal, dilution 1:10000 (IFL), Santa Cruz biotechnologies, CA, USA, code number sc52 (Oboti *et al*., 2011); anti-NeuN, mouse IgG monoclonal, dilution 1:1000 (IFL), Chemicon International, Temecula, CA, USA, code number MAB377 (Oboti *et al*., 2009); anti-GAD67, dilution 1:1000 (IFL), mouse IgG monoclonal, Chemicon International, Temecula, CA, USA, code number MAB5406 (Oboti *et al*., 2009). For BrdU immunostaining, sections were pre-treated with 2N HCl for 30 min at 37°C, for antigene retrieval, and neutralized with borate buffer, pH 8.5, for 10 min. For the avidin-biotin-peroxidase method, sections were incubated for 1 h at room temperature in biotinylated secondary antibody (anti-rat IgG; Vector Laboratories, Burlingame, CA) diluted 1:250 in 0.01 M PBS, pH 7.4 following by avidin-biotin-peroxidase complex (Vector Laboratories). To reveal immunoreactivity, we used 0.015% 3,3'-diaminobenzidine and 0.0024% $H_2O_2$ in 0.05 M Tris-HCl, pH 7.6. After adhesion on gelatin-coated glass slides, sections were mounted in DPX (Merck-Millipore, VWR International PBI, Milan, Italy). For immunofluorescence double-staining, the sections were incubated in a mixture of primary antibodies and appropriate blocking sera for 24h at 4°C, then incubated with appropriate fluorochrome-conjugated secondary antibodies (Cy3-conjugated secondary Ab, 1:800; 488-conjugated secondary Ab, 1:400; Jackson ImmunoResearch Laboratories, USA) and/or biotinylated secondary

antibodies (1:250; Vector Laboratories, Burlingame, CA, USA), and finally incubated with avidin-FITC (1:400, Vector Laboratories, Burlingame, CA, USA). The sections were then coverslipped with the anti-fade mounting medium Dabco (Sigma) and analysed with a laser scanning LAS AF Lite confocal system (Leica Microsystems).

### Cell counting and statistical analysis

The number of BrdU-positive nuclei in the AOB granule cell layer was established by counting peroxidase/DAB-stained 25μm thick parasagittal sections in all experimental groups. Three series (n=7 sections/animal) representing the whole AOB were used for each animal. Cells were counted in the AOB granule cell layer. The area of each examined section was measured with Neurolucida software (MicroBrightField, Colchester, VT). Cell densities were calculated by summing cell counts made on all sections per animal and referred to the proper cellular layer volume (Σ of sampled areas × 25μm). The percentage of double-labelled c-Fos/BrdU-positive cells, NeuN/BrdU-positive, GAD67/BrdU positive and triple-labelled c-Fos/NeuN/BrdUpositive and c-Fos/GAD67/BrdU positive in the AOB granule cell layer was established by counting labelled cells in parasagittal sections (25μm thick) with a 40x objective with a confocal microscope using a UPI an FL 40x (N.A. 1.3) Olympus objective. One series (n= 3–4 sections/ animal) representing the whole AOB was used for each animal. In each section, all of the BrdU-positive cells were analysed for co-expression with c-Fos, NeuN, GAD67, c-Fos/NeuN or c-Fos/GAD67, and ratios of double-labelled or triple-labelled cells were determined. All cell counts were performed blind to the treatment. For statistical analysis, unpaired Student's *t*-tests were used for simple 1:1 comparison of parametric data; paired Student's *t*-test were used for sex and individual preference tests, in which same mice were measured twice, before (P20) and after (P41 or P50) puberty; one-way or two-way ANOVA were used for multiple comparisons for parametric data; chi-squared test was used for comparing the percentage of females showing lordosis.

### Sex preference tests

The preference of female mice for sex related odors was assessed by placing at opposite sides of the homecage (female subjects were either P20 or P41), urine stimuli were derived from adult male or female subjects (pooled from different individuals, 10µl) on filter paper. The time spent investigating these stimuli was evaluated during 10 minute trials.

### Learning of familiar male odors

This assay evaluated the learned preference of a female mouse for male or female individual volatile scents that were previously experienced during a five-day exposure period (familiarization), either with or without direct physical access to the odor source. This attractive response was based on an olfactory associative learning between volatile and non-volatile urinary components. During the exposure step, two urine stimuli obtained from distinct CD1 males (or females; 50µl each) were placed on filter paper in the cage housing the female. While one of these filter papers was directly accessible to the female (leading to a conditioning learning of urine volatiles), the second one was placed in a meshed box to prevent direct physical contact with the odor source (thus preventing urine volatiles conditioning). Urine donors were adult CD1 males (or females; 4 to 5 months old) housed individually, with no kinship relation. Stimuli were delivered randomly two times a day each for 5 min within a total 4h interval allowing for a 1 h resting phase between stimulations. After this learning period, a recognition step consisting of a two-choice olfactory preference test without physical contact was performed. Filter papers containing 50µl of both directly (CS) or indirectly (US) previously experienced urine were deposited in meshed plastic boxes to prevent direct physical contact. During a 5 min trial period, stimulus investigation time was scored as the time spent in close contact with the stimulus source (distance of the snout from the box <1cm) as well as the time spent manipulating, chewing, and biting the meshed box in an attempt to reach the stimulus source. As a critical control to rule out any pre-existing preference prior to the learning phase, we examined whether a given female showed a preference for volatiles in the tested urine sources; only females showing no preference were used for this assay.

## Female sexual receptivity (lordosis)

Adult and juvenile female mice (N=11 per group; both sexually naive) were single housed for one week and the estrus cycle was determined in order to use only animals in estrus or proestrus prior to the test. Adult males (sexually experienced) were introduced to the female's home cage and were recorded for 1 hour during the dark cycle. The number of lordosis events (in which females show a receptive still posture or arching of the back, allowing or promoting male mounting) by the females was assessed. The number of mounting behaviors as well as the latency to mount shown by male individuals were scored. The lordosis quotient was calculated as the ratio between lordosis events and male mounts, previously described as an index of female reproductive receptivity (Keller *et al*., 2006; Leinders-Zufall *et al.*, 2014).

## SDS-PAGE

Mouse urine sample aliquots (2.5µl) were mixed with 4x LDS buffer and 20x Reducing Reagent (Thermo Fisher Scientific, Walthem, MA USA). Samples were then boiled and loaded on 4-12% SDS precast BisTris NuPage gels (Thermo Fisher Scientific, Walthem, MA USA). Gels were ran for one hour at 170V, fixed (50:10:40/ methanol: acetic acid: $H_2O$/ v:v:v), stained with Biosafe Comassie (BioRad, Hercules, CA USA), and washed extensively with water. Gels were scanned using ChemiDoc MP System. Differences in protein content among samples was estimated by comparing gel band intensities using ImageJ.

## Results

### Male odors promote granule cell survival in the AOB of postpubertal females

Immature neurons migrating to the AOB typically reach a mature phenotype around four weeks after genesis (Oboti *et al*., 2009) and are particularly sensitive to olfactory stimulations after reaching the olfactory bulb (seven days after BrdU injection), between their second and third week (Oboti *et al*., 2011). They integrate mainly in the granule cell layer (Oboti *et al*., 2009, 2011), which is populated for the most part by GABAergic inhibitory neurons (granule cells; Larriva-Sahd, 2008). Here, we further confirmed the fully mature phenotype of newborn granule cells in the AOB 28 days after their genesis (91.58±4.44% of BrdU-positive cells co-express NeuN, as in mature neurons; Miller *et al*., 1992). In addition, we show that the vast majority of the BrdU-labeled neurons found in the AOB four weeks after genesis do have a GABAergic phenotype (84.51±4.3% of BrdU-positive cells co-express GAD67, the GABA synthesis enzyme; Mugnaini *et al*., 1994; Suppl. Fig.1).

We previously found that exposure to male soiled bedding and male-derived urine compounds increases the survival of immature AOB GCs migrating from the SVZ in P50 female mice (a stage at which both brain circuits and neuroendocrine physiology can be considered mature; Finlay *and* Darlington, 1995), but not in prepubertal P20 females (Oboti *et al.*, 2011). However, whether this effect is due to the different sensitivity of sensory circuits in the female brain or to other behavioral mechanisms (e.g., increase motivation in exploring these cues) affecting the female detection of male stimuli through the VNO, is not known.

To address this issue, we first sought to define more precisely the time at which AOB inhibitory circuits become sensitive to male bedding exposure. We analyzed the effect of one week male bedding stimulation on 2-weeks-old GCs (more sensitive to sensory activity; Oboti *et al.*, 2011) birthdated by BrdU injections at different peripubertal stages: P21 and P28 (Figure 1A, B). BrdU systemic injection results in nuclear DNA labelling during cell division

(Nowakowski *et al.*, 1989) and therefore it has been used to localize newborn neurons at different times after genesis (Figure 1C). Following BrdU treatment, along with a Ca. 50% reduction in the basal level of AOB newborn neuronal survival (neurons born at P21 vs P28), we found that exposure to adult male soiled bedding promotes the survival of newborn GCs in the AOB after P35 (Figure 1B), but not earlier (Figure 1A). This corresponds to the age of menarch, when the first estrus cycle occurs, as shown by vaginal patency assessment (opening of the external genitalia) and cytological examination (Figure 1D; Goldman *et al*., 2007). Around P27 almost 60% of the females showed vaginal opening, the 100% being reached only at about P30 (Figure 1D), similarly to what has been previously observed in both the CD1 (Szymanski *and* Keller, 2014) and C57 mouse strains (Oboti *et al*., 2014; Nelson *et al*., 1990). Signs of first estrus cycle occurrence were found only between 3-10 days later (50% of the females reached estrus around P35, while the rest reached estrus around P40-P42, similar to what occurring in C57 mice; Figure 1D; Nelson *et al*., 1990).
Puberty onset is accompanied by a surge of luteinizing hormone, follicle stimulating hormone and estrogens (Swerdloff *and* Odwell, 1975) which affects both neuronal proliferation (e.g. prolactin, Larsen *et al*., 2008; Menezes *et al*., 1995; Mak *et al*., 2007; estrogens, Tanapat *et al*., 1999) and pubertal maturation (Koyama *et al*., 2013). Therefore, since exposure to male odor stimuli also modulates the production of ovarian hormones (Bronson *and* Maruniak, 1976), the survival of AOB GCs could depend on indirect hormone-mediated mechanisms, rather than VNO activity *per-se*. Although male odor exposure was delivered when GCs were not further proliferating (seven days after genesis) but began to integrate in the AOB circuits (Luskin, 1993; Lois *and* Alvarez-Buylla, 1994), we could not rule out hormone-mediated effects on cell division occurring during late stages of migration through the rostral migratory stream (*rms*; Smith *et al*., 2001) or cell survival itself, which indeed have been previously reported (Veyrac *and* Bakker, 2011; Schellino *et al*., 2016).

We addressed this issue by performing the same sensory stimulations (from P35 to P42) in P28 BrdU-injected animals, after removal of the ovaries at P21. At this stage female gonadal hormones are not yet at peak (Bronson *and* Maruniak, 1976; Bronson, 1981). Therefore, seven days after surgery, blood

hormone levels remain stable at prepubertal levels (Bronson *and* Maruniak, 1976; Bronson, 1981). A two-way ANOVA indicated a significative effect of ovariectomy (OVX) on BrdU levels (regardless of bedding treatments). However, although OVX showed on average lower levels of BrdU expression in the AOB (Figure 1E), male bedding exposure in ovariectomized mice seemingly affected GC survival as in sham-operated subjects, however without reaching statistical significance (see Supplementary Table 1 for statistical tests). Overall, these results suggest that gonadal hormones might modulate broad aspects of SVZ-OB neurogenesis (Larsen *et al*., 2008; Menezes *et al*., 1995; Mak *et al*., 2007; Tanapat *et al*., 1999), without critically affecting activity dependent modulation of granule cell survival (Figure 1E).

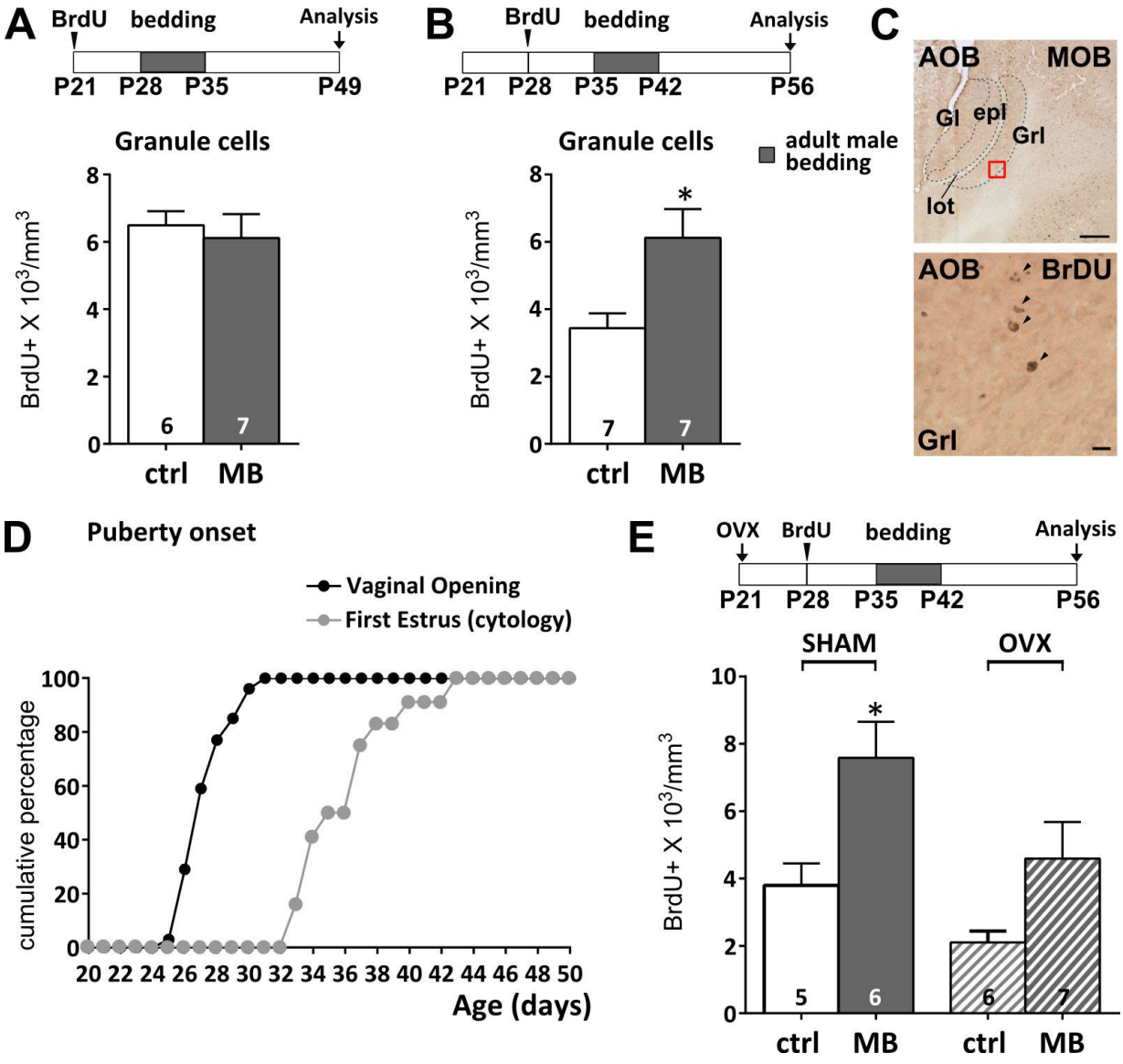



**Figure 1: Survival of newborn granule cells in the AOB is sensitive to vomeronasal inputs after puberty. (A,B)** Daily exposure of female mice to male soiled bedding (MB) increases granule cell survival after the onset of the first estrus cycle (P35-P42). Bedding stimulations were timed during the second week after granule cell genesis and their survival 28d.p.i. (unpaired Student's *t*-test, P28-P35 ctrl *vs* MB, *P*=0.669, P35-P42 ctrl *vs* MB, **P*=0.016). **(C)** Sagittal section of the AOB showing BrdU labeled nuclei (scale bar 100µm). The lower panel refers to the red squared area in the granule cell layer (scale bar 5µm). **(D)** Graph showing the onset of female puberty. This can be determined by examination of vaginal opening and the occurrence of the first estrus, assessed through cytological examination (see methods). **(E)** Bedding exposure in OVX prepubertal females (P21) does not modulate GC survival as significantly as in SHAM animals (two-way ANOVA, factors: ovariectomy, $F_{2,10}$=6.766, *P*=0.017; stimulus, $F_{2,10}$=12.14, *P*=0.002; interaction, $F_{2,10}$=0.520, *P*=0.479; Tukey's post-hoc, SHAM ctrl *vs* SHAM MB, **P*=0.045; OVX ctrl *vs* OVX MB, *P*=0.206). Abbreviations: ctrl, control; MB, male bedding; MOB, main olfactory bulb; AOB, accessory olfactory bulb; Gl, glomerular layer; epl, external plexiform layer; GrL, granule cell layer; lot, lateral olfactory tract; OVX, ovariectomy; d.p.i., days post-injection. Sample sizes are indicated on each histogram bar. The values shown are the mean ± s.e.m.

## Urine odors from mature, but not juvenile males, promote granule cell integration

Male urine has been repeatedly shown to be efficient in promoting survival of AOB newborn cells in female mice (Nunez-Parra *et al*., 2011; Oboti *et al*., 2009; Schellino *et al*., 2016). In CD1 swiss mice, stimuli comprised in the low molecular weight (LMW) fraction of male urine (mass lower than 3 kDa) are more efficient in promoting neuronal integration of immature cells in the AOB of adult females (Oboti *et al*., 2011). This fraction contains volatile and non-volatile molecules resulting from the metabolism of hormones like testosterone (Knopf *et al*., 1983). Stimuli in the high molecular weight (HMW) fraction of male urine include instead proteins and large peptidic complexes, which production depends on both age and testosterone levels (Garratt *et al*., 2011; Knopf *et al*., 1983; Chamero *et al.*, 2007). Therefore the composition of male chemostimuli varies with age, and urine protein content can be used as a proxy to assess these changes.

To clarify whether or not the maturity of male chemostimuli was differently affecting AOB GC density, we exposed female mice to bedding obtained from cages of coetaneous (cMB; i.e., same age of exposed females) but unrelated males. We found that such stimulation had no effect on BrdU-labeled GCs either at the time of vaginal opening (Figure 2A) or at the age of first estrus (one week later; Figure 2B), when stimuli from mature males began to be effective (Figure 1B). Conversely, around P50, exposure to bedding material from coetaneous males (P50, cMB) increased the number of newborn GCs in the AOB as more mature stimuli do (Figure 2C). Thus, odor stimuli affected those cells generated right after these treatments (P45), even though females received daily bedding from the same donors (Figure 2C). Since long term exposure to the same odor stimuli has no reported effect on neuronal survival in the OB (Magavi *et al*., 2005; Rochefort *et al.*, 2002), this suggests the occurrence of changes in the composition of male urine stimuli during peripubertal development. In addition, they also indicate that experience induced remodelling of AOB inhibitory circuits occurs in females at a time when donors (males) and receivers (females) of urine chemosignals are mature for mating. As a confirmation of reached sexual

maturity in P50 males, we assessed the presence of urinary proteins, which production and excretion through urine are known to be testosterone dependent (Chamero *et al.*, 2007; Knopf *et al.*, 1983). We used gel electrophoresis to test individual urine samples collected from male CD1 mice at different ages. We found that at P50 male urine was already enriched with a high proportion of HMW compounds (15-25kDa), while around P30 and younger ages urine protein content was lower, indicating a different composition of the stimuli (Figure 2D, E). Thus, male urine composition is likely to be a key factor affecting AOB GC survival, since female mice did not have previous experience with coetaneous donors in these experiments.

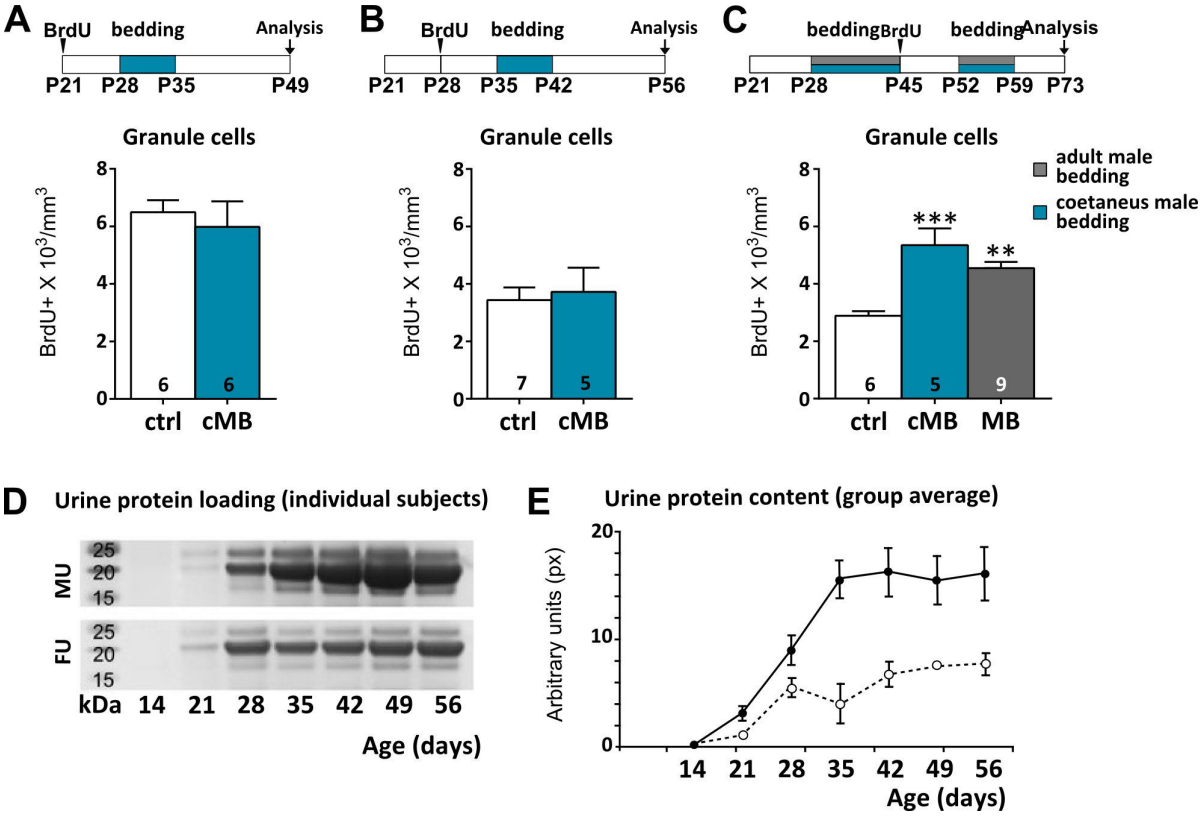



**Figure 2: Urine stimuli affecting AOB granule cells in females are produced by sexually mature males. (A-C)** Comparison of the effect of coetaneous male bedding (cMB) stimulations delivered to female mice at different postnatal ages (P28, P35, P52), compared to mature male bedding (MB). Survival of AOB granule cells is affected by odor exposures if the donors age is at least P52 (unpaired Student's *t*-test, P28-P35 ctrl *vs* CMB, $P$=0.614, P35-P42 ctrl *vs* cMB, $P$=0.751; one-way ANOVA, $F_{2,19}$=13.759, $P$=0.000; Tukey's post-hoc, ctrl *vs* cMB ***$P$=0.000; ctrl *vs* MB, **$P$=0.003). **(D)** PAGE of urine samples collected from male/female donors at different ages (P14-P56; the bands show the loading of proteins within the 15-27kDA range, which includes MUPs). **(E)** Average protein content by age in the two sexes (males, solid line; females, dotted line; repeated measures two-way ANOVA, suppl. Table 1). Abbreviations: MU, male urine; FU, female urine. Sample sizes are indicated on each histogram bar. The values shown are the mean ± s.e.m.

Alternatively, early postnatal odor experience (nest odors, littermates) might have biased successive AOB male odor responses simply because the wiring of sensory neurons might have needed to retune (Jones *et al*., 2008; Hovis *et al*., 2012; Xia *et al*., 2006) to a new set of stimuli (e.g. MUPs and MHC-related compounds change with age, Singer *et al.*, 1997; Sherborne *et al*., 2007). To verify this possibility, we have long-term exposed (P0-P42) female mice to the odors of kin-related coetaneous littermates, in order to evaluate GCs survival after puberty (as above). This was done by protracting bedding exposures a few days before (P42) or after (P50) BrdU injections (P45), in order to control for possible proliferative effects. We found that postpubertal exposure to coetaneous littermates affected GC survival, similarly to what occurred when mature unrelated male stimuli are used (Figure 3A).

Specifically, the amount of BrdU-labeled granule cells was significantly higher (compared to controls) when stimulations were interrupted ten days before (P42) the second exposure (thus not covering the proliferative phase of cells labeled with BrdU at P45; Figure 3A). Conversely, lower levels of BrdU-labeling were found in the AOB GC layer when bedding stimulations were interrupted only two days before (P50) the second exposure (during the proliferative phase of BrdU labeled cells; Figure 3A). This is in accordance to previous studies reporting a minor effect of long-term odor exposure on granule cells survival (Alonso *et al*., 2006; Magavi *et al*., 2005). Kin-related odors affected GC survival in the AOB even when females were separated before puberty (P21; Figure 3B), thus confirming that the maturation of male stimuli is likely to be the most relevant factor affecting AOB plasticity. Finally, to rule out possible effects of donor genetic identity on GC survival in the AOB, we exposed mature females to male soiled bedding material from either kin-related (kMU) or unrelated foster cage mates (foMU), housed together with females until P21. Both stimuli were effective in modulating the survival of newborn GCs (Figure 3C). Overall, these results indicate that the survival of newborn GCs in the AOB of postpubertal female mice is similarly affected by exposure to kin-related or unrelated males when they reach sexual maturity (P50).

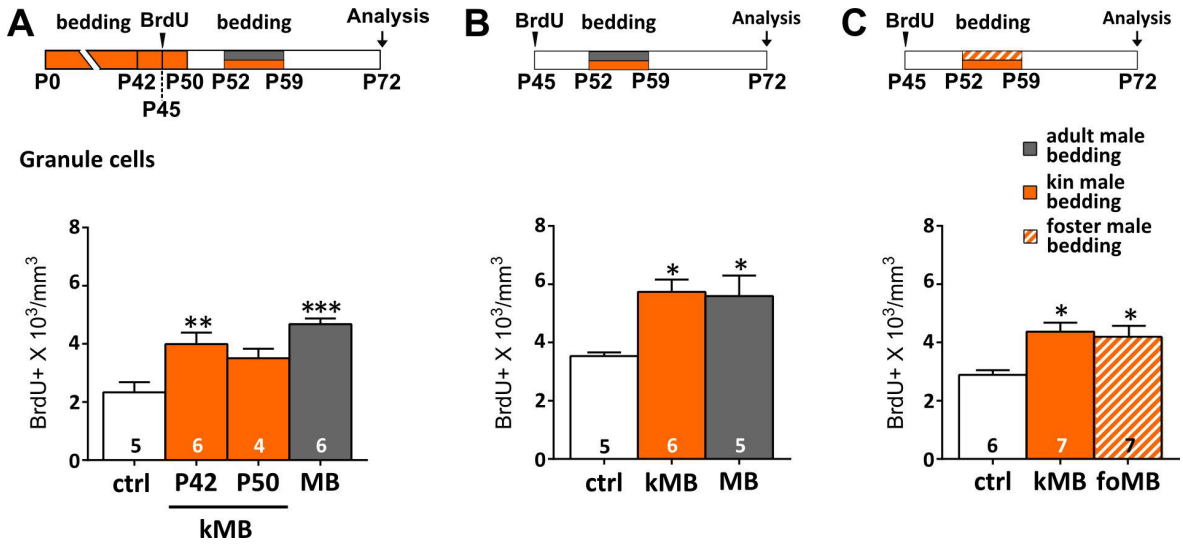



**Figure 3: Kinship does not prevent male odors to increase AOB granule cells. (A)** Long-term exposure (until P42) to kin-related male stimuli (kMB) increases AOB granule cells, after post-pubertal (P52-P59) exposure to the same stimuli, similarly to mature unrelated male cues (MB). By contrast, exposure to kMB cues until P50 does not increase AOB granule cells density (one-way ANOVA, $F_{3,19}$=10.47, *P*=0.000; Tukey's post hoc, ctrl *vs* P42 kMB, ***P*=0.008; ctrl *vs* P50 kMB, *P*=0.123, ctrl *vs* MB, ****P*=0.000). **(B)** Post-pubertal exposure to kin-related male stimuli (kMB) affects granule cell survival as do unrelated male odors (MB; one-way ANOVA, $F_{2,13}$=6.508, *P*=0.001; Tukey's post hoc, ctrl *vs* kMB **P*=0.02, ctrl *vs* MB, **P*=0.03). **(C)** Post-pubertal stimulation (P52-P59) to bedding material soiled by foster littermates (foMB) is as effective as bedding soiled by kin-related males (kMB) (one-way ANOVA, $F_{2,17}$=6.493; *P*=0.001; Tukey's post hoc, ctrl *vs* foMB **P*=0.024, ctrl *vs* kMB, *P=0.010). Sample size is indicated on each histogram bar. The values shown are the mean ± s.e.m.

## Differential responses of AOB circuits and newborn granule cells to familiar odor stimuli

Novel stimuli are shown to be most effective in promoting survival of newborn cells (Rochefort *et al.*, 2002). Once odor stimulations are repeated through familiarization, newborn cells in the OB have been shown to preferentially respond to familiar odors, showing higher levels of c-Fos expression than pre-existing neurons (Magavi *et al.*, 2005). Here, we reported similar effects occurring in the AOB (Figure 4A, B; Oboti *et al.*, 2011). By exposing female mice to different male individual odors, either previously experienced (one week odor familiarization) or novel, we evaluated odor-induced c-Fos expression in BrdU-positive GC neurons, when they are most responsive (i.e. around two weeks of age; Figure 4A; Oboti *et al.*, 2011). Not only did one week of

bedding exposure increase the amount of newborn GCs (BrdU-positive) in the AOB (Figure 4B), but also increased the percentage of those activated by familiar odors, as shown by a direct comparison between control group and familiarized females (c-Fos/BrdU co-expression; Figure 4C). Notably, c-Fos expression in AOB BrdU-positive cells was always associated to NeuN-positive/GAD67-positive inhibitory interneurons (Suppl. Fig.1). Interestingly, considering the entire AOB population, c-Fos activation in response to novel odors, in presence (unfamiliar) or absence (focal exposure) of previous familiarization, was always higher than the one evoked by either familiar odors or clean bedding (Figure 4D). This indicates that odor experience does not lead to a generalization in AOB responses to male cues but, instead, enhances the differences between different odor patterns, at least at the level of GCs (Figure 4E). Collectively, these results show that AOB immature GCs respond preferentially to familiar odor stimuli sensed during their integration into the local circuits, while the rest of the population shows increased responses to different novel stimuli, regardless of previous experience. Therefore, postpubertal exposure to male odors increases the number of GCs responding to male odor cues and promote their integration in specific circuits implied in male odor responses.

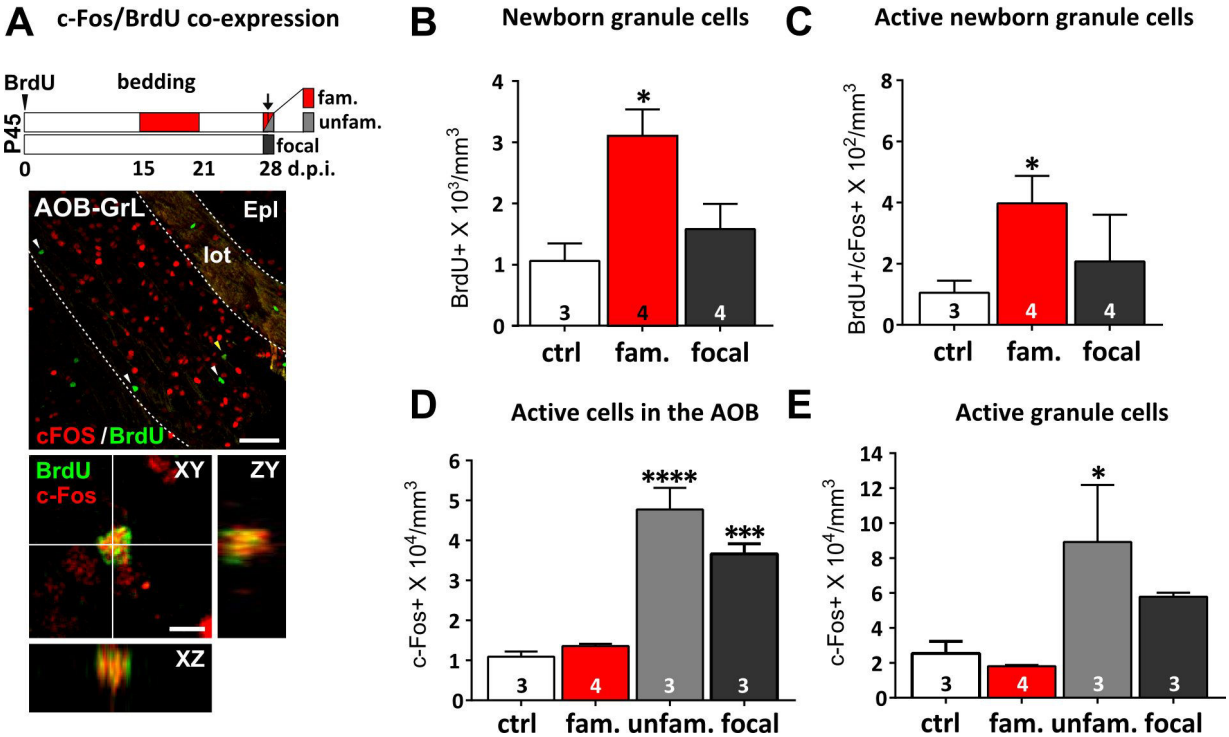


**Figure 4: Repeated exposure (familiarization) of females to male urine stimuli increases AOB newborn cell odor responses. (A)** On the top, protocol used for the analysis of AOB responsiveness to either familiar (fam; exposure to the same male bedding previously experienced from 15 to 21 d.p.i; red), unfamiliar (unfam; exposure to a different male bedding compared to the previously experienced; light gray) or focal odor exposures (without any previous experience; dark gray). Females were injected with BrdU at P45 and analyzed 28 d.p.i. On the bottom, co-expression of c-Fos (red) and BrdU (green) immunofluorescence in the AOB granule cell layer (GrL) measured 28 d.p.i. (scale bar 50µm). Arrowheads indicate c-Fos-negative/BrdU-positive cells. The yellow arrowhead indicates the c-Fos/BrdU double-stained cell magnified in the lower panel (scale bar 5µm). **(B)** The BrdU-positive cell density measured in the AOB granule cell layer increases after male odor familiarization (fam), but not focal odor stimulations (focal; one-way ANOVA, $F_{2,8}$=6.880, *P*=0.018; Tukey's post hoc, ctrl *vs* fam, **P*=0.021). **(C)** Odor-evoked activity (c-Fos expression) in the BrdU-positive cells is higher after exposure to familiar stimuli (red), compared to focal stimulations and to novel odors (dark gray; unpaired Student *t*-test, ctrl *vs* fam, **P*=0.046). **(D)** Evaluation of c-Fos expression in the entire cell population of the AOB after exposure of female mice to either familiar, unfamiliar or focal odor exposure (one-way ANOVA, $F_{3,9}$=40.12, *P*<0.0001; Tukey's post hoc, ctrl *vs* unfam *****P*=0.000, ctrl *vs* focal, ****P*=0.001). **(E)** This effect is evident even considering the granule cell layer only (one-way ANOVA, $F_{3,9}$=4.651, *P*=0.0315; fam *vs* unfam, Tukey's post hoc, **P*=0.032). Sample sizes are indicated on each histogram bar. The values shown are the mean ± s.e.m.

## VNO-dependent identity learning of male stimuli is displayed by females after puberty

Male urine odors sensed through the VNO exert higher activity levels in the female AOB, compared to female odors (Dudley *and* Moss, 1999; Kumar *et al*., 1999). In the AOB, neuronal survival was clearly affected by male odor stimuli at puberty, when these cues changed in composition (Figure 2D, E). However, the reasons why stimuli from adult males did not induce similar effects in the AOB of younger females were not clear. Since male cues are theoretically able to activate developing vomeronasal receptor neurons (Hovis *et al*., 2012; Oboti *et al*., 2015; Xia *et al*., 2006) and given that prepubertal hormone levels might not constrain eventual activity-dependent effects on AOB GC survival (Figure 1E), we hypothesized the presence of behavioral mechanisms limiting the contact of peripubertal female mice to adult male cues.

We tested this idea by first measuring the time spent by mixed housed female mice to investigate odors from both adult males and females, before (P20) and after (P41) puberty onset. While younger females spent more time investigating female odors (paired Student's *t*-test, *p*=0.029), after the onset of puberty this

preference is reversed, with P40 females displaying higher levels of investigation towards male cues (paired Student's *t*-test, $p$=0.025; Figure 5A). This implies that juvenile females might assign a different meaning to male odors, if compared to reproductively mature subjects. Therefore, it could be that male odor stimuli have an impact on neuronal survival in the AOB not only when they acquire a mature composition but also when they become meaningful mating signals. Since male odors promote female receptivity during mating (Roberts *et al*., 2010; Haga *et al*., 2010; Ramm *et al.*, 2008; Jemiolo *et al*., 1991), we addressed this point by evaluating the impact of peripubertal male odor exposure on female lordosis behavior towards male intruders. Female mice were exposed to male soiled bedding from P35 to P42, prior to behavioral tests. In general, juvenile females (P43-P45) showed low receptivity towards adult male intruders (Figure 5B-D and relative statistics in suppl. Table 1) as compared to adults, either in absence of any male stimuli, or after one week daily exposure to male urine, further supporting that at this age male odors do not represent mating signals promoting female receptivity. Nonetheless, given the role of AOB GCs in shaping mitral cell odor selectivity (Hendrickson *et al*., 2008), we hypothesized that the addition of new GCs to pre-existing AOB circuits might occur when the vomeronasal sensory neurons begins to tune their discriminative capabilities at puberty. In order to test this possibility, we quantified the female learned preference for individual male volatile odors: after repeated direct exposure to individual male chemostimuli, female mice prefer to investigate the volatile cues derived from the same male, if compared to other subjects. This selective preference is established through short-term experience of urine derived cues sensed through the vomeronasal pathway (Ramm *et al*., 2008; Moncho-Bogani *et al*., 2002; Oboti *et al*., 2014). Accordingly, we found that repeated direct exposure to male individual urine compounds induced in postpubertal females resulted in a higher interest towards the associated volatile stimuli, as compared to unexperienced odors (Figure 5E). Conversely, prepubertal exposure to male odors failed to elicit this effect in female mice (Figure 5E). However, exposure to mature female odors, had negligible effects in both juvenile and mature females (Figure 5F), indicating sex-specificity in this response. Overall, these results suggest that male stimuli used to establish male individual preference (or simply

discrimination of experienced individual odors) may not gain efficient access to the VNO sensory epithelium until more mature stages.

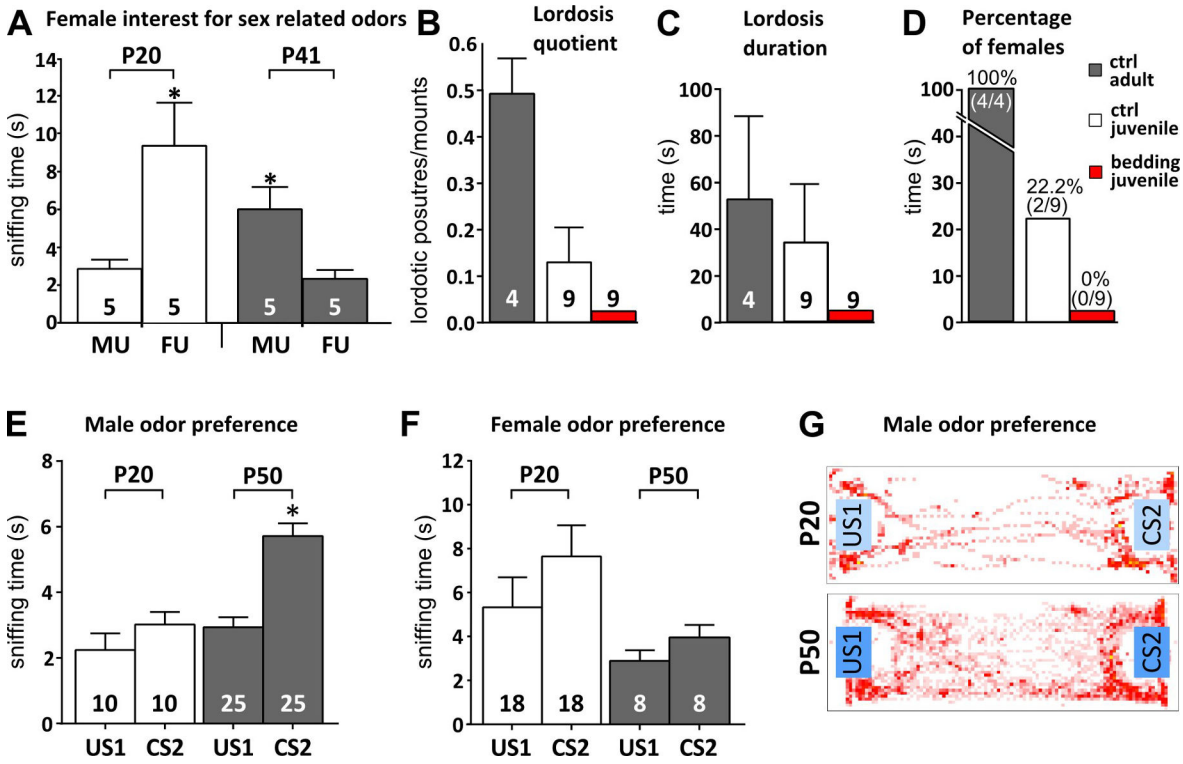



**Figure 5: Female mice learn to prefer familiar male odors after puberty**. **(A)** Reversal of female preference for sex odors (MU, male urine; FU, female urine) across puberty (P20 and P41; unpaired Student's *t*-test, P20 MU<FU, **P*<0.05; P41 MU>FU, **P*<0.05). **(B-D)** Evaluation of female receptivity (lordosis quotient, duration and percentage of females displaying lordosis) after peripubertal exposure to male soiled bedding compared to unstimulated controls (ctrl; B- one-way ANOVA, $F_{2,18}$=6.386, *P*=0.008; Tukey's post hoc, juv ctrl *vs* juv bed *P*=0.402, juv ctrl *vs* adult ctrl, *P*=0.048, juv bed *vs* adult ctrl, *P*=0.006; C- one-way ANOVA, $F_{2,18}$=1.672, *P*=0.217; Tukey's post hoc, juv ctrl *vs* juv bed *P*=0.383, juv ctrl *vs* adult ctrl, *P*=0.842, juv bed *vs* adult ctrl, *P*=0.244; D- chi-squared contingency test, $\chi^2$(2, N=22) = 14,16, *P*=0.008).Adult levels were calculated in P90 mature female mice. **(E)** Male odor preference in post-pubertal females after exposure to volatiles male cues. Volatile odors become preferred after repeated direct contact to non-volatile male stimuli (conditioned stimuli, CS) in respect to never experienced volatile odors (unconditioned stimuli, US). The graph shows an increase in preference for volatile male odors (CS2) after familiarization. Female mice develop this preference only after puberty (as P20 females show equal responses to CS and US stimuli; unpaired Student *t*-test, P50 CS2>US1, **P*<0.05). **(F)** Exposure to female odors in the same behavioral paradigm does not lead to preference learning. **(G)** Heatmaps representing the mouse presence in the cage during behavioral tests. Sample sizes are indicated on each histogram bar. The values shown are the mean ± s.e.m.

To clarify this point, we measured the access of male urine stimuli to the VNO of female mice at different peripubertal ages (P28, P35 and P52). To evaluate the extent up to which male cues reach the VNO epithelia in females, male urine was mixed with a fluorescent dye (rhodamine, 0.002%, Ogura *et al*., 2010). We found a positive correlation between rhodamine-based fluorescence and age (P28-P52), in both the VNO duct ($R^2$=0.726) and lumen ($R^2$=0.948), suggesting either an increased access or intake of male derived cues to the VNO sensory epithelia (no significant differences were evident using one-way ANOVA, see Supplementary Table 1; duct $F_{(2,14)}$=0.674, *P*=0.526; lumen $F_{(2,14)}$=0.609, *P*=0.558). At younger stages, rhodamine fluorescence was mainly present in the duct at the entrance of the VNO while at later stages, fluorescence was clearly visible across the whole extent of the inner lumen of the VNO (Figure 6A-D). We evaluated male urine intake bilaterally and analyzed rhodamine fluorescence in both VNOs (Figure 6B). In both the VNO duct and the lumen of the sensory epithelium, there was an increased tendency of rhodamine retention across puberty (by measuring fluorescence in arbitrary units ranged between 0 and 256, the maximum values were observed at older ages P35, P52), which is consistent with the role of the AOS in the detection of pheromones which coordinates reproduction. Overall these results suggest that the detection of male urine compounds through the VNO might increase during female puberty and therefore promote the addition of newborn GCs to AOB circuits involved in individual odor processing.

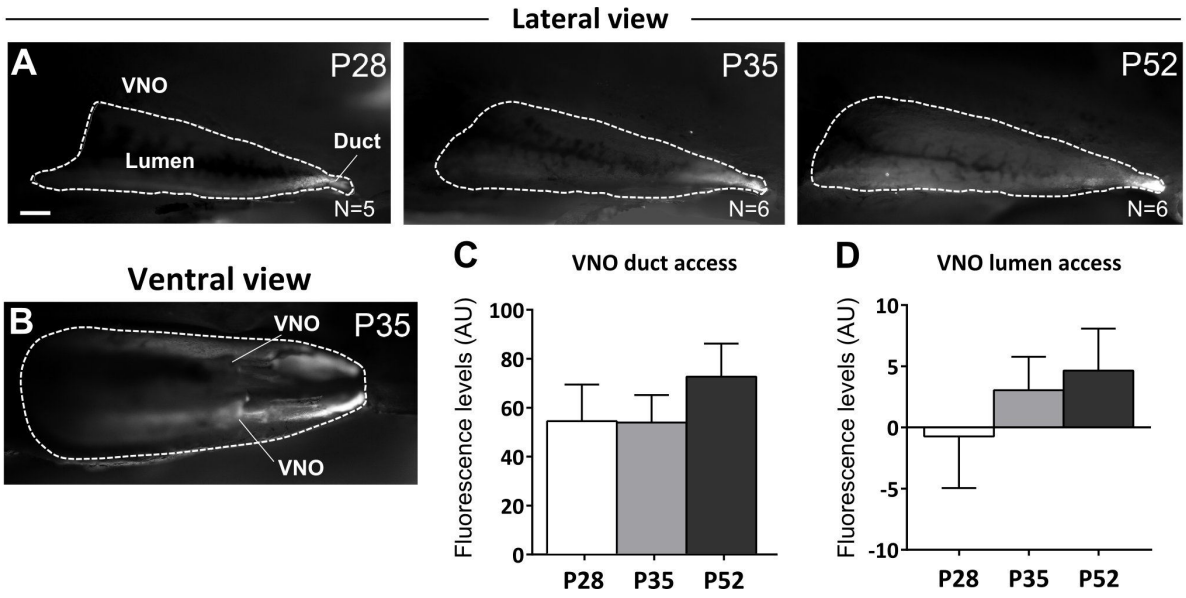



**Figure 6: VNO intake of male urine derived stimuli increases after puberty. (A-B)** Sagittal dissection of the nasal cavity showing rhodamine intake in the VNO upon nostril presentation of dyed-male urine to female mice at different postnatal ages (P28, P35, P52; A-lateral view, B-ventral view; scale bar 0.5mm). Fluorescent labeling inside the VNO indicates active stimulus intake in the VNO during investigation. **(C-D)** Fluorescence levels (AU), measured at the level of the VNO duct (C) and internal lumen (D) indicate appropriate stimulus delivery at all three postnatal ages (P28, P35, P52) and increased levels of stimulus intake (see text for major details). Abbreviations: AU, arbitrary units. The values shown are the mean ± s.e.m.

## Discussion

Previously, we have shown that survival of AOB inhibitory interneurons is modulated by sensory activity only in post-pubertal female mice (Oboti *et al.*, 2009, 2011). Here, we aimed to explore the physiological and behavioral parameters underlying the delayed sensitivity of granule cell (GC) survival to male urine odors. First, we show that male bedding exposure affects GCs at the onset of the first estrus cycle. Male stimuli produced by sexually mature males are more effective than stimuli from younger males. Moreover, the genetic identity of the donors does not affect GC survival, as kin-related and unrelated stimuli induced similar effects. The pubertal onset of GC sensitivity to male odor cues is unlikely mediated by hormonal changes occurring in females at this stage, although gonadal hormones might affect neuronal survival indirectly, by

regulating VNO pumping activity and sensory detection (Dey *et al*., 2015), or by modulating either cell proliferation or migration in the SVZ as previously reported (Larsen *et al*., 2008; Menezes *et al*., 1995; Mak *et al*., 2007; Tanapat *et al*., 1999). Conversely, this timing correlated with a higher intake of male signals by the female VNO, observed from the onset of puberty onwards, therefore directly linking the maturation of AOB inhibitory circuits to olfactory sensory activity. Consistently, after short-term exposure to male odors, newborn GCs showed higher odor specific responses. As a consequence of this experience, male cues become preferred over unfamiliar or novel stimuli (Ramm *et al*., 2008). Overall, our results indicate that the first brain relay of the vomeronasal system, the accessory olfactory bulb, is capable of rearranging its circuits by increasing the survival of newborn GCs in response to environmental stimuli once they become meaningful social signals during peripubertal development.

Puberty represents a phase in which the meaning of social signals is subjected to changes, due to the increased complexity of the behavioral contexts in which such signals are used (mating, territoriality, parental care). Because both the identity and the relative composition of social signals might be subject to contingent variation (Garratt *et al*., 2011; Sappington *and* Taylor 1990; Clark *et al*. 1997; Moore, 1997; Mucignat-Caretta *et al*., 1998), a reliable way to communicate information about social and reproductive status should imply a certain degree of adaptation in order to optimize the correspondence between chemical signals and behavioral response (Lemmen *and* Evenden, 2009; Villar *et al*., 2015). Neuronal turnover in other OB inhibitory networks has long been proposed to meet this demand by optimizing mitral cell odor representations (Alonso *et al*., 2006; Lepousez *et al*., 2014). Consistently, odor-evoked activity affects both the maturation and the integration of immature interneurons depending on the extent and value of odor experience (Lledo *et al*., 2006; Magavi *et al*., 2005; Rochefort *et al*., 2002; Schellino *et al*., 2016). Nonetheless, in most of the functional studies focused on the VNS, this plasticity has been often disregarded (but see for example Sakamoto *et al*., 2011, 2014; Imayoshi *et al*., 2008). The main reason for this is the misconception that animal social behaviors must be somehow constrained by the presence of highly conserved molecular signals and highly specialized olfactory receptors to which they bind.

In this scenario, increasing behavioral complexity necessarily requires a concurrent increase in the number of signals used to match behavioral needs to behavioral responses (for example adult sexual interactions are promoted by the peptide ESP1 and adult-juvenile interactions inhibited by ESP22; Haga *et al*., 2010; Ferrero *et al*., 2013). However, the presence of redundancy in both signal production and detection (Marino *et al*., 2000) provides evidence that single molecules alone cannot be reliable signals in social communication.

For instance, different molecules are used as puberty accelerating signals (DHT, DHB, MUPs: Mucignat-Caretta *et al*., 1995; Novotny *et al*., 1999a), signals for triggering aggression (MUPs, DHT, DHB: Chamero *et al*., 2007; Novotny *et al*., 1985) or signals for promoting female attraction (Darcin, ESP1, MUPs: Haga *et al*., 2010; Kimoto *et al*., 2007; Roberts *et al*., 2010; Oboti *et al*., 2014). Therefore, in the case of MUPs for example, the signaling properties depend probably more on the receiver's behavioral state, rather than on the specific ligand/receptor interactions.

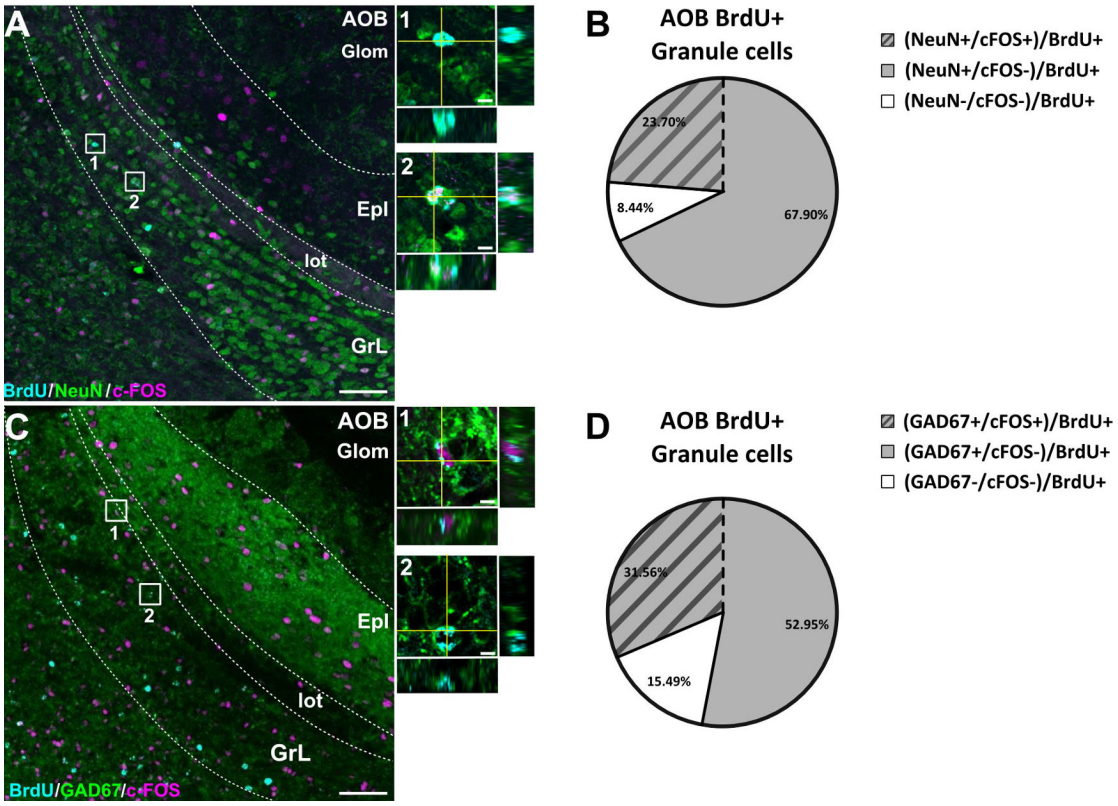
Given the high affinity of VNO receptors to their ligands, it would be both evolutionarily and metabolically expensive if this olfactory system were to maintain the potential to detect all potential ligands for its receptors. Our results show that the activation of AOB granules depends on the identity of experienced stimuli. Importantly, the inhibitory feedback they provide to AOB mitral cells is responsible for the highly selective responses shown by these output neurons to individual odors (Luo *et al*., 2003; Ben-Shaul *et al*., 2010; Hendrickson *et al*., 2008). Therefore, the activity dependent plasticity of AOB granule cells potentially represents a mechanism by which such odor specific responses are established through experience. Eventually, this further implies that social behavioral displays triggered by the vomeronasal system might involve a certain degree of adaptation to signal availability occurring through experience. However, future experiments are necessary to define both the contexts and the constraints of such plasticity.

Importantly, we do not want to argue that the VNO is not functional before puberty, as several reports indicated that prepubertal animals are indeed sensitive to stimuli detected through vomeronasal sensory neurons (Mucignat *et al*., 2004; Hovis *et al*., 2012; Lomas *and* Keverne, 1982; Mucignat-Caretta *et al*.,

1995; Nishimura *et al*., 1989; Novotny *et al*., 1999a,b; Price *and* Vandenbergh, 1992). Despite juvenile (pre-pubertal) female mice are probably able to sense male odorants through the VNO, they are likely to be less motivated to investigate them since male odors are repulsive for prepubertal females (Mucignat-Caretta *et al*., 1998). However, although these stimuli might affect the advancement of female puberty onset (Novotny *et al*., 1999a,b; Price *and* Vandenbergh, 1992; Lomas *and* Keverne, 1982), at this stage they might still be ineffective in promoting sexual receptivity, as shown by our behavioral assays. This is probably because, both behaviorally and physiologically, female mice might be still immature at this stage. In addition, it has been shown that the patency of the vomeronasal duct matures progressively during postnatal development and around puberty (Coppola *et al.*, 1993), suggesting the occurrence of changes in the general physiology of the VNS as well as in the female reproductive physiology, in a phase in which male chemical cues acquire the meaning of sexual signals utilized in the context of mating and reproductive behaviors. All these factors might be equally relevant in explaining the postpubertal sensitivity of AOB granule cell survival to sensory activity.

Overall, our results provide evidence in support of the pubertal activity-dependent tuning in sensory circuits conveying salient signals for mating and social behaviors, as proposed for higher order brain circuits as well (see for a review Piekarski *et al*., 2017). Moreover, they corroborate the idea that the functional maturation of the neural systems implied in goal-oriented behaviors might occur not only through modulatory mechanisms activated by hormones and neurotransmitters (see for example Anderson, 2016), but also through activity-dependent changes in the wiring of the underlying circuits (as shown also by Hovis *et al*., 2012; Jones *et al*., 2008).

## Supplementary informations




**Supplementary Figure 1. A)** Immunostaining of BrdU (cyan), NeuN (green) and cFos (magenta) immunofluorescence in the AOB granule cell layer (GrL) evaluated 28 d.p.i. (scale bar 50µm). Magnification panel 1 represents a c-Fos-negative/NeuN-positive/BrdU-positive granule cell and panel 2 a c-Fos-positive/NeuN-positive/BrdU-positive cell (scale bars 5µm). **B)** Percentage of newborn (BrdU) granule cells expressing NeuN (grey). Stripes indicate the percentage of newborn NeuN-positive cells activated (c-Fos-positive) by bedding exposure. **C)** Co-expression of BrdU (cyan), GAD67 (green) and cFos (magenta) immunofluorescence in the AOB granule cell layer (GrL) measured 28 d.p.i. (scale bar 50µm). Magnification panel 1 represents a c-Fos-positive/GAD67-positive/BrdU-positive granule cell and panel 2 a c-Fos-negative/NeuN-positive/BrdU-positive cell (scale bars 5µm). **D)** Percentage of newborn (BrdU) granule cells expressing GAD67 (grey). Stripes indicate the percentage of newborn GAD67-positive cells activated (c-Fos-positive) by bedding exposure. N=3 animals per group.

**Supplementary Table.** This table report all statistical tests performed in the experiments presented.

| FIGURE | *Experimental Paradigm* | GROUPS | STATISTICAL TEST | F value | P value |
|---|---|---|---|---|---|
| **1°** AOB Grl BrdU+ cells | *P28-P35 bedding exp* | ctrl *vs* MB | Unpaired Student's*t*-test | | *P*=0.669 |
| **1B** AOB Grl BrdU+ cells | *P35-P42 bedding exp* | ctrl *vs* MB | Unpaired Student's*t*-test | | *P*=0.016 |
| **1E** AOB Grl BrdU+ cells | *P35-P42 bedding exp* | SHAM ctrl *vs* SHAM MB *vs* OVX ctrl *vs* OVX MB | Two-way ANOVA | | |
| | | FACTORS | Ovariectomy | $F_{(1,20)}$=6.766 | *P*=0.017 |
| | | FACTORS | Stimulus | $F_{(1,20)}$=12.14 | *P*=0.002 |
| | | FACTORS | Interaction | $F_{(1,20)}$=0.520 | *P*=0.479 |
| | | SHAM ctrl *vs* SHAM MB | Tukey post hoc | | *P*=0.045 |
| | | SHAM ctrl *vs* OVX ctrl | Tukey post hoc | | *P*=0.587 |
| | | SHAM ctrl *vs* OVX MB | Tukey post hoc | | *P*=0.924 |
| | | SHAM MB *vs* OVX ctrl | Tukey post hoc | | *P*=0.002 |
| | | SHAM MB *vs* OVX MB | Tukey post hoc | | *P*=0.098 |
| | | OVX ctrl *vs* OVX MB | Tukey post hoc | | *P*=0.206 |
| **2A** AOB Grl BrdU+ cells | *P28-P35 bedding exp* | ctrl *vs* cMB | Unpaired Student's*t*-test | | *P*=0.614 |
| **2B** AOB Grl BrdU+ cells | *P35-P42 bedding exp* | ctrl *vs* cMB | Unpaired Student's*t*-test | | *P*=0.751 |
| **2C** AOB Grl BrdU+ cells | *P28-P45, P52-P59 bedding exp* | ctrl *vs* MB *vs* cMB | One-way ANOVA | $F_{(2,17)}$=13.76 | *P*=0.0003 |
| | | cMB *vs* MB | Tukey post hoc | | *P*=0.204 |
| | | ctrl *vs* MB | Tukey post hoc | | *P*=0.003 |
| | | ctrl *vs* cMB | Tukey post hoc | | *P*=0.000 |
| **2E** urine | | P14 *vs* P21 *vs* P28 *vs* P35 *vs* | Repeated measures Two- | | |

| | | | | | |
|---|---|---|---|---|---|
| protein content | | P42 *vs* P49 *vs* P56<br>Males *vs* Females | | way ANOVA | |
| | | | FACTORS | Postnatal days | $F_{(6,24)}$=15.67 *P*<0.0001 |
| | | | | Gender | $F_{(1,4)}$=5.737 *P*=0.075 |
| | | | | Interaction | $F_{(6,24)}$=3.306 *P*=0.016 |
| | *Males SDS-PAGE* | P14 *vs* P21 | | Bonferroni post hoc | *P*>0.999 |
| | | P14 *vs* P28 | | | *P*=0.366 |
| | | P14 *vs* P35 | | | *P*=0.001 |
| | | P14 *vs* P42 | | | *P*<0.0001 |
| | | P14 *vs* P49 | | | *P*<0.0001 |
| | | P14 *vs* P56 | | | *P*=0.004 |
| | | P21*vs* P28 | | | *P*>0.999 |
| | | P21*vs* P35 | | | *P*=0.003 |
| | | P21*vs* P42 | | | *P*<0.0001 |
| | | P21*vs* P49 | | | *P*<0.0001 |
| | | P21*vs* P56 | | | *P*=0.015 |
| | | P28 *vs* P35 | | | *P*=0.379 |
| | | P28 *vs* P42 | | | *P*=0.004 |
| | | P28 *vs* P49 | | | *P*=0.006 |
| | | P28 *vs* P56 | | | *P*>0.999 |
| | *Males SDS-PAGE* | P35 *vs* P42 | | | *P*>0.999 |
| | | P35 *vs* P49 | | | *P*>0.999 |
| | | P35 *vs* P56 | | | *P*>0.999 |
| | | P42 *vs* P49 | | | *P*>0.999 |
| | | P42 *vs* P56 | | | *P*=0.404 |
| **2E** urine protein content | | P49 *vs* P56 | | | *P*=0.526 |
| | *Females SDS-PAGE* | P14 *vs* P21 | | Bonferroni post hoc | *P*>0.999 |
| | | P14 *vs* P28 | | | *P*=0.976 |
| | | P14 *vs* P35 | | | *P*>0.999 |
| | | P14 *vs* P42 | | | *P*=0.113 |
| | | P14 *vs* P49 | | | *P*=0.026 |
| | | P14 *vs* P56 | | | *P*=0.236 |
| | | P21 *vs* P28 | | | *P*>0.999 |
| | | P21 *vs* P35 | | | *P*>0.999 |
| | | P21 *vs* P42 | | | *P*>0.999 |
| | | P21 *vs* P49 | | | *P*=0.407 |
| | | P21 *vs* P56 | | | *P*>0.999 |

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| **2E** urine protein content | | P28 *vs* P35 | | | *P*>0.999 |
| | | P28 *vs* P42 | | | *P*>0.999 |
| | | P28 *vs* P49 | | | *P*>0.999 |
| | | P28 *vs* P56 | | | *P*>0.999 |
| | | P35 *vs* P42 | | | *P*>0.999 |
| | | P35 *vs* P49 | | | *P*>0.999 |
| | | P35 *vs* P56 | | | *P*>0.999 |
| | | P42 *vs* P49 | | | *P*>0.999 |
| | | P42 *vs* P56 | | | *P*>0.999 |
| | | P49 *vs* P56 | | | *P*>0.999 |
| **3A** AOB Grl BrdU+ cells | *P0-P42/P50, P52-P59 bedding exp* | ctrl *vs* p42 kMB *vs* p50 kMB *vs* MB | One-way ANOVA | $F_{(3,19)}$=10.47 | *P*=0.0003 |
| | | ctrl *vs* p42 kMB | Tukey post hoc | | *P*=0.008 |
| | | ctrl *vs* p50 kMB | Tukey post hoc | | *P*=0.123 |
| | | ctrl *vs* MB | Tukey post hoc | | *P*=0.000 |
| | | p42 kMB *vs* p50 kMB | Tukey post hoc | | *P*=0.750 |
| | | p42 kMB *vs* MB | Tukey post hoc | | *P*=0.345 |
| | | p50 kMB *vs* MB | Tukey post hoc | | *P*=0.081 |
| **3B** AOB Grl BrdU+ cells | *P52-P59 bedding exp* | ctrl *vs* kMB *vs* MB | One-way ANOVA | $F_{(2,13)}$=6.508 | *P*=0.001 |
| | | ctrl *vs* kMB | Tukey post hoc | | *P*=0.015 |
| | | ctrl *vs* MB | Tukey post hoc | | *P*=0.028 |
| | | kMB *vs* MB | Tukey post hoc | | *P*=0.976 |
| **3C** AOB Grl BrdU+ cells | *P52-P59 bedding exp* | ctrl *vs* kMB *vs* foMB | One-way ANOVA | $F_{(2,17)}$=6.493 | *P*=0.001 |
| | | ctrl *vs* kMB | Tukey post hoc | | *P*=0.010 |
| | | ctrl *vs* foMB | Tukey post hoc | | *P*=0.024 |
| | | kMB *vs* foMB | Tukey post hoc | | *P*=0.903 |
| **4B** AOB Grl BrdU+ cells | *P60-P66 bedding exp* | ctrl *vs* fam *vs* focal | One-way ANOVA | $F_{(2,8)}$=6.880 | *P*=0.018 |
| | | ctrl *vs* fam | Tukey post hoc | | *P*=0.021 |
| | | ctrl *vs* focal | Tukey post hoc | | *P*=0.664 |
| | | fam *vs* focal | Tukey post hoc | | *P*=0.056 |
| **4C** AOB Grl BrdU/cFos cells | *P60-P66 bedding exp* | ctrl *vs* fam *vs* focal | One-way ANOVA | $F_{(2,8)}$=1.623 | *P*=0.256 |
| | | ctrl *vs* fam | Tukey post hoc | | *P*=0.250 |
| | | ctrl *vs* focal | Tukey post hoc | | *P*=0.820 |
| | | fam *vs* focal | Tukey post hoc | | *P*=0.474 |
| | | ctrl *vs* fam | Student *t*-test | | *P*=0.046 |
| **4D** AOB cFos cells | *P60-P66 bedding exp* | ctrl *vs* fam *vs* unfam*VS* focal | One-way ANOVA | $F_{(3,9)}$=40.12 | *P*<0.0001 |
| | | ctrl *vs* fam | Tukey post hoc | | *P*=0.901 |
| | | ctrl *vs* unfam | Tukey post hoc | | *P*<0.0001 |
| | | ctrl *vs* focal | Tukey post hoc | | *P*=0.001 |
| | | fam *vs* unfam | Tukey post hoc | | *P*<0.0001 |

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| | | fam *vs* focal | Tukey post hoc | | *P*=0.001 |
| | | unfam *vs* focal | Tukey post hoc | | *P*=0.093 |
| **4E** AOB Grl cFos cells | *P60-P66 bedding exp* | ctrl *vs* fam *vs* unfam *vs* focal | One-way ANOVA | $F_{(3,9)}$=4.651 | *P* =0.032 |
| | | ctrl *vs* fam | Tukey post hoc | | *P*=0.984 |
| | | ctrl *vs* unfam | Tukey post hoc | | *P*=0.074 |
| | | ctrl *vs* focal | Tukey post hoc | | *P*=0.500 |
| | | fam *vs* unfam | Tukey post hoc | | *P*=0.032 |
| | | fam *vs* focal | Tukey post hoc | | *P*=0.291 |
| | | unfam *vs* focal | Tukey post hoc | | *P*=0.525 |
| **5A** sniffing time (s) | *Female sex odors preference* | P20 FU > MU | Paired Student's*t*-test | | *P*=0.03 |
| | | P41 FU < MU | Paired Student's*t*-test | | *P*=0.03 |
| **5B** lordotic postures/ mounts | *Lordosis quotient* | juv ctrl *vs* juv bed *vs* adult ctrl | One-way ANOVA | $F_{(2,18)}$=6.386 | *P*=0.008 |
| | | juv ctrl *vs* juv bed | Tukey post hoc | | *P*=0.402 |
| | | juv ctrl *vs* adult ctrl | Tukey post hoc | | *P*=0.048 |
| | | juv bed *vs* adult ctrl | Tukey post hoc | | *P*=0.006 |
| **5C** time(s) | *Lordosis duration* | juv ctrl *vs* juv bed *vs* adult ctrl | One-way ANOVA | $F_{(2,17)}$=1,672 | *P*=0.217 |
| | | juv ctrl *vs* juv bed | Tukey post hoc | | *P*=0.383 |
| | | juv ctrl *vs* adult ctrl | Tukey post hoc | | *P*=0.842 |
| | | juv bed *vs* adult ctrl | Tukey post hoc | | *P*=0.244 |
| **5D** Lordosis expression | *Percentage of females showing lordosis* | juv ctrl *vs* juv bed *vs* adult ctrl | Chi-square | $\chi^2$(2, N=22) = 14,16 | *P*=0.0008 |
| **5E** sniffing time (s) | *Male odor preference* | P20 US1 = CS2 | Unpaired Student's*t*-test | | *P*>0.05 |
| | | P20 US1 < CS2 | Unpaired Student's*t*-test | | *P*<0.05 |
| **5F** sniffing time (s) | *Female odor preference* | P20 US1 = CS2 | Unpaired Student's*t*-test | | *P*>0.05 |
| | | P20 US1 = CS2 | Unpaired Student's*t*-test | | *P*>0.05 |
| **6C** fluorescence levels (AU) | *VNO Duct* | P28 *VS* P35 *VS* P52 | One-way ANOVA | $F_{(2,14)}$=0.674 | *P*=0.526 |
| | | P28 *VS* P35 | Tukey post hoc | | *P*=0.999 |
| | | P28 *VS* P52 | Tukey post hoc | | *P*=0.615 |
| | | P35 *VS* P52 | Tukey post hoc | | *P*=0.568 |

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| **6D** fluorescence levels (AU) | *VNO Lumen* | P28 *VS* P35 *VS* P52 | One-way ANOVA | $F_{(2,14)}$=0.609 | *P*=0.558 |
| | | P28 *VS* P35 | Tukey post hoc | | *P*=0.731 |
| | | P28 *VS* P52 | Tukey post hoc | | *P*=0.539 |
| | | P35 *VS* P52 | Tukey post hoc | | *P*=0.939 |
| **6C** fluorescence levels (AU) | *VNO Duct* | | Regression analysis | | $R^2$=0.726 |
| **6D** fluorescence levels (AU) | *VNO Lumen* | | Regression analysis | | $R^2$=0.948 |

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# CHAPTER III

# Unraveling a Link Between the GnRH System and Adult Neural Plasticity


## Abstract

The occurrence of adult neurogenesis (AN) in the mouse Subventricular Zone (SVZ)- Olfactory System (OS) and in the Dentate Gyrus of hippocampus (DG) gives a unique opportunity to investigate mechanisms/functions of neural plasticity in the adult brain. Social cues, modulate the process of AN in both adult neurogenic niches and elicit neuroendocrine responses and primary motivated behaviours. In turn, gonadal hormones can impact on the modulation of AN. In this context, we found that the pheromonal-dependent modulation of AN starts around puberty, a developmental critical period characterized by increased activity of the gonadotropin releasing hormone (GnRH) secretion, which controls the hypothalamic-pituitary-gonadal axis (HPG). We argue that the process of AN could be mediated, at least in part, by the GnRH System, through direct and/or indirect mechanisms. Accordingly, we found the occurrence of GnRH-immunoreactive (-ir) fibers and cells in the OB, and GnRH-receptor expression in both the OB and the DG. Furthermore, using transgenic mice that lose GnRH expression during postnatal development (*GnRH::Cre;Dicer$^{loxP/loxP}$ mice*) we found that a postnatally acquired dysfunction in the GnRH System affects the process of AN at different steps (i.e. proliferation and survival/integration) in the two neurogenic systems and in a dimorphic way. Finally, in *GnRH::Cre; Dicer$^{loxP/loxP}$* mice we found defects in olfactory discrimination of both social and non-social cues, further supporting a link between GnRH system and olfactory function.


## Introduction

The reproductive behaviour in mammals is controlled by the neuroendocrine circuits of the so-called HPG axis, which involves the hypothalamus (H), the pituitary (P), and the gonads (G). In turn, the activity and

function of the HPG axis is influenced by external and internal cues, which are sensed by neural pathways (e.g. olfactory system) featured by high levels of neural plasticity. Recent data indicate that the accomplishment of specific reproductive behaviors is achieved through a reciprocal crosstalk between the HPG circuits/factors and neural plasticity, which is necessary to integrate external and internal stimuli (Mak *et al*., 2007; Oboti *et al*., 2011; Brus *et al*., 2016; Schellino *et al*., 2016). Nevertheless, the molecular and anatomical bases of this complex integration remain still largely vague. The HPG axis is governed by the Gonadotropin-Releasing Hormone (GnRH) neurons, which are mostly located in the preoptic area (POA) of the hypothalamus. GnRH neurons are the final output of the brain controlling reproduction and they can sense peripheral signals that in turn modulate GnRH secretion (Roa, 2013). Thanks to the privileged position and characteristics of these neurons, they could be considered as the best candidate to relay plasticity in the adult brain to reproduction.

Adult Neurogenesis (AN), a striking form of brain plasticity, is restricted to two main areas: the subventricular zone (SVZ) of the forebrain, from where neuroblasts migrate along the so-called "rostral migratory stream" (RMS) to reach the main (MOB) and the accessory olfactory (AOB) bulb, and the dentate gyrus of hippocampus (DG; Alvarez-Buylla *and* Garcìa Verdugo, 2002; Ming *and* Song, 2005; Lledo *et al*., 2006; Gheusi *et al*., 2013; Bonfanti *et al*., 1997; Peretto *et al*., 2001; Oboti *et al*., 2009, 2011). Multiple experimental evidences have shown that social stimuli involved in the control of reproductive physiology (i.e. pheromones), enhance AN in both neurogenic niches (Mak *et al*., 2007; Larsen *et al*., 2008; Feierstein *et al*., 2010; Oboti *et al*., 2009; 2011). In turn, newborn neurons seem to optimize/adapt the function of brain circuits underlying social/reproductive behaviors through a complex mechanism, which implies multimodal integration and coordinated activity of the brain with the endocrine system (Feierstein, 2012; Larsen *and* Grattan, 2012; Peretto *et al*., 2014). It is well known that in rodents AN in both the OB and the DG is regulated by gonadal hormones in a sex- and experience- dependent way (Brock *et al*., 2010; Veyrac *and* Bakker 2011; Galea *et al*., 2013; McClure *et al*., 2013; Chan *et al*., 2014; Farinetti *et al*., 2015). Moreover, gonadal hormones modulate sensory inputs

(e.g. olfactory, pheromonal) to produce state- specific reproductive behaviors (Halem *et al*., 1999; Dey *et al*., 2015). These data indicate that AN and the endocrine system cooperate to adapt the organism to different environmental stimuli. In this context, we have recently demonstrated that the pheromonal-dependent modulation of adult neurogenesis (AN) starts around puberty, a developmental critical period characterized by increased activity of GnRH secretion (Oboti *and* Trova, 2017). One important open question is whether and how the GnRH system, which controls the release of gonadal hormones, can exert its influence on sensory activity modulating neural plasticity, and specifically the process of AN in the OB and the DG.

According to these questions in the present work we investigated the possible role of GnRH system in the modulation of olfactory cues eliciting reproductive behaviors and its direct/indirect impact on AN in both neurogenic niches. To address this issue, we firstly characterized the presence of GnRH cells and fibers in the OS and the expression of the GnRH and gonadal hormones receptors in both neurogenic systems. Moreover, we investigated the process of AN in a mouse model with impaired GnRH secretion, the *GnRH::cre;Dicer*$^{loxP/loxP}$ mice (Messina *et al*., 2016). These animals are characterized by hypogonadotropic hypogonadism and infertility, resulted from an impairment in microRNA synthesis into GnRH neurons (Messina *et al*., 2016). Interestingly, in these mice, GnRH deficiency is not due to a developmental lack of GnRH, but it is acquired postnatally, during the pubertal period, thus allowing to specifically evaluate GnRH deficiency during this critical period in which animals acquire reproductive competencies.

## Materials and Methods

### Animals

All animals were group-housed under specific pathogen-free conditions in a temperature-controlled room (21-22 °C) with a 12-h light-dark cycle and *ad libitum* access to food and water. C57Bl/6J *GnRH::cre*(Tg(*Gnrh1::cre*)1Dlc), C57Bl/6J *Dicer*$^{LoxP/LoxP}$ and C57Bl/6J GnRHR:IRES-Cre (GRIC)/eR26-$_T$GFP were a

generous gift of Dr. Catherine Dulac (Howard Hughes Medical Institute, Cambridge MA), Dr. Brian Harfe (University of Florida, FL) and Dr Ulrich Boehm (University of Saarland School of Medicine, D-66421 Homburg, Germany), respectively.

Animal studies were approved by the Institutional Ethics Committees for the Care and Use of Experimental Animals of the Universities of Lille, Cordoba and Turin; all experiments were performed in accordance with the guidelines for animal use specified by the European Union Council Directive of September 22, 2010 (2010/63/EU). The sex and the number of the animals used is specified in the text, figures and/or figure legends, except for real time-PCR analyses and for experiments assessing GnRHR and GnRH expression, in which animals of both sexes were used. Investigators were blind to the genotype or treatment group of animals. Heterozygous *Gnrh::cre;Dicer*$^{LoxP/+}$ littermates were excluded from the analyses. Experiments were designed to minimize the number of animals used.

### 5-Bromo-2′-deoxyuridine (BrdU) treatment

To identify newly generated cells in the AOB, MOB and DG, GnRH::Cre/Dicer$^{loxP/loxP}$ and Dicer$^{loxP/loxP}$ (controls) mice were intraperitoneally injected with BrdU in 0.1 M Tris (pH 7.4) twice a day (delay = 8 h, 100 mg/kg body weight) and killed 28 days later to evaluate neuronal survival.

### Tissue preparation for immunohistochemistry

Mice were deeply anesthetized via an intraperitoneal injection of a 3:1 ketamine (Ketavet; Gellini, Italy) and xylazine (Rompun; Bayer, Germany) solution. All the animals were transcardially perfused with a 0.9% saline solution followed by cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed from the skull and post-fixed for 4–6 h in 4% PFA at 4 °C. Post-fixing was followed by a cryopreservation step with a 30% sucrose solution in 0.1 M PB pH 7.4 at 4 °C. The two hemispheres were separated and embedded in OCT (Sakura Finetek, CA, USA) and then frozen and cryostat sectioned. Free-floating parasagittal and coronal sections (30 µm) were collected in multi-well dishes to provide representative series of the AOB and

MOB/SVZ/DG, respectively. The sections were stored at − 20 °C in an antifreeze solution (30% ethylene glycol, 30% glycerol, 10% PB: 189 mM NaH2PO4, 192.5 mM NaOH; pH 7.4) until use.

## Immunohistochemistry

Sections were rinsed in PBS to remove the antifreeze solution and incubated for 24 h at 4 °C in primary antibodies diluted in 0.01 M PBS, pH 7.4, 0.5% Triton X-100, and 1% normal sera that matched the host species of the secondary antibodies. The following primary antibodies were used: anti-Ki67, rabbit IgG polyclonal, dilution 1:1000, abcam (ab15580); anti-DCX, goat IgG polyclonal, dilution 1:2000, Santa Cruz Biotechnology (sc-8066); anti-BrdU, rat IgG monoclonal, dilution 1:5000 (ABC) AbD serotec, Bio-Rad Laboratories (OBT0030CX) (Liu et al., 2009); anti-GnRH, guinea-pig, dilution 1:10000, produced by Dr Erik Hrabovszky (Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary) (EH#1018); anti-GFP, chicken, dilution 1:1000, Aves Lab (GFP-1020); anti-TH, mouse, dilution 1:3000, Immunostar (22941); anti-cFos, rabbit IgG polyclonal, dilution 1:8000, Santa Cruz biotechnologies (sc5211) and anti-NCAM, mouse, dilution 1:1000, Sigma (C9672). For BrdU immunostaining, the sections were pre-treated with 2N HCl for 30 min at 37 °C for antigen retrieval and neutralized with borate buffer, pH 8.5, for 10 min. For the avidin-biotin peroxidase method, the sections were incubated for 1 h at room temperature in a biotinylated secondary antibody (anti-rat IgG; Vector Laboratories) diluted 1:250 in 0.01 M PBS, pH 7.4, followed by incubation with the avidin-biotin-peroxidase complex (Vector Laboratories). To reveal immunoreactivity, we used 0.015% 3,3′ -diaminobenzidine and 0.0024% $H_2O_2$ in 0.05 M Tris-HCl, pH 7.6. After adhesion on gelatin-coated glass slides, sections were mounted in DPX (Merck-Millipore, VWR International PBI, Milan, Italy). For immunofluorescence double-staining, the sections were incubated in a mixture of primary antibodies and appropriate blocking sera for 48 h at 4 °C, then incubated with appropriate fluorochrome-conjugated secondary antibodies for 1,5 h at Room Temperature (RT). Secondary antibodies were used as follow:

anti-rabbit and anti-mouse 647-conjugated (1:600; Jackson ImmunoResearch); anti-goat and anti-chicken 488-conjugated (1:400; Jackson ImmunoResearch); anti-guinea pig Cy3-conjugated (1:800; Jackson ImmunoResearch). The sections were then coverslipped with the anti-fade mounting medium Dabco (Sigma) and/or Mowiol and analysed with a laser scanning LAS AF Lite confocal system (Leica Microsystems).

### Cell counting and statistical analysis

Cell counting and image analysis were performed on either a Nikon microscope coupled with a computer-assisted image analysis system (Neurolucida software, MicroBrightField) and Leica DM600 CS LAS AF Lite confocal microscope (Leica Microsystems). Confocal image z-stacks were captured through the thickness of the slice at 1-µm optical steps and used for double cell counts. Brightness, colour, and contrast were balanced and assembled into panels with Inkscape (Free vector graphics editors). All cell counts were performed blind to the genotype and/or the gender. The number of Ki67-positive nuclei and double-labelled Ki67-positive/DCX-positive cells was evaluated in the SVZ and in the hippocampal DG; the number of DCX-positive cells was established only in the DG. Three representative coronal sections (anterior, medial, posterior) per animal were choose for both the SVZ and DG, respectively, and counted in a one-in-six series sections (30µm thickness) through a 40x objective. Cell number quantification was performed by using the ImageJ software. In the MOB, the number of BrdU-positive nuclei was established by counting peroxidase/DAB-stained nuclei in 3 representative OB coronal (anterior, medial and posterior) sections (30µm thickness) per animal in the granular cell layer, by using a systematic random sampling method. Sampling was conducted by overlaying each section with a virtual counting grid (squares size 80 x 80 µm). Cells were counted through the 30-µm thickness of the slice in one square of the grid (one of every two) through sequential translation of the counting frame until the area of interest was entirely covered (40x objective). This procedure allowed us to analyse about one-fourth of the area of interest. Cell density (number of labelled profiles/$mm^3$) was calculated by multiplying

cells x 4 and by multiplying the area measurements by the mean section thickness (30 μm) [Σ of sampled areas $\mu m^2$x25μm]. In the AOB, the number of BrdU-positive nuclei was established by counting peroxidase/DAB-stained nuclei in 7 para-sagittal (representing the entire AOB) sections (30μm thickness) per animal in the granular cell layer. In the hippocampal DG, the number of BrdU-positive nuclei was established by counting peroxidase/DAB-stained nuclei in 8 coronal (representing the entire DG) sections (30μm thickness) per animal, and counted in a one-in-six series sections through a 40x objective. The number of BrdU-positive cells was multiplied by 6 to provide an estimate number of positive cells. For both the AOB and the DG, the area of each examined section was measured by using the Neurolucida software (MicroBrightField, Colchester, VT, United States). Cell densities were calculated by summing cell counts made on all sections per animal and referred to the proper cellular layer volume (6 or 8 of sampled areas x 30μm). Numbers of animals used for each experiment is indicated in histogram bars in each table. Counts were performed blind to the treatment.

For statistical analysis, unpaired Student *t*-test and one-way ANOVA were used for 1:1 comparison and multiple comparisons for parametric data of Real Time PCR analyses, respectively. Two-way ANOVA was used for proliferation and survival analyses (with genotype and gender as main factors). Paired Wilcoxon-Mann-Whitney was used for the habituation/dishabituation test (for hab4 *vs* dishab comparison) and for the olfactory preference test. Unpaired Wilcoxon-Mann Whitney was used for the comparison of dishab WT *vs* dishab KO in the habituation/dishabituation test.

### 3D Reconstruction

For 3D reconstructions, one brain of a WT male mouse was serially coronally sectioned (40 μm thickness) and immunolabeled for anti-GnRH and DAPI. For the reconstruction of the GnRH cells and fibers in the olfactory bulbs, 50 consecutive sections were digitally captured and reciprocally aligned using TrakEM2 ImageJ plugin. Each slice was aligned to the next by using the DAPI staining, and then only GnRH fibers and cells were rendered visibles in the Movie1, 2 and in Fig.1.

## Real Time PCR analysis

For gene expression analyses, OBs were extracted and fresh collected from males (n=4) and females (n=16). OBs were then sectioned with a vibratome (400µm section thickness) and the MOB and the AOB were dissected and dissociated. Total mRNA was extracted from MOB and AOB separately by using the RNeasy SuperScript® Lipid Tissue Mini Kit – 74804 (QIAGEN) and reverse transcribed using SuperScript® III Reverse Transcriptase (Life Technologies). A linear preamplification step was performed using the TaqMan® PreAmp Master Mix Kit protocol (Applied Biosystems). Real-time PCR was carried out on a StepOnePlus TM Real Time-PCR System (Applied Biosystems) using the following exon-boundary-specific TaqMan® Gene Expression Assays (Applaied Biosystems): GnRHR (Mm00439143_m1), Er1 (Mm00433149_m1), Er2 (Mm00599821_m1), Ar (Mm00442688_m1) and ProgR (Mm00435626_m1). Quantitative real-time PCR was performed using TaqMan Low-Density Arrays (Applied Biosystems) on an Applied Biosystems 7900HT thermocycler using manufacturer's recommended cycling conditions. Gene expression data were analysed using ExpressionSuite Software v1.01.

## Habituation/dishabituation test

Odour habituation/dishabituation was used to measure MOE-mediated olfactory discrimination (de Chevigny et al., 2006; Breton-Provencher, 2009). Briefly, mice were familiarized with a first odour (habituation odour) in four successive sessions and then exposed once to a novel odour (dishabituation odour). Each session was 1 min long and was followed by a 10-min inter-session interval. Olfactory discrimination was analysed using two different odours, acetophenone (00790, Sigma) and octanal (05608, Sigma), which were diluted $10^{-3}$ % in mineral oil (M3516, Sigma) and delivered via patches of filter paper. Twenty microliters of odour were applied to each filter paper, which was then paced in a petri dish. The two odours were both alternatively tested as novel stimuli (during dishabituation) in separate sessions. Mouse sniffing activity was quantified using by BORIS software (Friard *and* Gamba, 2016).

### Olfactory preference test

For all behavioural tests, the animal subjects were coded so that the investigator was blind to the genotype of each animal. During the social olfactory preference test assessment, the WT ($Dicer^{loxP/loxP}$) and the KO (GnRH:cre/$Dicer^{loxP/loxP}$) male and female mice were first exposed to urine from an adult C57BL/6J WT stud male and an oestrus female for 30 min. Then, after 30 min in clean bedding, the mice were presented with the same urine samples, which were delivered on a piece of filter paper placed within a petri dish (enabling direct contact with the source). Male and female urine samples were placed on opposite sides of the cage, equidistant from the cage walls. Trials lasted 10 min, during which mouse behaviour directed towards the two urine sources was recorded. The amount of time spent sniffing the petri dish in which a test urine sample was presented was used as an indication of the mouse's interest gaining further information from a scent source. The tests were conducted in the animals' home cages to minimize both manipulation and exposure to external stimuli. For each test, approximately 50µl of either male or female urine were put on each piece of filter paper. Each session was video-recorded, and the time the animals spent sniffing the urine source was subsequently measured by BORIS software (Friard *and* Gamba, 2016).

## Results

### Anatomical and functional characterization of GnRH-ir elements in the olfactory bulb of adult mice

A recent work (Casoni *et al*., 2016) showed that in mice and humans during embryonic development GnRH cells besides reaching the hypothalamus migrate also into several extra-hypothalamic brain regions. In adult mice, it has been shown that one of these populations of GnRH-ir cells is located in the olfactory bulb (OB), organized as a peculiar ring-like structure that surrounds the bulbs. GnRH-ir cells were also described within the OB and in its medial dorsal portion, which corresponds to the AOB (Casoni *et al.*, 2016). Starting from these data in order to get more information on this population, we performed a 3D reconstruction analysis on the whole OB that was cut into 40µm thick coronal

slices. This approach allowed us to get a very precise information about the distribution of all GnRH-ir cells and fibers. Our results clearly showed that the ring of GnRH ir- elements around the OBs is mostly formed by a system of immunopositive fibers, and that this feature is evident in both male and female adult mice (Fig. 1 and Movie 1, Movie 2). Only a few GnRH-ir fibers were visible in the rostral part of the bulb, while they significantly increased in its caudal extent. Interestingly, in this OB region, the GnrH-ir fibers were found associated to both the olfactory and vomeronasal nerve layers (ONL/VNL; Fig. 2C), with many of them entering inside the AOB (Fig. 1A,B,D').

Besides fibers, we also identify OB GnRH-ir cells organized as small clusters, which show a very stable position in both male and female mice. Two main clusters were identified: one located in the rostral part of the OB (Bregma +3.85; Fig. 1C-C'), and one (larger) in the caudal part of the bulb (Bregma +2.62; Fig. 1E-E'). Moreover, isolated (individual) GnRH-ir cell bodies were found scattered from the rostral to caudal extent of the OB (Fig. 1D, 2A-A'), along with the MOB glomerular (GL) and the olfactory nerve layer (ONL). Some of these isolated cells were also visible in the AOB (Fig. 3B,B'). A preliminary quantification of the GnRH-ir cells in the OBs has revealed 209±23 cells in the female OBs (n=4) and 156 cells in the male OBs (n=1), with 6/7 clusters of GnRH-ir cells in both sexes.

Considering that most of the OBs GnRH-ir cells is located around the olfactory glomeruli, we examined whether these cells co-express markers of periglomerular (PGC) neurons. PGC cells are subdivided in diverse (phenotypic and functional) cell populations (Kosaka *et al*., 1995). We focused on two mayor PGC cell types: the calbindin-ir cells and the tyrosine hydroxylase (TH) expressing cells. TH is the rate-limiting enzyme for dopamine (DA) synthesis, which is strongly expressed in the MOB GL by type 1 PGC interneurons. These latter cells represent an important category of interneurons since have direct synaptic connections with the olfactory nerves (Kiyokage *et al*., 2010; Kosaka *et al*., 2007), and are likely involved in mediating salient olfactory stimuli such as those priming the reproductive activity (Serguera *et al*., 2008). Although we did not identify co-expression between GnRH and Calbindin or TH, we found close appositions between GnRH-ir fibers and TH-ir cells (Fig. 2A-A', B-B'). Close appositions were also found between GnRH-ir and olfactory nerve fibers,

identified by using the Neural Cell Adhesion Molecule (NCAM) as a marker of the olfactory nerve layer (Fig. 2C; Kafitz *and* Greer, 1998). Taken together these data suggest that the OBs GnRH cell population could be functionally involved in olfactory sensory perception.

To test this hypothesis, we firstly evaluated in females whether the OBs GnRH-ir cells express cFos, a marker of neuronal excitation (Morgan *et al*., 1987; Kovacs *et al*., 2008), after male pheromonal exposure. Indeed, exposure to opposite-sex cues are known to increase cFos in neurons involved in sensory perception (Oboti *et al*., 2009; 2011; Schellino *et al*., 2016). Although this stimulation significantly increased cFos-expression (in comparison to non-stimulated animals) in both MOB and AOB neurons (not shown) none of the OBs GnRH-ir cells were found cFos immunopositive (Fig. 2D). This result does not completely exclude a possible involvement of this cell population in olfactory sensory perception, since previous data on the hypothalamic GnRH neurons, known to integrate pheromonal cues (Boehm *et al*., 2005), already showed lack of cFos expression after exposure to opposite-sex pheromones (Taziaux *and* Bakker, 2015).

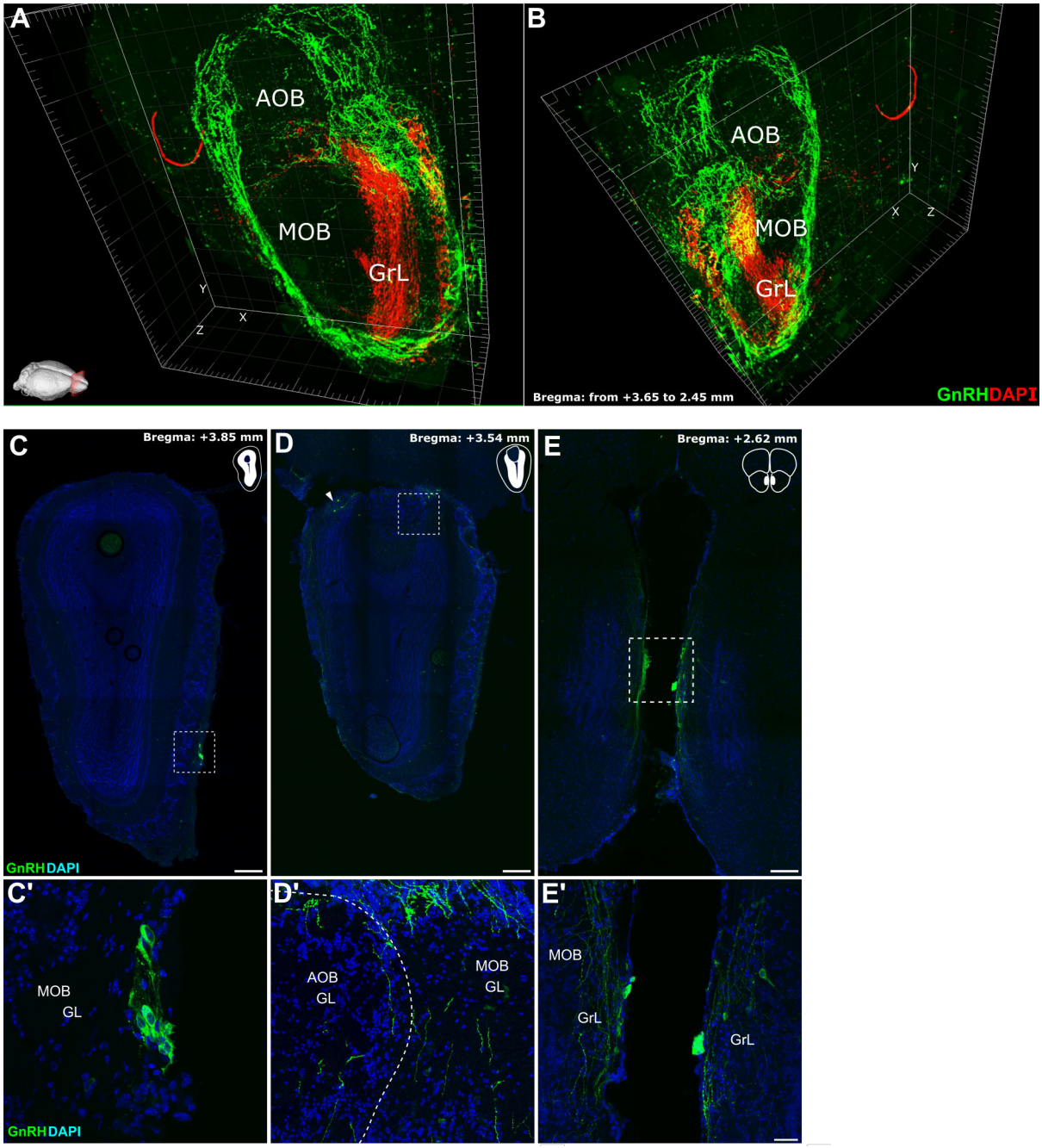


**Figure 1. GnRH-ir fibers in the Olfactory Bulb**

**A-B.** 3D reconstruction (1.2mm of thickness) of GnRH-ir fibers (green) around the olfactory bulb. Two orientations are depicted, from the front (A) and from the top (B). The reconstructed tissue corresponds to the trunk of the bulb (see drawing in A and coordinates in B). The Granular cell Layer (GrL, red) of the MOB is also evident. **C-E.** GnRH (green) expression in three coronal sections of the olfactory bulb at different caudal-rostral levels (Bregma: +3.85 in C-C', +3.54 in D-D', +2.62 in E-E') of an adult mouse. Insets represented in high magnification panels (63x in C', 40x in D' and E') indicate GnRH clusters in C' and E' and GnRH-ir fibers in D'. Arrowhead in D indicate GnRH-ir cell bodies in the ONL of the OB. Scale bars: 100µm (C,D), 50µm (E), 10µm (C'), 40µm (D',E'). Abbreviations: MOB, main olfactory bulb; AOB, accessory olfactory bulb; GL, glomerula layer; GrL, granular cell layer.

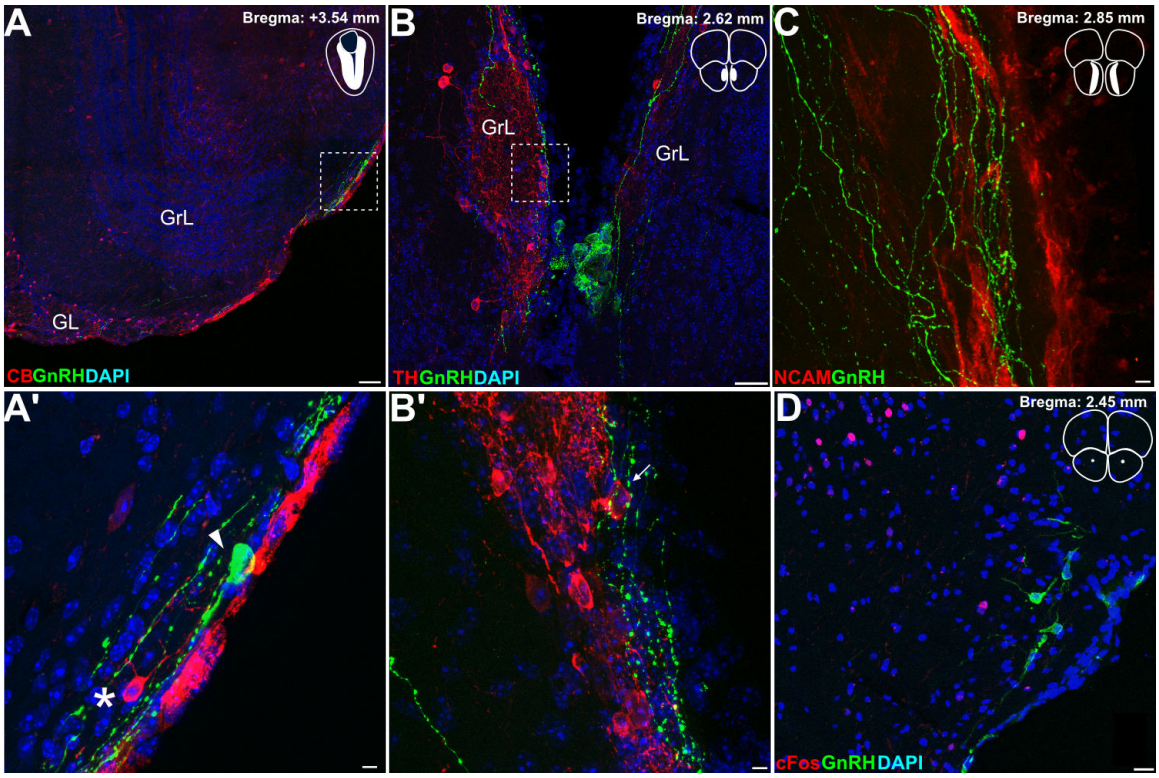


**Figure 2. Characterization of GnRH-ir cells in the OB**

**A.** GnRH (green) and CB (red) expression in the MOB in a coronal section of the olfactory bulb of an adult mouse (Bregma: +3.54). GnRH cells (arrowhead in A') do not coexpress CB (asterisk in A'). **B.** GnRH (green) and TH (red) expression in the Granular Layer of the MOB in a coronal section of the olfactory bulb of an adult mouse (Bregma: +2.62). GnRH fibers are in close contacts with TH cell bodies (arrow in B'). **C.** GnRH (green) and NCAM (red) expression in a coronal section of the olfactoy bulb of an adult mouse brain (Bregma: +2.85) indicates that GnRH-ir fibers in the OB travels in close association with olfactory nerve (immunopositive for NCAM). **D.** GnRH (green) and cFos (red) expression in a caudal coronal olfactory bulb section of an adult mouse brain (Bregma: +2.45) indicates that GnRH cells are not activated by pheromonal exposure. Scale bars: 50µm (A,B), 5µm (A',B',C,D). Abbreviations: MOB, main olfactory bulb; AOB, accessory olfactory bulb; GL, glomerula layer; GrL, granular cell layer.

## GnRH and gonadal receptors expression in the OB and DG of adult mice

Previous studies reported GnRH receptor (GnRHR) expression in several brain regions involved in olfactory sensory processing and sexual behaviour (Albertson *et al.*, 2009; Wen *et al.*, 2011). Here, by using the GRIC/R26-YFP mouse model (Wen *et al.*, 2008; Sirinivas *et al.*, 2001), we studied GnRHR expression in the two major sites of adult neurogenesis, the OB and DG of hippocampus. GRIC mice carry a mutant GnRHR allele, which is transcribed into a bicistronic messenger RNA, from which the GnRHR and Cre recombinase are independently translated (Wen *et al.*, 2008). When bred to R26-YFP mice, Cre

recombinase will activate YFP expression in GnRHR-expressing cells. Immunohistochemical analyses, showed GnRHR expression in cells of both the main (not shown) and AOB (Fig. 3A, B). In addition, GnRHR-ir fibers were identified in the vomeronasal nerve (Fig. 3A-B). Interestingly, in the AOB, close appositions between GnRHR-ir and GnRH-ir fibers were identified (Fig. 3B, B').

In the hippocampus, we found GnRHR-ir elements, mostly located in the CA1 (Fig. 3C, C'). Only a few cells in the CA2 were identified and no cells were detected in the CA3. Interestingly, some GnRHR-ir cell bodies were found in the granule cell layer of the DG (Fig. 3C, C'''). Finally, in agreement with previous data (Wen *et al.*, 2011), numerous GnRHR-ir cells were found also in the thalamus (Fig. 3C''').

The expression of GnRHR was also investigated through Real Time PCR analysis in the MOB and AOB. In order to get more information concerning a possible direct versus indirect role of GnRH in the modulation of OB plasticity, we coupled this study to the analysis of gonadal receptor expression (indeed, a possible effect of GnRH is expected also through activation of the HPG axis). We performed this study in both males and females, since no data were already available regarding possible gender-related differences in this region. This study showed expression of GnRH and gonadal hormones receptors (androgen receptor, Ar; estrogen receptor alpha and beta, Erα, Erβ; and progesterone receptor, PR) in both the MOB and AOB, without differences between males and females (Fig. 3D,E,F). Overall, although these data are not conclusive to state if GnRH can exert its action on neural circuits underlying olfactory sensory perception directly or via the activation of gonadal hormones, they suggest this activity occurs through also a complex interplay between endocrine factors and circuits underlying neural plasticity in adult animals. We argue that this effect could also involve a modulation of the process of adult neurogenesis.

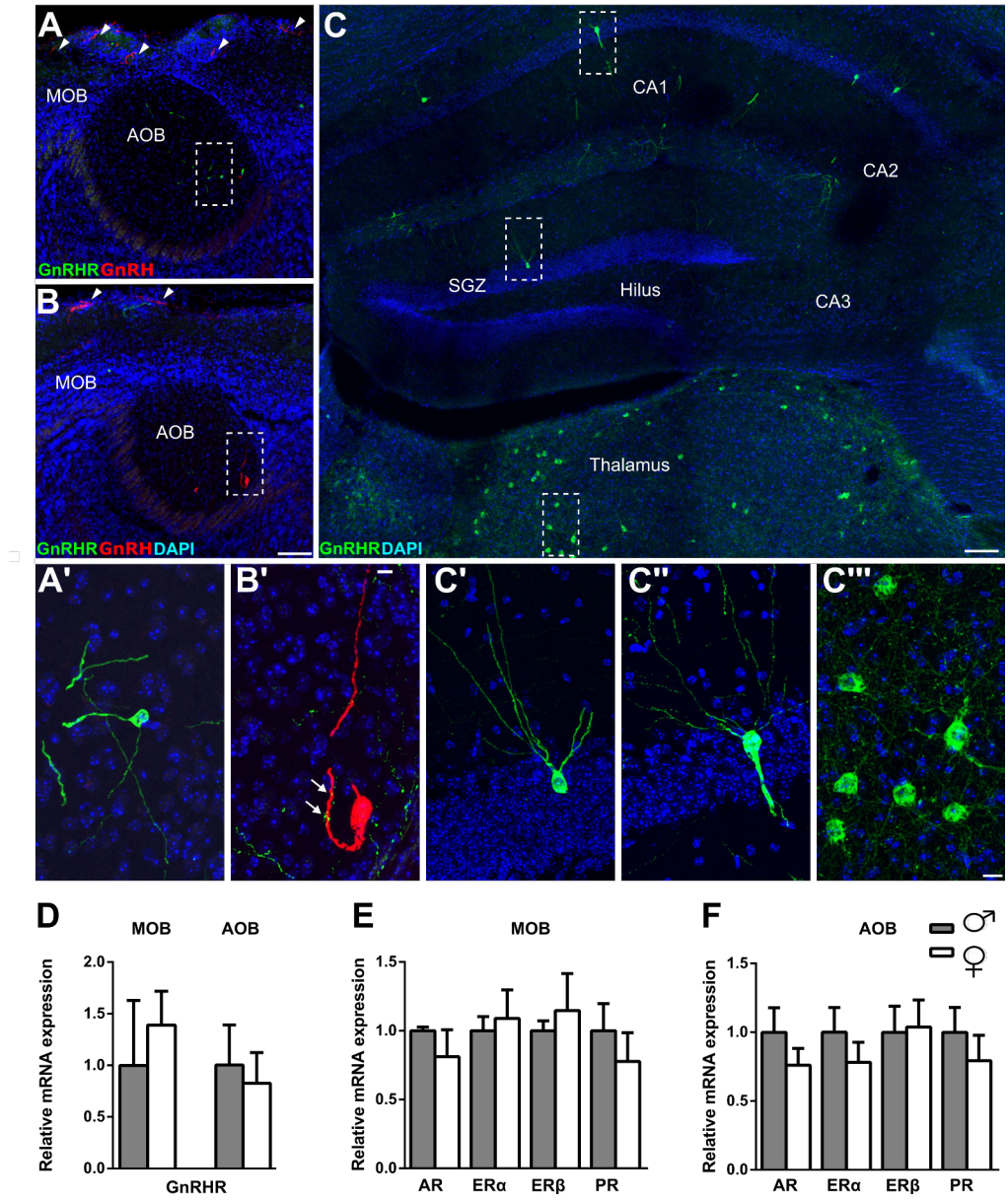


**Figure 3. GnRHR and gonadal hormones receptors in both neurogenic systems**

**A-B.** Immunofluorescence for GnRHR (green), GnRH (red) and DAPI (blue) in a coronal section of the olfactory bulb (Bregma +4.045 for A and +3.645 for B) of an adult GRIC/R26-YFP mouse. Scale bar 100µm. Insets, shown in high magnification panels in A' and B', indicate GnRHR-ir cells in A' and a GnRH-ir cell with contacts (arrows) with GnRHR-ir fibers in B', in the AOB GL. Arrowheads indicate GnRH-ir fibers in the ONL of the OB. Scale bar 10µm. **C** Immunofluorescence for GnRHR (green), and DAPI (blue) in a coronal section of the hippocampus of an adult GRIC/R26-YFP mouse. Scale bar 100µm. Insets show GnRHR-ir cells in the DG (C'), in the CA1 (C'') and in the thalamus (C'''). **D-F** RT-PCR analysis for GnRHR-expression in the MOB and in the AOB (D) and for AR-, ERα-, ERβ-, PR-expression in the MOB (E) and in the AOB (F). Values are expressed relative to males set at 1. n=4 for males and n=16 for females. The values shown are the mean ± SEM. Abbreviations: MOB, main olfactory bulb; AOB, accessory olfactory bulb; SGZ, subgranular zone; CA1-2-3, Cornu Ammonis 1-2-3.

## The role of GnRH secretion in modulating adult neurogenesis

In order to get insights about a possible link between the GnRH system and adult neurogenesis, we investigated different steps of this process in the *GnRH::cre;Dicer*$^{loxP/loxP}$ mice. In this model GnRH is ablated soon before the onset of puberty, thus preserving the possible impact of GnRH during the critical organizational stage of early postnatal development, and resulting in animals characterized by a marked reduction of adenohypophyseal hormones and a severe hypogonadism during adulthood (see the introduction and Messina *et al.*, 2016).

## Analysis of progenitor cells proliferation in *GnRH::cre;Dicer*$^{loxP/loxP}$ *mice*

Firstly, in *GnRH::cre;Dicer*$^{loxP/loxP}$ mice (males and females) we examined the rate of cell proliferation and cell differentiation in both adult neurogenic niches, by combining Ki67, an endogenous marker of cell proliferation expressed during all active phases of the cell cycle (G1, S, G2 and mitosis) and absent in the G0 cells (Zacchetti *et al.*, 2003), with DCX, a brain-specific microtubule-associated protein which is expressed by neuroblasts and young differentiating neurons (Francis *et al.*, 1999; Gleeson *et al.*, 1999). In particular, we quantified the population of progenitors not yet committed toward a specific glial *vs* neuronal fate (Ki67+/DCX- cells), the population of proliferating cells already committed toward a neuronal fate (Ki67+/DCX+ cells), and finally (only in the DG of hippocampus) the population of post-mitotic neuroblasts (Ki67-/DCX+). This kind of analysis allowed us to establish possible effects of a GnRH inactivation on the early critical steps of the process of AN.

In the SVZ, this analysis did not show any difference between *GnRH::cre;Dicer*$^{loxP/loxP}$ mice and control animals when this region was considered as an homogeneous domain (not shown). By contrast, we found significant differences when the SVZ was subdivided into the ventral (vSVZ) and dorsal (dSVZ) subdomains. Indeed, previous studies have demonstrated that in the OB,

different interneuron subtypes are produced by distinct SVZ micro domains (Merkle *et al*., 2014; Merkle, *et al*., 2005; Fig. 4B).

In the vSVZ no differences between the *GnRH::cre;Dicer*$^{loxP/loxP}$ and control mice were identified in both sexes, as concern the number of proliferating progenitors (Ki67+/DCX-), the number of young post-mitotic neuroblasts (Ki67-/DCX+), and the total number of proliferating cells (Ki67+/DCX- plus Ki67+/DCX+ cells; Fig. 4C,F,G; supplementary Tables 1.1, 1.2, 1.3). By contrast, a significant decrease in proliferating DCX+ cells (Ki67+/DCX+ cells) was found in the dSVZ of *GnRH::Cre/Dicer*$^{loxP/loxP}$ male mice (Fig. 4C,D, E; Two-way ANOVA, gender $F_{(3,24)}$=1.114, P=0.363; genotype $F_{(1,24)}$=3.005, P=0.096; interaction $F_{(3,24)}$=2.948, P=0.053; Tukey *post hoc*, dSVZ control males *vs* dSVZ *GnRH::Cre/Dicer*$^{loxP/lox}$ males, p=0.039; supplementary Tables 1.1, 1.2).

In addition, in this micro domain while in control animals a gender-related difference in the total number of proliferating cells (Ki67+/DCX- plus Ki67+/DCX+ cells) was found, this was not evident in the *GnRH::cre;Dicer*$^{loxP/loxP}$ mice (Fig. 4C; Two-way ANOVA, gender $F_{(3,24)}$=2.510, P=0.828; genotype $F_{(1,24)}$=0.030, P=0.863; interaction $F_{(3,24)}$=1.496, P=0.240; Tukey *post hoc*, dSVZ control males *vs* dSVZ *GnRH::Cre/Dicer*$^{loxP/loxP}$ females, p=0.047; supplementary Table 1.3).

In the DG of hippocampus no differences between *GnRH::cre;Dicer*$^{loxP/loxP}$ and control animals in both sexes were identified, as for the number of proliferating cells not yet committed to the neuronal lineage (Ki67+/DCX-) as the total number of proliferating cells (Ki67+/DCX- plus Ki67+/DCX+ cells; Fig. 5C,E; supplementary Table 2.1, 2.3). By contrast, a significant difference in the total number of already neuronal committed cells (Ki67+/DCX+ and Ki67-/DCX+) was identified in the *GnRH::Cre/Dicer*$^{loxP/loxP}$ males when compared with control and *GnRH::Cre/Dicer*$^{loxP/loxP}$ females (Fig. 5A,B,D,F; DCX+/Ki67- cells: two-way ANOVA, gender $F_{(1,13)}$=8.118, P=0.014; genotype $F_{(1,13)}$=3.338, P=0.090; interaction $F_{(1,13)}$=0.990, P=0.338; Tukey *post hoc*, *GnRH::Cre/Dicer*$^{loxP/loxP}$ males *vs* control females, p=0.029; total number of DCX+ cells: two-way ANOVA, gender $F_{(1,13)}$=9.219, P=0.009; genotype $F_{(1,13)}$=4.002, P=0.067; interaction $F_{(1,13)}$=1.433, P=0.252; Tukey *post hoc*, *GnRH::Cre/Dicer*$^{loxP/loxP}$ males *vs* control females, p=0.019, *GnRH::Cre/Dicer*$^{loxP/loxP}$ male *vs* control females, p=0.039;

supplementary Table 2.2, 2.4). Overall, these data indicate that the absence of GnRH secretion during the post-pubertal/adult life affects specific stages of the early process of adult neurogenesis in both SVZ and DG in a gender-specific manner.

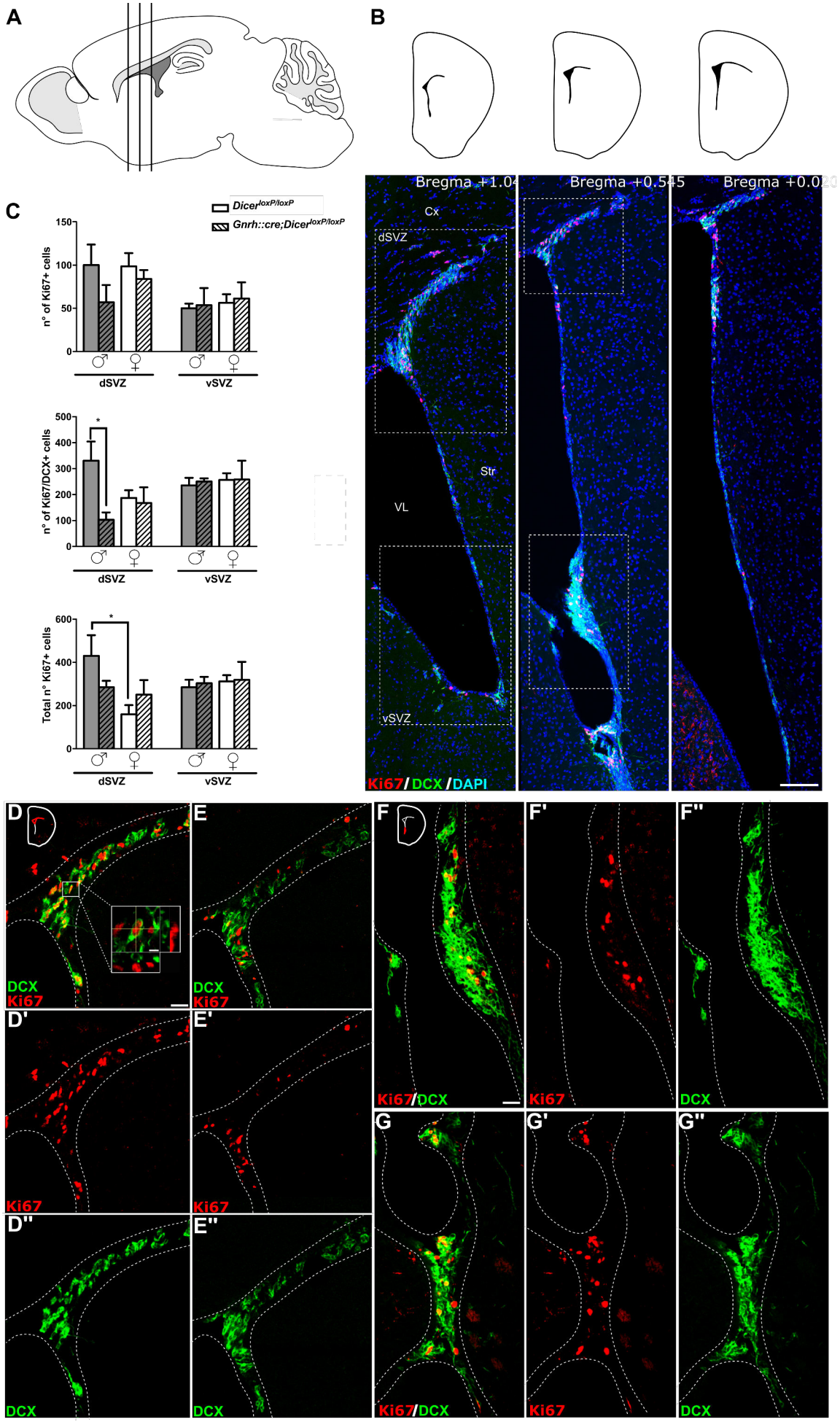
A
B
C
Dicer loxP/loxP
Gnrh::cre;Dicer loxP/loxP
n° of Ki67+ cells
n° of Ki67/DCX+ cells
Total n° Ki67+ cells
dSVZ
vSVZ
Bregma +1.0
Bregma +0.545
Bregma +0.020
Cx
dSVZ
Str
VL
vSVZ
Ki67/DCX/DAPI
D
E
F
F'
F''
G
G'
G''
DCX
Ki67
Ki67/DCX

**FIGURE 4_ Analysis of proliferation in the SubVentricular Zone**

**A.** Schematic lateral view of an adult mouse brain indicating the approximate spatial extent of the SVZ (dark grey). Vertical lines indicate three regions (used for analyses) from which the outline of the lateral ventricle is shown in coronal sections in B. **B.** Schematic representation of coronal sections at three different caudal-rostral levels (top) and the respectively representations in immunofluorescence for Ki67, DCX and DAPI in an adult (P90) *Dicer*$^{loxP/loxP}$ male mouse (bottom). **C.** Quantification of Ki67+/DCX- cells, Ki67+/DCX+ cells and the total number of Ki67 cells (Ki67+/DCX- _Ki67+/DCX+) in *GnRH::cre/Dicer*$^{loxP/loxP}$ and *Dicer*$^{loxP/loxP}$ mice in both males and females, at the level of the d-SVZ and the v-SVZ (see insets in B, Bregma +1.0). **D,E.** Immunofluorescence for DCX and Ki67 (D and E), for Ki67 only (D' and E') and for DCX only (D'' and E'') at the level of the d-SVZ (Bregma +0.545) comparing a *Dicer*$^{loxP/loxP}$ male (D-D'') with a *GnRH::cre/ Dicer*$^{loxP/loxP}$ male mouse (E-E''). Inset in D shows double-labelled cell (DCX+/Ki67+) in the dSVZ. **F-G.** Immunofluorescence for DCX and Ki67 (F and G), for Ki67 only (F' and G') and for DCX only (F'' and G'') at the level of the v-SVZ (Bregma +0.545) comparing a *Dicer*$^{loxP/loxP}$ male (F-F'') with a *GnRH::cre/ Dicer*$^{loxP/loxP}$ male mouse (G-G''). Dashed lines mark dorsal and ventral SVZ in **D-G**. Dashed boxes in B represent d-SVZ and v-SVZ at the level of Bregma 1.045. Dashed boxes in B (Bregma +0.545) are shown in higher magnification in D-D'' and F-F'' representing the d-SVZ and v-SVZ, respectively. Scale bars represent 100µm for **B** and 20µm for **D**,**E**,**F** and **G**. The values shown are the mean ± SEM. Abbreviations: d-SVZ, dorsal subventricular zone; v-SVZ, ventral subventricular zone; Cx, cortex; Str, striatum; VL, lateral ventricle. Two-way ANOVA, *p<0.05.

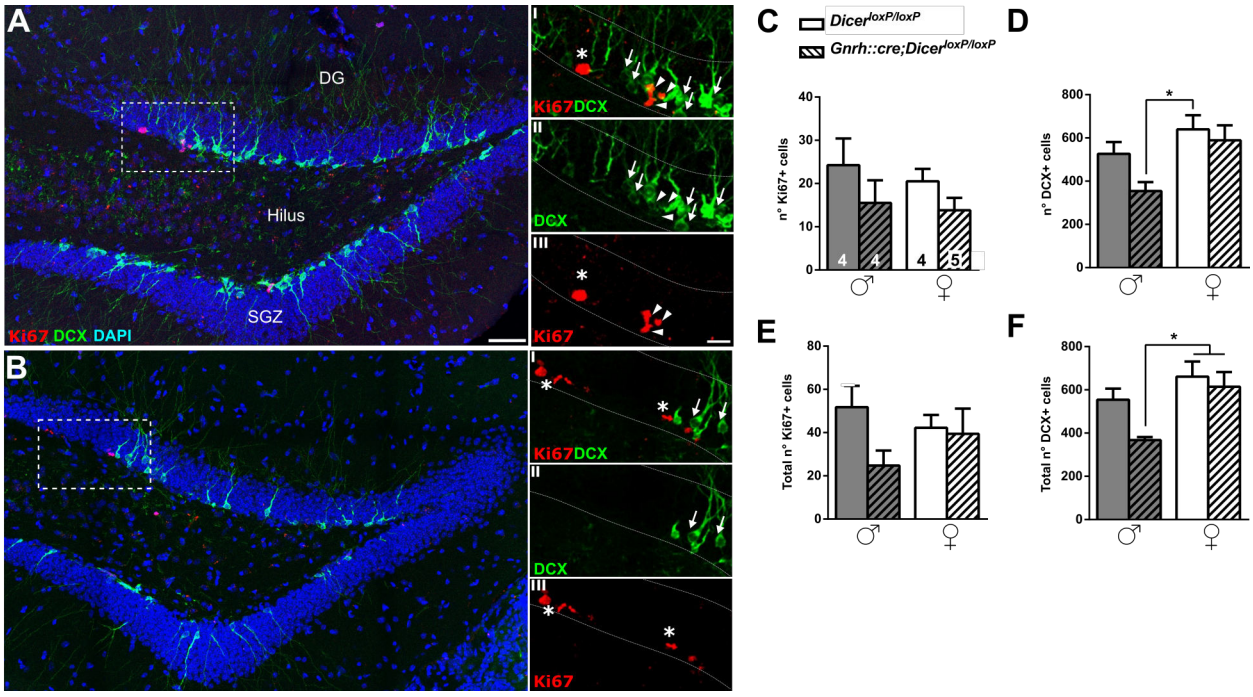


**Figure 5. Analysis of proliferation in the SubGranular Zone**

**A-B.** Immunofluorescence for DCX, Ki67 and DAPI, comparing a *Dicer*$^{loxP/loxP}$ female (A) with a *GnRH::cre/Dicer*$^{loxP/loxP}$ male mouse (B). Insets in A and in B are represented in high magnification panels on the right to the respective picture. Insets indicate Ki67+ cells (asterisks), DCX+ cells (arrows) and double-labelled Ki67+/DCX+ cells (arrowheads) (|-||-|||). Dashed lines mark the SGZ upper layer. Scale bars represent 50µm (A) and 10µm (|||). **C-F.** Quantification of

Ki67+/DCX- cells, DCX+/Ki67- cells, total number of Ki67+ cells (Ki67+/DCX- _Ki67+/DCX+) and total number of DCX+ cells (Ki67-/DCX+ _Ki67+/DCX+) in *GnRH::cre/Dicer*$^{loxP/loxP}$ and *Dicer*$^{loxP/loxP}$ mice in both males and females, at the level of the SGZ. The values shown are the mean ± SEM. Abbreviations: DG, dentate gyrus; SGZ, subgranular zone. Two-way ANOVA, *p<0.05.

## Analysis of newborn cell survival in the OB region and DG of hippocampus in the *GnRH::Cre/Dicer*$^{loxP/loxP}$ mice

Next, in *GnRH::Cre/Dicer*$^{loxP/loxP}$ mice we examined the survival of newborn neurons in the pre-existing circuits. To this aim, we quantified the number of BrdU-ir cells in the MOB, AOB and DG of hippocampus 30 days after BrdU injection (Figs. 6-8).

In the MOB (Fig. 6) of the *GnRH::Cre/Dicer*$^{loxP/loxP}$ mice we found a significant reduction of BrdU cell density in the granule cell layer of females, while no differences were found in males (Fig. 6D; two-way ANOVA, gender $F_{(1,21)}$=3.663, P=0.069; genotype $F_{(1,21)}$=2.150, P=0.157, interaction $F_{(1,21)}$=8.160, P=0.009; Tukey *post-hoc*, females *GnRH::cre/Dicer*$^{loxp/loxP}$ *vs* females control, p=0.013; supplementary Table 3.1). This result is intriguing considering that in males, but not in females, we found a decreased rate of cell proliferation (Fig. 4, 5). In addition, this analysis revealed an unexpected sexual dimorphism in control animals, with females showing a higher level of BrdU cell density in the MOB GCL (Fig. 6C, D; females control *vs* males *GnRH::cre/Dicer*$^{loxP/loxP}$ , P=0.016). Similar results were obtained by considering the total number of BrdU+ cells (data not shown). Importantly, the GrL surface examined does not change among the all experimental groups (Fig. 6E), further confirming that the difference in BrdU+ cell density found was due to a diverse number of BrdU+ cells integrated in the MOB GrL, and not to a reduction/increase on the olfactory bulb volume.

In the AOB GrL in contrast to what observed in the MOB, we did not found any significant difference in the survival of newborn neurons by considering both gender and genotype, even if in control females we found in average higher values (not significant) of BrdU cell density (Fig. 7C; supplementary Table 3.2). Interestingly, such difference becomes significant when the AOB was considered in its anterior and posterior functional subdivisions (Sugai *et al*. 1997; Dudley *and* Moss 1999; Martínez-Marcos *and* Halpern 1999; Kumar *et al*. 1999).

Specifically, the Two-way ANOVA analysis showed a significant effect considering the gender factor alone (two-way ANOVA, gender $F_{(1,12)}$=5.499, P=0.037; genotype $F_{(1,12)}$=1.635, P=0.225, interaction $F_{(1,12)}$=1.001, P=0.337; supplementary Tables 3.3, 3.4), in which control females show an higher density on BrdU+ cells compared to the other experimental groups (Fig. 7D). Volumes of the AOB are similar among all experimental groups (data not shown).

In the DG of hippocampus, the survival of newborn neurons was not found altered in the absence of GnRH secretion by considering both gender and genotype (Fig. 8A-D; supplementary Table 3.5). As for the previous regions, the volumes of the DG were similar in all experimental groups (Fig. 8D).

Overall, the analysis of the multi-step process of adult neurogenesis in the *GnRH::Cre/Dicer*$^{loxP/loxP}$ mice clearly show that GnRH secretion during adulthood is involved in the regulation of this process in both neurogenic niches. This activity occurs in a complex and non-linear way (e.g., effects on proliferation in males and survival in females). Whether this modulation could be due to a direct versus indirect (gonadal hormones) effect remains to be clarified.

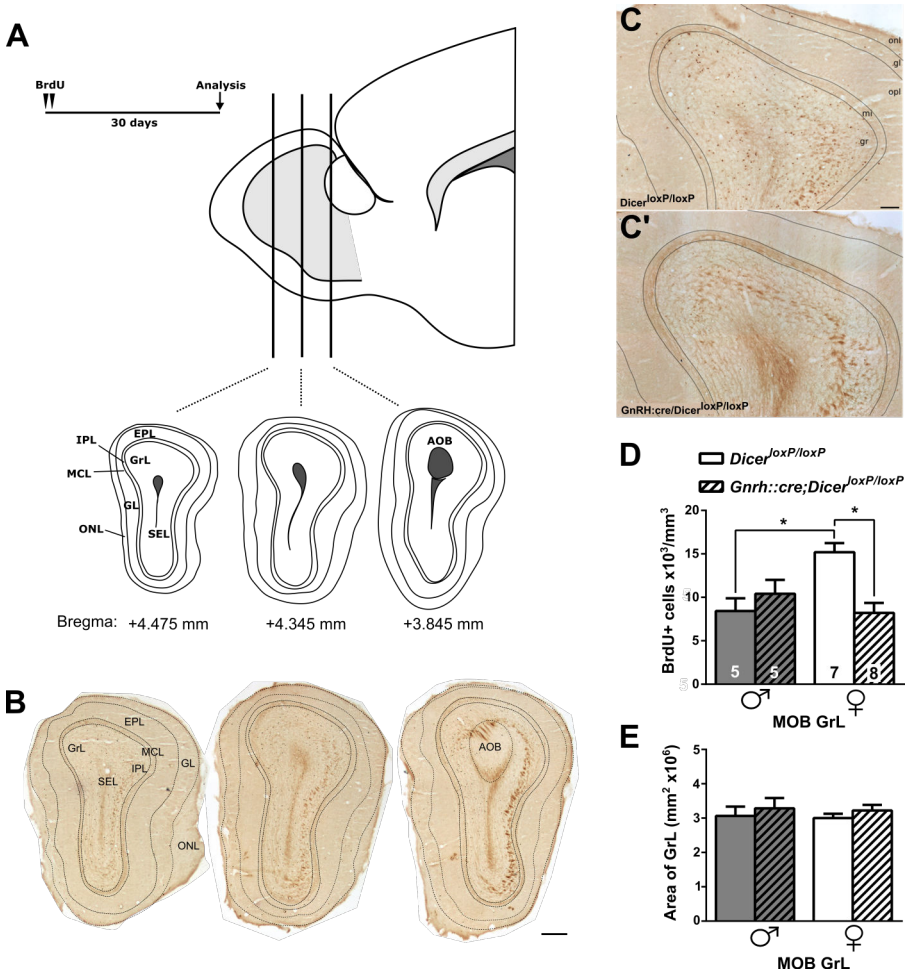


**Figure 6. Survival of newborn granule cells (GCs) in the Main Olfactory Bulb (MOB)**
**A.** Experimental protocol (top left): newborn cells are quantified in the GrL of the MOB 30 days after BrdU injection. Schematic lateral view of the rostral part of an adult mouse brain (top right) indicating the spatial extent of the MOB. Vertical lines indicate three regions (used for analyses) from which the outline of the MOB is shown in coronal sections (bottom) with the Bregma coordinates. **B.** Coronal sections of three MOB slices corresponding to coordinates shown in A, immunolabeled for BrdU. Scale bar 200µm. **C-C'**. Representative images of MOB sections showing BrdU-positive newborn neurons in controls (*Dicer*$^{loxP/loxP}$) (C) and *GnRH::cre/Dicer*$^{loxP/loxP}$ (C') females, 1 month after BrdU administration. Scale bar 100µm. **E.** Quantification of BrdU positive cell density in the MOB GrL indicating a significant decrease in newborn neurons in *GnRH::cre/Dicer*$^{loxP/loxP}$ females compared to *Dicer*$^{loxP/loxP}$ females and sexual dimorphism in control mice (two-way ANOVA, see the text and supplementary Table 3.1). **F.** The total area ($mm^2$) of GrL is not different among all groups. Sample size are indicated on each histogram bar in E. The values shown are the mean ± SEM. Abbreviations: ONL, olfactory nerve layer; GL, glomerular layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GrL, granule cell layer; SEL, subependymal layer.

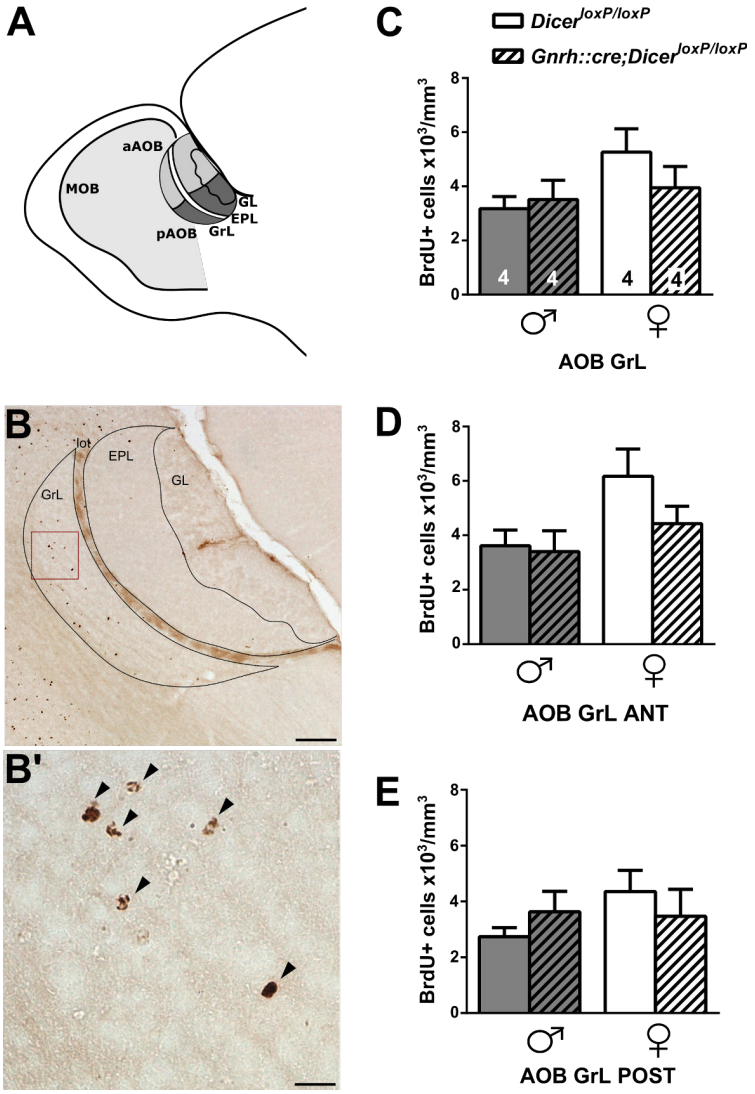


**Figure 7. Survival of newborn granule cells (GCs) in the Accessory Olfactory Bulb (AOB)**
**A.** Schematic lateral view of the rostral part of an adult mouse brain indicating the spatial extent of the AOB, showing the anterior AOB (aAOB, light grey) and the posterior AOB (pAOB, dark grey). **B-B'.** Sagittal section of the AOB showing BrdU labeled nuclei (scale bar 50µm). The lower panel in B' refers to the red squared area in the Granule cell layer (scale bar 10µm). The arrows indicate BrdU+ nuclei. **C-E**. Quantification of BrdU positive cell density in the AOB GrL (C) and in its subdivisions (D,E) indicates no differences among groups in the AOB GrL or in the pAOB GrL. A significant variation between genders is present in the aAOB GrL (two-way ANOVA, see the text and supplementary Table 3.2, 3.3, 3.4). The values shown are the mean ± SEM. Abbreviations: aAOB, anterior AOB; pAOB, posterior AOB; GrL, Granular cell Layer; EPL, External Plexiform Layer; GL, Glomerular Layer.

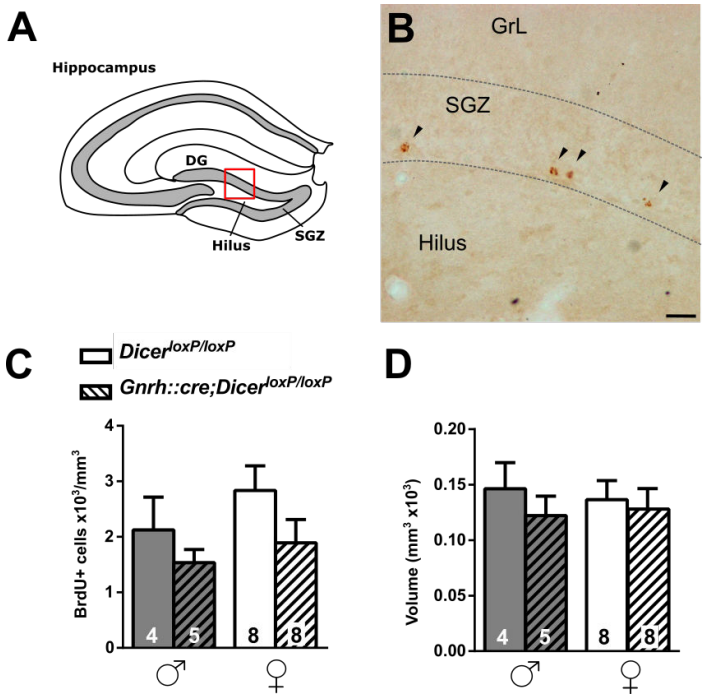


**Figure 8. Survival of newborn granule cells (GCs) in the DG**
**A.** Schematic coronal view of the hippocampus of an adult mouse brain indicating the Dentate Gyrus (DG), the Subgranular Zone (SGZ) and the Hilus. **B.** Coronal section of the upper layer of the DG (see red inset in A) showing BrdU labeled nuclei (arrowheads) in *Dicer*$^{loxP/loxP}$ females, 1 month after BrdU administration. Scale bar 20µm. **C**. Quantification of BrdU positive cell density in the SGZ in *GnRH::cre/Dicer*$^{loxP/loxP}$ and *Dicer*$^{loxP/loxP}$ mice in both males and females. **D.** The total volume (mm$^3$) of SGZ is not different among groups. Sample size is indicated on each histogram bar in C and in D. The values shown are the mean ± SEM. Abbreviations: DG, dentate gyrus; SGZ, subgranular zone; GrL, granule cell layer.

## The role of GnRH secretion in mediating olfactory discrimination of non-social and social cues.

We previously demonstrated the presence of GnRH-ir cells and fibers and the expression of GnRHR in the olfactory bulb region (MOB and in the AOB). We also identified several alterations of the process of AN in the adult *GnRH::cre/Dicer*$^{loxP/loxP}$ mice, which include reduced proliferation in the SVZ of males and reduced survival in the MOB of females. Therefore, in order to unravel a direct link between GnRH secretion (during post pubertal and adulthood) and olfactory perception/discrimination, we performed two olfactory tests useful to evaluate the olfactory competences for social and non social stimuli in the in the *GnRH::cre/Dicer*$^{loxP/loxP}$ mice. To this aim, we used the habituation-dishabituation

paradigm (de Chevigny *et al*., 2006; Breton-Provencher *et al*., 2009), which is tailored to evaluate the main olfactory system activity, and the preference for the opposite-sex cues, which implies a cooperation between the main and accessory olfactory system (Hurst, 2009).

To perform the habituation-dishabituation test. we used acetophenone and octanal (non-social odours stimuli). While controls (males and females), were able to perform discrimination between the two odors, the *GnRH::Cre/Dicer*$^{loxP/loxP}$ females were not able to discriminate the novel odor (Fig.9A, B). This result is intriguing considering that in females, but not in males, we found a decrease of newborn cells survival in the MOB (Fig. 5).

As regards the ability to discriminate social cues in *GnRH::Cre/Dicer*$^{loxP/loxP}$ mice, we exploited the "natural" preference exhibited by adult mice to prefer cues from the opposite sex. Therefore, we evaluated the responses elicited by urinary scents/pheromones from sexually active males versus oestrous females. This was done allowing direct nasal-urine contact, which implies activation of both the accessory and main olfactory systems (perception of volatile and non volatile cues). As expected, both control males and females spent more time investigating urine from the opposite-sex (Fig. 9 C,D). On the contrary, *GnRH::Cre/Dicer*$^{loxP/loxP}$ mice of both genders, never showed any preference for male or female scents after direct contact with familiar urine (Fig. 9 C,D).

Overall, these results on olfactory perception/discrimination indicate GnRH secretion during adulthood is key to the olfactory function. In addition, although further analyses are still required to exactly define the mediators (e.g., gonadal hormones and/or GnRH), it is clear that this regulative activity also implies the modulation of mechanisms of neural plasticity, such as adult neurogenesis.

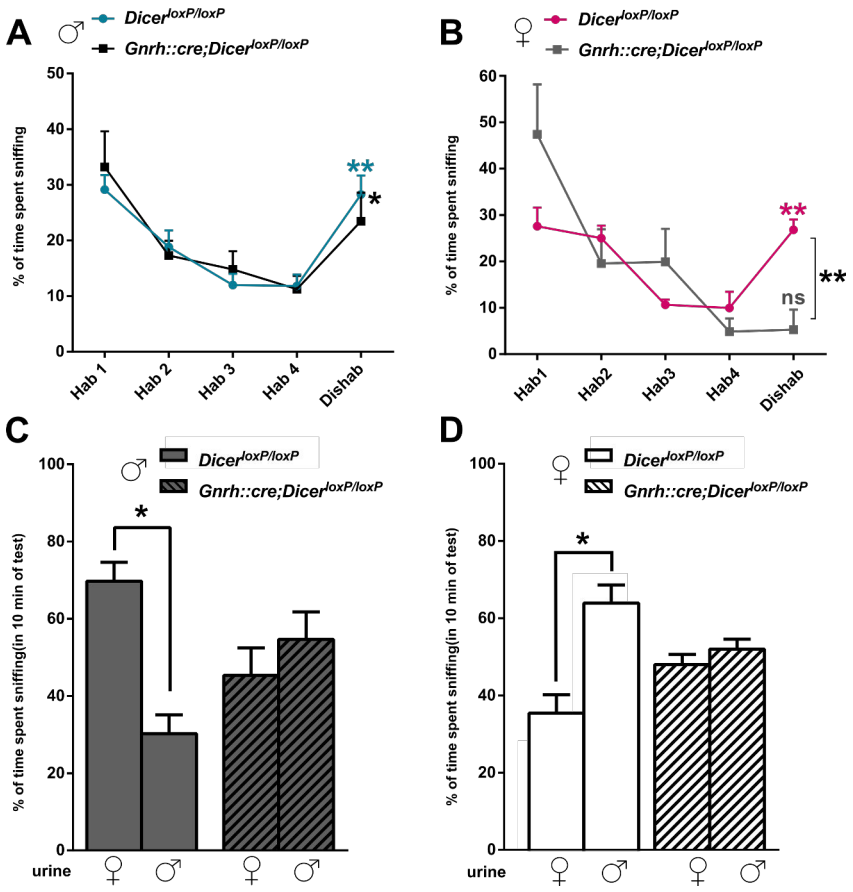


**Figure 9. Olfactory discrimination of non-social and social cues**

**A-B.** The habituation/dishabituation paradigm indicates that *GnRH::cre/Dicer*$^{loxP/loxP}$ males (black line) are able to discriminate habituation odours from dishabituation odours as well as control (*Dicer*$^{loxP/loxP}$) male mice (blue line) (A; control males: n=9; paired Wilcoxon-Mann-Whitney test, hab4 *vs* dishab P=0.008; *GnRH::cre/Dicer*$^{loxP/loxP}$ males: n=9; paired Wilcoxon-Mann-Whitney test, hab4 *vs* dishab P=0.023). *GnRH::cre/Dicer*$^{loxP/loxP}$ females (grey line) are not able to discriminate habituation odours from dishabituation odours, in contrast to control females (pink line) (B; *GnRH::cre/Dicer*$^{loxP/loxP}$ females: n=4; paired Wilcoxon-Mann-Whitney test, hab4 *vs* dishab P= 0.875; control females: n=6; paired Wilcoxon-Mann-Whitney, hab4 *vs* dishab P=0.006; unpaired Wilcoxon-Mann-Whitney, dishab control *vs* dishab *GnRH::cre/Dicer*$^{loxP/loxP}$ P=0.004). **C-D.** During direct contact with pheromones (volatile compounds), both control male and female mice spend more time sniffing the odours of the opposite sex (n=7 control males; paired Wilcoxon-Mann-Whitney test, P=0.015; n=6 control females: paired Wilcoxon-Mann-Whitney test, P=0.031), while both *GnRH::cre/Dicer*$^{loxP/loxP}$ males and females show no preference (n=8 *GnRH::cre/Dicer*$^{loxP/loxP}$males: paired Wilcoxon-Mann-Whitney test, P=0.546; n=4 *GnRH::cre/Dicer*$^{loxP/loxP}$ females: paired Wilcoxon-Mann-Whitney, P=0.375).

## Discussion

Recent data suggest that the accomplishment of some olfactory-dependent behaviors requires interplay among social (environmental) cues, hormones and neural plasticity. Indeed, pheromones increase adult neurogenesis and elicit sexual behaviors, and in turn gonadal hormones modulate the activity of nuclei underlying reproduction and the process of adult neurogenesis. In order to enlighten some of the players/mechanisms involved in this complex integration, we addressed the role of GnRH secretion during adulthood. Accordingly, the hypothalamic GnRH neurons orchestrate reproduction, through a fine tuned control of the HPG axis, and thus the production and secretion of gonadal hormones. Interestingly, recent evidence indicates the occurrence of a GnRH extrahypothalamic population in the OB (Casoni *et al*., 2016), suggesting GnRH secretion could be also directly involved in mediating some aspects of the olfactory system physiology, plasticity and function. Although, further steps are still necessary to completely clarify how GnRH can impact on the olfactory function, this work clearly shows its importance in this activity. In the next paragraphs we report an extended discussion of our data and preexisting literature in order to sustain our hypothesis and to delineate future studies/experiments aimed at defining how GnRH can play its control on the olfactory function.

### The olfactory GnRH System

In vertebrates, the GnRH neurons control reproduction by directing the activity of the HPG axis. GnRH neurons control the release of gonadotropins, which lead in turn the release of adenohypophyseal and gonadal hormones. The release of GnRH is pulsatile and sensitive to a multitude of external and internal stimuli (Sisk *and* Foster, 2004). GnRH neurons (about 800 in mice) are mostly located into the POA. A recent study (Casoni *et al*., 2016) has disclosed that during embryonic development, GnRH neurons, in addition to the well-known ventral migratory pathway that drives these cells to the POA, also migrate to subpallial and pallial regions, including the developing olfactory bulb. They

further show a persistence of GnRH immunoreactivity around the adult olfactory bulb of mice. Presence of GnRH-ir cells and fibers associated to the OB were previously described also in adult hamsters and prairie voles (Wirsig *and* Leonard, 1986; Wirsig-Wiechmann *and* Wiechmann, 2001). Here, by using multiple approaches, including a 3D reconstruction analysis, not only we corroborate previous observations, but also we provide new precise anatomical and functional data on this system, here referred as to the olfactory bulb GnRH system. Specifically, we demonstrated that: i) the OB GnRH immunoreactivity is mostly due to a peculiar system of fibers which forms a ring-like structure around the bulbs at their most caudal level; ii) that these fibers are often in close contact with them of both the main and accessory OB nerves, and with cell bodies and processes of the dopaminergic TH-ir periglomerular neurons; iii) that clusters of GnRH-ir cells are scattered within the main and AOB; iv) that these cells are about 150-200 in both male and females. In addition, we also demonstrated through GRIC/R26-YFP transgenic mice and quantitative RT-PCR analysis the OB (and DG of hippocampus) express GnRHR. These set of data strongly support GnRH can modulate olfaction. This activity could be direct (through a local release from fibers and cells of the OB GnRH population) or mediated by HPG activation (gonadal hormones) or through a direct and indirect mechanism. It is to note that in both main and AOB we also demonstrated strong expression of gonadal hormones receptors in both males and females.

One interesting point related to the topography of OB GnRH-ir population is that it bears to a group of olfactory glomeruli called “necklace glomeruli”, which surround the caudal end of the OBs (Shinoda *et al*., 1989). The function of the necklace glomeruli throughout animal’s life is not yet known, but there is evidence that it may play a role in pheromone detection, for example during suckling in neonatal rats (Greer *et al*., 1982). The density of GnRH fibers in proximity to these glomeruli suggests these fibers may be directly involved in the modulation of olfactory inputs eliciting olfactory-dependent reproductive activity. This is further sustained by the close apposition of these fibers with both the olfactory and vomeronasal nerves and with the dopaminergic PG neurons. Indeed, it is known that dopamine in the MOB of mice is involved in the perception of social odours contained in male urine (Serguera *et al*., 2008), and

that the expression of TH in the MOB is modulated by gonadal hormones (Dluzen *et al*., 2002; Arbogast *et al*., 1993; Tashiro *et al*., 1990). For example, the OB GnrH-ir population could be involved in the modulation of the initial detection of pheromones by the olfactory system and the setting up of odor attraction. This role would imply a local release of GnRH by GnRH neurons/fibers, the presence of the GnRH receptor in the olfactory system as well as a neuromodulator/neurotransmitter role of GnRH decapeptide. Accordingly, several studies in vitro support GnRH can actually exert a neuromodulatory role in the olfactory neurons among different vertebrate classes. For example, in rodents, GnRH can specifically modulate olfactory and vomeronasal neuron responses to odours (Wirsigh-Wiechmann *et al*., 2000). In mudpuppies, GnRH modulates olfactory neuron voltage-gated responses (Eisthen *et al*., 2000). Interestingly, these responses are sensible to hormonal fluctuations during seasons. In fact, during breeding seasons, twice as many olfactory neurons of these animals respond to GnRH as at other times. This fact may depend on factors controlling GnRH synthesis. For example, estrogen can increase the synthesis of GnRH in brain neurons (Rosie *et al*., 1990) and also in the nervus terminalis in Xenopus laevis (Wirsig-Wiechmann *and* Lee, 1999). On the contrary, ovariectomy leads to a depletion of GnRH in nervus terminalis neurons (Wirsig-Wiechmann *and* Lee, 1999). These findings support that GnRH can influence the processing of chemosensory information at the receptor cell level (Eisthen *et al*., 2000; Wirsig-Wiechmann et al., 2000).

The involvement of the OB GnRH-ir fiber and cell population in olfactory sensory perception is further supported by the wide expression of GnRHR in the whole olfactory system. Besides our results, expression of GnRH-ir cells have been previously described in the mitral cell layer of both the main and the accessory olfactory bulb, in the anterior olfactory nucleus, in the tenia tecta, in the piriform cortex, in the olfactory tubercle and in entorhinal cortex (Albertson *et al*., 2009). In addition, GnRHR-ir cells were also found in numerous limbic regions, especially the CA1, CA2, CA3, CA4 and the dentate gyrus of the hippocampus (Albertson *et al*., 2009; Wen *et al*., 2011; our study). These data suggest that there may be an intracerebral modulation of olfactory function by GnRH (Lin *et al*., 2004).

A possible local release by the OB GnRH-ir elements is supported by the expression of the GnRHR in this region. As for the hypothalamic GnRH cell population, we did not find any GnRH cell or fiber in the bulb co-expressing the GnRH receptor, or *vice versa*. Nevertheless, in the olfactory bulb we identified GnRH fibers surrounded by GnRHR-ir fibers. Potential contacts between fibers of GnRH- and GnRHR-ir cells have been previously identified in the preoptic area (POA), where the majority of GnRH- and GnRHR-ir cells are found (Wen *et al*., 2011). Moreover, we found many GnRHR-ir cells into the hippocampus. In accordance to this, previous data reported that the hippocampus has the highest number of GnRH receptors in the brain (Ruebi *and* Maurer, 1985; Jennes *et al*., 1988). All these data strongly support that the OB GnRH-ir population can act directly on the olfactory system function through a local release. However, how the olfactory bulb GnRH-ir population can be induced to secrete GnRH is unknown. The major regulator of hypothalamic GnRH secretion is Kisspeptin, a peptide released by a group of neurons mainly located in the hypothalamic arcuate nucleus and in the anteroventral periventricular nucleus (AVPV; Clarkson *et al*., 2009; Gottsch *et al*., 2004; Mikkelsen *et al*., 2009). A third population of Kisspeptin neurons is also located in the amygdala (Kim *et al*., 2011), a key part of the limbic system involved in complex social behaviors (Baxter *et al*., 2002; Roozendal *et al*., 2009; Walf *et al*., 2006). Kisspeptin mediates GnRH release by acting through a G-protein coupled receptor Kiss1r (also known as GPR54; Muir *et al*., 2001; Irwin *et al*., 2004). A recent study (Pineda *et al*., 2017) reveals a feedback loop existing between the amygdala neurons Kiss and mitral and granule cells into the accessory olfactory bulb (Pineda *et al*., 2017). The authors found that the action of Kiss on the AOB is inhibitory. They also demonstrated that Kisspeptin amygdaloid neurons project both retrogradely to the AOB and then to the POA, where they are in contact with GnRH neurons to control reproductive behaviour. This reciprocal link may explain several behaviours related to pheromonal cue-induced re-organization of the gonadotropic axis. Since either Kiss mRNA transcript, Kiss fibers and GnRH fibers are found in the AOB, we can hypothesize a direct action of Kisspeptin also on GnRH cells residing into the olfactory bulb. Moreover, if such action involves GPR54 or other receptors expressed by these GnRH cells, will deserve further

investigation. Despite these evidences, further studies are still necessary to demonstrate whether and how (molecular mechanisms) the OB GnRH-ir cell population can affect sensory olfactory function. In the chapter "future perspectives" I will illustrate some of the experimental approaches planned to address these issues.

## Adult Neuoregenesis is modulated in *GnRH::Cre/Dicer$^{loxP/loxP}$* mice

Adult neurogenesis is a dynamic process, which is regulated by many intrinsic and extrinsic factors, acting at different stages of the neurogenic process, from the proliferation of progenitor cells, their fate specification/differentiation into neuronal or glial cells, to the migration, survival and finally integration of new-born neurons into the preexisting circuits (Alvarez-Buylla *and* Lim, 2004; Ming *and* Song, 2005; 2011). Among the intrinsic factors involved in this multi-step regulatory process, gonadal hormones have been recently indicated as major players (Galea *et al*., 1999; 2008; 2013). Indeed, several studies have proved their direct involvement in the modulation of AN in both neurogenic systems (Brock *et al*., 2010; Veyrac *and* Bakker 2011; Galea *et al*. 2013; McClure *et al*. 2013; Chan *et al*. 2014; Farinetti *et al*. 2015; Galea, 2008; Brus *et al*., 2016 and see introduction). In turn, AN is implicated in specific aspects of the reproductive behaviour (Oboti *et al*., 2011; Peretto *et al*., 2014). These data strongly support the existence of a mutual cross talk between AN and reproductive physiology. However, the cellular and molecular mechanisms underlying such link remain mostly unknown.

The release of gonadal hormones is the final output of the activity of the GnRH system, which in turn is regulated by a complex interaction between the central nervous system (CNS) and the gonads, which implies integration of different circuits and sensory stimuli. The GnRH system is re-activated at puberty by Kisspeptin signaling (Han *et al*., 2005). At puberty, GnRH induces the release of adenohypophysary gonadotropin hormones and in turn gonadal activity. In addition to this main role in regulating the HPG axis, multiple experimental evidences support that GnRH is also potentially involved in other physiological functions. For example, it is involved in mediating olfactory

receptors activity acting as a neurotransmitter rather than a neurohormone (see previous paragraph). In addition, a recent study (Zhang *et al*., 2013) have demonstrated that the hypothalamus is also implicated in aging development via immune-neuroendocrine control, despite its primarily roles in growth, reproduction and metabolism. The physiological effects of aging induce the decline of many processes, including adult neurogenesis and GnRH secretion, but GnRH treatment during aging restores adult neurogenesis in the brain (Zhang *et al*., 2013). This set of data is consistent with the hypothesis that GnRH, besides through the HPG axis (activation of gonadal hormones) can be involved directly in modulating neurogenesis during adulthood. To prove this hypothesis, we investigated the process of AN in a mouse model with impaired GnRH secretion, the *GnRH::cre/Dicer*$^{loxP/loxP}$ mice, recently developed in the laboratory of Development & Plasticity of Neuroendocrine Brain in Lille, France (Messina *et al*., 2016). In these mice, GnRH impairment is not the result of a developmental lack of GnRH neurons; rather, it is acquired during the peri-pubertal period, caused by an alteration in the delicate balance in inductive and repressive signals into the GnRH promoter (Messina *et al*., 2016). This characteristic is intriguing since the critical perinatal action of gonadal and adenohypophisary hormones in brain organization (Bakker *et al*., 2003; Poling *et al*., 2013) is not altered in these mice. Therefore, the modulation/alteration in AN could be attributable to a specific lack of GnRH and/or gonadal hormones occurring during the peri-pubertal period, rather than aberrant organizational effects of hormones that are responsible for shaping the brain during the peri-natal period. For the same reason, we did not use the hypogonadal (*hpg*) mice, in which the primary hypogonadism derives from an autosomal genetic mutation into GnRH gene, which leads in turn to the absence of GnRH secretion from birth (Cattanach *et al*., 1977).

In the *GnRH::cre/Dicer*$^{loxP/loxP}$ mice, we focussed on different steps of the neurogenic process in both adult niches: the proliferation of progenitor cells, the specification into a neuronal lineage and the survival/integration of new neurons into pre-existing circuits. Our data showed that in the *GnRH::cre/Dicer*$^{loxP/loxP}$ mice the specification/differentiation of progenitor cells toward a neuronal fate (number of proliferating cells already expressing DCX)

was affected only in males, in both dSVZ and DG of hippocampus. By contrast, in females was found reduced survival in the MOB and in the anterior AOB. Therefore, in the absence of GnRH secretion the process of AN is differentially modulated in its diverse steps in males and females. Although, we can not rule out any possible direct effect of GnRH on this sex-dimorphic regulation, these results seem attributable to gonadal hormones, and thus in our model by a GnRH-dependent alteration of the HPG axis. Accordingly, alteration in the early steps of AN and DCX expression have been previously linked to the level of circulating testosterone (T). For example, mice castration leads to a decrease in the number of DCX positive cells in the DG without impairing proliferating cells (Benice *et al*., 2010). Similar results have been obtained also in male canaries (Balthazart *et al*., 2008). In addition, treating female canaries with T, and its metabolites, dihydrotestosterone (DHT), or with DHT associated with E2, increases the size of vocal control nuclei (which are higher in males than females) and the number of neurons in these structures. The mechanism by which T could influence the progenitor cell fate is not completely clear. Indeed, the expression of androgen receptor, at least in vivo, is not associated to progenitor cells and immature neurons of the DG, although robustly expressed in other areas of the hippocampus, such as the CA1 (Hamson *et al*., 2013). Therefore, whether this action is mediated by T or by its derivatives, as recently suggested (Farinetti *et al*., 2015), needs further investigation. By contrast, females *GnRH::cre/Dicer*$^{loxP/loxP}$ mice did not show any impairment neither in the proliferation, nor in the specification/differentiation of progenitor cells, in both neurogenic systems. This support that neither GnRH nor gonadal hormones influence these processes in females. This result is in line with previous studies in rodents showing that the treatment with T, E2 or a combination of both in females does not modify neurogenesis in the SVZ (Farinetti *et al*., 2015), and that in absence of aromatase (the enzyme responsible for the conversion of androgens into estrogens), the level of proliferation of progenitor cells is not affected in the adult female SVZ (Veyrac *and* Bakker, 2011). Nevertheless, it is also important to underline that the action of estrogens on neurogenesis in rodents is rather complex and not homogeneous in different species, strains, pathological conditions and type of experimental paradigm (e.g., time and dose-

dependent; Tanapat *et al.*,1999; Galea *et al*., 1999; Duarte-Guterman *et al.*, 2015; Zhang *et al*., 2016).

The second main result of this work concerns the decreased survival of newborn neurons in the MOB and in the anterior AOB of *GnRH::cre/Dicer*$^{loxP/loxP}$ females. This data is consistent with a role of estradiol and/or progesterone in promoting cell survival (Brock *et al*., 2010; Veyrac *and* Bakker 2011; Galea *et al*., 2013; McClure *et al*., 2010; Chan *et al*., 2014; Farinetti *et al*., 2015). Interestingly, *GnRH::cre/Dicer*$^{loxP/loxP}$ male mice do not show any impairment in the survival of newborn neurons, despite a significant reduction of proliferating neuroblasts in the dorsal SVZ. Different alternative hypothesis could explain such effect. First, the process of selection and integration of newborn neurons is dynamically regulated at all stages of the neurogenic process and therefore, an enhanced cell survival in the OB could rescue the loss of neuroblasts production during the early stages of the neurogenic process. In fact, about 50% of newborn cells die in the OB between 15 – 45 days after their genesis (Petreanu *et al*., 2002). A second explanation is related to the existence of distinct SVZ microdomains, which contribute to diverse interneuron subtypes in the olfactory bulb (Merkle *et al*., 2007; Merkley *et al*., 2014). The dorsal regions of the SVZ produces mostly TH+ PG cells and superficial granule cells (Merkle *et al*., 2007). Thus, impairments in granule cell survival in the MOB of *GnRH::cre/Dicer*$^{loxP/loxP}$ male mice could be evident only by specifically focussing on the survival of these bulbar populations.

Finally, in control animals our study disclosed an unexpected sex-dimorphism related to the number of newborn neurons integrated in the MOB granule cell layer and in the anterior AOB, in which females show higher newborn cell density than males. One explanation of these sex differences could be attributable to the fluctuation of sex hormones (oestrus cycle). In rats, during pro-estrus, when oestradiol levels are highest, cell proliferation is increased compared to estrus or dioestrus (Tanapat *et al*., 1999). It is to note that sexually dimorphism related to the survival of BrdU+ cells in the OB region was already described in previous studies performed in rats (Peretto *et al*., 2001) or in other mouse strains (Nunez-Parra *et al*., 2011), although with opposite results. These discrepancies among different studies could be reasonably attributed to difference in methodology and species.

Overall, the data so far collected confirm the gonadal hormones as major factors involved in modulating the AN process, suggesting that GnRH can impact on AN indirectly, through its activity on the HPG axis. Nonetheless, our experimental approach can not exclude a concomitant action of GnRH and gonadal hormones in shaping the process of adult neurogenesis. In example, the GnRH is present in the cerebrospinal fluid (CSF) at concentration levels proportional to those detected in the portal blood vessels (Van Vugt *et al*., 1985), and as previously demonstrated for other factors contained in the CFS, influence the process of olfactory bulb AN. In addition, one appealing possibility is suggested by a recent work (Paul *et al*., 2017), that demonstrated the hypothalamic proopiomelanocortin (POMC) neurons (which control appetite) can modulate adult OB neurogenesis depending on feeding necessity/behavior, through projections directed to the anterior ventral V-SVZ. Though a similar mechanism the hypothalamic GnRH system might influence the OB neurogenesis based on social interaction/reproduction. In addition, a further possibility for a direct action of GnRH on AN, at least in the OB, could be sustained by the OB GnRH cell population above described.

## Olfactory function is modulated by GnRH

The data obtained regarding the ability to discriminate social and non social stimuli in the *GnRH::cre/Dicer*$^{loxP/loxP}$ mice clearly demonstrate the importance of a postnatal/adult secretion of GnRH to perform appropriate olfactory-dependent behaviors. This fact is in agreement with several evidences indicating the GnRH neurons as a central system involved in the integration of multiple sensory signals, including the olfactory cues, to regulate the reproductive behavior (Boehm *et al*., 2005; Yoon *et al*., 2005; Moss *and* McCann, 1973; Pfaff, 1973). Specifically, we found a disrupted olfactory discrimination of non-social odours in females, and an altered attraction to the opposite-sex odours in both sexes. Interestingly, taking into account the role of newborn neurons in the OB, the result in females is consistent with the reduction of newborn cell survival identified in the *GnRH::cre/Dicer*$^{loxP/loxP}$ female mice. In addition, the loss of preference for the opposite sex showed by these animals in

both genders, suggests GnRH could act as a primary players of this socio-sexual behaviour, independently by the gonadal hormones. Whether this is the case, and whether this activity could be mediated by the hypothalamic and/or OB GnRH cell populations, represent an important aim of my future studies.

## Conclusions

In conclusion, the data here presented although still in progress, indicate GnRH is involved in the olfactory function, not only because many GnRH cells and fibers are present along the olfactory pathways, but also because the absence of GnRH alters olfactory behaviors related to social and non-social stimuli. Moreover, we also demonstrated that the GnRH secretion during adulthood negatively affect adult neurogenesis, a striking form of neural plasticity, although this function seems mediated by its activity on the HGP axis. Additional experiments will be performed to better clarify the molecular and cellular mechanisms through which GnRH mediates olfaction, and neural plasticity in the adult brain.

## Future Perspectives

We are aware that our data on the possible role of GnRH in regulating olfactory-dependent behaviors and adult neural plasticity need further investigation. Here, we indicate some main points and experimental procedures that we intend to address to clarify many of the still open questions.

**- Clarify the role of GnRH on the olfactory function**. To this aim we have already planned some experiments based on a viral strategy combined with transgenic animals, in order to selectively inhibit olfactory GnRH cells without affecting the hypothalamic GnRH neurons. Specifically, we have the AAV (Adeno Associated Virus) DREADDS (Designed Receptor Exclusively Activated by

Designer Drugs - inhibitor) that are activated after Cre recombination. We have already injected these viruses in the olfactory bulb of *GnRH::cre* mice (n=4 males and females). Once we will confirm the specificity of cell population infected by the AAV-DREADDS, we will selectively inhibit olfactory GnRH cells by administrating the CNO (Clozapine-N-Oxyde) and we will perform olfactory tests towards social and non-social cues in both adult males and females.

**- Validate if TH+ PGC cells, which are in close apposition with GnRH neurons, express the GnRH receptor.** This experiment will prove a direct action of GnRH on this olfactory cell population, which is involved in olfactory-mediated reproductive behaviours;

**- Elucidate the role of distinct hormones on the specific steps of the adult neurogenesis process**. Specifically, to address the role of testosterone in the differentiation of progenitors cells toward a neuronal lineage, we intend to inject testosterone and/or its metabolites (dihydrotestosterone and estradiol) in both males and females *GnRH::cre/Dicer*$^{loxP/loxP}$ mice, and then evaluate the differentiation of progenitor cells; to unravel the role of estradiol in the survival of newborn neurons, we have planned to inject estradiol combined or not with progesterone in both males and females *GnRH::cre/Dicer*$^{loxP/loxP}$ mice, and then evaluate the survival of newborn neurons; to address a possible direct effect of GnRH on AN, we have planned intracerebral injections of the GnRH peptide in gonadectomized *GnRH::cre/Dicer*$^{loxP/loxP}$ mice to exclude involvement of adenohypophyseal and gonadal hormones;

**- Evaluate whether the alteration in the dorsal SVZ can affect specific subpopulations of interneurons in the MOB**. We aim to selectively quantify different newborn cells subpopulation (TH+, CB+ and CR+) in superficial and deep granular cell layers of the MOB.

## Supplementary Tables

### Table 1.1

| Ki67+ cells in the SVZ | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 7,797 | 0,4511 | ns | No | |
| Gender | 20,5 | 0,0936 | ns | No | |
| Genotype | 3,128 | 0,3059 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 2993 | 3 | 997,5 | F (3, 24) = 0.9096 | P=0.4511 |
| Gender | 7867 | 3 | 2622 | F (3, 24) = 2.391 | P=0.0936 |
| Genotype | 1201 | 1 | 1201 | F (1, 24) = 1.095 | P=0.3059 |
| Residual | 26320 | 24 | 1097 | | |

| Tukey's multiple comparisons test | 95.00% CI of diff. | Summary | P Value |
|---|---|---|---|
| dSVZ FEMALE:WT vs. dSVZ FEMALE:KO | -63.05 to 92.05 | ns | 0,9982 |
| dSVZ FEMALE:WT vs. dSVZ MALE:WT | -79.05 to 76.05 | ns | >0.9999 |
| dSVZ FEMALE:WT vs. dSVZ MALE:KO | -36.05 to 119.1 | ns | 0,6431 |
| dSVZ FEMALE:WT vs. vSVZ FEMALE:WT | -35.3 to 119.8 | ns | 0,6233 |
| dSVZ FEMALE:WT vs. vSVZ FEMALE:KO | -40.3 to 114.8 | ns | 0,751 |
| dSVZ FEMALE:WT vs. vSVZ MALE:WT | -29.05 to 126.1 | ns | 0,4594 |
| dSVZ FEMALE:WT vs. vSVZ MALE:KO | -32.55 to 122.6 | ns | 0,5503 |
| dSVZ FEMALE:KO vs. dSVZ MALE:WT | -93.55 to 61.55 | ns | 0,9967 |
| dSVZ FEMALE:KO vs. dSVZ MALE:KO | -50.55 to 104.6 | ns | 0,9375 |
| dSVZ FEMALE:KO vs. vSVZ FEMALE:WT | -49.8 to 105.3 | ns | 0,9286 |
| dSVZ FEMALE:KO vs. vSVZ FEMALE:KO | -54.8 to 100.3 | ns | 0,9743 |
| dSVZ FEMALE:KO vs. vSVZ MALE:WT | -43.55 to 111.6 | ns | 0,8241 |
| dSVZ FEMALE:KO vs. vSVZ MALE:KO | -47.05 to 108.1 | ns | 0,8891 |
| dSVZ MALE:WT vs. dSVZ MALE:KO | -34.55 to 120.6 | ns | 0,6034 |
| dSVZ MALE:WT vs. vSVZ FEMALE:WT | -33.8 to 121.3 | ns | 0,5835 |
| dSVZ MALE:WT vs. vSVZ FEMALE:KO | -38.8 to 116.3 | ns | 0,7141 |
| dSVZ MALE:WT vs. vSVZ MALE:WT | -27.55 to 127.6 | ns | 0,4222 |
| dSVZ MALE:WT vs. vSVZ MALE:KO | -31.05 to 124.1 | ns | 0,5108 |
| dSVZ MALE:KO vs. vSVZ FEMALE:WT | -76.8 to 78.3 | ns | >0.9999 |
| dSVZ MALE:KO vs. vSVZ FEMALE:KO | -81.8 to 73.3 | ns | >0.9999 |
| dSVZ MALE:KO vs. vSVZ MALE:WT | -70.55 to 84.55 | ns | >0.9999 |

| | | | |
|---|---|---|---|
| dSVZ MALE:KO vs. vSVZ MALE:KO | -74.05 to 81.05 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ FEMALE:KO | -82.55 to 72.55 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ MALE:WT | -71.3 to 83.8 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ MALE:KO | -74.8 to 80.3 | ns | >0.9999 |
| vSVZ FEMALE:KO vs. vSVZ MALE:WT | -66.3 to 88.8 | ns | 0,9997 |
| vSVZ FEMALE:KO vs. vSVZ MALE:KO | -69.8 to 85.3 | ns | >0.9999 |
| vSVZ MALE:WT vs. vSVZ MALE:KO | -81.05 to 74.05 | ns | >0.9999 |

**Table 1.2**

| Ki67+/DCX+ cells in the SVZ | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 22,57 | 0,0531 | ns | No | |
| Gender | 8,524 | 0,3631 | ns | No | |
| Genotype | 7,668 | 0,0958 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 78354 | 3 | 26118 | F (3, 24) = 2.948 | P=0.0531 |
| Gender | 29593 | 3 | 9864 | F (3, 24) = 1.114 | P=0.3631 |
| Genotype | 26623 | 1 | 26623 | F (1, 24) = 3.005 | P=0.0958 |
| Residual | 212602 | 24 | 8858 | | |

| Tukey's multiple comparisons test | 95.00% CI of diff. | Summary | P Value |
|---|---|---|---|
| dSVZ FEMALE:WT vs. dSVZ FEMALE:KO | -200.7 to 240.2 | ns | >0.9999 |
| dSVZ FEMALE:WT vs. dSVZ MALE:WT | -363.9 to 76.92 | ns | 0,4103 |
| dSVZ FEMALE:WT vs. dSVZ MALE:KO | -136.2 to 304.7 | ns | 0,9025 |
| dSVZ FEMALE:WT vs. vSVZ FEMALE:WT | -289.7 to 151.2 | ns | 0,9629 |
| dSVZ FEMALE:WT vs. vSVZ FEMALE:KO | -291.4 to 149.4 | ns | 0,9578 |
| dSVZ FEMALE:WT vs. vSVZ MALE:WT | -268.7 to 172.2 | ns | 0,9953 |
| dSVZ FEMALE:WT vs. vSVZ MALE:KO | -283.7 to 157.2 | ns | 0,9772 |
| dSVZ FEMALE:KO vs. dSVZ MALE:WT | -383.7 to 57.17 | ns | 0,2621 |
| dSVZ FEMALE:KO vs. dSVZ MALE:KO | -155.9 to 284.9 | ns | 0,9747 |
| dSVZ FEMALE:KO vs. vSVZ FEMALE:WT | -309.4 to 131.4 | ns | 0,8754 |
| dSVZ FEMALE:KO vs. vSVZ FEMALE:KO | -311.2 to 129.7 | ns | 0,8645 |
| dSVZ FEMALE:KO vs. vSVZ MALE:WT | -288.4 to 152.4 | ns | 0,9663 |
| dSVZ FEMALE:KO vs. vSVZ MALE:KO | -303.4 to 137.4 | ns | 0,909 |
| dSVZ MALE:WT vs. dSVZ MALE:KO | 7.334 to 448.2 | * | 0,0393 |

| | | | |
|---|---|---|---|
| dSVZ MALE:WT vs. vSVZ FEMALE:WT | -146.2 to 294.7 | ns | 0,9469 |
| dSVZ MALE:WT vs. vSVZ FEMALE:KO | -147.9 to 292.9 | ns | 0,953 |
| dSVZ MALE:WT vs. vSVZ MALE:WT | -125.2 to 315.7 | ns | 0,8341 |
| dSVZ MALE:WT vs. vSVZ MALE:KO | -140.2 to 300.7 | ns | 0,9224 |
| dSVZ MALE:KO vs. vSVZ FEMALE:WT | -373.9 to 66.92 | ns | 0,3303 |
| dSVZ MALE:KO vs. vSVZ FEMALE:KO | -375.7 to 65.17 | ns | 0,3173 |
| dSVZ MALE:KO vs. vSVZ MALE:WT | -352.9 to 87.92 | ns | 0,5077 |
| dSVZ MALE:KO vs. vSVZ MALE:KO | -367.9 to 72.92 | ns | 0,3772 |
| vSVZ FEMALE:WT vs. vSVZ FEMALE:KO | -222.2 to 218.7 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ MALE:WT | -199.4 to 241.4 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ MALE:KO | -214.4 to 226.4 | ns | >0.9999 |
| vSVZ FEMALE:KO vs. vSVZ MALE:WT | -197.7 to 243.2 | ns | >0.9999 |
| vSVZ FEMALE:KO vs. vSVZ MALE:KO | -212.7 to 228.2 | ns | >0.9999 |
| vSVZ MALE:WT vs. vSVZ MALE:KO | -235.4 to 205.4 | ns | >0.9999 |

**Table 1.3**

| **Total Ki67+ cells in the SVZ** | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 12,45 | 0,2408 | ns | No | |
| Gender | 20,89 | 0,0828 | ns | No | |
| Genotype | 0,08398 | 0,8633 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 59171 | 3 | 19724 | F (3, 24) = 1.496 | P=0.2408 |
| Gender | 99247 | 3 | 33082 | F (3, 24) = 2.51 | P=0.0828 |
| Genotype | 399 | 1 | 399 | F (1, 24) = 0.03027 | P=0.8633 |
| Residual | 316336 | 24 | 13181 | | |

| Tukey's multiple comparisons test | 95.00% CI of diff. | Summary | P Value |
|---|---|---|---|
| dSVZ FEMALE:WT vs. dSVZ FEMALE:KO | -360.4 to 177.4 | ns | 0,9442 |
| dSVZ FEMALE:WT vs. dSVZ MALE:WT | -539.6 to -1.886 | * | 0,0476 |
| dSVZ FEMALE:WT vs. dSVZ MALE:KO | -394.6 to 143.1 | ns | 0,7741 |
| dSVZ FEMALE:WT vs. vSVZ FEMALE:WT | -421.6 to 116.1 | ns | 0,5752 |
| dSVZ FEMALE:WT vs. vSVZ FEMALE:KO | -428.4 to 109.4 | ns | 0,5237 |
| dSVZ FEMALE:WT vs. vSVZ MALE:WT | -394.4 to 143.4 | ns | 0,7758 |
| dSVZ FEMALE:WT vs. vSVZ MALE:KO | -412.9 to 124.9 | ns | 0,6422 |
| dSVZ FEMALE:KO vs. dSVZ MALE:WT | -448.1 to 89.61 | ns | 0,3817 |
| dSVZ FEMALE:KO vs. dSVZ MALE:KO | -303.1 to 234.6 | ns | 0,9999 |
| dSVZ FEMALE:KO vs. vSVZ FEMALE:WT | -330.1 to 207.6 | ns | 0,994 |
| dSVZ FEMALE:KO vs. vSVZ FEMALE:KO | -336.9 to 200.9 | ns | 0,9889 |
| dSVZ FEMALE:KO vs. vSVZ MALE:WT | -302.9 to 234.9 | ns | 0,9999 |
| dSVZ FEMALE:KO vs. vSVZ MALE:KO | -321.4 to 216.4 | ns | 0,9977 |
| dSVZ MALE:WT vs. dSVZ MALE:KO | -123.9 to 413.9 | ns | 0,6346 |
| dSVZ MALE:WT vs. vSVZ FEMALE:WT | -150.9 to 386.9 | ns | 0,8234 |
| dSVZ MALE:WT vs. vSVZ FEMALE:KO | -157.6 to 380.1 | ns | 0,8616 |
| dSVZ MALE:WT vs. vSVZ MALE:WT | -123.6 to 414.1 | ns | 0,6327 |
| dSVZ MALE:WT vs. vSVZ MALE:KO | -142.1 to 395.6 | ns | 0,7674 |
| dSVZ MALE:KO vs. vSVZ FEMALE:WT | -295.9 to 241.9 | ns | >0.9999 |
| dSVZ MALE:KO vs. vSVZ FEMALE:KO | -302.6 to 235.1 | ns | 0,9999 |
| dSVZ MALE:KO vs. vSVZ MALE:WT | -268.6 to 269.1 | ns | >0.9999 |
| dSVZ MALE:KO vs. vSVZ MALE:KO | -287.1 to 250.6 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ FEMALE:KO | -275.6 to 262.1 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ MALE:WT | -241.6 to 296.1 | ns | >0.9999 |
| vSVZ FEMALE:WT vs. vSVZ MALE:KO | -260.1 to 277.6 | ns | >0.9999 |
| vSVZ FEMALE:KO vs. vSVZ MALE:WT | -234.9 to 302.9 | ns | 0,9999 |
| vSVZ FEMALE:KO vs. vSVZ MALE:KO | -253.4 to 284.4 | ns | >0.9999 |
| vSVZ MALE:WT vs. vSVZ MALE:KO | -287.4 to 250.4 | ns | >0.9999 |

**Table 2.1**

| Ki67+ cells in the DG | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 0,3284 | 0,8189 | ns | No | |
| Gender | 2,321 | 0,5453 | ns | No | |
| Genotype | 18,65 | 0,1018 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 4,424 | 1 | 4,424 | F (1, 13) = 0.05458 | P = 0.8189 |
| Gender | 31,27 | 1 | 31,27 | F (1, 13) = 0.3858 | P = 0.5453 |
| Genotype | 251,3 | 1 | 251,3 | F (1, 13) = 3.100 | P = 0.1018 |
| Residual | 1054 | 13 | 81,04 | | |

| Tukey's multiple comparisons test | 95% CI of diff. | Summary | P Value |
|---|---|---|---|
| male :WT vs. male :KO | -9.934 to 27.43 | ns | 0,5355 |
| male :WT vs. female:WT | -14.93 to 22.43 | ns | 0,9336 |
| male :WT vs. female:KO | -7.275 to 28.17 | ns | 0,348 |
| male :KO vs. female:WT | -23.68 to 13.68 | ns | 0,8597 |
| male :KO vs. female:KO | -16.02 to 19.42 | ns | 0,9918 |
| female:WT vs. female:KO | -11.02 to 24.42 | ns | 0,6903 |

**Table 2.2**

| DCX+ cells in the DG | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 3,953 | 0,3378 | ns | No | |
| Gender | 32,41 | 0,0137 | * | Yes | |
| Genotype | 13,32 | 0,0907 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 15488 | 1 | 15488 | F (1, 13) = 0.9903 | P = 0.3378 |
| Gender | 126966 | 1 | 126966 | F (1, 13) = 8.118 | P = 0.0137 |
| Genotype | 52206 | 1 | 52206 | F (1, 13) = 3.338 | P = 0.0907 |
| Residual | 203308 | 13 | 15639 | | |

| Tukey's multiple comparisons test | 95% CI of diff. | Summary | P Value |
|---|---|---|---|
| male :WT vs. male :KO | -87.55 to 431.5 | ns | 0,2575 |
| male :WT vs. female:WT | -372.5 to 146.5 | ns | 0,5918 |
| male :WT vs. female:KO | -308.5 to 183.9 | ns | 0,8781 |
| male :KO vs. female:WT | -544.5 to -25.45 | * | 0,0298 |
| male :KO vs. female:KO | -480.5 to 11.93 | ns | 0,0643 |
| female:WT vs. female:KO | -195.5 to 296.9 | ns | 0,9289 |

**Table 2.3**

| Total Ki67+ cells in the DG | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 9,556 | 0,2252 | ns | No | |
| Gender | 0,4346 | 0,7903 | ns | No | |
| Genotype | 14,6 | 0,1396 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 613,9 | 1 | 613,9 | F (1, 13) = 1.621 | P = 0.2252 |
| Gender | 27,92 | 1 | 27,92 | F (1, 13) = 0.07372 | P = 0.7903 |
| Genotype | 937,9 | 1 | 937,9 | F (1, 13) = 2.477 | P = 0.1396 |
| Residual | 4923 | 13 | 378,7 | | |

| Tukey's multiple comparisons test | 95% CI of diff. | Summary | P Value |
|---|---|---|---|
| male :WT vs. male :KO | -13.39 to 67.39 | ns | 0,2512 |
| male :WT vs. female:WT | -30.89 to 49.89 | ns | 0,8989 |
| male :WT vs. female:KO | -25.97 to 50.67 | ns | 0,7812 |
| male :KO vs. female:WT | -57.89 to 22.89 | ns | 0,5954 |
| male :KO vs. female:KO | -52.97 to 23.67 | ns | 0,6829 |
| female:WT vs. female:KO | -35.47 to 41.17 | ns | 0,9961 |

**Table 2.4**

| Total DCX+ cell in the DG | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 5,27 | 0,2526 | ns | No | |
| Gender | 33,89 | 0,0095 | ** | Yes | |
| Genotype | 14,71 | 0,0668 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 20529 | 1 | 20529 | F (1, 13) = 1.433 | P = 0.2526 |
| Gender | 132023 | 1 | 132023 | F (1, 13) = 9.219 | P = 0.0095 |
| Genotype | 57318 | 1 | 57318 | F (1, 13) = 4.002 | P = 0.0668 |
| Residual | 186169 | 13 | 14321 | | |

| Tukey's multiple comparisons test | 95% CI of diff. | Summary | P Value |
|---|---|---|---|
| male :KO vs. male :WT | -434.9 to 61.87 | ns | 0,1734 |
| female:WT vs. male :WT | -141.1 to 355.6 | ns | 0,5979 |
| female:KO vs. male :WT | -175.2 to 296.0 | ns | 0,874 |
| female:WT vs. male :KO | 45.38 to 542.1 | * | 0,0189 |
| female:KO vs. male :KO | 11.28 to 482.5 | * | 0,0389 |
| female:KO vs. female:WT | -282.5 to 188.8 | ns | 0,9353 |

**Table 3.1**

| Tukey's multiple comparisons test | 95.00% CI of diff. | Summary | P Value |
|---|---|---|---|
| male :KO vs. male :WT | -4154 to 8105 | ns | 0,8058 |
| female:WT vs. male :WT | 1103 to 12452 | * | 0,0156 |
| female:KO vs. male :WT | -4889 to 6161 | ns | 0,9882 |
| female:WT vs. male :KO | -872.3 to 10477 | ns | 0,1166 |
| female:KO vs. male :KO | -6864 to 4186 | ns | 0,9052 |
| female:KO vs. female:WT | -11157 to -1126 | * | 0,0129 |

| Density of BrdU+ cells in the MOB GrL | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 22,3 | 0,0094 | ** | Yes | |
| Gender | 10,01 | 0,0693 | ns | No | |
| Genotype | 5,875 | 0,1574 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 98645436 | 1 | 98645436 | F (1, 21) = 8.16 | P=0.0094 |
| Gender | 44286121 | 1 | 44286121 | F (1, 21) = 3.663 | P=0.0693 |
| Genotype | 25989349 | 1 | 25989349 | F (1, 21) = 2.15 | P=0.1574 |
| Residual | 253862540 | 21 | 12088692 | | |

**Table 3.2**

| Density of BrdU+ cells in the total AOB GrL | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 7,776 | 0,2751 | ns | No | |
| Gender | 18,16 | 0,1061 | ns | No | |
| Genotype | 2,709 | 0,5125 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 2723057 | 1 | 2723057 | F (1, 12) = 1.308 | P=0.2751 |
| Gender | 6358697 | 1 | 6358697 | F (1, 12) = 3.053 | P=0.1061 |
| Genotype | 948619 | 1 | 948619 | F (1, 12) = 0.4555 | P=0.5125 |
| Residual | 24989306 | 12 | 2082442 | | |

| Tukey's multiple comparisons test | 95.00% CI of diff. | Summary | P Value |
|---|---|---|---|
| male:WT vs. male:KO | -3368 to 2691 | ns | 0,9868 |
| male:WT vs. female:WT | -5115 to 943.6 | ns | 0,2259 |
| male:WT vs. female:KO | -3803 to 2256 | ns | 0,8714 |
| male:KO vs. female:WT | -4777 to 1282 | ns | 0,3592 |
| male:KO vs. female:KO | -3465 to 2594 | ns | 0,9727 |
| female:WT vs. female:KO | -1717 to 4342 | ns | 0,5883 |

**Table 3.3**

| Density of BrdU+ cells in the Anterior AOB | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 4,971 | 0,3369 | ns | No | |
| Gender | 27,31 | 0,0371 | * | Yes | |
| Genotype | 8,118 | 0,2252 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 2341384 | 1 | 2341384 | F (1, 12) = 1.001 | P=0.3369 |
| Gender | 12863701 | 1 | 12863701 | F (1, 12) = 5.499 | P=0.0371 |
| Genotype | 3824028 | 1 | 3824028 | F (1, 12) = 1.635 | P=0.2252 |
| Residual | 28073842 | 12 | 2339487 | | |

| Tukey's multiple comparisons test | 95.00% CI of diff. | Summary | P Value |
|---|---|---|---|
| male:KO vs. male:WT | -3424 to 2998 | ns | 0,9972 |
| female:WT vs. male:WT | -652.6 to 5769 | ns | 0,1374 |
| female:KO vs. male:WT | -2395 to 4027 | ns | 0,8732 |
| female:WT vs. male:KO | -439.9 to 5982 | ns | 0,0997 |
| female:KO vs. male:KO | -2183 to 4239 | ns | 0,7788 |
| female:KO vs. female:WT | -4954 to 1468 | ns | 0,4087 |

**Table 3.4**

| Density of BrdU+ cells in the Posterior AOB | | | | |
|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | |
| Alpha | 0,05 | | | |
| Source of Variation | % of total variation | P value | P value | Significant? |

| | | | | | |
|---|---|---|---|---|---|
| Interaction | 10,08 | 0,251 | ns | No | |
| Gender | 6,823 | 0,3405 | ns | No | |
| Genotype | 0,0001843 | 0,996 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 3133537 | 1 | 3133537 | F (1, 12) = 1.455 | P=0.2510 |
| Gender | 2121947 | 1 | 2121947 | F (1, 12) = 0.9852 | P=0.3405 |
| Genotype | 57,3 | 1 | 57,3 | F (1, 12) = 2.661e-005 | P=0.9960 |
| Residual | 25845227 | 12 | 2153769 | | |

| **Tukey's multiple comparisons test** | **95.00% CI of diff.** | **Summary** | **P Value** |
|---|---|---|---|
| male:KO vs. male:WT | -2192 to 3970 | ns | 0,8266 |
| female:WT vs. male:WT | -1467 to 4694 | ns | 0,4379 |
| female:KO vs. male:WT | -2349 to 3813 | ns | 0,893 |
| female:WT vs. male:KO | -2356 to 3805 | ns | 0,8958 |
| female:KO vs. male:KO | -3238 to 2924 | ns | 0,9987 |
| female:KO vs. female:WT | -3962 to 2200 | ns | 0,8301 |

**Table 3.5**

| **Density of BrdU+ cells in the DG** | | | | | |
|---|---|---|---|---|---|
| Two-way ANOVA | Ordinary | | | | |
| Alpha | 0,05 | | | | |
| Source of Variation | % of total variation | P value | P value | Significant? | |
| Interaction | 0,5602 | 0,6989 | ns | No | |
| Gender | 5,165 | 0,2468 | ns | No | |
| Genotype | 10,6 | 0,1025 | ns | No | |
| ANOVA table | SS | DF | MS | F (DFn, DFd) | P value |
| Interaction | 186211 | 1 | 186211 | F (1, 22) = 0.1535 | P = 0.6989 |
| Gender | 1,72E+06 | 1 | 1,72E+06 | F (1, 22) = 1.416 | P = 0.2468 |
| Genotype | 3,52E+06 | 1 | 3,52E+06 | F (1, 22) = 2.904 | P = 0.1025 |

| | | | |
|---|---|---|---|
| Residual | 2,67E+07 | 22 | 1,21E+06 |

| **Tukey's multiple comparisons test** | **95.00% CI of diff.** | **Summary** | **P Value** |
|---|---|---|---|
| Males:wt vs. Males:ko | -1384 to 2564 | ns | 0,8397 |
| Males:wt vs. Females:wt | -2584 to 1162 | ns | 0,7199 |
| Males:wt vs. Females:ko | -1641 to 2104 | ns | 0,9858 |
| Males:ko vs. Females:wt | -2953 to 350.5 | ns | 0,1579 |
| Males:ko vs. Females:ko | -2010 to 1293 | ns | 0,93 |
| Females:wt vs. Females:ko | -586.7 to 2471 | ns | 0,3419 |

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# CHAPTER IV

## Concluding Remarks and Future Perspectives

Ongoing studies performed during the last few years are showing that the accomplishment of social-reproductive behaviors is achieved through a complex interaction among adult brain plasticity, endocrine system and environmental cues. The way this integration occurs represents a step ahead in understanding how the brain processes external and internal information to produce appropriate behaviors. This issue was the focus of my PhD thesis that approached this goal by studying the modulation of adult neurogenesis in the olfactory bulb, as an elective mechanism of neural plasticity. The study focussed on puberty (a critical postnatal developmental time-window), and employed analyses on wild type mice as well as models showing reduced or absence of gonadal hormones and GnRH secretion. The data here collected clearly confirm that gonadal hormones and adult neurogenesis are cross-linked, although the drastic change in responses to sex cues occurring in the female olfactory bulb after puberty seems independent by the rise of gonadal hormones that typically takes place with the onset of puberty. Rather, our results suggest that the responsiveness to the male cues in female mice is an activity-dependent mechanism that drives, and in parallel implies, modulation of newborn neurons in the AOB, and thus increases motivation in exploring opposite-sex cues. Nevertheless, it is reasonable to suppose that the level of gonadal hormones in the post-pubertal females is involved in this motivational aspect. Another important result here established is concerning the involvement of GnRH in modulating adult neural plasticity and, in turn, olfactory-dependent reproductive behaviours. This result is quite original, but at the same time consistent with several experimental evidences supporting multiple roles for the GnRH system in the adult brain. Important points that should be addressed in the close future concern whether and how the GnRH system acts to modulate adult neural plasticity, besides the HPG axis activation. We propose that a more functional study on the olfactory bulb population, and GnRH replacement therapy in deprived models will allow to unravel the above questions.

# CHAPTER V

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# CHAPTER VI

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# Personal Information

## Articles

- Giulia Nato, Alessia Caramello, **Sara Trova**, Valeria Avataneo, Chiara Rolando, Verdon Taylor, Annalisa Buffo, Paolo Peretto* and Federico Luzzati* "*Striatal astrocytes produce neuroblasts in an excitotoxic model of Huntington's disease*", Development, 2015 Mar 1;142(5):840-5. doi: 10.1242/dev.116657. Epub 2015 Feb 5. *co-last-authors.

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- **Sara Trova***, Livio Oboti*, Roberta Schellino, Marilena Marraudino, Natalie R Harris, Olubukola M Abiona, Mojca Stampar, Weihong Lin and Paolo Peretto, "*Activity Dependent Modulation of Granule Cell Survival in the Accessory Olfactory Bulb at Puberty*", Frontiers in Neuranatomy, 2017 May 1st, Accepted. *co-first-authors.

## Oral Presentations

- Seminars entitled "Epigenetic in the Nervous System", Course in Deveolpmental Biology (Chair: Prof. Aldo Fasolo), Turin (Italy), May 2014;

- "The involvment of the GnRH System in mediating Olfactory Bulb physiological plasticity", 4th Scientific Meeting/Training School of the European GnRH Network, Budapest, Hungary, 6-9 March, 2016;

- Seminars entitled "The development of the GnRH System", Course in Deveolpmental Biology (Chair: Prof. Silvia De Marchis), Turin (Italy), 18 May 2016;

- Seminars entitled "The development of neurons controlling reproduction", Course in Deveolpmental Biology (Chair: Prof. Silvia De Marchis), Turin (Italy), 9 May 2017;

- "Activity Dependent Modulation of Granule Cell Survival in the Accessory Olfactory Bulb at Puberty", 63° Convegno Gruppo Embriologico Italiano (GEI), Rome (Italy), 12-15 June 2017.

## Attended Congresses

- FENS (Federation of European Neuroscience Societies) Forum 5-9 July 2014, Milan, Italy;

- Joint Scientific Meeting & Training School, COST-GnRH Network, PRATO, Italy, 27-29 April, 2015;

- Adult Neurogenesis: Evolution, Regulation and Function, DRESDEN, Germany, 6-8 May, 2015;

- British Society For Neuroendocrinology Annual Meeting 2015 - 3rd BSN-SNE Joint Meeting, LILLE, France, 23-25 September, 2015;

- 4th Scientific Meeting/Training School of the European GnRH Network, Budapest, Hungary, 6-9 March, 2016;

- Neuroscience 2016 - Society for Neuroscience, San Diego, California (USA), 12-16 November, 2016;

- 9th International Meeting Steroids and Nervous System, Torino, 11-15 February, 2017;

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# APPENDIX

## Semiochemicals

**Pheromones** (from the Greek "pherein", which means to carry or transfer and "hormone", which means to stimulate or excite) are chemicals that influence members within the same species. They provide a way for animals to communicate socially, and they can strongly induce or suppress sexual behavior (Liberles, 2014). The first pheromone ever to be characterized chemically was the insect pheromone bombykol, which is released by the female silkworm *Bombyx mori* to attract mates. Pheromones can be classified as either releasers or primers. Releaser pheromones initiate an immediate behavioral response upon reception. Primer pheromones modulate the endocrine system causing a delayed behavioral response. Pheromones are present in male and female urine (e.g., MUPs, Trimethylamine, modified steroids- estrogens and androgens derivates) or in tears (exocrine-gland secreting peptides; ESP1, ESP24). In mice, the production of some pheromones depend on the hormonal state. Some pheromones are produced at near constant level throughout life without influence on the internal state, such as those that signal individual identity (genotype and kinship; Bohem *and* Zufall, 2006). Production of other pheromones is striking altered at puberty: levels of adult male and female pheromones increase while levels of a juvenile pheromone decrease (Stowers *and* Kuo, 2015; Ferrero *et al*., 2010, 2013; Haga-Yamanaka *et al*., 2014; Kimoto *et al*., 2007).

**Kairomones** ("kairos" meaning the right or opportune moment) are interspecific chemical messengers benefitting only the receiver, by improving its fitness. For example, cats and rats emit specific chemical cues that elicit innate fear behavior in their natural preys, mice (Bakker *and* Leinders-Zufall, 2016).

**Allomones** ("allos" meaning another or different, implying something unfamiliar or foreign) are interspecific chemical messengers that benefit the emitter, but not the receiver. They are usually produced by plants as repellent against insect herbivores (Bakker *and* Leinders-Zufall, 2016).

**Synomones** ("syn" meaning acting or considered together) are chemicals released by members of one species, which evokes in the receiver of another species a behavioral or physiological response that is adaptatively favorable to

both organisms (Sbarbati *and* Osculati, 2006). Two examples of synomones are floral odors that attract pollinators (in which the pollinator gains food whereas the flower is pollinate) and the chemical interaction between anemone fish (clownfish) and sea anemones.

## GnRH Neurons Development

### Origin

The noticeable properties of GnRH neurons in postnatal development and adult life are related to their singular embryological origin and development. In fact, while the majority of neural cells arise from neurons within the developing nervous system itself, GnRH neurons are unusual in that they are derived from progenitor cells in the epithelium of the olfactory placode, entwining their development with that of the olfactory system (Boehm *et al*., 2015). In 1989, two independent studies in mice (Wray *et al*., 1989; Schwanzel-Fukuda *et al*., 1989) reported for the first time the origin of GnRH-1 cells. By combining *in situ* hybridization histochemistry and immunocytochemistry, they detected LHRH mRNA expressing cells in the olfactory pit at embryonic day 11.5 (E11.5), without any presence of these cells into the forebrain. From E12.5 to E15.5, these cells were located in a rostral-to-caudal gradient in forebrain areas, and during this period, their location shifts, being the majority of cells in the nasal compartment at E12.5, and in the forebrain at E15.5. The total number was established at ~800 cells (equalled the number of adult animals). Thus, these two studies showed that LHRH cells in the central nervous system arise from a discrete group of progenitor cells in the olfactory placode and that a subpopulation of these cells migrate in association with developing sensory axons of the olfactory/vomeronasal/terminal nerve system into forebrain areas, where they subsequently establish an adult-like distribution.

In all vertebrates, nasal placodes (also known as olfactory placodes or nasal pit; a structure that forms as the olfactory placode invaginates) develop as thickenings of the ectoderm coming from the anterior end of the neural plate. In humans, olfactory placode develop around the fifth week of gestation (Muller

*and* O'Rahilly, 2004). Starting from embryonic day 10.5 (E10.5) in mouse, the nasal placodes invaginate and give rise to cell types including GnRH-1 neurons that migrate to the brain, olfactory sensory neurons (OSNs) that project to the brain (Wray *et al*., 1989) and olfactory ensheathing cells (OECs) that provide essential growth and guidance for olfactory sensory neurons (OSN) axons (Su *and* He, 2010).

Key developmental studies, based on ablation experiment of the olfactory pit or removal of the respiratory epithelium in mice (Akutsu *et al*., 1992; Murakami *et al*., 1992; Dellovade *et al*., 1998b; Abraham *et al*., 2010; el Amraoui *and* Dubois, 1993), or based on mutation of trophic factors coming from the respiratory epithelium (Kawauchi *et al*., 2005; Chung *et al*., 2008; Forni *et al*., 2013), identified the origin of GnRH neurons in the olfactory pit, coinciding with that of the olfactory system, thus contributing to highlight why in some diseases, like Kallman syndrome, defects of the sense of smell coexist with hypogonadism. Nevertheless, more recent studies (Sabado *et al*., 2011; Forni *et al*., 2011; Forni *and* Wray, 2012) thanks to cell and lineage tracing experiments, confirmes that GnRH-1 cells derive from progenitor cells whithin the placode, but also that the embryonic origin and/molecular profile of the GnRH-1 neuronal progenitors is not homogeneous, since some GnRH-1 cells could have also a neural crest origin (Forni *et. al*., 2011; Forni *and* Wray, 2012). Therefore, neural crest could contribute also to the cells of the olfactory pit (olfactory ensheathing cells and subpopulations of GnRH-1 neurons, olfactory and vomeronasal cells; Forni *et al*., 2011).

**Migration**

The peculiarity of GnRH cells resides in the fact that they are born outside the CNS then they migrate inside the CNS, independent to their final location, the majority project to the median eminence. In rodents and in humans, early GnRH neurons migrate together with heterogeneous coalescence of placode-derived and neural crest-derived migratory cells (Forni *et al*., 2011; Casoni *et al*., 2016) and olfactory axons, collectively called the 'migratory mass' (MM; Miller *et al*., 2010; Valverde *et al*., 1992). These pioneer neurons of the MM are postmitotic, since they express delta/notch-like EGF repeat containing (DNER), a

transmembrane protein specifically localized in the dendrites and cell bodies of postmitotic neurons (Casoni *et al*., 2016). The GnRH journey start in the nasal compartment, they migrate through the cribriform plate with large numbers of olfactory sensory axons as well as blood vessels, and then turn caudally on a transient pathway into the developing forebrain. In mice, the first GnRH cells leave the nasal placode around embryonic day (E)11. In humans, they are first detected at GW 5 and 6 and they start to migrate at this stage. The migration of GnRH cells during development is an axophylic migration, since it shares both characteristics of tangential and radial migration, the two typical migrations that undertake neurons from their birthplace to their final location during development. The axophilic migration means that neurons migrate along the anterior-posterior axis of the body use existing axons tract to migrate along. Moreover, GnRH-1 neurones migrate in clusters that resemble the chains seen in homotypic neuronal migration.

The pathway support of GnRH neurons is caracterized by vomeronasal nerve (VNN) and the nervus terminalis (TN). Neurons from these nerves belong to the accessory olfactory system (AOS) and they develop simultaneously from the medial part of the nasal disc, together with GnRH neurons (Muller *and* O' Rahilly, 2004; Verney et al., 2002). The VNN and TN travel together across the nasal region (Brown, 1987), emerging from the vomeronasal epithelium and running medially from the olfactory epithelium towards the dorsal region of the OB, namely the developing Accessory Olfactory Bulb (AOB), whereas the TN project both ventrally and dorsally within the forebrain, primarily to the septal, hypothalamic and limbic areas (Pearson, 1941; Schwanzel-Fukuda *and* Silverman, 1980; Wirsing-Wiechmann *and* Oka, 2002). In both rodents and humans, the VNN and TN pathway express: peripherin, a neural type III intermediate filament protein (Wray *et al*., 1994; Casoni *et al*., 2016); TAG1, a transient axonal glycoprotein, transiently expressed during development (contactin 2; Yoshida *et al*, 1995; Casoni *et al*., 2016) and the neuron-specific beta III tubulin (Giacobini *et al*., 2008; Casoni *et al*., 2016). Prior to GnRH-1 neurons migration, 'pioneer' olfactory axons cross the nasal region and target the base of the developing telencephalon. The caudal branch is also termed nervus terminalis or the caudal branch of the vomeronasal nerve. During their

migration, GnRH neurons are tighly coupled to their pathway substrate via multiple membrane-bound cell adhesion molecules. In the nasal region, olfactory/vomeronasal axons express TAG-1 and DCC (deleted in colorectal cancer; receptor for the guidance molecule netrin-1) but not on the branches of these nerves that enter the forebrain and course rostrally into the olfactory bulb / accessory regions. At the junction between the nasal compartment and the forebrain, both extracellular factors and membrane molecules are involved in directing GnRH cells to follow the caudal branch of VNN. Several molecules are downregulated in the GnRH neurons as they enter into the forebrain. Spatio-temporal events suggest that a combination of molecules, with some turning on and other turning off, plays an important role in establishing the appropriate GnRH-1 adult-like distribution. Interestingly, at the nasal/forebrain junction GnRH neurons appear to pause: for mature; for establishing their migratory pathway to appropriate forebrain regions; changing in extracellular milieu composition. Molecules that are involved in this process are: HGF (hepatocyte growth factor) and SDF-I (stromal cell derived factor I). Intriguingly, Casoni and coworkers recently showed that, in addition of the canonical ventral migratory pathway, a considerable subset of TN fibers sprouted dorsally and laterally to telencephalic regions corresponding to the NCX and to the developing hippocampus. GnRH neurons, that are juxtaposed to these fibers, migrate also to these telencephalic areas (Casoni *et al*., 2016). So in humans, at 53$^{rd}$ day of gestation, GnRH neurons migrate both dorsally and laterally towards the septum, OB and cortex other than ventrally toward the developing hypothalamus (Casoni *et al*., 2016). Interestingly, in both rodents and humans, some GnRH cells form an unexpected ring-like distribution, forming two symmetric circles around the OB, which will be maintained (at least in rodents) during adulthood (see Casoni *et al*., 2016; and CHAPTER III of this thesis).

At the final step of their journey, GnRH neurons migrate to the forebrain and appear to the detach to peripherin-positive fibers before entering the septal-preoptic area (Yoshida *et al*., 1995). As expected, in both rodents and humans, the number of GnRH cells in the nose during development progressively decreased with a concomitant increase in cells in dorsal and ventral migratory pathways. Casoni and coworkers demonstrated also that at GW12 in humans, a

wide distribution of GnRH cells bodies and fibers were located in the hypothalamus, as well as into several extrahypothalamic areas, including the OB, cerebral cortex, hippocampus, piriform cortex, amygdala and habenula (Casoni *et al*., 2016).

Once in place, these GnRH neurons extend axonal projections to the median eminence where they coordinate their secretion of GnRH in a pulsatile pattern that is absolutely required by the gonadotrope to evoke physiologic gonadotropin secretion and gonadal function.

**Molecules involved in the GnRH-1 neuronal migration in nasal regions**

Several cues are involved in the GnRH – 1 migration: diffusible molecule (e.g. netrins, slits, reelin, semaphorins); growth factors and chemokines, which play a role as a chemotactic gradients (attractive or repellent); adhesion molecules (e.g. N-CAM, Neural Cell Adhesion Molecule; PSA, Polysialic Acid; NELF, Nasal Embryonic LHRH Factor; axonal surface glycoprotein TAG-1) and neurotransmitters and peptides (e.g. GABA, Glutamate, colecistochinine). These cues regulate both radial and tangential migration (Messina *et al*., 2013).

**GnRH isoforms**

GnRH is a decapeptide present in nature as 3 isoforms encoded by three paralogous of GnRH genes (*gnrh1*, *gnrh2* and *gnrh3*) arose from two rounds of genome duplication (Okubo *and* Nagahama, 2008). GnRH-1 (the hypothalamic form) is present in different vertebrates (in rodents in chromosome 14 and in humans in chromosome 8) and it plays an endocrine role (hypophysiotropic function). It is the only form expressed in rodents (Fernald *and* White, 1999), while it is absent in modern teleost (zebrafish; Zohar *et al*., 2010; Palevitch *et al*., 2007), in which GnRH 3 isoform takes the hypophysiotropic function. GnRH-2 decapeptide differs in several aminoacids (usually 5, 7 and 8) from GnRH-1 decapeptide, and it is present in tetrapods (excluding rodents), teleost fishes and Lampreys. Since the GnRH-2 cells are located in the midbrain and do not appear to project to the median eminence (D'Aniello *et al*., 1994) they could play

different roles compared to the GnRH1 cells. For example, as suggested by Riskind and Moss in 1979, they are involved in the reproductive behavior, that it is consistent with the role of the midbrain in lordosis behavior. GnRH-3 cells have been identified in the telencephalon of ancient and modern teleosts, but it is absent in tetrapods lineage (Tostivint, 2011). This isoform is expressed by neurons of the terminal nerve/olfactory region and it functions as a neuromodulator, thus making it indirectly linked to the reproductive neuroendocrine axis (Oka, 2009).

## Anatomy of the hippocampus

The hippocampus is a major component of the limbic system of the vertebrates. The hippocampus is a prominent C-shaped structure bulging in the floor of the temporal horn of the lateral ventricle. The hippocampus proper consists of three main regions: CA1, CA2 and CA3. The other parts are the dentate gyrus, the subicular complex, and the enthorinal cortex. We can also distinguish a ventral and a dorsal part. The dentate gyrus is composed of unidirectional projections dispersed towards CA3 pyramidal cells of the hippocampus. It consists of three layers of neurons: molecular, granular and polymorphic. The granular neurons are the associative elements between the dentate gyrus itself and the CA3 and their axons form the so-called mossy fibers. Based on its location and in turn on its extrinsic connectivity, the hippocampus receives a vast amount of highly processed multimodal sensory information that is processed by the entorinhal cortex. The entorinhal cortex is in turn connected to associational neocortical areas in a reciprocal manner (Schultz *and* Engelhardt, 2014).

The hippocampus is involved in processes like formation of spatial memory and navigation, episodic or autobiographical memory and also general declarative memory; it also seems that the dorsal region plays a larger role in cognitive faculties, whereas the ventral region is more involved in emotionality (Galea *et al*., 2013).

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RESEARCH REPORT

STEM CELLS AND REGENERATION

# Striatal astrocytes produce neuroblasts in an excitotoxic model of Huntington's disease

**Giulia Nato[1,2], Alessia Caramello[1,2], Sara Trova[1,2], Valeria Avataneo[1,2], Chiara Rolando[3], Verdon Taylor[3], Annalisa Buffo[2,4], Paolo Peretto[1,2,*,‡] and Federico Luzzati[1,2,*,‡]**

## ABSTRACT

In the adult brain, subsets of astrocytic cells residing in well-defined neurogenic niches constitutively generate neurons throughout life. Brain lesions can stimulate neurogenesis in otherwise non-neurogenic regions, but whether local astrocytic cells generate neurons in these conditions is unresolved. Here, through genetic and viral lineage tracing in mice, we demonstrate that striatal astrocytes become neurogenic following an acute excitotoxic lesion. Similar to astrocytes of adult germinal niches, these activated parenchymal progenitors express nestin and generate neurons through the formation of transit amplifying progenitors. These results shed new light on the neurogenic potential of the adult brain parenchyma.

**KEY WORDS: Neural stem cells, Parenchymal progenitors, Stem cell quiescence, Lesion-induced neurogenesis, Huntington's disease, Mouse**

## INTRODUCTION

In adult neurogenic niches, astrocytic cells produce neurons throughout life (Fuentealba et al., 2012). Parenchymal astrocytes can become neurogenic *in vitro* when isolated from the lesioned neocortex (Buffo et al., 2008; Sirko et al., 2013) or *in vivo* after overexpression of specific transcription factors (Niu et al., 2013). However, whether parenchymal astrocytes can spontaneously generate neurons *in vivo* is unclear (Dimou and Götz, 2014). The adult brain parenchyma has been generally considered gliogenic and not permissive for the activity of neuronal progenitors (Lim et al., 2000; Ninkovic and Götz, 2013; Shihabuddin et al., 2000). Nonetheless, examples of parenchymal neurogenesis are emerging (Bi et al., 2011; Luzzati et al., 2006, 2011b; Ohira et al., 2010). For instance, we previously demonstrated that clusters of proliferating cells with features of transient amplifying progenitors (TAPs) produce neurons in the striatum of rabbits (Luzzati et al., 2006) and in a mouse model of striatal degeneration (Luzzati et al., 2011b).

Here, we analysed striatal neurogenesis in the quinolinic acid (QA) lesion mouse model of Huntington's disease (Fan and Raymond, 2007). We show that such a lesion activates striatal astrocytes to produce neurons.

## RESULTS AND DISCUSSION

At 5 weeks post-QA lesion (w.p.l.), numerous $DCX^+$ neuroblasts were present in the striatum and organised into clusters or as individual cells (Fig. 1A-E). As in other models of striatal neurogenesis (Liu et al., 2009; Luzzati et al., 2011b), these neuroblasts expressed SP8, a transcription factor typical of lateral/caudal ganglionic eminence-derived interneurons (Ma et al., 2012; Waclaw et al., 2006), and some of them expressed NeuN (RBFOX3 – Mouse Genome Informatics; data not shown) and attained complex morphologies (supplementary material Fig. S1). The clusters of $DCX^+$ cells were closely associated to clusters of cells expressing the proliferation marker Ki67 (MKI67 – Mouse Genome Informatics), with numerous cells colabelled for DCX (Fig. 1B,C,E).

Based on clustering and the differential expression of Ki67 and DCX we could define four striatal cell types that were induced by QA: clustered $Ki67^+/DCX^-$ cells (cK), clustered $Ki67^+/DCX^+$ cells (cKD), clustered $DCX^+/Ki67^-$ cells (cD) and individual $DCX^+/Ki67^-$ cells (iD) (Fig. 1C-F). The cK, cKD and cD cells appeared between 2 and 3 w.p.l. (2 versus 3 w.p.l., Tukey's post-hoc test: cK, $P$=0.010; cKD, $P$=0.005; cD, $P$=0.021; Fig. 1F) and, although their number remained constant after 3 weeks (ANOVA: cK, $F_{2,7}$=2.464, $P$=0.155; cKD, $F_{2,7}$=0.383, $P$=0.695; cD, $F_{2,7}$=0.419, $P$=0.673), at all time points a high proportion incorporated BrdU when injected 4 days before sacrifice (supplementary material Fig. S2A,D). This indicates that cK, cKD and cD cells have a high turnover rate. By contrast, iD cells showed a delayed increase that peaked at 4 w.p.l. (Tukey's post-hoc test: 2 versus 3 weeks, $P$=0.226; 3 versus 4 weeks, $P$=0.001; Fig. 1F) accompanied by a reduction in the fraction of $BrdU^+$ cells over time (Tukey's post-hoc test: 3 versus 4 weeks, $P$=0.031; supplementary material Fig. S2C,D). Thus, this latter population appears later and has a lower turnover rate.

Interestingly, most cK cells expressed the TAP markers ASCL1 (Parras et al., 2004) and SOX9 (Cheng et al., 2009) (Fig. 1G-I; data not shown). Collectively, these data suggest that, as proposed in other models of striatal neurogenesis (Luzzati et al., 2006, 2011b), QA stimulates the appearance of TAP-like progenitors (cK cells) that give rise to neuroblasts that initially cluster (cKD, cD cells) and subsequently disperse as individual cells (iD cells). At 6 months after QA, striatal TAPs and neuroblasts were still present and could incorporate BrdU (supplementary material Fig. S3), suggesting that QA results in the long-term establishment of an intrastriatal neurogenic niche.

The induction of neurogenic potential in resident parenchymal cells was further supported by the appearance of self-renewing multipotent neurospherogenic cells in the striatum at 5 w.p.l. (supplementary material Fig. S4; data not shown). Interestingly, clusters of cK, cKD and cD cells were generally closely associated to $GFAP^+$ astrocytes, which were occasionally proliferating, as assessed through both Ki67 and BrdU (supplementary material Fig. S5). Using hGFAP-GFP mice (Platel et al., 2009; Zhuo et al., 1997) we could establish that $GFP^+/Ki67^+$ proliferating astrocytes represented 8±3% of all cK cells at 5 w.p.l. (Fig. 1I-L). These observations support the contention that cK cells and their progeny originate from striatal astrocytes.

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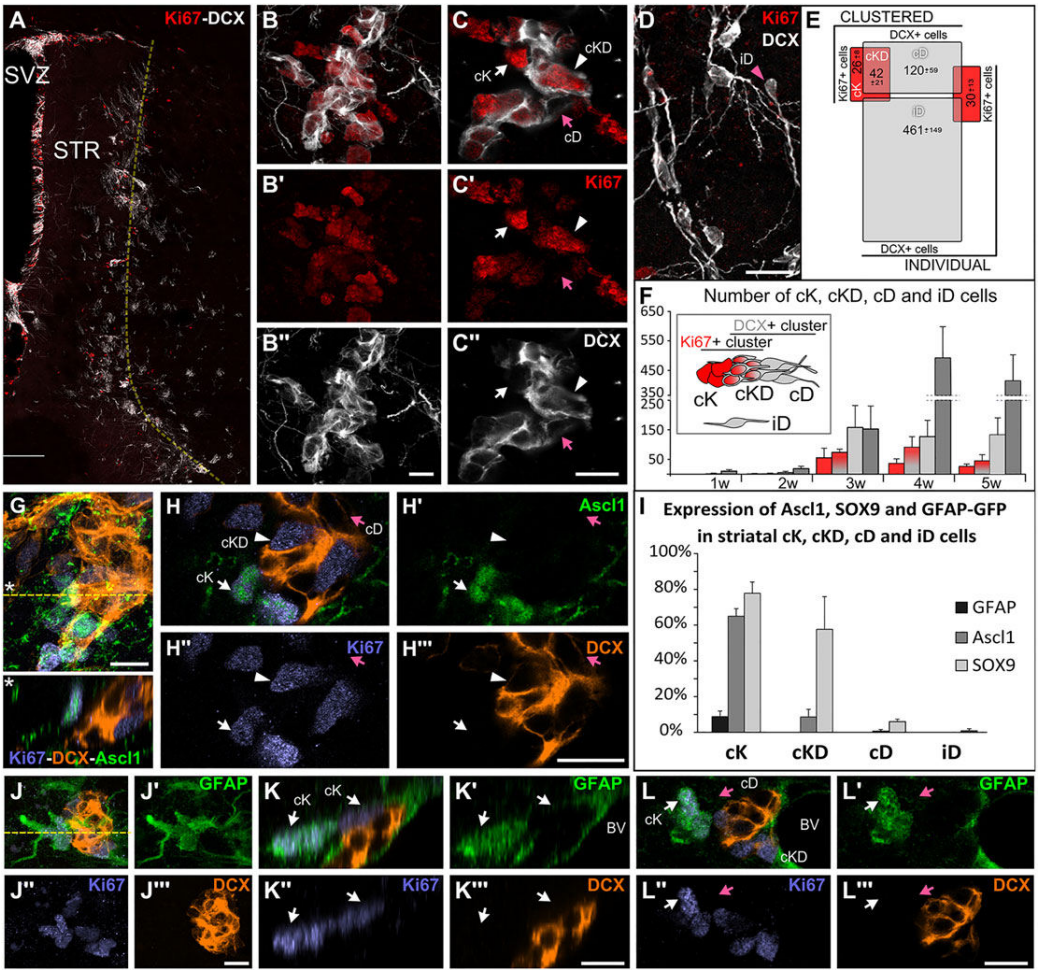


**Fig. 1. Ki67$^+$ and DCX$^+$ cells in the 5 w.p.l. striatum.** (A) Coronal section showing striatal Ki67$^+$ (red) and DCX$^+$ (white) cells densely packed at the lesion border (yellow dashed line). (B-C″) *z*-projection (B-B″) and single confocal plane (C-C″) of a Ki67$^+$ cluster partially overlapping with a DCX$^+$ cluster. (D) iD cells. (E) Each square represents the number of individual (bottom right) or clustered (top left) DCX$^+$ (grey) and Ki67$^+$ (red) cells in the striatum at 5 w.p.l. The overlap of these populations is in dark red. (F) Number of cK, cKD, cD and iD cells in the striatum at 1, 2, 3, 4 and 5 w.p.l. A schematic view of the cell types is shown in the inset. (G-H‴) *z*-projection (G), reslice (asterisk in G marks plane of section beneath) and single confocal plane (H-H‴) of a Ki67$^+$ (violet) and DCX$^+$ (orange) cluster immunolabelled for ASCL1 (green). (I) Percentage of cK, cKD, cD and iD cells expressing GFAP-GFP, ASCL1 and SOX9. (J-L‴) *z*-projection (J-J‴), reslice (K-K‴, at yellow dashed line in J) and single confocal plane (L-L‴) of a Ki67$^+$ (violet) and DCX$^+$ (orange) cluster showing cK cells labelled for GFAP-GFP (green). Error bars indicate s.d. Scale bars: 200 µm in A; 10 µm in B-C″,G-H‴; 20 µm in D 10 µl in J-L‴.

To explore this possibility further, we first performed cell fate-mapping analysis using transgenic mice expressing the tamoxifen-inducible recombinase CreER$^{T2}$ under the control of diverse cell type-specific promoters, namely *Glast* (*Slc1a3* – Mouse Genome Informatics), nestin (*Nes*) and *Ng2* (*Cspg4* – Mouse Genome Informatics). GLAST is a pan-astrocytic marker (Dimou and Götz, 2014), whereas nestin more specifically associates with active neurogenic astrocytes (Codega et al., 2014) and some oligodendrocyte progenitors (Boda et al., 2015), while NG2 is specifically expressed by oligodendrocyte progenitors (Zhu et al., 2011). Accordingly, in intact animals 1 week after tamoxifen, recombined YFP$^+$ cells represented 44±3% of all S100b$^+$ striatal astrocytes in GLAST-CreER$^{T2}$ mice and 10±6% in Nestin-CreER$^{T2}$ animals. In the NG2-CreER$^{T2}$ line, astrocytes were not targeted (supplementary material Fig. S6).

To determine whether cells expressing these genes are the source of intrastriatal TAPs and neuroblasts after lesion, tamoxifen was administered 1 week before QA (bQA). In addition, for each genotype a second group of animals was treated with tamoxifen at 4 w.p.l. (aQA) to identify possible injury-related changes in the phenotype of the neurogenic progenitors after their activation. In all cases animals were analysed at 5 w.p.l. (Fig. 2A). In NG2-CreER$^{T2}$ animals, we never observed DCX$^+$ neuroblasts expressing the reporter YFP, either in the striatum or in the subventricular zone-olfactory bulb (SVZ-OB) system (data not shown), indicating that NG2$^+$ cells are not neurogenic in our model. By contrast, in the SVZ of GLAST-CreER$^{T2}$ and Nestin-CreER$^{T2}$ animals, YFP$^+$ cells included putative TAPs (Ki67$^+$/DCX$^-$), proliferating (DCX$^+$/Ki67$^+$) and postmitotic (DCX$^+$/Ki67$^-$) neuroblasts (Fig. 2A-C). The percentage of YFP$^+$ cells did not differ between strains (supplementary material Table S1), indicating similar efficiency of nestin- or GLAST-driven recombination in SVZ neurogenic astrocytes.

In the striatum of GLAST-CreER$^{T2}$ bQA and aQA animals, YFP was expressed by cK, cKD, cD and iD cells, indicating that these cells originate from astrocytes that express GLAST both before and after the QA lesion (Fig. 2A,D,E; supplementary material Fig. S7A,B). YFP$^+$ postmitotic neuroblasts (cD and iD) were less numerous in the

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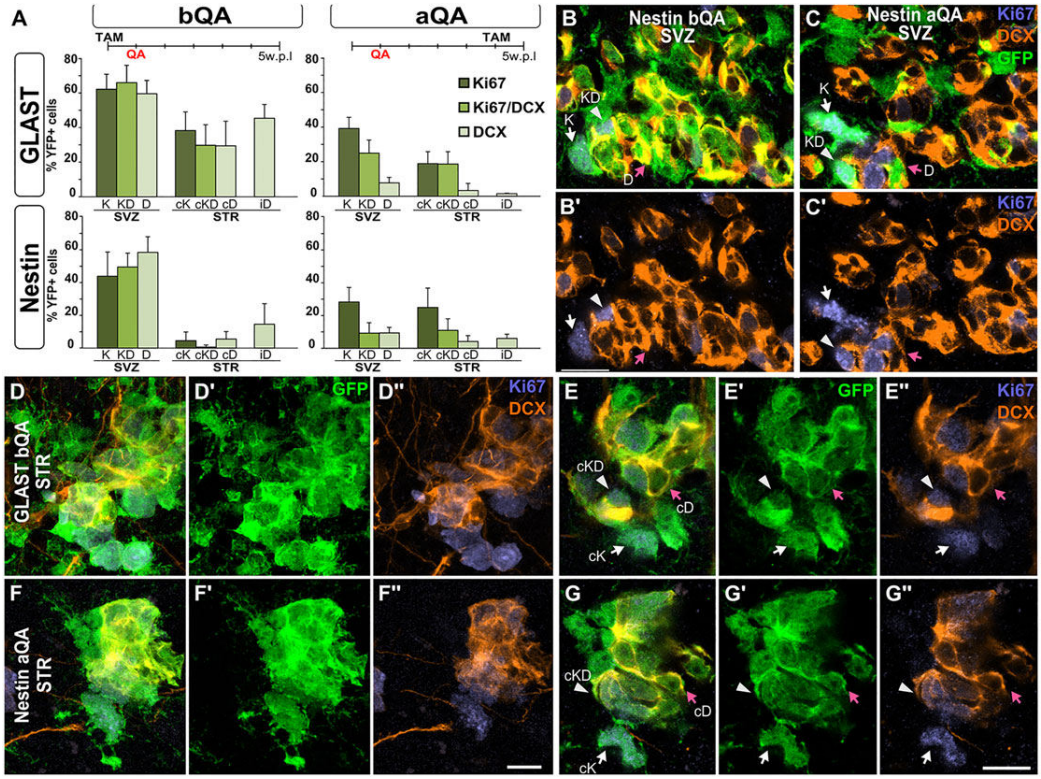


**Fig. 2. Genetic lineage tracing.** (A) Fraction of SVZ and striatal (STR) putative TAP cells (Ki67$^+$; K, cK), proliferating (Ki67$^+$/DCX$^+$; KD, cKD) and post-mitotic (DCX$^+$; D, cD, iD) neuroblasts that expressed YFP in 5 w.p.l. GLAST-CreER$^{T2}$ (top row) and Nestin-CreER$^{T2}$ (bottom row) animals that received TAM before (bQA, left column) or after (aQA, right column) lesion. Error bars indicate s.d. (B-C′) Single confocal plane of the SVZ of a Nestin-CreER$^{T2}$ bQA (B) and aQA (C) labelled for Ki67 (violet), DCX (orange) and GFP (green). (D-G″) *z*-projections (D,F) and single confocal planes (E,G) of recombined Ki67$^+$/DCX$^+$ clusters. Scale bars: 10 µm.

striatum of GLAST-CreER$^{T2}$ aQA animals, but increased to levels comparable to those of YFP$^+$ proliferative cells (cK and cKD) in the GLAST-CreER$^{T2}$ bQA animals (Fig. 2A; supplementary material Table S1A), further supporting that cK and cKD cells are early stages of the striatal neurogenic lineage. Three-dimensional reconstructions in GLAST-CreER$^{T2}$ bQA animals indicated that the morphology of the GFP$^+$ iD cells was comparable to that of their GFP$^-$ counterparts (supplementary material Fig. S8). Thus, GLAST$^+$ astrocytes are the source of QA-induced intrastriatal TAPs and neuroblasts.

In Nestin-CreER$^{T2}$ bQA mice, YFP$^+$ cells corresponding to the striatal cell types (cK, cKD, cD and iD) were very rare (Fig. 2A). However, in Nestin-CreER$^{T2}$ aQA animals, the levels of genetic labelling of cK and cKD cells were greatly increased and reached similar levels to those seen in GLAST-CreER$^{T2}$ aQA animals (Fig. 2A,F,G; supplementary material Table S1C). Interestingly, although nestin was mostly absent from striatal astrocytes under normal conditions, several YFP$^+$ cells with astrocytic morphology appeared in Nestin-CreER$^{T2}$ aQA animals (supplementary material Fig. S7C,D). These findings suggest that the resident GLAST$^+$ striatal astrocytes upregulate the expression of nestin after lesion and generate cK, cKD, cD and iD cells.

To directly confirm both the striatal origin of the neurogenic progenitors and their astrocytic identity, we performed intrastriatal injections of either a GFP-tagged lentiviral vector (VSVG-GFP; $n$=3) or an adenoviral vector carrying Cre recombinase under the control of the mouse *Gfap* promoter (Ad:GFAP-Cre; $n$=3; Merkle et al., 2007) 1 week before QA lesion. Whereas VSVG-GFP showed broad cellular tropism (data not shown), injection of Ad:GFAP-Cre into the striatum of R26R reporter mice resulted in the expression of YFP almost exclusively in astrocytes (supplementary material Fig. S9). Only animals with no YFP staining in the SVZ-OB system were analysed. In both cases, at 5 w.p.l. serial section 3D reconstructions of the whole striatum revealed multiple examples of YFP$^+$ or GFP$^+$ cK, cKD, cD and iD cells (Figs 3 and 4). The morphology of these latter cells was consistent with that of GFP$^-$ iD cells (Fig. 4B-E). These data indicate that striatal astrocytes generate TAPs and neuroblasts after lesion.

Notably, in both our genetic and viral fate-mapping analyses about 85% of the striatal Ki67$^+$ clusters exhibiting reporter expression were entirely composed of cells expressing YFP or GFP (Fig. 2D-G, Fig. 3C,D and Fig. 4F,G; see Materials and Methods for details), indicating that proliferative clusters originate mostly from the clonal expansion of a single striatal astrocytic progenitor.

SVZ progenitors have been shown to generate neuroblasts for the lesioned striatum (Liu et al., 2009). To examine whether these progenitors can further contribute to the intrastriatal TAPs, we injected Ad:GFAP-Cre or a TAT-Cre to respectively target the dorsolateral and the periventricular SVZ of R26R mice 1 week before QA. In the striatum of these animals at 5 w.p.l. we observed only a few iD cells expressing YFP (supplementary material Fig. S10), suggesting that striatal TAPs originate only from local astrocytes.

Taken together, these results indicate that some striatal astrocytes are quiescent neuronal progenitors that become activated after QA lesion. Like neurogenic astrocytes of other neurogenic niches, these cells upregulate nestin in their active state (Codega et al., 2014) and

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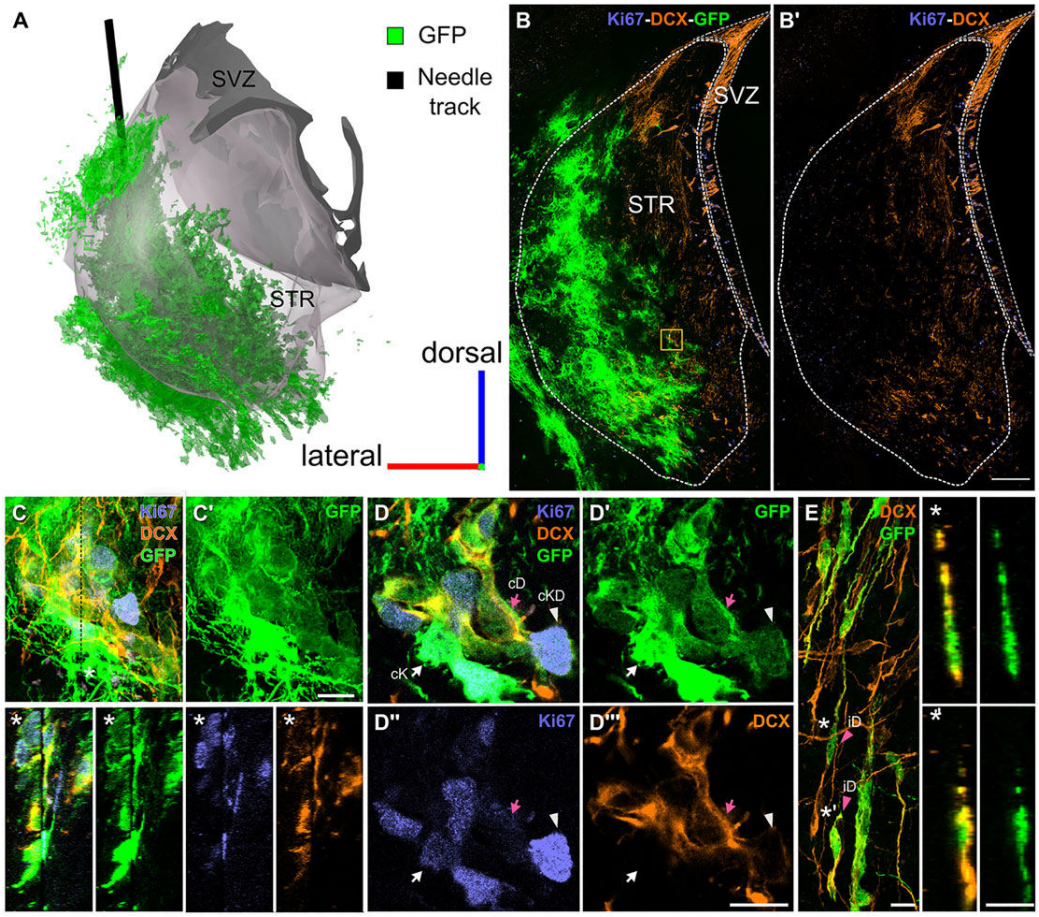


**Fig. 3. Viral lineage tracing.** (A) 3D reconstruction of SVZ (grey), striatum (transparent grey) and GFP staining (green) of a 5 w.p.l. animal injected with VSVG-GFP 1 week before QA. (B,B′) Coronal section at the level of the injection site labelled for DCX (orange), Ki67 (white) and GFP (green). (C-D‴) *z*-projection (C), reslice (asterisk, at black dotted line) and single confocal plane (D-D‴) of a cluster (box in B) made by cK, cKD and cD cells reconstructed from two successive 50 µm serial sections. (E) *z*-projection and reslices (asterisk and asterisk with prime) of $GFP^+$ iD cells. Scale bars: 200 µm in B,B′; 10 µm in C-E.

produce neurons through $ASCL1^+$ and $SOX9^+$ TAPs (Dimou and Götz, 2014; Fuentealba et al., 2012). However, striatal progenitors and/or their microenvironment may possess unique features that enable them to produce neurons in the brain parenchyma. Unravelling these features might help to unleash the full neurogenic potential of the adult brain, a fundamental prerequisite in order to design cell replacement therapies for brain repair. Interestingly, while this study was under revision the activation of neurogenic potential in striatal astrocytes was also demonstrated in a model of stroke (Magnusson et al., 2014) and under physiological conditions during guinea pig development (Luzzati et al., 2014). Thus, in contrast to SVZ and dentate gyrus neuronal progenitors, which are constitutively active, other populations of neurogenic astrocytes are activated only under specific conditions. The fate potential of these progenitors remains an important issue. Most neuroblasts generated in both the normal and lesioned striatum have a short life-span, but attain complex and specific morphologies (Luzzati et al., 2011a,b, 2014). These transient neurons might sustain a new type of adult brain plasticity that merits further exploration.

## MATERIALS AND METHODS

### Animals

All animal experiments were approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin. Experiments were performed on 8- to 12-week animals. C57BL/6 lesioned mice received two intraperitoneal injections (6 h apart) of 5-bromo-2-deoxyuridine (BrdU, Sigma-Aldrich; 50 mg/kg in 0.1 M Tris pH 7.4) 4 days before sacrifice. Tamoxifen (TAM, Sigma-Aldrich T5648-1G) was dissolved in corn oil (Sigma-Aldrich C8267) and 2.5 mg was administered by forced feeding (oral gavage) twice with a 24 h interval.

### Histology

Animals were anesthetised with a ketamine (100 mg/kg ketavet, Gellini) and xylazine (33 mg/kg rompun, Bayer) solution and perfused with a solution of 4% paraformaldehyde (PFA) and 2% picric acid (AnalytiCals, Carlo Erba 409302) in 0.1 M sodium phosphate buffer (PB) pH 7.4. Brains were then post-fixed for 3 h, cryoprotected in 30% sucrose (Fluka 84100) in 0.1 M PB pH 7.4, embedded at −80°C in Killik/OCT (Bio-Optica 05-9801), and cryostat sectioned in a series of 50 µm-thick sections.

### Generation of viral vectors and TAT-Cre

VSVG-GFP vector stocks were produced by transient transfection of the transfer plasmid expressing eGFP under the control of the CMV promoter, the packaging plasmids pMDLg/pRRE and pRSV.REV, and the VSV envelope plasmid pMD2.VSV-G in HEK293T cells as described (Follenzi et al., 2000). Viral particles were purified and concentrated by ultracentrifugation as described (Dull et al., 1998). Vector titre on HeLa cells was $2\times10^9$ TU/ml. The virus was then diluted 1/20 in PBS containing 0.6% glucose and frozen.

Generation of Ad:GFAP-Cre virus was described previously (Merkle et al., 2014). Briefly, HEK293 cells were infected to produce replication-defective adenovirus, which was purified using the Fast-Trap

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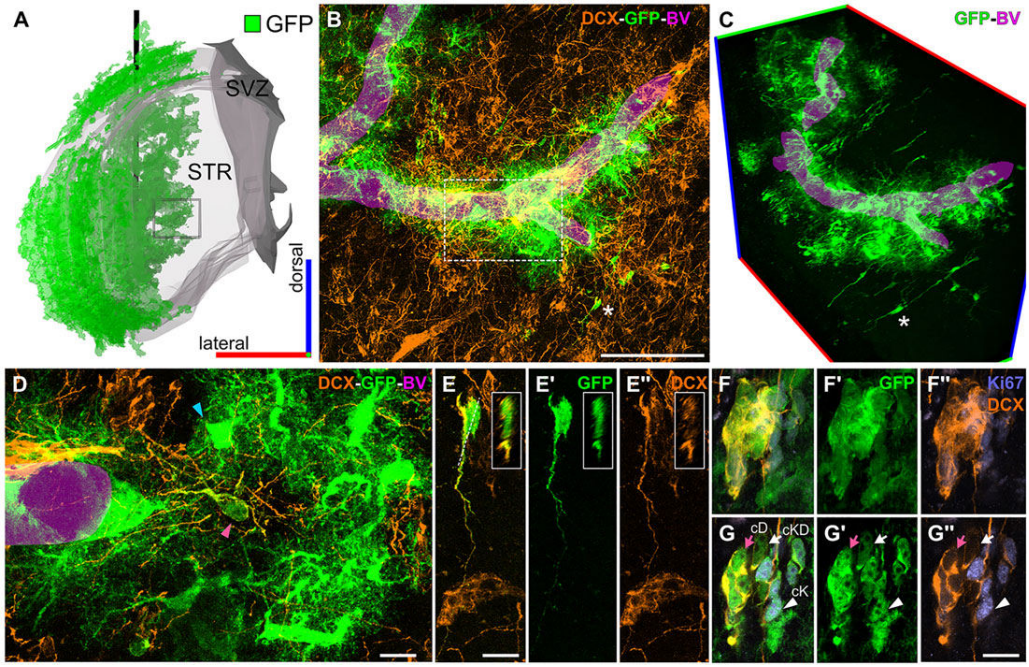


**Fig. 4. Intrastriatal injection of Ad:GFAP-Cre.** (A) 3D reconstruction of SVZ (grey), striatum (transparent grey), GFP staining (green) and needle track (black bar) of a 5 w.p.l. R26R-YFP animal injected with Ad:GFAP-Cre 1 week before QA. (B,C) *z*-projection (B) and perspective view (C) of a 3D reconstruction comprising four 50 µm-thick sections labelled with YFP (green) and DCX (orange) (box in A). Most $YFP^+$ cells are distributed around blood vessels (BV, magenta). However, some $DCX^+$ cells are also found deeper in the striatal parenchyma. (D) *z*-projection of a single 50 µm section (box in B) showing $YFP^+/DCX^+$ (pink arrowhead) and $YFP^+/DCX^-$ (blue arrowhead) cells near a blood vessel. (E-E″) Higher magnification of an individual $YFP^+/DCX^+$ cell, as indicated by the asterisk in B,C. The inset shows a reslice. (F-G″) *z*-projection (F-F″) and single confocal plane (G-G″) of a cluster made by cK, cKD and cD cells. Scale bars: 100 µm in B; 10 µm in D-G″.

Adenovirus Purification and Concentration Kit (Millipore). The titre was $1\times10^{10}$ infectious particles/ml. TAT-Cre recombinant protein was produced as previously described (Peitz et al., 2002).

### Stereotaxic injections

Mice were anesthetised with 0.3 ml/kg ketamine and 0.2 ml/kg xylazine, positioned in a stereotaxic apparatus (Stoelting) and injected with a pneumatic pressure injection apparatus (Picospritzer II, General Valve Corporation). Injection coordinates: QA (Sigma-Aldrich P6,320-4; 1 µl diluted to 120 mM in 0.1 M PB), +0.1 mm AP, −2.1 mm ML and −2.6 mm DV; VSVG-GFP and Ad:GFAP-Cre, 0.8 mm AP, −2.1 mm ML and −3.2 mm DV ($n$=3 for each vector). Ad:GFAP-Cre virions driving Cre recombinase expression in $GFAP^+$ cells were injected into R26YFP reporter mice. For both vectors, we analysed only animals in which the SVZ and OB were entirely free of reporter-positive cells. To target the SVZ, VSVG-GFP and TAT-Cre were injected respectively at +1.2 mm AP, −1 mm ML and −1.3 mm DV and at +3 mm AP, −0.8 mm ML and −2.9 mm DV.

### Immunofluorescence

Sections were incubated for 48 h at 4°C in 0.01 M PBS pH 7.4 containing 2% Triton X-100, 1:100 normal donkey serum and primary antibodies (supplementary material Table S2). For BrdU staining, sections were pre-incubated in 2 M HCl for 30 min at 37°C and then rinsed in 0.1 M borate buffer pH 8.5. Sections were incubated overnight with appropriate secondary antibodies (supplementary material Table S2) and coverslipped with antifade mounting medium Mowiol (4-88 reagent, Calbiochem 475904).

### Image processing and 3D reconstructions

Images were processed using ImageJ (NIH) and Photoshop 7.0 (Adobe Systems). Confocal microscopy serial section 3D reconstructions were performed as described (Luzzati et al., 2011a). Briefly, images from each section were stitched in Fiji (Preibisch et al., 2009), aligned with Reconstruct 1.1 (Fiala, 2005) or with TracKEM2 (Cardona et al., 2010) and neurons were traced in NeuronStudio (Wearne et al., 2005). 3D models were rendered in Blender 2.6. (Blender Foundation) and Vaa3D (Peng et al., 2014).

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

F.L., P.P. and G.N. designed the experiments. A.B. contributed to the experimental design. F.L. and G.N. performed the experiments and analysed the data. A.C. contributed to the lineage-tracing study. S.T. and V.A. contributed to the stereological analyses. V.T. provided the Ad:GFAP-Cre viral vector. C.R. produced and tested the viral vectors. A.B. contributed to the neurosphere assays. F.L., P.P. and A.B. wrote the paper. V.T. critically commented on the paper.

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### Supplementary material

Supplementary material available online at
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# Opposite-sex attraction in male mice requires testosterone-dependent regulation of adult olfactory bulb neurogenesis

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**Opposite-sex attraction in most mammals depends on the fine-tuned integration of pheromonal stimuli with gonadal hormones in the brain circuits underlying sexual behaviour. Neural activity in these circuits is regulated by sensory processing in the accessory olfactory bulb (AOB), the first central station of the vomeronasal system. Recent evidence indicates adult neurogenesis in the AOB is involved in sex behaviour; however, the mechanisms underlying this function are unknown. By using Semaphorin 7A knockout (Sema7A ko) mice, which show a reduced number of gonadotropin-releasing-hormone neurons, small testicles and subfertility, and wild-type males castrated during adulthood, we demonstrate that the level of circulating testosterone regulates the sex-specific control of AOB neurogenesis and the vomeronasal system activation, which influences opposite-sex cue preference/attraction in mice. Overall, these data highlight adult neurogenesis as a hub for the integration of pheromonal and hormonal cues that control sex-specific responses in brain circuits.**

The regulation of reproductive physiology in rodents involves the integration of salient chemosensory stimuli (i.e., pheromones) with endocrine signals[1,2]. In particular, olfactory cues appear crucial for the expression of 'appetitive' or pre-copulatory sexual behaviours[3,4], which include the investigation of and approach towards the opposite sex. Appropriate reproductive responses to pheromones are triggered by the action of gonadal hormones on brain circuits, whose sex-specific organization is established early during a developmental critical period[3,5]. These neural circuits include several nuclei of the vomeronasal system (VNS) and hypothalamus, and the integration of pheromonal and endocrine signals in these circuits is required to regulate the activity of the hypothalamic-pituitary-gonadal (HPG) axis and thus reproduction[6]. Pheromones and sex hormones are also key regulators of adult neurogenesis, the process of the continuous generation of new neurons that occurs in restricted regions of the adult brain, namely the olfactory bulb (OB) and the dentate gyrus of the hippocampus[7,8]. In the accessory olfactory bulb (AOB; i.e., the first relay station in the VNS) of female mice, adult neurogenesis is positively regulated by male pheromonal stimuli and is essential for one of the best-known examples of neuroendocrine responses elicited by pheromones: the exteroceptive block of pregnancy or Bruce effect[9–11]. In addition, pheromonal perception in both sexes, as well as pregnancy and lactation in females, drives the secretion of adenohypophyseal

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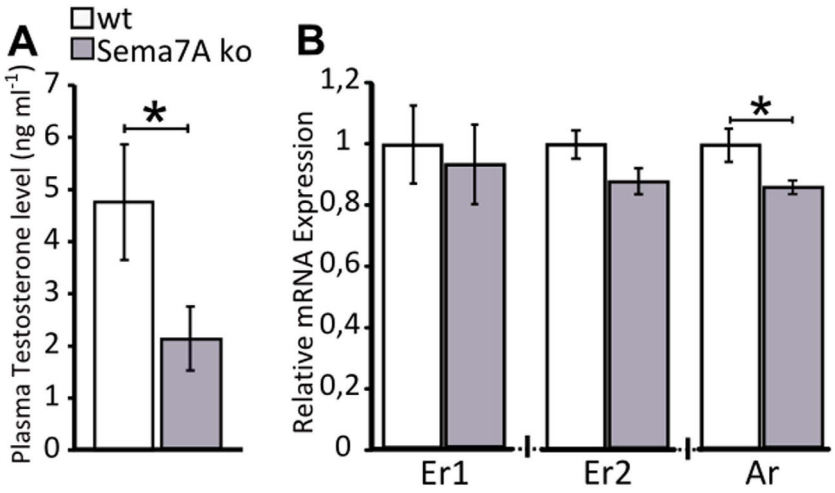


**Figure 1. Reduced testosterone levels and androgen receptor expression in Sema7A ko male mice.** (**A**) Plasma testosterone (TST) levels in wt and Sema7A ko male mice (n = 8 wt, 8 ko; unpaired Student's t-test, P = 0.024) indicate a reduction in TST levels in ko mice. (**B**) Real-time PCR analysis of oestrogen receptor (Er 1 and 2) and androgen receptor (Ar) expression in the olfactory bulb, normalized to values in wt mice (=1), indicates decreased Ar receptor expression in Sema7A ko mice (n = 8 wt, 8 ko; Wilcoxon-Mann-Whitney test, P = 0.015) and no differences in Er expression (Wilcoxon-Mann-Whitney test, P > 0.05). The values shown are the mean ± s.e.m.

hormones (e.g., prolactin and luteinizing hormone) and sex steroids (e.g., oestradiol and testosterone -TST), which in turn influence adult neurogenesis[12–15]. Thus, increasing evidence points to a role for adult neurogenesis in the control of reproduction through the integration of pheromonal and sex hormonal cues[8,16,17], yet the mechanisms underlying this integration remain largely elusive. To address this issue, here, we investigated adult AOB neurogenesis and activation of the VNS in the context of pre-copulatory sexual behaviour in Sema7A ko mice[18]. These animals are characterized by defective migration of gonadotropin releasing hormone (GnRH) neurons during development, which results in a significant reduction of GnRH neurons in the adult hypothalamus as well as reduced testis size and subfertility[19]. GnRH neurons are the master regulators of reproductive functions in all vertebrates and orchestrate the HPG axis by integrating multiple sensory signals, including olfactory cues[20–22]. Accordingly, defective GnRH function is related to abnormal olfactory and reproductive responses[23]. Here, we show that in the Sema7A ko males, as well as in wild-type males castrated during adulthood, the level of circulating TST in adult animals plays a critical role in modulating pheromone-induced adult neurogenesis in the AOB and, in turn, neuronal activity in downstream VNS nuclei that control sex-specific reproductive responses. These results indicate that the expression of suitable sexually dimorphic responses to opposite-sex pheromones requires fine-tuned cooperation between gonadal hormones and adult neurogenesis.

## Results

**Sema7A ko male mice show reduced circulating testosterone levels and altered opposite-sex odour preference.** Sema7A ko mice show a reduced number of GnRH neurons, reduced testis size and subfertility[19]. Here, to assess the impact of this phenotype on olfactory-dependent reproductive behaviour, we first evaluated the concentration of blood TST and the expression of gonadal hormone receptors in the OB of adult ko males. Indeed, appropriate levels of circulating TST and gonadal hormone receptor expression are required to sustain the typical male preference for female odours[24–27].

We found a strong decrease in circulating TST levels in Sema7A ko compared to wild-type (wt) mice (Fig. 1A). TST acts either directly, via binding to androgen receptor (Ar), or through its aromatization, by activating Er1 and Er2 oestrogen receptors[28,29]. Using real-time PCR, we found a significant reduction of Ar expression in the OB of the Sema7A ko mice (Fig. 1B), whereas the expression of the oestrogen receptor-encoding genes Er1 and Er2 did not significantly differ between the two genotypes (t-test P > 0.05; Fig. 1B).

Next, the opposite-sex preference of Sema7A ko mice was evaluated by comparing the responses to urinary scents/pheromones from sexually active males versus sexually receptive females. This was done by exposing the mice to the airborne volatiles of familiar urine only, and after direct nasal-urine contact. Indeed, it is known that sex recognition through the main olfactory system (airborne volatiles) in female mice requires earlier activation of both vomeronasal and olfactory epithelia through urine direct contact (contact with familiar urine)[30], implying a functional crosstalk between the two olfactory systems. As expected, wt males spent more time investigating urine from females (Fig. 2A,B). This was true both after exposure to the airborne volatiles of the familiar urine only (indirect contact, Fig. 2A) and after direct nasal-urine contact (Fig. 2B). By contrast, the Sema7A ko males never showed any preference for male or female scents after either indirect or direct contact with familiar urine (Fig. 2A,B), although the behaviour of the Sema7A ko females did not differ from that of wt females (Fig. 2A,B). These results point to a sexually dimorphic role of Sema7A for opposite-sex preference. Indeed, the lack of Sema7A affected the preference for opposite-sex odour in males but not in females. The response of males

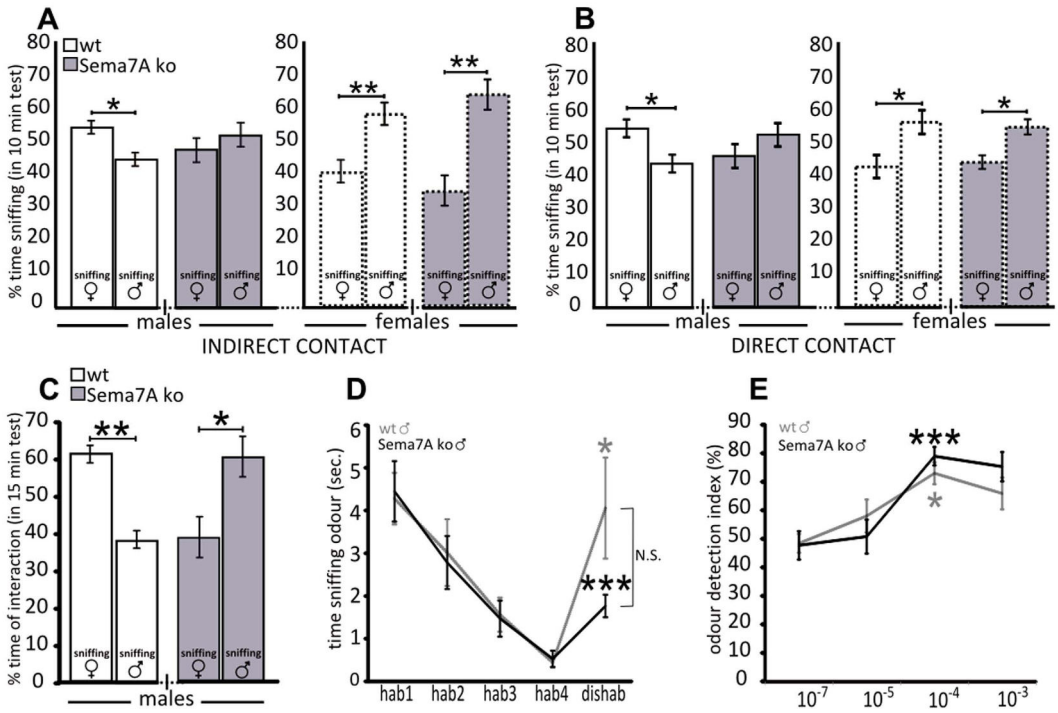


**Figure 2. Altered opposite-sex responses in Sema7A ko males.** (**A**) During indirect contact with pheromones (volatile compounds), wt male mice spend more time sniffing female odours (n = 8; Wilcoxon-Mann-Whitney test, P = 0.015), while Sema7A ko males show no preference (n = 8; Wilcoxon-Mann-Whitney test, P > 0.05). Both wt and Sema7A ko females spend more time in contact with the odour of the opposite sex (n = 8 wt, 8 ko; Wilcoxon-Mann-Whitney test, P = 0.001). (**B**) The same behaviours are observed during direct contact with the pheromonal source: the wt males and females and Sema7A ko females spend more time in contact with the opposite-sex odours (n = 8; Wilcoxon-Mann-Whitney test, P = 0.020 for wt males; P = 0.015 for wt females; P = 0.011 for ko females), while the Sema7A ko males do not show any preference (n = 8; Wilcoxon-Mann-Whitney test, P > 0.05). (**C**) In the individual preference test, the wt males prefer the opposite sex (female) (n = 8; Wilcoxon-Mann-Whitney test, P = 0.0003), whereas the Sema7A ko males spend more time in contact with the same sex (male) (n = 8; Wilcoxon-Mann-Whitney test, P = 0.021). (**D**) The habituation/dishabituation paradigm indicates that Sema7A ko males (black line) are able to discriminate habituation odours from dishabituation odours (n = 8; paired Student's t-test, P = 0.0001), as observed in wt mice (grey line; n = 8; paired Student's t-test, P = 0.014). No statistical significant difference (N.S.) is evident between the dishabituation time-points of Sema7A ko and wt mice (unpaired Student's t-test, P = 0.08). (**E**) Odour detection threshold test shows that both Sema7A ko (black line) and wt mice (grey line) can detect odour (octanal) starting from $10^{-4}$ concentration (Sema7A ko, n = 7; paired Student's t-test, P = 0.0004; wt, n = 8; paired Student's t-test, P = 0.014). Normalized values are expressed as the mean ratio between the time spent investigating the odour and the total sniffing time (odour plus oil). The values shown are the mean ± s.e.m.

was further investigated using a three-chamber social interaction assay[31] in which the subject can interact directly with two stimulus animals. Again, wt males spent more time interacting (sniffing and contacting) with receptive females, whereas the Sema7A ko males showed significant preference to interact with the male (Fig. 2C), further supporting an altered sexual preference in ko males.

Because the main olfactory system has been shown to participate in sex discrimination[32], we conducted habituation-dishabituation olfactory tests and odour detection threshold analyses to measure main olfactory epithelium-mediated olfactory discrimination[33,34] using non-social odour stimuli (i.e., acetophenone and octanal; Fig. 2D,E). These experiments did not show any significant impairment in the odour discrimination and olfactory sensory perception of Sema7A ko males (Fig. 2D,E), suggesting that the alteration in sex-preference in Sema7A ko males is unlikely to depend on the main olfactory system. The lack of defects in olfactory sensory perception in these animals is further supported by the lack of difference between ko versus wt males in the total time of sniffing of urinary scents (Suppl. Fig. 1A). Similarly, no differences were found when considering the total time of individual interaction in the two groups of males (Suppl. Fig. 1D).

Overall, these data suggest that altered opposite sex-preference in Sema7A ko males is not attributable to a general reduction of either olfactory sensory perception or motivation to explore social stimuli, but rather can imply the involvement of the accessory olfactory system.

**Feminized neurogenic response to male pheromones in the AOB of Sema7A ko males.** Work by our and other laboratories has demonstrated that exposure to male chemosensory stimuli increases adult

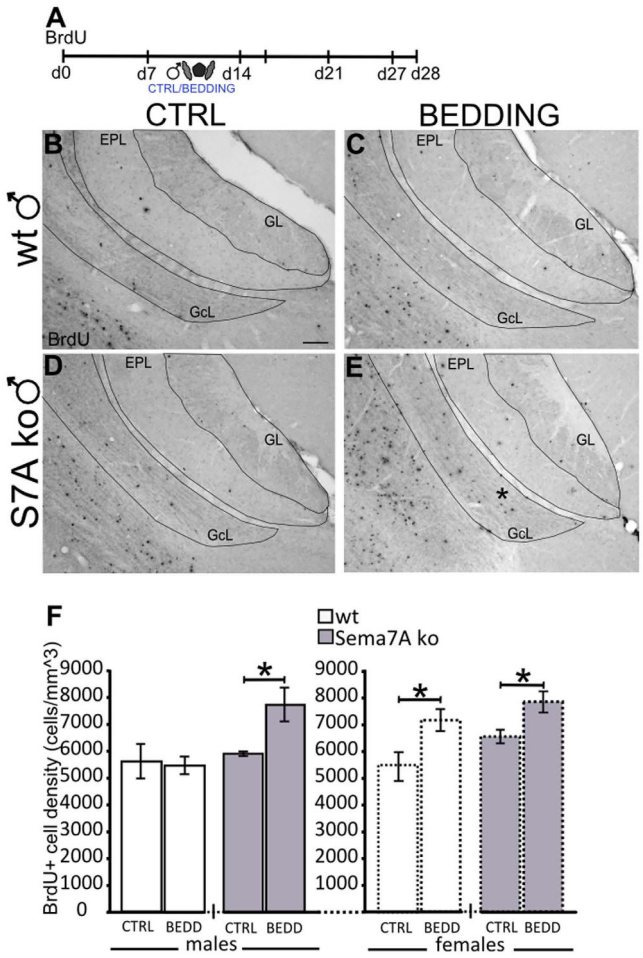


**Figure 3. Sema7A ko males display feminized-like neurogenesis in response to male pheromones.** (**A**) Experimental protocol: newborn cells were quantified in the GcL of the AOB 28 days after BrdU injection. From day 7 to day 14, the mice were familiarized with male-soiled bedding, while the control groups received clean bedding. (**B–E**) Representative images of AOB sections showing BrdU-positive newborn neurons in wt (**B,C**) and Sema7A ko (**D,E**) males after bedding familiarization. (**F**) Quantification of BrdU-positive cell density in the AOB GcL indicates an increase in newborn neurons in the Sema7A ko males after male-soiled bedding (BEDD) exposure compared to the clean bedding (CTRL) condition ($n = 4$; unpaired Student's t-test, $P = 0.030$); no differences are observed in wt males ($n = 4$ CTRL, $n = 6$ BEDD; unpaired Student's t-test, $P > 0.05$). Both wt and Sema7A ko females show increased BrdU-positive cell density in the BEDD condition ($n = 4$ wt, 4 ko) compared to the CTRL condition ($n = 4$ wt, 5 ko; unpaired Student's t-test, $P = 0.043$, $P = 0.024$, respectively). The values shown are the mean ± s.e.m. Abbreviations: AOB: accessory olfactory bulb; GcL: granule cell layer; GL: glomerular layer; EPL: external plexiform layer; S7A: Semaphorin 7A; BrdU: bromodeoxyuridine. Scale bar: 100 μm.

neurogenesis in female rodents, optimizing intersexual interaction[11,13,35,36]. In wt female mice, one-week exposure to stud-male pheromones (familiarization) promotes the survival/integration of newborn granule cells in the AOB[10,11]. Notably, this effect was not found in males[11]. Based on the altered (feminized) preference to opposite-sex cues observed in the Sema7A ko males, we evaluated adult neurogenesis in the OB of these animals. The number of newborn cells was quantified 28 days after 5-bromo-2′-deoxyuridine (BrdU) injection in mice exposed for 1-week to either clean bedding or stud-male urine-soiled bedding, following a previously established experimental paradigm that allows to evaluate newborn cell survival in the AOB[11] (Fig. 3A). The clean bedding condition revealed no statistically significant difference in the density of BrdU-positive cells in the AOB of ko versus wt males (Fig. 3B,D,F). Similarly, no difference was found in the main olfactory bulb (MOB; Suppl. Fig. 2A). Moreover, unchanged rate of cell proliferation was observed in the SVZ-RMS of Sema7A ko, evaluated at two hours after a single BrdU pulse (Suppl. Fig. 2B). These data indicate that in the basal condition (absence of stud-male pheromones), a normal rate of newborn cell proliferation/survival occurs in the olfactory system of Sema7A ko males. By contrast, the ko males exposed to stud-male pheromones showed increased survival of BrdU + granule cells in the AOB (Fig. 3D–F), without any change in the AOB granule cell layer (GcL) volume (wt: $0.0421 \pm 0.003\ mm^3$; Sema7A ko: $0.0451 \pm 0.003\ mm^3$; unpaired Student's t-test, $P = 0.5$). Notably, this increase in newborn cell density was comparable to that found in wt and ko females exposed to male pheromones (Fig. 3F). In contrast, these cues did not enhance AOB neurogenesis in wt males (Fig. 3B,C,F), or in the MOB

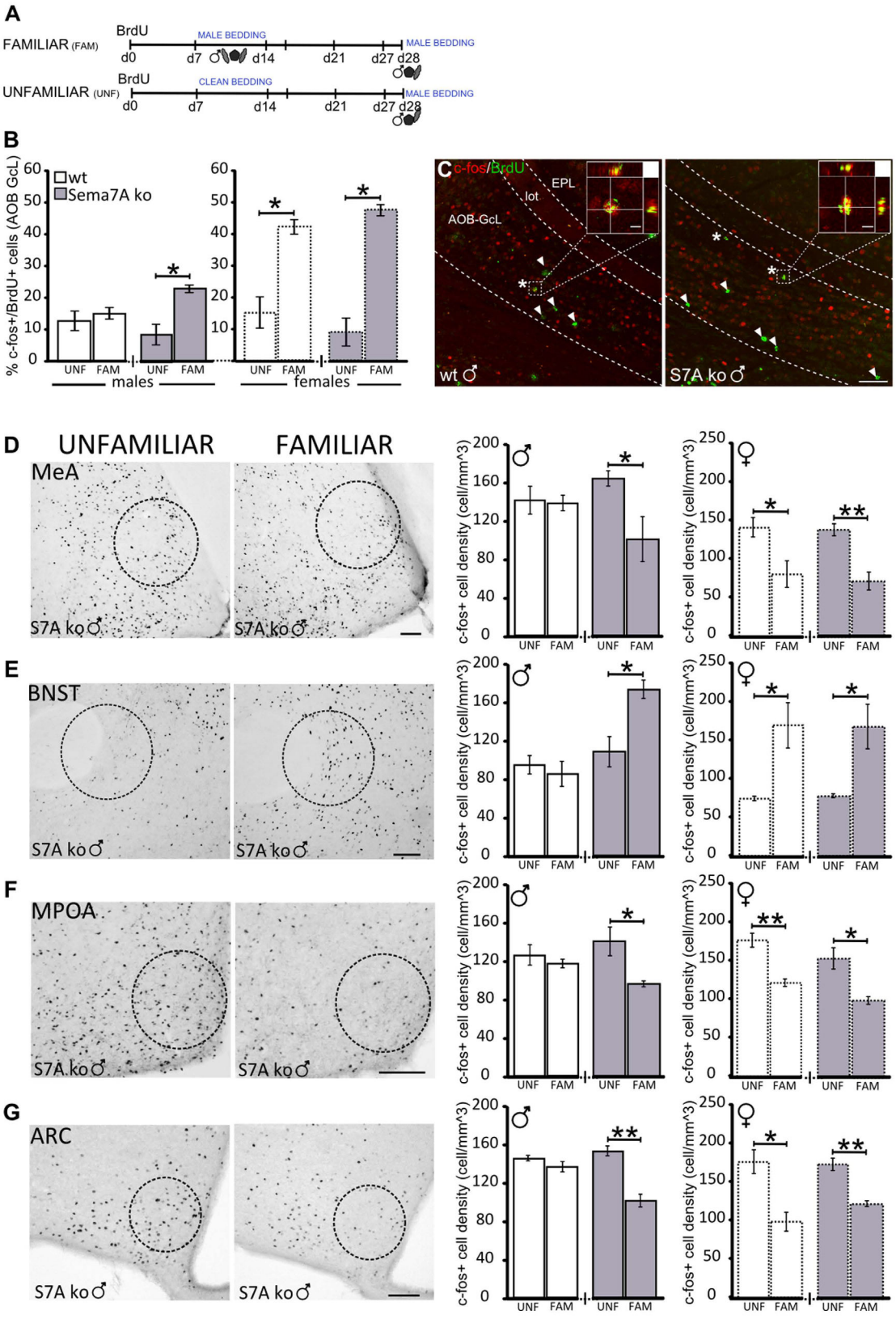


**Figure 4. The vomeronasal pathway of Sema7A ko males shows a feminized c-fos response to pheromones.** (**A**) Experimental protocol: the familiar groups were stimulated with male-soiled bedding during the 2nd week after BrdU administration, from day 7 to day 14, whereas the unfamiliar groups received clean bedding. Both groups were then stimulated with male bedding 90 min before animal perfusion. (**B**) Quantification of c-fos/BrdU double-labelled cells in the AOB GcL reveals an increase in the familiar Sema7A ko group compared to the unfamiliar Sema7A ko group (n = 4; Wilcoxon-Mann-Withney test, P = 0.028). No differences are

observed in wt males (n = 4, Wilcoxon-Mann-Withney test, $P > 0.05$). An increase in c-fos/BrdU+ cells is also observed in the familiar versus unfamiliar group of wt females (n = 4; Wilcoxon-Mann-Withney test, $P = 0.029$) and Sema7A ko females (n = 4; Wilcoxon-Mann-Withney test, $P = 0.028$). (**C**) BrdU (green) and c-fos (red) immunofluorescence in the AOB-GcL of wt and Sema7A ko males. Asterisks indicate representative c-fos/BrdU double-stained cells. Arrowheads indicate c-fos-negative/BrdU-positive cells. Scale bars: main panels 50 μm; magnified panels 5 μm. (**D–G**) Representative images of Sema7A ko males and histograms showing changes in c-fos expression throughout the VNS in Sema7A ko and wt mice after bedding familiarization. The quantification of c-fos+ cell density shows no differences between the unfamiliar and familiar groups of wt males (unpaired Student's t-test, $P > 0.05$). In Sema7A ko males and in wt and Sema7A ko females, familiarization decreases c-fos cell density in the medial amygdala (MeA, **D**), medial preoptic area (MPOA, **F**) and arcuate nucleus (ARC, **G**) and increases c-fos expression in the bed nucleus of the stria terminalis (BNST, **E**). (Sema7A ko males n = 3; MeA $P = 0.034$; BNST $P = 0.024$; MPOA $P = 0.044$; ARC $P = 0.003$; wt females n = 3; MeA $P = 0.047$; BNST $P = 0.033$; MPOA $P = 0.006$; ARC $P = 0.016$; Sema7A ko females n = 3; MeA $P = 0.009$; BNST $P = 0.037$; MPOA $P = 0.020$; ARC $P = 0.004$). The values shown are the mean ± s.e.m. Scale bar: 100 μm.

of both wt and ko males (Suppl. Fig. 2C). Overall, these findings show that the increase of newborn neurons in the Sema7A ko males is associated with an effect of male soiled bedding in cell survival specifically in the AOB, indicating Sema7A ko males exhibit a feminized AOB neurogenesis phenotype as well as a feminized sexual preference phenotype.

**The male cue-elicited c-fos expression pattern along the vomeronasal pathway is feminized in Sema7A ko males.** In female mice, exposure to the pheromones of familiar males increases the percentage of newborn AOB granule cells that express the immediate early gene c-fos[11], which is a marker of neuronal activation[37,38]. In turn, the same stimuli elicit a sex-specific c-fos expression pattern in the downstream nuclei of the VNS[11,39,40], wherein the integration of pheromonal and endocrine signals modulates opposite-sex attraction/preference[6,41]. Therefore, considering the feminized responses to male pheromones observed in the Sema7A ko males, both at the behavioural and cellular level, we investigated c-fos expression in the VNS of these mice.

In Sema7A ko males, focal exposure (30-min bedding/urine presentation 90 min before sacrifice, Fig. 4A) to previously experienced (familiar) male cues induced a net increase in the percentage of c-fos/BrdU-positive newborn AOB neurons in comparison to focal exposure with unfamiliar cues (Fig. 4B,C). By contrast, wt males did not show any difference in c-fos expression in response to familiar versus unfamiliar male cues (Fig. 4B). As expected, in wt females, we found an increase in c-fos expression in response to familiar male cues (Fig. 4B), and a similar pattern was also found in the Sema7A ko females (Fig. 4B).

In the VNS nuclei downstream of the AOB, the Sema7A ko males showed an increase in c-fos-positive cells in the bed nucleus of the stria terminalis (BNST, Fig. 4E), while the number of c-fos positive cells was reduced in the medial amygdala (MeA), medial preoptic area (MPOA), and arcuate nucleus (ARC) (Fig. 4D,F,G). In wt males, there was no difference in c-fos expression in response to familiar versus unfamiliar male cues in these nuclei (Fig. 4D–G), whereas both wt and Sema7A ko females showed the same pattern observed in the Sema7A ko males (Fig. 4D–G). Thus, the lack of Sema7A impacts male sexual behaviour, possibly through abnormal (feminized) neuronal activation along the VNS pathway.

**Testosterone administration reverses the changes in sex-preference, neurogenic and VNS c-fos responses in Sema7A ko mice.** We speculated that the sex-specific pheromonal-dependent response observed in the Sema7A ko males at both the behavioural and cellular levels could be directly related to the reduced circulating TST found in these animals. To test this hypothesis, one cohort of adult ko males received, in addition to BrdU injection and bedding familiarization, a TST replacement treatment[42] that involved daily subcutaneous injections of TST proprionate (Fig. 5A). One week before the TST treatment, the Sema7A ko males showed a feminized sex-preference response to opposite-sex scents or individuals, as expected (Fig. 5B,C). Sixteen days of TST treatment in these animals was sufficient to restore the typical preference for opposite-sex stimuli (Fig. 5B',C'). This occurred without changes in the total time of sniffing in olfactory and individual preference tests (Suppl. Fig. 1B,E), suggesting that the level of circulating TST mediates opposite-sex preference without affecting sensory perception or motivation to investigate social stimuli. In addition, TST administration did not modify the level of Ar expression neither in the OB, where a decrease was observed in the Sema7A ko (Figs 1B and 5G), nor in the hypothalamic preoptic area, which includes several nuclei involved in the control of sex behaviour[6], and where no difference in Ar expression between wt and ko males were found (Fig. 5H).

Strikingly, following familiarization with male pheromones, the density of newborn cells in the AOB of the TST-treated Sema7A ko mice at 28 days after BrdU injection was similar to that in wt (oil-treated) males (Fig. 5D, Suppl. Fig. 3A). In line with these findings, we did not observe any difference in the pattern of c-fos expression in newborn AOB neurons or in downstream VNS nuclei between the TST-treated Sema7A ko males and wt males (Fig. 5E,F and Suppl. Fig. 3B–F). These data indicate that the feminized phenotype observed in the Sema7A ko males is fully reversible by TST treatment in adult life. In addition, they reveal a link between levels of circulating TST, the sex-dependent modulation of newborn cell survival in the AOB, and cellular responses in VNS nuclei involved in the control of sexual behaviour.

**Testosterone inhibits the neurogenic response to male pheromones in the AOB.** To further elucidate the role of TST in regulating sex-specific pheromonal-dependent responses in adult animals, we performed

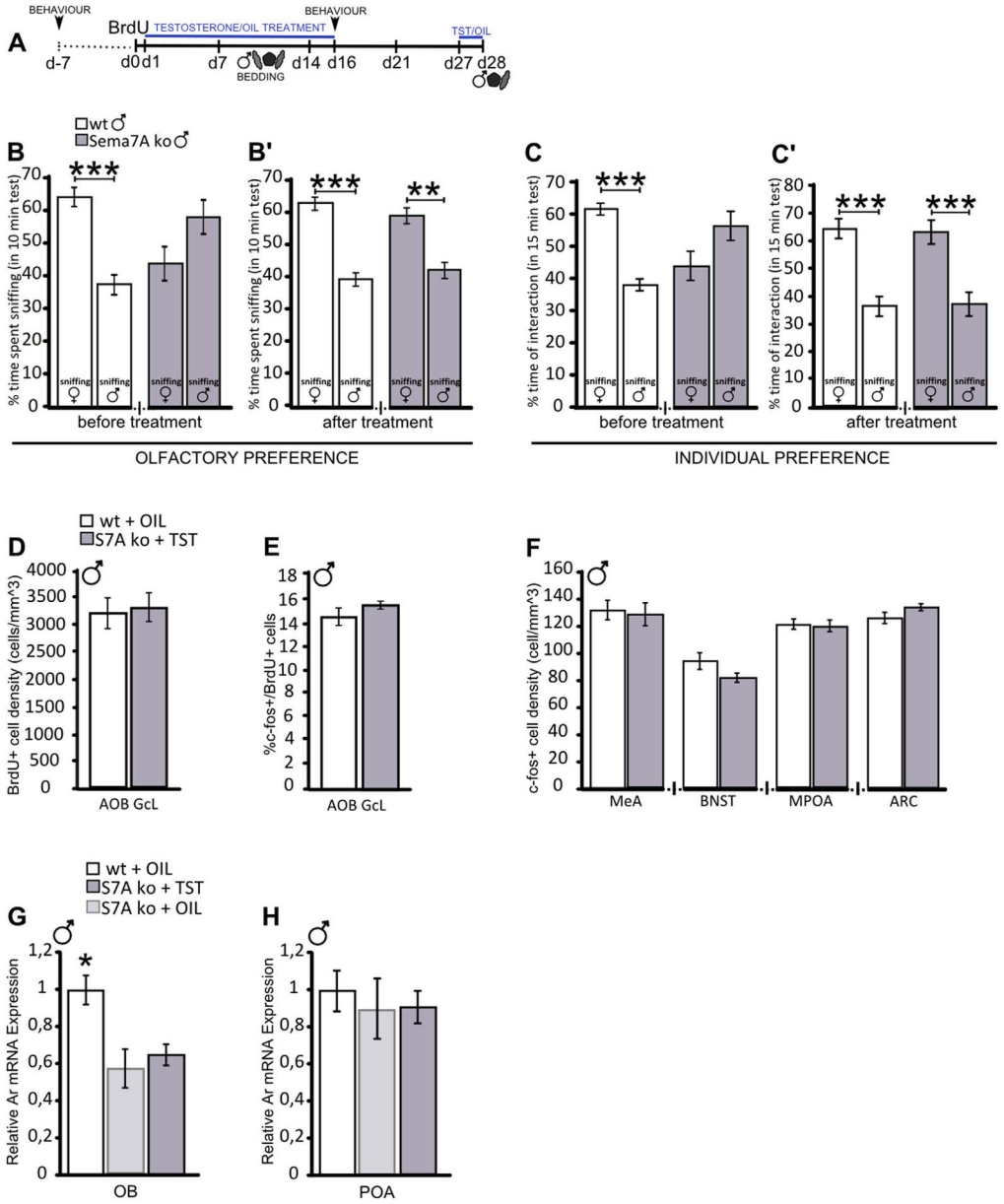


**Figure 5. Administration of testosterone in Sema7A ko male mice restores opposite-sex preferences, neurogenic activity and the c-fos response to pheromones.** (**A**) Experimental protocol: testosterone (TST; for Sema7A ko) or oil (for wt) was administered for 16 days starting one day after BrdU injection ( d1–d16). Two additional pulses were given 2 days before animal perfusions. The mice were familiarized with male bedding between d7–d14 after BrdU injection, then received a focal stimulus with male bedding 90 min before perfusion. Behaviour was assessed one week before, and at the end of the TST/oil treatment (d16). (B-B') Olfactory preference behaviour. Before treatment (**B**), wt mice prefer female odours (n = 7; Wilcoxon-Mann-Whitney test, P = 0.0006), while Sema7A ko males do not show any preference (n = 7; Wilcoxon-Mann-Whitney test, P > 0.05). After treatment (B'), both wt and Sema7A ko males show a preference for the opposite sex (Wilcoxon-Mann-Whitney test, wt P = 0.0006; ko P = 0.001). (C-C') Individual preference behaviour. Before treatment (**C**), wt mice prefer female odours (n = 7; Wilcoxon-Mann-Whitney test, P = 0.0006), while Sema7A ko males do not show any preference (n = 7; Wilcoxon-Mann-Whitney test, P > 0.05). After treatment (C'), both wt and Sema7A ko males show a preference for females (Wilcoxon-Mann-Whitney test, wt P = 0.0006; ko P = 0.0006). (**D**) After treatment, no difference in BrdU+ cell density is observed between the wt and Sema7A ko mice (n = 6 wt, 6 ko; unpaired Student's t-test, P > 0.05). (**E**) After treatment, no difference in cfos/BrdU+ cell percentage is observed between the wt and Sema7A ko males (n = 4 wt, 4 ko; Wilcoxon-Mann-Whitney test, P > 0.05). (**F**) After treatment, no differences in cfos+ cell density in the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST), medial preoptic area (MPOA) and arcuate nucleus (ARC) were found between the wt and Sema7A ko males. (**G,H**) Real-time PCR analysis indicates that TST administration does not restore the level of androgen receptor (Ar) in the OB of ko males (n = 4wt, 4Sema7A, 5Sema7A + TST; Kruskall-Wallis Test; P = 0.023). In the preoptic area (POA) no differences are visible between wt and oil- or TST-treated ko males (n = 4wt, 3Sema7A, 4Sema7A + TST; Kruskall-Wallis Test; P > 0.05). The values are normalized to wt mice (= 1). The values shown are the mean ± s.e.m.

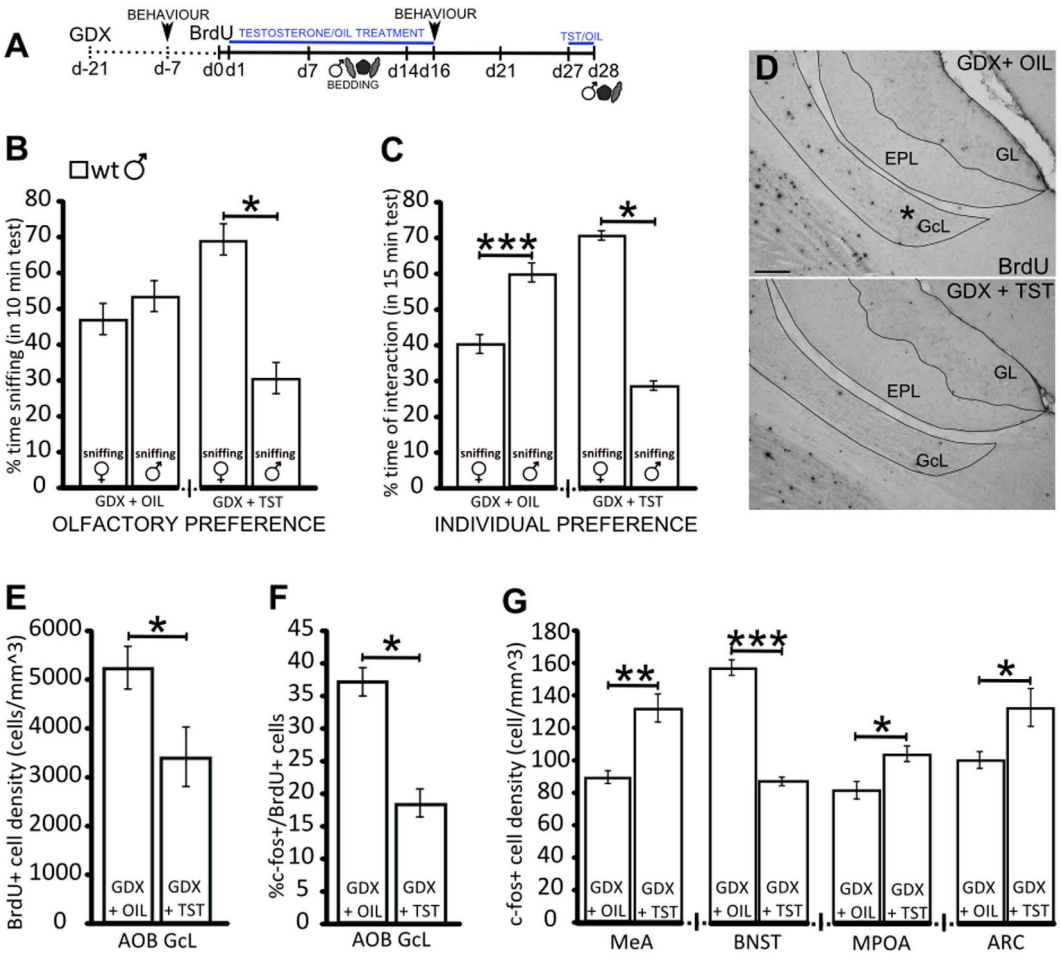


**Figure 6. Gonadectomy and testosterone administration in wt mice mimic the behavioural and cellular responses that characterize the phenotype of the Sema7A ko males.** (**A**) Experimental protocol: wt males were gonadectomized (GDX) 3 weeks before BrdU administration. Testosterone (TST)/oil treatment was administered for 16 days (d1–d16) starting one day after BrdU injection. Two additional pulses were given 2 days before animal perfusions. Male bedding was given the 2nd week after BrdU injection (d7–d14), and as a focal stimulus 90 min before perfusion. The behaviour of the GDX mice was assessed one week before TST/oil treatment and at the end of the treatment (d16). (**B**) Olfactory preference behaviour indicates no preference between female and male odours in the oil-treated GDX wt males (n = 10; Wilcoxon-Mann-Whitney test, $P > 0.05$), whereas the TST-treated GDX wt males show a preference for female odours (n = 4; Wilcoxon-Mann-Whitney test, $P = 0.028$). (**C**) Individual preference behaviour analysis shows oil-treated GDX males spend more time sniffing male than female odours (n = 10; Wilcoxon-Mann-Whitney test, $P = 0.0002$), while TST-treated GDX males prefer female than male odours (n = 4; Wilcoxon-Mann-Whitney test, $P = 0.028$). (**D**) AOB sections of oil- and TST-treated GDX wt mice showing the increase in BrdU+ cells in the AOB GcL of oil-treated animals. (**E**) Quantification of BrdU+ cell density in the AOB GcL shows increased newborn neuron density in oil-treated compared to TST-treated GDX wt mice (n = 6 oil treated, 4 TST treated; unpaired Student's t-test, $P = 0.036$). (**F**) Quantification of c-fos/BrdU+ cells shows an increase in oil-treated compared to TST-treated GDX wt mice (n = 6 oil treated, 4 TST treated; Wilcoxon-Mann-Whitney test, $P = 0.014$). (**G**) Oil-treated GDX wt male mice show a lower density of c-fos+ cells in the MeA, MPOA and ARC and a higher density in the BNST compared to TST-treated GDX wt animals (n = 6 oil treated, 4 TST treated; unpaired Student's t-test, MeA $P = 0.002$; BNST $P = 0.0006$; MPOA $P = 0.019$; ARC $P = 0.044$). The values shown are the mean ± s.e.m. Abbreviations: GcL: granule cell layer; GL: glomerular layer; EPL: external plexiform layer; MeA: medial amygdala; BNST: bed nucleus of the stria terminalis; MPOA: medial preoptic area; ARC: nucleus arcuatus;. Scale bar: 100 μm.

the same set of experiments in a cohort of wt males that were castrated during adulthood (and thus had normally developed sex-related brain circuits) and treated with TST or vehicle (sesame oil) alone (Fig. 6A). The oil-treated castrated males showed reduced or no interest in opposite-sex urine scents or individuals, whereas the TST-treated castrated males exhibited a clear preference for the opposite-sex cues, behaving as control males (Fig. 6B,C). Again, also in these groups no differences were found in the total time of sniffing in olfactory and individual preference (Suppl. Fig. 1C,F), further supporting these aspects are not related to the level of circulating TST during adulthood.

Notably, familiarization with male pheromones induced increased neurogenesis in the AOB of castrated oil-treated males in comparison to the TST-treated castrated males (Fig. 6D,E), indicating that in males, TST is necessary and sufficient to inhibit the AOB neurogenic response elicited by male pheromones. In line with these results, the c-fos expression pattern observed in the newborn AOB neurons and in the downstream nuclei of the VNS of the oil-treated castrated males under familiar male odour exposure was reminiscent of that observed in the Sema7A ko mice and significantly different from that in the TST-treated castrated males (Fig. 6F,G, and Suppl. Fig. 3). Overall, these data indicate that the level of circulating TST during adulthood is critical for the fine-tuned and sex-specific control of AOB neurogenesis and VNS activation, which influences opposite-sex cue preference/attraction.

## Discussion

In mammals, pheromones and gonadal hormones are known to work in concert to elicit sex-specific patterns of neuronal activity in the brain circuits that control sexual behaviour. In addition, studies conducted over the last decade in rodents have shown that correct pheromonal perception requires the integration and activity of newborn interneurons in the AOB, the first central station of the VNS pathway. Here, we demonstrate that in male mice, the level of circulating TST plays a critical role in regulating the process of adult neurogenesis in the AOB and, in turn, the pattern of neuronal activity in the downstream VNS nuclei that drives attraction towards females. Overall, these results strongly suggest that cooperation between the endocrine system and adult neurogenesis is needed for appropriate responses to social reproductive stimuli.

**Sema7A ko males show feminized behavioural and c-fos responses to opposite-sex stimuli.** In vertebrates, GnRH neurons control reproduction by directing the activity of the HPG axis through the integration of a multitude of external and internal stimuli[20]. Accordingly, defects in the organization and/or function of the GnRH system cause heterogeneous reproductive disorders that are characterized by a reduction or failure of sexual competences[43,44]. In Sema7A ko males, a suboptimal number of GnRH neurons leads to impaired testicular growth[19], which we found to be associated with lowered levels of circulating TST, reduced androgen receptor expression in the OB, and the absence of a preference/attraction towards opposite-sex smells and/or individuals that is not associated to altered olfactory perception and/or attraction to social cues. In mice, attraction to the opposite sex is mediated by pheromonal perception[45] and is considered part of appetitive, or pre-copulatory, sexual behaviour[3,4,46]. The neural circuits responsible for sex behaviour involve several brain areas where the integration of olfactory input and endocrine stimuli occurs[6,41]. Importantly, in these circuits, pheromonal exposure triggers a sexually dimorphic pattern of excitation[25,39,40,47–50]. Here, we demonstrated that in Sema7A ko males, exposure to male cues elicited feminized c-fos responses in the AOB, MeA, BNST, MPOA, and ARC, which are target nuclei of the vomeronasal pathway involved in the control of sexual behaviour[39,40,51]. This pattern of c-fos expression is thus consistent with the altered attraction to opposite-sex stimuli found in the ko males. By contrast, in the Sema7A ko females, male pheromonal exposure elicited attraction and the expected c-fos expression pattern in sex-specific brain nuclei, indicating that in Sema7A ko mice, altered responses to opposite-sex stimuli occur only in males.

**Sexually dimorphic Testosterone-dependent regulation of AOB neurogenesis.** One key point of our study is that in the Sema7A ko males, exposure to male pheromones increased the integration/survival of newborn interneurons in the AOB, a phenomenon that is normally restricted to females[10,11] and that is upstream of the cascade of sexually dimorphic activation patterns that take place in the VNS nuclei[11,39,40,51]. Strikingly, in adult Sema7A ko males, chronic TST treatment was sufficient to block the effects elicited by male pheromonal exposure, repressing the increase in adult AOB neurogenesis and reversing the feminized phenotype, in terms of both the c-fos expression pattern along the VNS pathway and appetitive behaviour. Although we did not examine whether this effect is purely androgen dependent or mediated by the TST metabolites oestradiol and/or dihydrotestosterone, our data clearly indicate that the sexually dimorphic integration of newborn neurons in the AOB of adult mice involves a gonadal-dependent mechanism.

Altered sexual behaviour, including a shift of cellular and behavioural responses to opposite-sex pheromones, has been previously described in male mice with genetic alteration/elimination of androgen or oestrogen signalling[24,26,27,29,52–57]. Activation of these signalling pathways during the perinatal critical period is fundamental for brain masculinization and depends on a perinatal surge of TST from the testis and its conversion to oestrogens[3,5], although activation of the Ar is also required[29]. Thus, it is conceivable that the low circulating TST level, as well as the reduction of Ar expression observed in the OB in the Sema7A ko mice could affect the perinatal organization of male sex-specific brain circuits and, in turn, attraction to females during adulthood. However, sexual motivation in male mice, which is related to pre-copulatory behaviour, also depends on the level of circulating TST during adulthood[58–60]. Accordingly, TST administration in adult Sema7A ko males was sufficient to restore the typical male pattern of cellular and behavioural responses along the VNS, although it was inefficient in restoring a normal level of Ar expression in the OB.

The castration of adult animals allows organizational effects to be distinguished from activational effects, which can be reversed by replacement of the active steroid[5]. Using this strategy in wt mice, we further demonstrated that an appropriate level of circulating TST during adulthood is both necessary and sufficient to modulate AOB neurogenesis and the activity of downstream pheromonal-sensitive brain circuits that control opposite-sex attraction/preference in male mice.

**Fine-tuned gonadal-dependent regulation of AOB neurogenesis controls opposite-sex attraction in mice.** The role of the AOB as one of the primary integrative regions for sensory stimuli controlling reproduction has been well established by several studies[2]. AOB neurons encode the sexual and genetic status of conspecifics based on signals contained in body fluids, such as urine and saliva[30,61], and their activity is required

for several neuroendocrine responses to opposite-sex pheromones, such as the selective block of pregnancy or "Bruce effect"[9]. In addition, and of particular interest considering our results, in males, the activation of AOB circuits is important for the encoding of the motivational value of opposite-sex chemo-signals[60,62]. Key elements in the AOB sensory processing are the inhibitory interneurons, the granule and periglomerular cells, which gate excitatory inputs from the AOB that are sent to downstream nuclei of the VNS and hypothalamus. In both the MOB and AOB, these cells are continuously refreshed by the addition of 'young and excitable' elements, which become involved in sensory perception soon after their activity-dependent integration[11,63–65].

Exposure to male chemo-signals that trigger sexual behaviour significantly enhances newborn cell integration in female mice[10,11,13], and importantly the ablation of OB neurogenesis through chemical[11,13], genetic[35] or physical irradiation[36] approaches always leads to abnormal socio-sexual behaviours in male and female mice[17]. These data strongly suggest that the number of newborn neurons that are integrated in the OB region, and particularly in the AOB, is critical for the optimization of pheromone-elicited reproductive responses. Although sex behaviour involves a complex interaction and activity of multiple steroid-sensitive brain circuits, in this context our results add two new important pieces of evidence: i) an increase in the integration of newborn neurons in the AOB drives attraction/preference for male cues (influencing the activity of downstream VNS circuits) independently of the genetic sex of mice; and ii) this (pheromone-elicited) increase depends strictly on low circulating TST levels, a condition typical of females[66].

The interaction of gonadal hormones and adult-generated neurons in promoting 'appropriate' opposite-sex attraction also sheds new light on the way sex differences in the anatomy and function of the VNS are achieved[60,67], at least in the circuits involved in the regulation of pheromone-evoked reproductive responses. Based on our data, we propose that sexual dimorphism in adult VNS circuits is sustained by adult neurogenesis, through which a diverse number of newborn neurons, key elements for sensory perception, are integrated based on sex-specific internal (gonadal hormones) and external cues (pheromones). Although we cannot rule out the possibility that this mechanism could be a peculiar feature of mice because sex hormones differentially modulate adult neurogenesis depending on species, sex and age[68,69], our study revealed a key role of adult neural plasticity in the gonad-dependent regulation of sensory cue-elicited sex behaviours.

## Methods

**Animals.** The experiments employed adult, 3- to 5-month-old male and female mice. The Semaphorin 7A homozygous mutant constitutive knockout mice (C57BL6/J strain) were generated by Alex Kolodkin (John Hopkins University School of Medicine) and provided by Prof. Pasterkamp (Utrecht University). Mutant phenotypes were obtained by crossing heterozygous mice, and wt mice from the same litters were used as the control group[18]. After DNA was isolated from tail samples, genotypes were determined using PCR amplification. Animals of the same sex were maintained 4–6 per cage in rooms with a 12:12 light/dark cycle, with standard diet and water provided ad libitum. The male subjects that were used for behavioural analysis were kept in isolation for the duration of the behavioural experiments. All experimental procedures were conducted in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC), Recommendation 18/06/2007, Dir. 2010/63/UE, and with the University of Turin's institutional guidelines on animal welfare (DL116/92). The experiments were also approved by the Italian Ministry of Health and the Bioethical Committee of the University of Turin (Protocol Number DGSAF0007085-A05/04/2013). All experiments were designed to minimize the number of animals used.

**5-Bromo-2′-deoxyuridine (BrdU) treatment.** To identify newly generated cells in the OB, mice were intraperitoneally injected with BrdU in 0.1 M Tris (pH 7.4) twice a day (delay = 8 h, 100 mg/kg body weight) and killed 28 days later for to evaluate neuronal survival. Cell proliferation in the SVZ-RMS in the clean bedding condition was analysed 2 hours after a single intraperitoneal injection of BrdU (0.1 M Tris, pH 7.4; 100 mg/kg body weight).

**Tissue preparation.** Mice were deeply anesthetized via an intraperitoneal injection of a 3:1 ketamine (Ketavet; Gellini, Italy):xylazine (Rompun; Bayer, Germany) solution. All of the animals were transcardially perfused with a 0.9% saline solution followed by cold 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed from the skull and post-fixed for 4–6 h in 4% PFA at 4 °C. Post-fixing was followed by a cryopreservation step with a 30% sucrose solution in 0.1 M PB pH 7.4 at 4 °C. The two hemispheres were separated and embedded in OCT (Bio-Optica) and then frozen and cryostat sectioned. Free-floating parasagittal and coronal sections (25 μm) were collected in multi-well dishes to provide representative series of the AOB and MOB, respectively. The sections were stored at −20 °C in an antifreeze solution (30% ethylene glycol, 30% glycerol, 10% PB: 189 mM $NaH_2PO_4$, 192.5 mM NaOH; pH 7.4) until use.

**Immunohistochemistry.** After the sections were rinsed in PBS to remove the antifreeze solution, they were incubated for 24 h at 4 °C in primary antibodies diluted in 0.01 M PBS, pH 7.4, 0.5% Triton X-100, and 1% normal sera that matched the host species of the secondary antibodies. The following primary antibodies were used: anti-BrdU, rat IgG monoclonal, dilution 1:5000 (ABC), 1:1000 (IFL), AbD serotec, Bio-Rad Laboratories, code number OBT0030CX (Liu *et al.*, 2009); and anti-cfos, rabbit IgG polyclonal, dilution 1:10000 (IFL), Santa Cruz biotechnologies, CA, USA, code number sc52[11]. For BrdU immunostaining, the sections were pre-treated with 2N HCl for 30 min at 37 °C for antigen retrieval and neutralized with borate buffer, pH 8.5, for 10 min. For the avidin-biotin peroxidase method, the sections were incubated for 1 h at room temperature in a biotinylated secondary antibody (anti-rat IgG, anti-goat IgG; Vector Laboratories, Burlingame, CA) diluted 1:250 in 0.01 M PBS, pH 7.4, followed by incubation with the avidin-biotin-peroxidase complex (Vector Laboratories). To reveal immunoreactivity, we used 0.015% 3,3′-diaminobenzidine and 0.0024% $H_2O_2$ in 0.05 M Tris-HCl, pH 7.6. After

the sections were adhered onto gelatin-coated glass slides, they were dehydrated and mounted in Sintex (Nuova Chimica, Cinisello Balsamo, Italy).

For immunofluorescence double-staining, the sections were incubated in a mixture of primary antibodies and appropriate blocking sera for 24 h at 4 °C, then incubated with appropriate fluorochrome-conjugated secondary antibodies (Cy3-conjugated secondary Ab, 1:800; 488-conjugated secondary Ab, 1:400; Jackson ImmunoResearch Laboratories, USA) and/or biotinylated secondary antibodies (1:250; Vector Laboratories, Burlingame, CA, USA), and finally incubated with avidin-FITC (1:400, Vector Laboratories, Burlingame, CA, USA). The sections were then coverslipped with the anti-fade mounting medium Dabco (Sigma) and analysed with a laser scanning LAS AF Lite confocal system (Leica Microsystems).

**Familiarization with male bedding.** The experiment was carried out by defining eight groups of animals characterized by a specific combination of genotype (Sema7A ko or wt), gender (male or female) and treatment (exposure to male-soiled bedding or clean bedding).

The groups exposed to male-soiled bedding (familiarized groups) were exposed to a mixture of soiled beddings, collected from the cages of C57BL6/J stud males, from day 7 to day 14 after BrdU injection. The soiled bedding was renewed daily. The control groups (unfamiliarized animals) were kept in a separate room from day 0 to day 28 after BrdU injection and treated with clean bedding in the same way. In all animals (the familiarized and unfamiliarized groups), the stimulus (soiled bedding) was presented focally (30 min) 90 min before the animals were perfused to detect c-fos activation.

**Olfactory preference test.** For all behavioural tests, the animal subjects were coded so that the investigator was blind to the phenotype of each animal. During the social olfactory preference test assessment, the wt and Sema7A ko mice (males and females) were first exposed to urine from an adult C57BL6/J wt stud male and oestrus female, respectively, for 30 min. Then, after 30 min in clean bedding, the mice were presented with the same urine samples, which were delivered on a piece of filter paper placed within a petri dish (enabling direct contact with the source) or in a drilled box (enabling contact only with the volatile compounds). Male and female urine samples were placed on opposite sides of the cage, equidistant from the cage walls. Trials lasted 10 min, during which mouse behaviour directed towards the two urine sources was recorded. The amount of time spent sniffing the petri dish or the drilled box in which a test urine sample was presented was used as an indication of the mouse's interest in gaining further information from a scent source. The tests were conducted in the animals' home cages to minimize both manipulation and exposure to external stimuli. For each test, approximately 50 μl of either male or female urine was put on each piece of filter paper. The duration of the sniffing of the tested mouse was quantified using JWatcher Behavioral Software (UCLA, USA).

**Individual preference test.** Sex preference was assessed using a three-chamber cage[31]. The dividing walls were made from clear Plexiglas, with an open middle section, allowing free access to each chamber. Two mice, one male and one female, were placed inside two identical wire cup-like containers with removable lids that were large enough to hold a single mouse. These containers were placed vertically inside the apparatus, one in each side chamber. Each container consisted of metal wires to allow for air exchange between the interior and exterior of the cylinder but was small enough to prevent direct physical interactions between the animal on the inside with the animal on the outside. The main principle of this test is based on a subject mouse's free choice to spend time in contact with the mice in the containment cups. Before starting the test, a female mouse in oestrus was chosen to be held in one of the containers. To determine the ongoing oestrous cycle phase of the female mice used in the behavioural experiments, vaginal cytology was evaluated through optical microscope observations of smears. Samples were collected non-invasively via glass Pasteur pipettes after flushing with a saline solution. Adult C57BL6/J wt and Sema7A ko male mice that were previously cage-isolated for at least one week were tested. The morning before the test, the mice were accustomed to the empty cage during a 12-min acclimation period. The amount of time spent in direct contact with the cup-like container (containing a male or female mouse) was evaluated over a 15-min test period, and the mouse's social odour preference was defined as the propensity to spend more time sniffing one containment cup than the other. The duration of direct (active) contact between the tested mouse and the containment cup was quantified using JWatcher Behavioral Software (UCLA, USA). The percentage of time spent investigating one of the two animals (male or female) relative to the total amount of time spent investigating both animals (sniffing period) was evaluated.

**Habituation/dishabituation test.** Odour habituation/dishabituation was used to measure MOE-mediated olfactory discrimination[33,34]. Briefly, mice were familiarized with a first odour (habituation odour) in four successive sessions and then exposed once to a novel odour (dishabituation odour). Each session was 1 min long and was followed by a 10-min inter-session interval. Olfactory discrimination was analysed using two different odours, acetophenone (00790, Sigma) and octanal (O5608, Sigma), which were diluted $10^{-3}$% in mineral oil (M3516, Sigma) and delivered via patches of filter paper. Twenty microliters of odour was applied to each filter paper, which was then placed in a petri dish. The two odours were both alternatively tested as novel stimuli (during dishabituation) in separate sessions. Mouse sniffing activity was quantified using JWatcher Behavioral Software (UCLA, USA).

**Odour detection threshold test.** The test was performed as previously described[34] with minor modifications. Five days prior to the test, mice (Sema7A ko and wt) were single housed in their home cages, then used as testing chambers. During the test, each animal was exposed to two drilled boxes, placed at the opposite sides of the cage, containing a piece of filter paper (1,5 × 1,5 cm) soaked one with 25 μl of odorless mineral oil (M3516, Sigma), as a control, and the other with 25 μl of octanal (O5608, Sigma), diluted in mineral oil. Sniffing time was measured

for four concentrations of the odour ($10^{-7}$, $10^{-5}$, $10^{-4}$, $10^{-3}$) tested in separate sessions in ascending order. Each session was 5 min long and was separated by 15 min intersession interval. Each session was video-recorded, and the time the animals spent sniffing the box, defined as a nasal contact with the dish within a 0.5 cm distance, was subsequently measured by BORIS software[70]. For statistical analysis, an 'odour preference' index was calculated, as the ratio between the time spent investigating the odour, and the total sniffing time (odour plus mineral oil). The values between 0.50 and 1.00 were indicative of a preference for the odour, compared to mineral oil.

**Castration procedure.** Adult (3 months old) wt C57BL6/J mice were deeply anaesthetized with a solution of ketamine and xylazine, and using aseptic procedures, both testes were removed by a small incision in the scrotum. The spermatic cord was then blocked with a silk suture to prevent haemorrhage during dissection of the testis. The mice were then allowed to recover for 3 weeks before further treatments.

**Testosterone treatment.** For the analysis of adult neurogenesis, the TST treatment was applied in one group of intact Sema7A ko males and in castrated wt C57BL6/J males (24 h after BrdU administration, see Figs 5A and 6A). Another group of Sema7A ko males was treated with TST for the analysis of Ar expression. The treatment consisted of daily subcutaneous injections of testosterone propionate (1 mg/day), diluted to 10 mg/ml in sesame oil[42] for 16 consecutive days. Control animals received sesame oil treatment only. During the TST/oil treatment, the animals underwent behavioural analyses and familiarization with stud-male bedding. Ten days after the behavioural analyses, two additional daily pulses of TST/oil were given before focal bedding presentation and sacrifice.

**Testosterone assays.** Trunk blood was collected directly from the mouse's heart into a sterile eppendorf tube with a needle and left on ice until centrifugation; the plasma was frozen and stored at −80 °C until use. Plasma TST was measured using a sensitive testosterone rat/mouse ELISA (Demeditec Diagnostics REF DEV9911) based on the principle of competitive binding. The lowest analytically detectable level of TST that can be distinguished from the Zero Calibrator is 0.066 ng/ml. The intra- and interassay coefficient of variation was 8.06% and 9.3%, respectively.

**Quantitative RT-PCR analyses.** For gene expression analyses, OBs and POA were extracted and fresh-collected from wt and Sema7A ko male mice (treated with TST and untreated). Total mRNA was extracted from these bulbs using the RNeasy® Lipid Tissue Mini Kit–74804 (QIAGEN) and reverse transcribed using SuperScript® III Reverse Transcriptase (Life Technologies). A linear preamplification step was performed using the TaqMan® PreAmp Master Mix Kit protocol (Applied Biosystems). Real-time PCR was carried out on a StepOnePlus TM Real Time-PCR System (Applied Biosystems) using the following exon-boundary-specific TaqMan® Gene Expression Assays (Applied Biosystems): Er1 (Mm00433149_m1), Er2 (Mm00599821_m1), and Ar (Mm00442688_m1). Relative gene expression levels were normalized against the mRNA levels of the housekeeping genes β-actin (Mm01205647_g1) and RN18S (Mm03928990_g1). Quantitative real-time PCR was performed using TaqMan Low-Density Arrays (Applied Biosystems) on an Applied Biosystems 7900HT thermocycler using the manufacturer's recommended cycling conditions. Gene expression data were analysed using ExpressionSuite Software v1.01.

**Cell count and statistical analysis.** Cell counts and image analyses were performed on a Nikon microscope equipped with a computer-assisted image analysis system (Neurolucida software; MicroBrightField, Colchester, VT) and on a Leica DM600 CS LAS AF Lite confocal microscope (Leica Microsystems). Confocal image z-stacks were captured through the thickness of the slice at 1-μm optical steps. These images were used for cell counting or assembled into extended-focus photographs. Brightness, colour, and contrast were balanced and assembled into panels with Inkscape (Free vector graphics editors). All cell counts were performed blind to the genotype and/or the treatment.

In the AOB, the number of all newborn neurons was established by counting peroxidase/DAB-stained or fluorescence-labelled BrdU-positive nuclei in 25-μm-thick parasagittal sections. Two series (n = 7/8 sections/animal) representing the whole AOB were used for each animal. Cells were counted in the AOB granule cell layer. The area of each examined section was measured with Neurolucida software. Cell densities were expressed relative to the volume of the entire AOB granule cell layer; this volume was obtained by multiplying the area measurements by the mean section thickness (25 μm).

The percentage of double-labelled c-fos/BrdU-positive cells in the AOB granule cell layer was established by counting labelled cells in parasagittal sections (25 μm thick) with a 40x objective. One series (n = 3–4 sections/animal) representing the whole AOB was used for each animal. In each section, all of the BrdU-positive cells were analysed for co-expression with c-fos, and ratios of double-labelled cells were determined.

In the MOB, the number of BrdU-positive nuclei was established by counting peroxidase/DAB-stained nuclei in three representative OB coronal (anterior, medial and posterior) or parasagittal (from lateral to medial) sections per animal using a systematic random sampling method. Newborn cells were quantified in the granule cell layer. Sampling was conducted by overlaying each section with a virtual counting grid (squares size of 80 × 80 μm). Cells were counted through the 25-μm thickness of the slice in one square of the grid (one of every two) through sequential translation of the counting frame until the area of interest was entirely covered (40x objective). This procedure allowed us to analyse about one-fourth of the area of interest. The number and position of each cell in the counted area were marked by the software. Cells contacting a line on the upper or left edge of the counting square were excluded from the counts, whereas those contacting the lower or right edge of the square were considered in the counts[71]. Cell density (number of labelled profiles/mm$^3$) was calculated by multiplying the area measurements by the mean section thickness (25 μm) [$\Sigma$ of sampled areas μm$^2$ ×25 μm]. To evaluate cell

proliferation in the SVZ-RMS region, newborn cells were quantified 2 hours after BrdU injection. Cell counting was performed on one-in-six parasagittal sections 25 μm thick. Eight different levels, along the rostro-caudal axis of the brain, were selected, based on stereotaxic atlas, and the total number of labelled cells was evaluated for each level. The number of BrdU-labelled cells was then multiplied by 6 to provide an estimate for the total number of newborn cells in the RMS-SVZ region.

For statistical analysis, unpaired student's t-tests were used for simple 1:1 comparisons of parametric data, while the Wilcoxon-Mann-Whitney test was used for non-parametric analyses. One-way Anova were used for multiple comparisons in case of parametric data; Kruskall-Wallis Test was used in case of non-parametric data (Supplementary Figures).

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## Author Contributions

P.P., S.D.M. and R.S. designed the experiments and wrote the paper. P.G. contributed to the experimental design and critically commented the manuscript. R.S. performed most of the experiments and analysed the data. S.T., I.C., B.C.J. and A.F. contributed to the experiments. R.J.P. provided Sema7A ko mice and critically commented the manuscript. G.P. contributed to the statistical analysis and critically commented the manuscript.

## Additional Information

**Supplementary information** accompanies this paper at http://www.nature.com/srep

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