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INDIRIZZO DI NEUROBIOLOGIA
CICLO XXVI

# Formation and function of neural circuitry in the olfactory bulb of mice with reduced afferent spontaneous activity


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# SOMMARIO

Nei sistemi sensoriali, i neuroni periferici proiettano i loro assoni in specifici loci del cervello.

La segregazione spaziale delle afferenze sensoriali provvede a creare mappe topografiche che definiscono la qualità e la localizzazione di complessi stimoli sensoriali.

L'attività elettrica gioca un ruolo chiave nella formazione di specifici contatti sinaptici tra i neuroni, sebbene resti ancora da definire il tipo di attività richiesta. In particolare il ruolo dell'attività elettrica spontanea nell'organizzazione topografica del sistema olfattivo, non è noto.

Per rispondere a questa domanda abbiamo studiato il ruolo dell'attività elettrica spontanea nella formazione e nella funzione dei circuiti neurali nel bulbo olfattivo.

Per raggiungere questo obiettivo abbiamo utilizzato una linea di topi geneticamente modificati, nei quali l'attività afferente spontanea è ridotta a causa della sovra-espressione di un canale potassio *inward rectifier* (Kir2.1), in tutti i neuroni olfattivi sensoriali (i topi Kir2.1).

Abbiamo analizzato la formazione della mappa sensoriale, in particolare se la convergenza dei neuroni sensoriali esprimenti il medesimo recettore olfattivo avveniva correttamente nei topi Kir2.1

La convergenza dei neuroni sensoriali in specifici loci del bulbo olfattivo, che porta alla formazione di glomeruli omogenei, cioè glomeruli formati esclusivamente da assoni esprimenti lo stesso recettore olfattivo, è una caratteristica critica della mappa sensoriale. Infatti i glomeruli definiscono le unità funzionali o colonne odorose del sistema.

Abbiamo trovato che in assenza di attività spontanea, gli assoni dei neuroni sensoriali non convergono a formare un unico glomerulo ma proiettano in molti siti dando luogo a ulteriori glomeruli. Questi addizionali glomeruli sono caratterizzati da una organizzazione eterogenea, risultano cioè formati da assoni di neuroni sensoriali esprimenti recettori olfattivi diversi.

Per capire se l'attività afferente spontanea potesse avere un ruolo anche sulle cellule postsinaptiche del bulbo olfattivo, abbiamo analizzato le cellule mitrali, i principali

neuroni di output, e le cellule dei granuli, i principali neuroni inibitori, del bulbo olfattivo.

Analizzando lo sviluppo morfologico del dendrite apicale delle cellule mitrali non abbiamo trovato alcuna differenza significativa nei topi Kir2.1 rispetto ai controlli. Per quanto concerne le cellule dei granuli, studiando la neurogenesi e la migrazione delle cellule dei granuli di nuova generazione, non abbiamo riscontrato differenze significative nei topi Kir2.1 rispetto ai controlli. Tuttavia l'analisi morfologica dell'arborizzazione dendritica delle cellule dei granuli ha messo in evidenza una ridotta densità di filopodi/spine nei topi Kir2.1 rispetto ai controlli.

Per analizzare le conseguenze funzionali di queste alterazioni anatomiche abbiamo eseguito specifici test comportamentali. I dati che abbiamo ottenuto indicano chiaramente che i topi Kir2.1 non erano in grado di discriminare tra due odori che attivano glomeruli che hanno distribuzione spaziale molto simile, quali gli enantiomeri. Tuttavia i topi Kir2.1 mantenevano la capacità di distinguere odori che attivano glomeruli posti in aree molto diverse del bulbo, quali l'acido 2-metilbutirrico e l'acido ciclobutancarbossilico (2Mb e CB).

Dato l'elevato grado di plasticità del sistema olfattivo, ci siamo chiesti se la manipolazione dell'attività elettrica in età adulta poteva influenzare la mappa sensoriale. Sfruttando la possibilità di indurre l'espressione del gene Kir2.1 in momenti diversi della vita dell'animale, abbiamo fatto esprimere il gene Kir2.1 solo in animali adulti per 4 settimane. Abbiamo trovato che l'espressione del gene Kir2.1 in animali adulti alterava l'organizzazione della mappa sensoriale, cioé la specifica convergenza degli assoni dei neuroni sensoriali nel bulbo olfattivo. I dati ottenuti indicano che l'assenza di attività spontanea nell'età adulta causa una "regressione" nell'organizzazione dei glomeruli. Abbiamo infatti trovato un elevato numero di glomeruli eterogenei che coesistevano coi principali glomeruli omogenei.

I nostri dati suggeriscono che l'attività elettrica spontanea è richiesta per lo sviluppo e il mantenimento della mappa sensoriale. Inoltre abbiamo trovato che le alterazioni morfologiche della circuiteria neuronale nel bulbo olfattivo contribuiscono ad alterare il comportamento olfattivo.

# SUMMARY

In the sensory systems, peripheral neurons project axons in specific loci of the brain. The spatial segregation of the sensory afferents provides topographic maps that define the quality and the location of complex sensory stimuli. Electrical activity plays a critical role in the formation of specific synaptic contacts among neurons, although the type of activity required remains a matter of significant debate. In particular the role of spontaneous activity in the formation of the topographic organization of the olfactory system remains unknown.

To address this question we investigated the role of spontaneous electrical activity in circuit formation and function in the olfactory bulb. To accomplish this goal, we took advantage of a line of mice engineered to have very little afferent spontaneous activity due to the over expression of the inward rectifying potassium channel Kir2.1 in the olfactory sensory neurons (Kir2.1 mice). We analyzed the formation of the sensory map, in particular whether the convergence of olfactory sensory neurons expressing the same odorant receptor took place properly in mice with reduced afferent spontaneous activity. The conflation of sensory axons to form homogeneous glomeruli, i.e. glomeruli formed exclusively by axons expressing the same olfactory receptor, in specific loci of the olfactory bulb is a critical feature of the sensory map that in turn defines functional units, i.e. odor columns. We found that in absence of spontaneous activity, the convergence of sensory neurons to form homogenous glomeruli took place but it was coarser than in controls. In particular we observed axons mistargeting that resulted in multiple heterogeneous glomeruli that persist also in adulthood.

To ascertain the role of spontaneous activity on the post synaptic elements of the olfactory bulb, we analyzed the mitral cells, the principal output neurons, and the granule cells, the major component of the inhibitor interneurons of the olfactory bulb. We found no difference in the developmental refinement of the apical dendrite of mitral cells in Kir2.1 and in control mice. The neurogenesis and the migration of granule cells was unaltered. However the filopodia-spine density on the dendritic tree of the granule cells was significantly reduced in Kir2.1 mice.

To analyze the functional outcome of these anatomical alterations, we performed behaviour experiments. We demonstrated that Kir2.1 mice were unable to discriminate between two odors, such as couple of enantiomers, that elicit very similar spatial patterns of activated glomeruli, while retained the ability to differentiate between odorants, such as 2-methylbutyric acid and cyclobutanecarboxylic acid (2Mb and CB) that activate patterns of glomeruli spatially very distinct.

Due to the high degree of plasticity of the olfactory system, we asked whether manipulation of electrical activity in adulthood could affect the already refined neural circuitry in the olfactory bulb. Taking advantage of the inducible nature of the Kir2.1 construct, we allowed the expression of the Kir2.1 channels only in adulthood, for 4 weeks. We found that the expression of the Kir2.1 channel in adults disrupted the organization of the sensory map, namely the convergence of olfactory sensory neuron axons. The absence of spontaneous afferent activity in adults induced a regression in the glomeruli organization. We found supernumerary heterogeneous glomeruli that coexist with the main homogeneous glomeruli.

All together our data suggest that spontaneous activity is required for the developmental refinement and maintenance of the sensory map. Furthermore we found that the unrefined connectivity of the neural circuitry of the olfactory bulb could affect olfactory discrimination behaviour.

# 1 INTRODUCTION

## 1.1 The olfactory system

The olfactory system contains the main and the accessory olfactory system (Figure 1).

**The main olfactory system** is constituted by the olfactory epithelium, the main olfactory bulb and the relative cortical areas including: the piriform cortex, the anterior olfactory nuclei, the olfactory tubercle, the anterior cortical amygdaloid nucleus and the lateral entorinal cortex. It

detects odorants. Most animals relies on odor cues for vital functions such as searching food, identifying a predator and prey and mark territory (Firenstein, 2001).

**The accessory olfactory system** consists of the vomeronasal organ, the accessory olfactory bulb and the relative cortical areas including the postero-medial cortical nucleus of the amygdala (Gutierrez-Castellanos et al. 2013). It recognizes pheromones: specie-specific non volatile chemicals, secreted in urine or other bodily fluids (Lin et al., 2005) that influence behavioural or physiological responses.

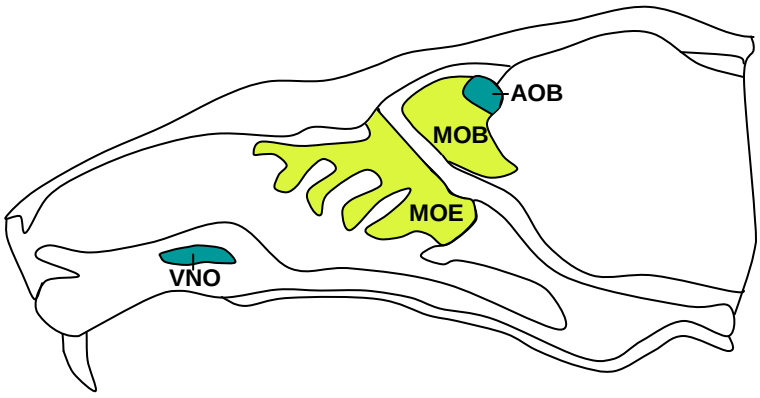


**Figure 1** Schematic diagram of half a mouse head. The main olfactory epithelium (MOE), the main olfactory bulb (MOB), the vomeronasal organ (VNO) and the accessory olfactory bulb (AOB) are represented with colours.

In my PhD work I focused my attention on circuit formation in the main olfactory system, in particular I studied the role of afferent spontaneous activity in circuit formation and function of the main olfactory bulb in mice.

## 1.2 The olfactory epithelium

The olfactory epithelium is a pseudostratified columnar epithelium that lines a series of cartilaginous structures called turbinates, localized in the posterior-dorsal part of the nasal cavities (Figure 2). It contains three principal cells types: the olfactory sensory neurons, the basal cells and the sustentacular (supporting) cells (Farbman, 1992).

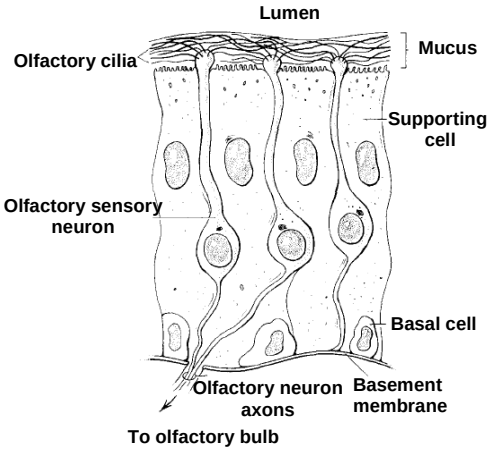


**Figure 2** Cross-section of the main olfactory epithelium in which are present: the basal cells, the supporting cells and the olfactory sensory neurons. Modified from Buck and Axel, 1991.

**The olfactory sensory neurons** represent the 70-80% of the entire cellular population of the olfactory epithelium. Olfactory sensory neurons constantly regenerate throughout the life of the individual. They have an half-life of 60-90 days. Olfactory sensory neurons are bipolar neurons with a single apical dendrite that reaches up to the surface of the epithelium and a small-diameter, unmyelinated axon that projects directly to the olfactory bulb, the first retransmission station of the olfactory system (Figure 3). The apical dendrite ends in a swelling-like structure called knob, from which several cilia

depart (Firenstein, Nature 2001). Olfactory receptors are expressed on the cilia, fine extensions that protrude in the nasal cavity. Cilia are immersed in the mucus that is produced by Bowman's glands distributed throughout the epithelium. The mucus protects the olfactory epithelium and creates the optimal environment for the odor perception. It contains olfactory binding proteins, (low-molecular-weight soluble proteins, secreted by nasal glands that convey the hydrophobic odorant molecules through the aqueous nasal mucus (Pelosi, 1994; Steinbrecht, 1998).

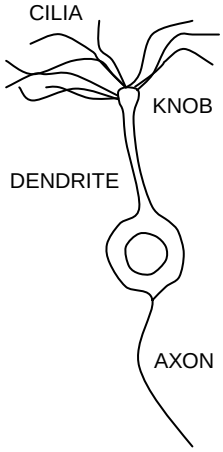


**Figure 3** Drawing of an olfactory sensory neuron. It consists of a soma, an axon and a single, unbranched-dendrite ending in the knob from which several cilia protrude.

**The sustentacular (supporting) cells** act as the glia of the olfactory epithelium. They perform several functions: they stabilize the epithelium giving it a structural matrix for support; they separate and electrically isolate sensory neurons; they are thought to participate in regulation of the ionic composition of the mucus layer (Getchell et al., 1984).

**The basal cells** are the precursors of olfactory sensory neurons. They rest on the basal membrane. Based on morphological and biochemical properties, two types of basal cells have been identified: 1) the dark/horizontal basal cells, which are in direct contact with the basal lamina. 2) the light/globose basal cells which are mainly situate superficial to the horizontal basal cells (Huart and Schwob, 1995).

## 1.3 The olfactory receptor

The olfactory receptor (OR) belongs to the large family of G-protein-coupled receptors (GPCRs). Like other GPCRs, olfactory receptors present: a coding region that lacks introns; a seven-α helical membrane-spanning domains connected by intracellular and extracellular loops of variable lengths; a conserved aspartate-arginine-tyrosine amino acid motif within the second intracellular loop (Spehr and Mungert, 2009), (Figure 4).

The olfactory receptors present also specific characteristics such as an unusually-long second extracellular loop and a hypervariable region in the third, fourth and fifth transmembrane regions, that may form the ligand-binding pocket (Mombaerts, 1999; Firestein, 2001).

The olfactory receptor gene family is one of the largest mammalian gene families, which comprises more than 1000 genes and represents 3-5% of all the genome.

In olfactory sensory neurons, the expression of a given receptor derives exclusively from one allele. This mechanism is thought to assure the expression of only one type of receptor in a given cell (Chess et al., 1994).

Olfactory receptor genes are distributed mainly in cluster on almost all mouse chromosomes (Zhang and Firestein, 2002; Godfrey et al., 2004). They present a high degree of homology ranging from 40% to over 90% identity (Firestein, 2001).

The number of functional activated genes differs in vertebrates. A high fraction of the human odorant receptor repertoire (~60%) has degenerated to pseudogenes. In contrast only about 20% of mouse ORs are pseudogenes (Zhang, and Firestein, 2002; Young and Trask, 2002) giving mice over three times as many intact genes as human (Young et al, 2002).

Each olfactory sensory neuron expresses only a single type of odor receptor. The odorant receptor therefore define the identity of the olfactory sensory neuron (Chess et al., 1994; Nef et al., 1992).

**Figure 4** Schematic structure of an odorant receptor. Residues that are most highly conserved are shown in shades of blue and those that are most variable are shown in shades of red. The seven –helical regions (boxed) are connected by intracellular and extracellular loops. Modified from Firestein, 2001

## 1.4 Odor transduction in olfactory sensory neurons

The olfactory transduction mechanism begins when odorant molecules bind the odorant receptors. The ligand bound receptor activates a stimulatory olfactory-specific G protein, $G_{olf,}$ (Jones and Reed, 1989) which in turn activates an adenylyl cyclase type III (ACIII) (Bakalyar and Reed,1990). The cyclase converts intracellular ATP into cyclic AMP (cAMP).

Cyclic AMP in turn binds the cyclic nucleotide-gated (CNG) channel, that produce a influx of $Na^+$ and $Ca^{2+}$. The calcium ions entering through the CNG channels bind and activate a chloride channel causing an efflux of $Cl^-$ ions. In combination this signalling pathway leads to olfactory sensory neuron depolarization (Firenstein, 2001; Kleene and Gesteland,1991) (Figure 5)

$Ca^{2+}$ has a dual role in the signalling pathway coupled to the olfactory receptor i.e., it plays a role in olfactory adaptation, in addition to its excitatory function (Matthews and Reisert, 2003).

$Ca^{2+}$ reduced the sensitivity of the CNG channel to cyclic nucleotide through a direct interaction between CNG channels and calcium-calmodulin (Matthews and Reisert, 2003; Cheng, 1994). Furthermore, the increase of $Ca^{2+}$ stimulates cAMP hydrolysis by a $Ca^{2+}$-dependent ciliary form of phosphodiesterase (PDE1C2) (Borisy,1992). $Ca^{2+}$ has also an inhibitory effect on type III adenylyl cyclise (Wayman, 1995).

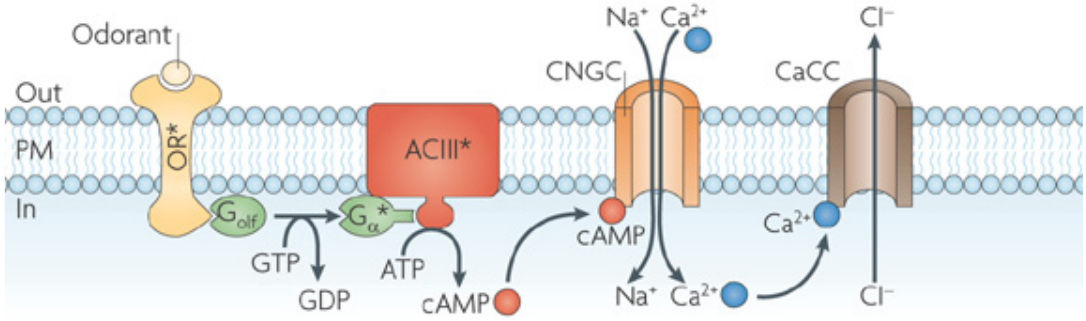


**Figure 5** The odour-induced signal transduction pathway. The binding of an odorant to the olfactory receptor (OR) successively activates the trimeric, olfaction-specific G protein ($G_{olf}$), adenylyl cyclase type III (ACIII), the olfactory cyclic nucleotide-gated channel (CNGC) and a $Ca^{2+}$-activated $Cl^-$ channel (CaCC). Activation of both channel types finally leads to depolarization. Image from Kaupp, 2010.

## 1.5 The combinatorial receptors code for odors

The olfactory system is a highly sophisticated system capable of detecting and discriminate a large number of odors. Each olfactory sensory neuron expresses only one type of olfactory receptor gene out of a repertoire of more than 1000 genes. Each odorant receptor is able to recognize specific structural features of the odor molecules (odotopes). The specific structural features include: the stereochemical structure of the hydrocarbon chain and the type and the position of the functional group. Each odor molecule typically exhibits several odotops that can activate different types of odor receptors. A single odor molecule can bind different odorant receptors, with different affinity, in turn an odorant receptor can bind several odors.

In this way, the mammalian olfactory system uses a “combinatorial code” to identify a myriad of different odorants (Malnic et al., 1999; Mori et al., 1999), (Figure 6).

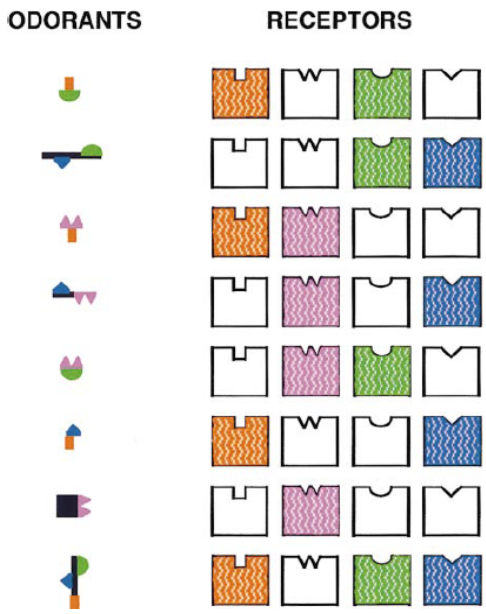


**Figure 6** Combinatorial receptorial code for odors. The identities of different odorants (left) are encoded by different combination of receptors (right). Each odorant receptor can serve as one component of the combinatorial receptor codes for many odorants. Image from Malnic, 1999.

## 1.6 The olfactory bulb

The olfactory bulb (OB) is an outgrowth of the forebrain and it is the first retransmission station of the olfactory system.

The olfactory bulb is a well layered structure. From the external to the internal layer we found:

the olfactory external nerve layer; the glomerular layer; the external plexiform layer; the mitral cells layer; the internal plexiform layer and the granule cells layer (Shepherd, 2004), (Figure 7).

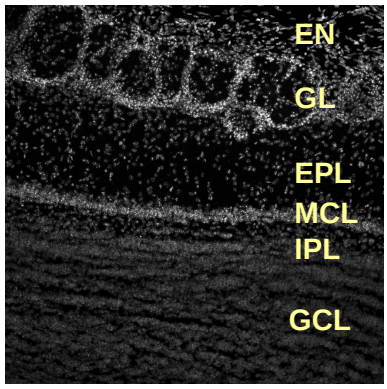


**Figure 7** Horizontal section of the olfactory bulb immunostained with the nuclear marker DAPI.
ENL, external nerve layer; GL, glomeruli layer; EPL, external plexiform layer; MCL, mitral cell layer; IPL, internal plexiform layer; GCL, granule cell layer. Image from Lodovichi's lab.

The neuronal elements of the olfactory bulb fall into three categories: 1) inputs, 2) outputs and 3) intrinsics.

1) The inputs consist of the axons of the olfactory sensory neurons that form the olfactory nerve layer. The axons of olfactory sensory neurons expressing the same odorant receptor converge in specific zones of the olfactory bulb to form glomeruli. Glomeruli are structure of neuropil formed by the axons of the olfactory sensory neurons that make synapse with the dendrites of postsynaptic elements: the mitral cells, the tufted cells and the periglomerular cells.
The olfactory bulb received also inputs from the brain, in particular from: the olfactory cortex, the anterior olfactory nucleus, the horizontal limb of the diagonal band, the locus coeruleus and the raphe nucleus.

2) The outputs consist of the axons of mitral and tufted cells.
**The mitral cells** are excitatory glutamatergic neurons. Their cell bodies (15-30 µm in diameter) lie in the mitral cell layer, a thin sheet 200-400 µm deep to the glomerular layer. The cell bodies are 15-30 µm in diameter. Each mitral cell tends to give rise to a single primary (apical) dendrite, which traverse the external plexiform layer (EPL) and terminates within a glomerulus in a tuft of branches. The tuft has a diameter of 30-150µm, extending throughout most of its glomerulus. The length of dendrite is 200-800

µm depending on how much it angles across the extemal plexiform layer. Each mitral cell also gives rise to several laterally direct secondary dendrite, which terminate in the external plexiform layer and form dendro-dendritic synapses with the granule cells. The primary and secondary dendrites are aspiny. The mitral cell axons proceed to the depths of the bulb and then pass caudally to form the lateral olfactory tract (LOT). The output axons in the LOT give off numerous collateral branches that terminate in layer Ia of the olfactory cortex. (Shepherd, 2004). One type of collateral is emitted from the axon within the olfactory bulb (Ojima et al., 1984).

**The tufted cells** are similar to mitral cells but located more superficially in the external plexiform layer. According to the position of their cell body, they have been subdivided in three main groups: the middle tufted cells ($T_m$), the external tufted cells ($T_e$) and the internal tufted cells ($T_i$).

The main population, the middle tufted cells, lies near the middle of the external plexiform layer. These cells have a cell body diameter of 15-20 µm, several thin basal dendrites (300-600 µm) and a primary dendrite (200-300 µm) ending in a relatively confined tuft of branches in a glomerulus. The axons gives off collaterals that form the lateral olfactory tract that projects to the olfactory cortex. The external tufted cells have the cell body located around the glomeruli. Their axons project in specific loci of the internal plexiform layer, where they constitute a topographically ordered intrabulbar association system that link glomeruli receiving input from sensory neurons expressing the same odorant receptor, (Schoenfeld et al., 1985; Belluscio et al. 2002; Lodovichi et al 2003). The external tufted cells forming the intrabulbar associational system form excitatory synapses (cholecystokinin) with the granule cells connected to the homologous (isofunctional) glomerulus, in the internal plexiform layer on the opposite side of the bulb (Liu et Shipley, 1994), (Figure 8).

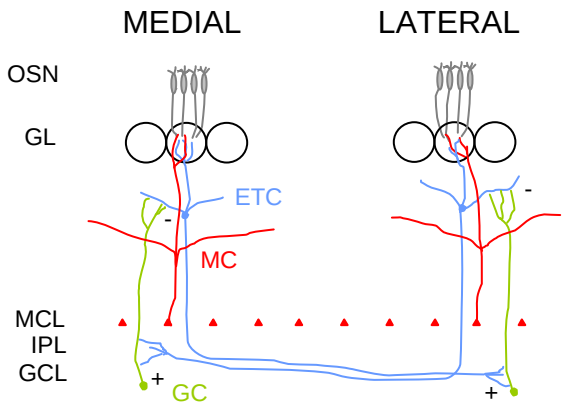


**Figure 8** Scheme of the intrabulbar circuit. Axons of external tufted cells (ETC) connected to a given glomerulus form excitatory synapses (+) onto the dendrites of the granule cells (GC) in a restricted region of the internal plexiform layer on the opposite side of the bulb. These granule cells in turn form inhibitory synapses (-) on the ETC connected to the homologous glomerulus. This connection is reciprocal. OSN, olfactory sensory neuron, GL, glomeruli layer, EPL external plexiform layer; MCL mitral cell layer, IPL internal plexiform layer, GCL granule cell layer. Modified from Lodovichi, 2003.

Internal tufted cells overlap in distribution and morphology with mitral cells.

3) the intrinsics include consist of periglomerular cells and granule cells.

The **periglomerular cells** are a heterogeneous population in which we can discriminate 3 principal types: the periglomerular cells, the short axons cells and the external tufted cells.

**The periglomerular cells** have a short bushy dendrite that arborizes to an extent of 50-100 µm within a glomerulus. The dendritic branches intermingle with the terminals of olfactory axons and the dendritic branches of mitral and tufted cells. The periglomerular cell axons distributes laterally within extraglomerular region, extending as far as five glomeruli away. Some periglomerular cells appear to lack axons. periglomerular cells are morphologically homogeneous but biochemically different. They could release different inhibitory neurotransmitter such as γ-amino-butyric acid (GABA), glycine or modulatory transmitter such as dopamine. Many periglomerular cells present a dual co-transmission of dopamine and GABA. (Maher and Westbrook, 2008; Borisovska et al.,

2013). The periglomerular cells are continuously generated also in adulthood. They originate in the anterior horn of the lateral ventricle and migrate through the rostral migratory stream to the glomerular layer of the bulb. Here they undergo a maturational process to become mature interneurons and to be incorporated into the olfactory bulb circuitry.

**The short axons cells (juxtaglomerular) cells** have axons that reach glomeruli further away than the smaller classical short axons periglomerular cells and dendrites that branch the interglomerular spaces. They are also believed to be glutamatergic.
**The external tufted cells** were previously described ( above p.10 ).

**The granule cells,** the main inhibitory population of the olfactory bulb, are localized in the granule cells layer. They are inhibitory interneuron (they release GABA) (Ribak et al., 1977) with a very small cell body (6-8 µm in diameter); the cell bodies are grouped in horizontal cluster celled isle. Each granule cell gives rise to a superficial process that extend radially toward the surface and ramified and terminates in the external plexiform layer, the branching field extending laterally some 50-200 µm. There are also deep processes that branch sparingly in the granule cells layer (Shepherd 2004). On the basis of morphological and molecular criteria granule cells can be divided in: superficial, deep and intermediate granule cells (Mori et al., 1983; Orona et al., 1983; Mori 1987; Greer 1987; Imamura et al, 2006).
Superficial granule cells have peripheral dendrites that ramify mainly in the superficial external plexiform layer, among the dendrites of tufted cells. Deep granule cells have their dendritic arbors restricted to the deep external plexiform layer, where they are believed to synapse predominantly with the secondary dendrite of mitral cells. Intermediate granule cells have dendrite that ramify at all levels of the external plexiform layer. (Shepherd 2004; Shepherd et al., 2007). Granule cells are axonless and present basal and an apical dendrites with numerous spines. The apical dendrite can be divided into an unbranched segment emerging from the soma followed by a branched segment (distal domain). The basal dendrites (basal domain) and unbranched apical dendrite, in its portion proximal to the soma, receive axo-dendritic inputs from axons collaterals of the olfactory bulb's projection neurons and from the olfactory cortex. The distal domain of the apical dendrites form bidirectional dendro-dendritic synapses. These bidirectional synapses receive glutamatergic input synapse from the lateral

dendrites of the olfactory bulb 's projections neurons ( i.e. mitral and tufted cells) and release GABA back onto these projection neurons. These dendro-dendritic synapses in the distal domain are the exclusive output of granule cells, and are responsible for local inhibition of the projection neurons of the olfactory bulb (Kelsch et al., 2007), (Figure 9).

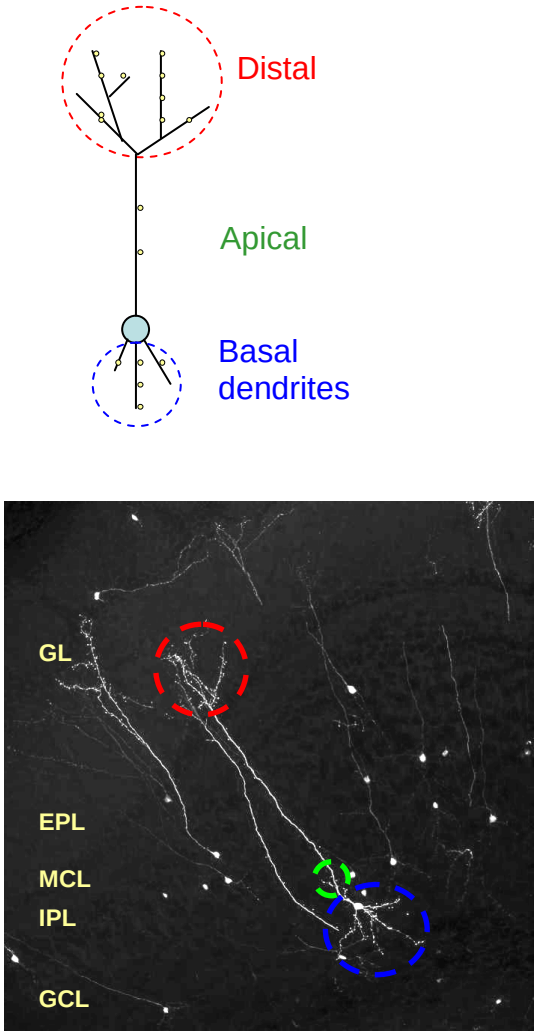


**Figure 9 Top**. Schematic representation of the different domain of granule cells: a basal domain, in blue (=basal dendrites) and different domains in the apical dendrite. The apical dendrite divides into an unbranched domain emerging from the soma followed by a more distal branched segment, in red (= distal domain) The 15% of the unbranched domain is defined the proximal domain, in green. **Bottom**. Corresponding different domains of a granule cell in coronal section of the olfactory bulb. Image from Lodovichi's lab.

Like periglomerular cells, also granule cells continuously regenerate during all life (see also below, p.10).

## 1.7 Organization of the olfactory epithelium and the olfactory bulb

The specificity of connectivity in the central nervous system is essential to translate electrical activity into meaningful neuronal codes. In the sensory systems, peripheral neurons project axons in specific loci of the brain to create an internal representation of the external word. The spatial segregation of the sensory afferents provide a topographic map that defines the quality and the location of complex sensory stimuli (Udin and Fawcett, 1988; Wang et al.,1998; Kaas, 1997). A topographic map is therefore an ordered projection of the sensory surface to higher brain regions. It is typically found in sensory or motor system. For instance, neighbouring points in visual space activate neighbouring points on the retina, and this relation is preserved at higher brain areas (Murthy, 2011).

The organization of the olfactory system differs from this general plan.

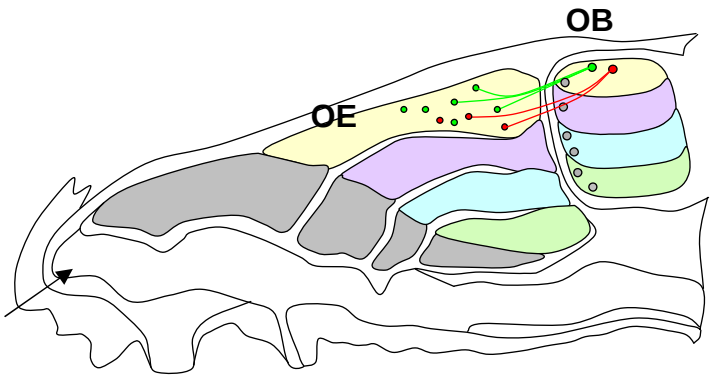


**Figure 9** Scheme of a sagittal section of the nasal cavity and the olfactory bulb. OE = the olfactory epithelium, where the olfactory sensory neurons are located; the olfactory epithelium has been subdivided in zones, indicated with different colors. OB = the olfactory bulb where olfactory sensory neurons send their axonal projections. Arrows indicates the entry of the air.

### 1.7.1 Organization of the olfactory epithelium

The mammalian olfactory system detects and discriminates thousands of odor molecules. The initial event of odor perception occurs in the olfactory sensory neurons located in the olfactory epithelium.

In the olfactory epithelium is present only a coarse topographic organization. Several studies

demonstrated that neurons that express the same olfactory receptor gene are confined to a circumscribed but broad area of the olfactory epithelium. Within a zone olfactory sensory neurons expressing different odorant receptor are randomly intermingled (Vassar et al., 1993; Ressler et al., 1993; Sullivan et al., 1996). The epithelium was subdivided in four zones along the dorso-ventral axes (Vassar et al., 1993; Ressler et al., 1993). More recente studies, however, have demonstrated that different receptors are expressed in distinct zones that are continuous and highly overlapping (Miyamichi, 2005; Zhang et al., 2004).

### 1.7.2 Topographic organization of the olfactory bulb

Spatial order is achieved in the olfactory bulb. Olfactory sensory neurons expressing a given odorant receptor project their axons to form one or two glomeruli on specific loci on the medial and the lateral side of each olfactory bulb. Each glomerulus occupies a specific location in the two bulbs of the same animal and this location is maintained among different animals. The projections of sensory axons provide therefore a glomerular map or sensory map, that represent the first level of topographic organization of the olfactory bulb.

As a result of the spatial segregation of sensory afferents to form glomeruli on the medial and the lateral side of each bulb, the olfactory bulb presents two mirror symmetric maps of isofunctional glomeruli. It has been demonstratated that the two maps of isofunctional glomeruli are reciprocally connected by an inhibitory link related to the external tuft cells (Belluscio et al., 2002; Lodovichi et al., 2003). Namely, the axons of the external tufted cells connected to a given glomerulus on the lateral side of the olfactory bulb form excitatory synapses onto the dendrites of the granule cells in a restrict area of the internal plexiform layer on the opposite side of the olfactory bulb.

These granule cells, in turn, form inhibitory synapses on the external tufted cells connected to the medial homologous glomerulus. This connection is reciprocal and create a single integrated map in which isofunctional odor columns are connected through an intrabulbar link. (Lodovichi et al., 2003, Figure 10). The intrabulbar link represents the second level of topographic organization of the bulb.

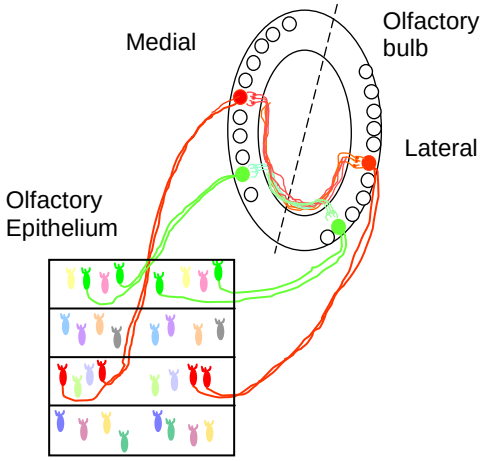


**Figure 10** Schematic representation of the axonal connectivity between the olfactory sensory epithelium and the main olfactory bulb. The OSNs expressing the same odorant receptor (green and red) are randomly intermix in one of the four regions of the olfactory epithelium but project their axons to glomeruli on the medial and lateral sides of each olfactory bulb. Dashed line indicates the line of symmetry between the two mirror-symmetric maps in horizontal sections. Lodovichi et al., 2003

Mombaerts and colleagues in 1996, designed a new genetic strategy to visualize the sensory map. They modified the P2 olfactory receptor gene by targeted mutagenesis in the germline of mice. In this way the P2 locus encoded a bicistronic mRNA that allowed the translation of P2 receptor along with tau-LacZ, a fusion of the microtubule-associated protein tau with β-galactosidase. Olfactory sensory neurons that transcribed the modified P2 receptor also expressed tau-LacZ. These subpopulation of sensory neurons exhibited a blue coloration, after X-Gal staining, in their dendrite, cell bodies and axons and were directly visualized (Figure 11).

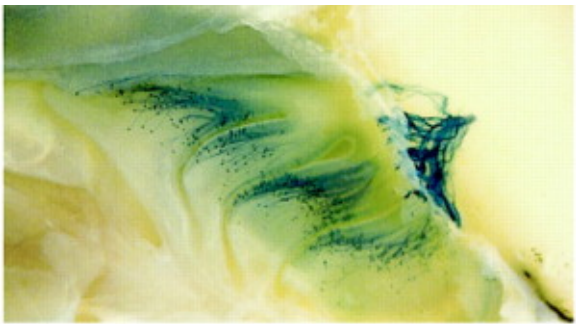

**Figure 11** Whole mount view of the wall of the nasal cavity and the medial aspect of the bulb. Blue neurons stained with X-Gal are distributed in a single zone in the olfactory epithelium. Their axons converge on a single glomerulus on the medial side of the bulb close to the cribriform plate. Image from Mombearts et al., 1996.

In successive studies, LacZ was replaced by the green fluorescence protein (GFP) that can be immediately observed also in *in vivo* animals (Potter et al., 2001; Treloar et al., 2001), (Figure 12).

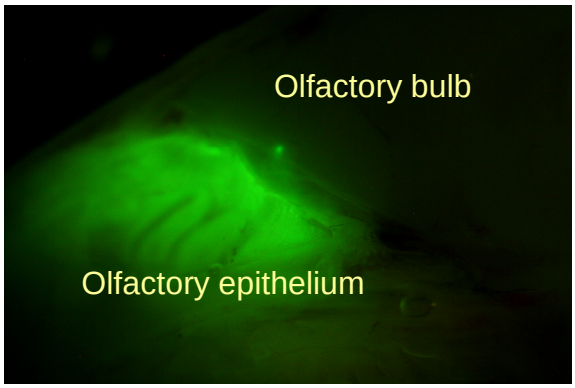


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**Figure 12** Whole mount view of the olfactory epithelium and the medial aspect of the bulb. Axons of P2-GFP sensory neurons converge on a single glomerulus on the medial side of the bulb. Image from Lodovichi's lab.

## 1.8 The functional glomeruli maps

The first experiments aimed to understand how an odor is represented in the in the olfactory bulb employed the technique of [$^{14}$C]-2-deoxyglucose (2-DG). Namely in this experimental approach the olfactory bulb activity in response to odorants was performed by using the 2-DG autoradiography of fixed tissue. (Coopersmith and Leon, 1984; Johnson et al.,1999). These technique is used to visualize the spatial pattern of increased metabolic activity, through the uptake of [$^{14}$C]-2-DG, in glomeruli, in response to odor exposure. However 2DG is unable to track real-time changes in a functional sensory representation and is limited to only one assessment per animal. To overcome this limitation, Rubin and Katz (1999) optimized the technique of optical imaging of intrinsic signals to visualize *in vivo* how odorants are represented in the rat olfactory bulb. Intrinsic signal imaging relies on activity-dependent changes in properties of the tissue such as change in blood volume, haemoglobin absorption and/or light scattering, etc (Frostig et al., 1990; Hill and Keynes, 1949; Chance et al., 1962; Jobsis et al., 1977; LaManna et al., 1987; Bonhoeffer and Grinvald, 1995). These processes give the active tissue different optical properties respect to the surrounding non-active tissue, due to the fact that active neurons reflect light in a different way from non active neurons.

Rodents can discriminate a wide range of odors including enantiomers. Enantiomers are pairs of mirror-symmetric, nonsuperimposable molecules that differ only in optical activity and their interaction with other chiral molecules. Imaging experiments revealed that couples of enantiomers, (+) and (-), elicited a very similar functional maps that differ for the activation of a few glomeruli in a very restricted area of the olfactory bulb. These results, indicated that the spatial pattern of glomerular activity provides sufficient information to discriminate molecular shape (Figure 13, Rubin and Katz, 2001, Linster et al., 2001).

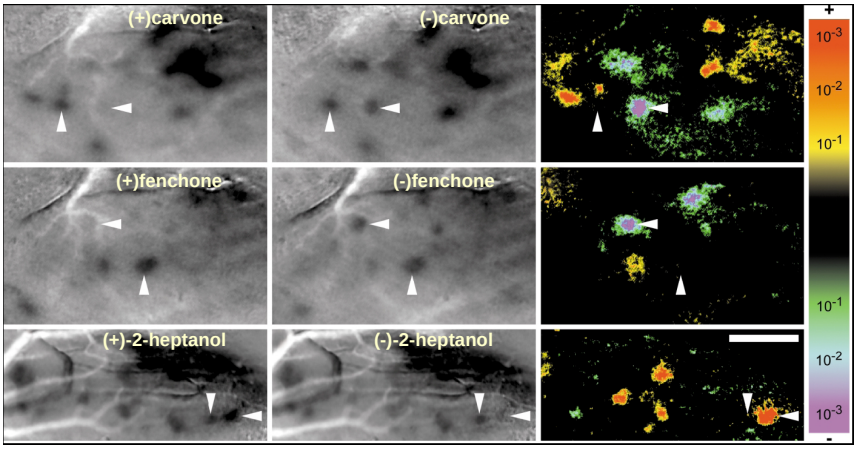



**Figure 13** Enantiomers activate distinct pattern of glomeruli on the dorsal surface of dorsal surface of rodents (Left and middle panels). The color of each pixel represents the probability that the two enantiomes evoked the same response with red indicating a preferential (+)-enantiomer response and violet inidicating a preferential (-)-enantiomer response (right panels) Vertical arrowheads show glomeruli that were equally activated by both members of a pair, and horizontal arrowheads show glomeruli that responded differentially. Bar 250 µm. Modified from Rubin and Katz, 2001.

Functional maps were obtained also using calcium-sensitive dyes. To visualize odorant representations on the mouse olfactory bulb, olfactory sensory neurons were loaded with calcium-sensitive dyes and odorant evoked responses from their axon terminals were imaged (Wachowiak and Cohen, 2001).

More recently, Bozza et al (2004) designed the use of Synapto-pHluorin (SpH) as indicator of neurotransmitter release, and therefore of activity. SpH is a fusion of pH-sensitive green fluorescent protein with the mouse synaptic vesicle-associated protein VAMP2. SpH labels presynaptic terminals of olfactory sensory neurons. The fluorescent domain is located inside the vesicles where the pH is acidic, leading to a low fluorescence level in the basal state. The fluorescence increases during vesicle release. Odorant stimulation evokes large amplitude fluorescence increases of the olfactory sensory neurons terminals in individual glomeruli *in vivo.*

## 1.9 The development of olfactory sensory neurons axons and the formation of glomeruli

A hallmark of mature glomeruli is that they are homogeneous, that is, innervated exclusively by axons of olfactory sensory neurons expressing the same olfactory receptor. In this way the glomerulus defines a functional unit that processes the information related to a given sensory receptor. What I just described is the mature form of the glomerulus that results from a developmental process. In early stages of development olfactory sensory neurons expressing the same olfactory receptor project to a restrict area of the olfactory bulb to form multiple glomeruli. In this stage, a glomerulus could be formed by axons of olfactory sensory neurons expressing different olfactory receptor (heterogeneous glomerulus).

Each type of glomerulus matures with different timing. For example, olfactory sensory neurons expressing the P2 receptor project their axons to specific site in the olfactory bulb as early as embryonic day 15.5 (E15.5). At this age the P2 glomerulus arises from an initially broad area that contains a mixed subpopulation of axons. By E 17.5, the P2 axons begin to cluster and target a specific region in the glomerular layer. By E18.5, the P2 axons form two to three tufted that are interconnected by bundles of axons. By postnatal day 0.5 (PD 0.5) glomeruli adopt a rounded morphology although they remain joined to each other by a band of axons. By PD 14.5 all interconnected glomeruli are separated and formed discrete glomeruli and no mistargeting fibers nor heterogeneous glomeruli are observed. (Royal and Key, 1999).

In the newborn mice, at postnatal day 0 (PD0), M71 and M72 axons reach two spatially conserved posterior-dorsal areas in each bulb. At this stage the axons are widely scattered and M71 and M72 glomeruli are not identifiable. By PD10 M71 and M72 expressing axon coalesce to form multiple glomeruli. At later stages, a (>P40) single M71 and M72 glomeruli are observed in both the medial and the lateral side of olfactory bulb. M71 and M72 glomeruli mature with a different time course. M71 glomeruli undergo a prolonged maturation than M72. The average number of M71 glomeruli per half-bulb did not reach the mature level until PD60. In contrast, the average number of M72 glomeruli per half-bulb rapidly decreased to the level of mature animals by PD20. (Zou et al., 2004).

## 1.10 The development of mitral cells

Along with tufted cells, mitral cells are the principle output neuron of the olfactory bulb. They originate in the ventricular zone and they migrate radially toward the pial surface of the developing olfactory bulb, finally reaching their destination in the mitral cell layer (Hinds and Ruffet, 1973). From their genesis at embryonic day E11 until they attain mature morphology, mitral cells undergo many morphological changes (Malun and Brujes, 1996). In mitral cells the maturational process occur in three steps: 1) postneurogenesis (E11-E13), when the cells migrate radially toward the olfactory bulb border before undergoing a tangential reorientation. The cells extend clearly defined axon processes that course torwards the lateral olfactory tract; 2) a sensitive period (E14-E16), in which the cells begin to exhibit the characteristic radial orientation toward the surface of the olfactory bulb following the arrival of the olfactory sensory neuron's axons; 3) cell refinement (E17), where mitral cells acquire their mature morphology. In this phase the mitral cells display the characteristic primary dendrites that contribute to the formation of the glomerulus and the tangential expansion of the secondary dendrites along the external plexiform layer (Blanchart et al., 2006).

At birth (P0), nearly all mitral cells possess multiple dendrites that extend radially from the cell body into the external plexiform layer. The selection of one process to become the primary dendrite is accompanied by the gradual remodelling of the surrounding apical dendrites. During this transient period most of the mitral cells present two primary apical dendrites in contact with two glomeruli. By P6 most mitral cells possess the mature form with a single, primary dendrite with a well-developed tuft that extends into a single glomerulus (Santacana et al., 1992; Malun and Brunjes, 1996 ), (Figure 14).

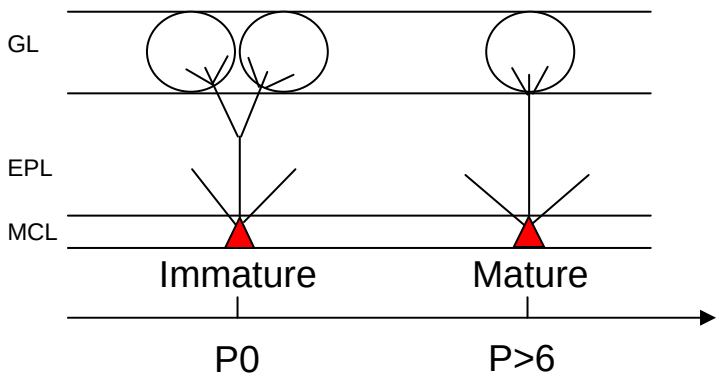


**Figure 14** Schematic representation of the developmental withdrawal of the extra apical dendrites in mitral cells.

## 1.11 Adult neurogenesis in the olfactory bulb: the granule cells

Adult neurogenesis is a process encompassing the generation, migration, maturation and synaptic integration of new neurons in the adult brain. It represents a form of adult neuronal structural plasticity (Katagiri et al, 2011).

Adult neurogenesis occurs in two regions of the adult mammalian brain: the subgranular zone of the dentate gyrus of the hippocampus and in the subventricular zone. The subventricular zone is a layer of dividing cells extending along the lateral wall of the lateral ventricle.

New neurons are born throughout the subventricular zone and join a network of chains of migrating neurons that coalesce to form the rostral migratory stream leading to the olfactory bulb, where they differentiate into granule and periglomerular neurons.

Within the adult subventricular zone, different types of cells can be identified. Namely the astrocyte-like cells that express the glial fibrillary acid protein (GFAP)- also called B-type cells. They act as neural stem cells (Doetsch et al., 1999, Doetsch, 2003). These cells are slowly dividing progenitors which give rise to transiently amplifying progenitor cells, or C-type cells, expressing the epidermal growth factor receptor (EGFR). C-type cells then generate the neuroblasts, i.e. the neural progenitor cells also called A-type cells, which express doublecortin (DCX) and PSA-NCAM.

These neuroblasts migrate as elongated aggregates of cells, called chains, from the subventricular zone along the rostral migratory stream to the olfactory bulb (Lois and Alvarez-Buylla, 1994; Doetsch et Alvarez-Buylla 1996; Doetsch et al., 1997).

When these cells arrive in the core of the olfactory bulb they begin to migrate radially to the surface of the olfactory bulb . During this phase they undergo a maturational process during which they pass from the typical morphology of neuroblasts to fully mature interneurons. In this process, the cells enlarge their cell body and grows several dendrites that extend beyond the mitral cell layer and branch in the external plexiform layer. They also grow basal dendrites, that remain mostly in the granule cell layer. At later time points, spines appears on the dendrites.

The differentiation and maturation occur in five stages (Petreanu and Alvarez-Buylla, 2002).

Cells in stage 1 and 2 (days 2-7 post-retroviral infection) are migratory cells with a leading process and multiple small ramification. The leading process is the precursors of the apical dendrite. Cells in stage 3 (days 9-13) reach the granule cell layer, have a larger round cell body with a prominent process that extend toward the external plexiform layer but don't cross the mitral cell layer. Stage 4 cells (days 13-22) exhibit elaborated dendritic arbors without spines. Stage 5 cells (day 15-30) have mature morphology with spiny dendrites, (Figure 15).

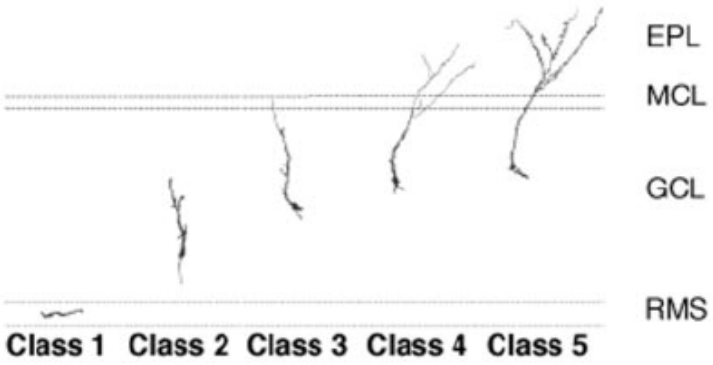



**Figure 15** Time coarse of the development of adult-generated olfactory bulb granule cells. Five stages are described: Class 1) tangential migrating neuroblast (days 2-7); class 2) radially migrating young neurons (days 5-7); class 3)GCs with a simple unbranched dendrite that does not beyond the mitral cell layer; class 4) GCs with a nonspiny branched dendrite in the external plexiform layer (days 11-22); Class 5) mature GCs (days 15-30). EPL, external plexiform layer; MCL. Mitral cell layer; GCL, granule cell layer; RMS, rostral migratory stream. From Petreanu and Alvarez-Buylla, 2002.

A remarkable phenomenon is that not all the newly generated granule cells survive for the entire life of the individual. It has been demonstrated that between 15 and 45 days after the generation of new granule cells, 50% of these adult-generated granule cells die. After 45 days, the number of cells stabilized and their number does not differ significantly during the following 3 months. After 1 year, one-third of these surviving cells survive in the granule cell layer. The early wave of cell death occurs shortly after the newly formed cells develop their dendritic arbors and synaptic spines. The more gradual decline that occurs 3 months after cell birth may be related to the rate of neuronal replacement in the granule cell layer (Petreanu and Alvarez-Buylla, 2002).

## 1.12 Role of electrical activity in the development of the pre and postsynaptic neurons of the olfactory bulb

The specificity of synaptic contacts among neurons is essential for normal brain function.

The development of precise synaptic connections between pre and postsynaptic neurons is regulated by spatially and temporally regulated axon guidance molecules as well as by specific pattern of activity between the pre and the post synaptic elements. Different guidance cues operate in a coordinated way to generate distinct axonal trajectories of individual neurons and to establish a correct topographic map during embryogenesis and postnatal development. The identity and the role of axon guidance molecules in circuit formation in the olfactory system were explored in several studies summarized in a few excellent recent reviews

(Sakano 2010; Mori e Sakano 2011; Lodovichi and Belluscio 2012).

In my Ph D work I focalized my attention on the role of the spontaneous afferent activity in the formation of neural circuits in the olfactory bulb.

Neuronal electrical activity can be divided in spontaneous and evoked activity. Spontaneous electrical activity consists of action potentials generated in absence of sensory stimuli. Evoked electrical activity consists of action potentials generated in response to specific sensorial stimuli i.e. odor stimuli. Both type of activities play a role in the development of neuronal circuits although the specific contribution of each type of activity remains a matter of significant debate. In several sensory systems such as the visual system, the role of spontaneous and evoked activity has been thoroughly investigated (Wiesel and Hubel, 1963; Shatz and Katz, 1996; Zhang and Poo, 2001). However the role of electrical activity in the formation of neural circuits in the olfactory system remains largely to be investigated.

Odorant receptors play a key role not only in the detection of odors but also in the formation of the sensory map in the olfactory bulb (Wang et al., 1998; Belluscio et al., 2002; Feinstein et al., 2004; Sakano, 2010). Furthermore the organization of the olfactory bulb hinges on the identity of the odorant receptor. Odour columns are identified by a given olfactory receptor and the intrabulbar link is between glomeruli receiving the same odorant receptor (Lodovichi et al., 2003). The evoked activity however, does not plays a significant role in the development of the topographic organization of the olfactory bulb.

To assess the role of the odor evoked activity in the formation of the sensory map in the olfactory bulb several transgenic lines of mice have been generated. In these lines of mice, different components of the signalling cascade coupled to the odorant receptor has been modified, hampering the transduction of the chemical signals (odors) into an electrical signals.

Mutant mice lacking key elements of the odorant transduction cascade such as $G_{olf}$ (Belluscio et al., 1998) or a functional olfactory cyclic nucleotide –gated (CNG) channel (Brunet et al., 1996) do not exhibit response to odors, when tested electrophysiologically. The sensory map formation in these mutants however occurred normally (Lin et al., 2000; Zheng et al.,2000). In $G_{olf}$ mutant mice the activation of adenylyl cyclise is probably mediated by another G protein, $Gs_{\alpha}$ , normally predominant in cilia of immature olfactory sensory neurons (Menco et al.,1994). These mice indeed present some evoked activity although reduced respect to controls.
Differently, mice knockout for adenylyl cyclise 3 (AC3), a critical component in the odor-evoked cAMP-dependent signalling pathway, presented an altered sensory map. Namely the axonal projection from olfactory sensory neurons of $AC3^{-/-}$ mice terminated in the glomerular layer of the olfactory bulb, but they were not organized into distinct glomeruli. (Trinh and Storm, 2003; Dal Col et al., 2007; Chesler et al., 2007; Zou et al., 2007).

The role of spontaneous electrical activity in circuit formation has been thoroughly investigated in other sensory modalities, such as vision, but remains unknown in olfaction.
To begin to investigate the role of spontaneous afferent activity in the organization of the olfactory bulb, Yu et al., (2004) generated a line of mice genetically modified to have very little afferent spontaneous activity. In this line of mice all sensory neurons are hyperpolarized because of the overexpression of the inwardly rectifying potassium channel Kir2.1. As a consequence of this genetic manipulation their spontaneous firing is almost completely abolished. They found that the over-expression of Kir2.1 results in a significant delay in the innervation of the olfactory bulb and in the formation of multiple additional glomeruli as opposed to the one medial and one lateral glomeruli in control mice.

However, many questions related to circuit formation and function in the olfactory bulb of Kir2.1 mice remained open. The effect of the afferent spontaneous activity on the size of the olfactory bulb was not investigated. A reduction of the size of the olfactory bulb, as observed in CNG ko mice, could compressed and/or altered its layered structure. Yu demonstrated the presence of a higher number of glomeruli per bulb. However the location and the organization of these glomeruli were not investigated. These features are critical for the topographic organization of the sensory map. The role of spontaneous electrical activity on the developmental refinement of the post synaptic cells, i.e. the mitral cells was not address. Whether afferent spontaneous activity could affect the neurogenesis in the OB remained an unexplored territory. Addressing these question is essential to understand the role of afferent spontaneous activity on circuit formation and function in the olfactory system.

In my PhD work I addressed these open questions by studying the topographical organization of the olfactory bulb, in particular the organization of the sensory map, the developmental maturation of the post synaptic cells, i.e. mitral cells and the neurogenesis in the olfactory bulb, in mice with reduced spontaneous afferent activity, the Kir2.1 mice.

# 2 MATERIALS AND METHODS

## 2.1 Mutant mouse lines

Experiments were performed on six genetically modified lines of mice: C57/BL6, OMP-IRES tTa, $Tet_0$-Kir2.1-IRES-tau-LacZ (Kir2.1), P2-IRES-GFP (P2-GFP), M71-IRES-GFP (M71-GFP) and OR23-IRES-GFP (OR23). The OMP-IRES tTa line was crossed with $Tet_0$-Kir2.1-IRES-tau_LacZ line to drive the expression of the KIR2.1. OMP-IRES tTa x $Tet_0$-Kir2.1-IRES-tau_LacZ (Kir2.1) were crossed with the P2-IRES-GFP (P2-GFP), M71-IRES-GFP (M71-GFP) and OR23-IRES-GFP (OR23)

The $Tet_0$-Kir2.1-IRES-tau-LacZ (Kir2.1)and the P2-IRES-GFP (P2-GFP), generously provided by JA Gogos, were described in details previously (Gogos et al., 2000; Yu et al., 2004). The M71-IRES-GFP (M71-GFP) mice, described in details previously (Feinstein et al., 2004), were purchased from the Jackson Laboratory.

## 2.2 Immunohistochemistry

Mice were killed with an overdose of a mixture of Zoletil 100 (a combination of Zolazapam and Tilemine, 1:1, Laboratoire Virbac) and Xilor (Bio98) and transcardically perfused with 0.9% saline followed by 4% paraformahaldeyde in 1X phosphate buffer saline (PBS). Olfactory bulbs were removed, post-fixed overnight, and sectioned (60 µm horizontal sections) at the vibratome (Leica, VT 1000S). sections were blocked with 0.5% Triton X-100 + 5% normal donkey serum in 1 X PBS for 60 min. Sections were then reacted with primary antibody against OMP (1:500, Wako, Germany) at 4°C overnight. The primary antibody was visualized with Cy3-conjugated donkey anti goat (Jackson laboratories) antibody after 2 hr of incubation. DAPI (Molecular Probe) was used as nuclear counter stain. Images were acquired at the confocal microscope (Leica SP 5) using a HC PL Fluotar 20X/0.5 NA objective (Leica).

## 2.3 Modulation of Kir2.1 channel expression

To express the Kir2.1 only in adults, two experimental procedures were used. In the first one, animals were fed with doxycycline (Sigma-Aldrich. 6mg/g pellet food) through gestation till P30 (to abolish Kir2.1 expression). The animals were killed and horizontal

section of olfactory bulb were processed with antibodies against OMP as described above.

In the second experimental procedure, the doxycycline administration was suspended at P30, the animals at P60 were killed and horizontal sections were immunostained for omp.

To assess the Kir2.1 expression, sections of the olfactory bulb of Kir2.1 and control animals at P30 and P60, were reacted with goat antibody specific for β galactosidase (βgal) (1:1000, Morphosys). The primary bound antibody was the visualised unsing anti-goat Cy3 coniugated, Jackson Laboratories)

## 2.4 Mitral cell labelling

Mice heterozygouse for OMP-IRES tTa, $Tet_0$-Kir2.1-IRES-tau_LacZ and wild type C57B/6 mice at postnatal age P6, P7 and P8 were sacrificed by decapitation. A small incision was made in the presumptive olfactory tract and a small crystal of DiI $_{18}$ (3) (Molecular Probes) was applied. Brains were replaced in fixative and incubated at 37° C for 7-10 days. Brains were then sectioned at the vibratome (60 µm thickness sections). Sections were imaged at a Leica SP 5 fluorescence confocal microscope with a HC PL Fluotar 20X/0.50 NA objective (Leica). Z stacks through selected mitral cells were performed and projected on the xy plane to obtain the maximum intensity projections, containing the reconstruction of the entire cells.

## 2.5 BrdU labelling and detection

To determine the number of newly generated cells, a DNA synthesis marker, BrdU (Sigma-Aldrich, 50 mg/Kg body) was administered intraperitoneally. Four injections, every 2 hours, were performed in mice heterozygous for $Tet_0$-Kir2.1-IRES-tau-LacZ and wild type C57B/6 mice at p30. to assess the neurogenesis and the survival of newborn granule cells, animals were sacrified by Xilor-Zoletil overdose 15 and 50 days, respectively, after BrdU injections and then transcardially perfused with 0.9 saline, followed by 4% Paraformahaldehyde in 0.1M phosphate buffer saline (PBS). Brains were then postfixed over night and then sectioned at vibratome (60 µm thickness section). Sections were pretreated with the DNA denaturating agent HCL (2N, at 37°C).

To detect the newly generated granule cells (i.e. cells double positive for BrdU and the neuronal marker NeuN) olfactory bulb sections were first incubated with a rat monoclonal anti BrdU (1:200 Abcam) and with a mouse monoclonal anti NeuN (1:200 Millipore) primary antibodies. The bound primary antibodies were then visualized using Cy3 conjugate anti-rat IgG and DyLight 488 conjugate anti-mouse IgG, respectively (Jackson laboratories).

## 2.6 Stereotaxic injection

GFP encoded lentiviruses (gift of Roman Hudec) were stereotaxically injected in the subventricular zone in mice heterozygous for $Tet_0$-Kir2.1-IRES-tau_LacZ and wild type C57B/6 at p30.

Stereotaxic injections were performed using a picopump (WPI), at the following coordinates (relative to bregma): anterior =1 mm, lateral =1 mm, depth: 2.2 mm (Petreanu and Alvarez-Buylla, 2002). The mice were killed by Xilor-Zoletil overdose 25 days after lentivirus injection and then transcardially perfused with 0.9 % saline solution, followed by 4% paraformahaldehyde in 0.1M phosphate buffer saline (PBS). Brains were then postfixed in 4% paraformahaldehyde over night and then sectioned at vibratome (60 µm thickness section) and mounted (Aquapolymount, Polysciences).

## 2.7 Image analysis and quantification

To assess the number of newborn granule cells, Brdu and NeuN double-labeled cells were counted in the entire granule cell layer on every two sections (120 µm) of the OB . To assess the total number of newly generated cells, all BrdU immunopositive nuclei were counted in the entire granule cells layer on every two sections of the OB . The ratio Brdu-NeuN/BrdU tot cells (%) where the Brdu –NeuN = the number newly generate neurons, BrdU tot = total number of newly generated cells, was used to determine the fate of the newly generated cells. Immunofluorescent sections were analyzed using a Leica SP5 confocal microscope with HC PL Fluotar 20X/0.50 NA objective (LEICA).

To analyze the morphology and the filopodia/spine density of the dendritic tree of the newborn GFP-labeled granule cells, 60 µm thick sections of the OB were analyzed at

the confocal microscope (Leica SP5) with a HC PL Fluotar 20X/0.5 NA and with a HCxPL Apo LambdaBlu 63X/1.40 NA oil immersion objectives (Leica). Quantitative data analysis on dendritic and filopodia/spine density was performed using Imagej (NIH) and Neuronstudio software (Rodriguez et al., 2008).

## 2.8 Behaviour

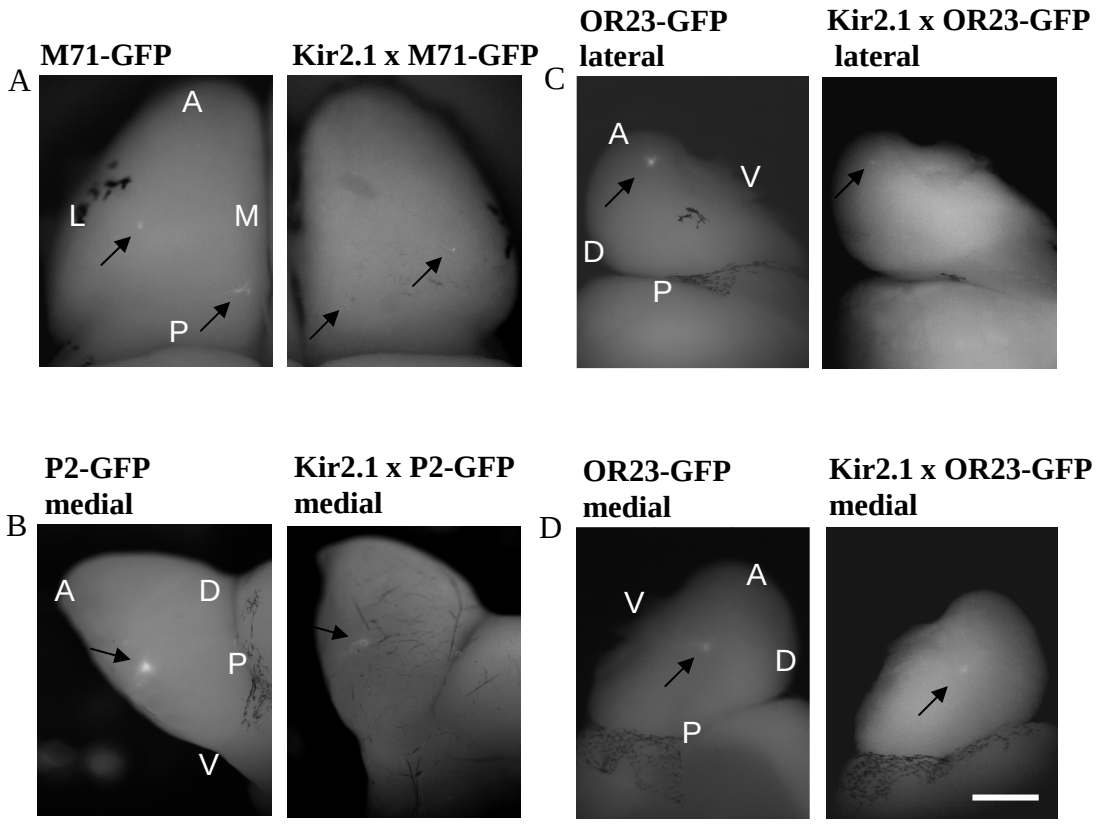
Wild type control (littermates) and Kir2.1 male mice, age postnatal day 30-50 (P30-P50), were trained to assess their ability to discriminate between pairs of odors: fenchone (+) and (-), carvone (+) and (-), 2-heptanol (+) and (-), 2-methylbutyric acid (2MB) and cyclobutanecarboxylic acid (cb) (all from Sigma-Aldrich). The behavioural paradigm was slightly modified from the one used by Shellick et al., 2001. Briefly, four days prior to training the animals were put on a food restriction schedule to maintain 80-85% of their free-feeding weight. Water was avaible *ad libitum*. Mice were trained for 4 days in the following way. Each mouse was exposed in 4 x 10 min sessions to a conditional stimulus (CS+) contained in a odor pot hidden in the bedding of the cage, together with a piece of sugar (0,05g). Each mouse was also exposed to the unconditioned stimulus (CS-) present in an odor pot hidden in the bedding of the cage, without sugar. The CS+ and CS- were presented in different cages and each mouse was exposed randomly to CS- and CS+ during the course of the training days. Odor stimulus were used pure. On the fifth day, mice were tested in a three chamber apparatus. In the left and the right compartment the CS+ and CS-, in odor pots, were hidden in the bedding. Sugar was not present in the testing phase. The animal was introduced in the chamber apparatus, without odors, for 5 minutes for habituation. After that odors were hidden in the lateral chambers and the animal introduce in the central chamber, having free access to the side compartments of the apparatus. The ability of the mice to discriminate CS+ versus CS- was assess scoring the time spent digging and nosing around each odor pot, within a 2 minutes time window. The discrimination test was done blind to the conditioned odor.

# 3 RESULTS

## 3.1 The sensory map and the organization of glomeruli in the olfactory bulb of Kir2.1 mice

### 3.1.1 Size of the olfactory bulb in controls and kir2.1 mice

To study the convergence of olfactory sensory neurons (OSNs) with reduced spontaneous activity to form glomeruli in the olfactory bulb (OB), mice heterozygous for OMP-IRES-tTA/teto-Kir2.1-IRES-tauLacZ were crossed with mice homozygous for the P2-IRES-GFP, OR23-IRES-GFP or M71-IRES-GFP allele (Kir2.1xP2-GFP, Kir2.1xOR23-GFP, or Kir2.1xM71-GFP mice). In these mice, sensory neurons expressing the odorant receptor P2, OR23 or M71 co-express the green fluorescent protein (GFP). As the result of this genetic manipulation the corresponding glomeruli can be easily identified in the OB. We measured the area of the dorsal, the medial and the lateral surface of the OB and we found that the each surface of the OB was comparable in Kir2.1 and control mice (Figure 1, area of dorsal surface of the OB (control mice n=7, Kir2.1 mice n= 9), area of medial surface of the OB (control mice n= 6, Kir2.1 mice n= 6) and lateral surface of the OB (control mice n= 3 , Kir2.1 mice = 3).

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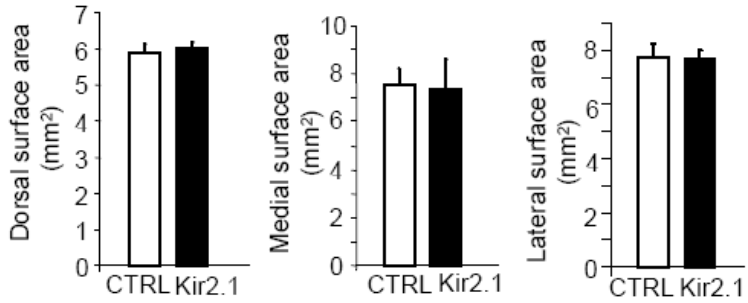



**Figure 1** **A** Dorsal view of olfactory bulbs (OBs) of M71-GFP, left, and Kir2.1x M71-GFP mice, right. **B** medial view of olfactory bulb of P2-GFP, left, and Kir2.1 x P2-GFP mice, right. **C-D** Top, lateral view of olfactory bulbs of OR23-GFP, left, and Kir2.1 x OR23-GFP mice, right. Bottom, medial view of olfactory bulb of OR23-GFP, left, and Kir2.1 x OR23-GFP mice, right. A = anterior, P = posterior, L = lateral, M = medial. Bar = 1 mm E) summary of the results, data are presented as mean ± SD.

### 3.1.2 Number of glomeruli in controls and Kir2.1 mice

To assess the number of the M71-GFP, OR23-GFP and P2-GFP glomeruli, we analyze consecutive horizontal section of olfactory bulbs in controls and Kir2.1 mice and we count the medial and lateral GFP-labelled glomeruli.

By postnatal day 30 (P30), in Kir2.1 mice, the P2-expressing axons and the OR23-expressing axons, form one or two distinct glomeruli on the medial and on the lateral side of each bulb (as in controls); however they also project to several additional glomeruli (Figure 2A-B). All the additional glomeruli were located in a restricted area, within 5 glomeruli from the main glomerulus. The number of glomeruli was analyzed in : P2-GFP mice n= 7, Kir2.1x P2-GFP mice n=7, OR23-GFP mice n= 3, Kir2.1x P2-GFP n= 4; t test, P2-GFP control vs Kir2.1x P2-GFP p= 0,002 **, OR23-GFP control vs Kir2.1x OR23-GFP p<0,001 ***. Due to the late maturation of the M71 glomeruli (Zou et al., 2004), we examined M71-GFP mice at P30 and P50. At both these temporal points, the Kir2xM71-GFP mice presented one glomerulus on each side of the bulb (same as controls) although, occasionally, a few M71-expressing axons entered also 1 or 2 adjacent glomeruli (Figure 2C, M71-GFP mice n= 7, Kir2.1x M71-GFP mice n= 6; t test, M71-GFP control mice vs Kir2.1 x M71-GFP mice p= 0,59).

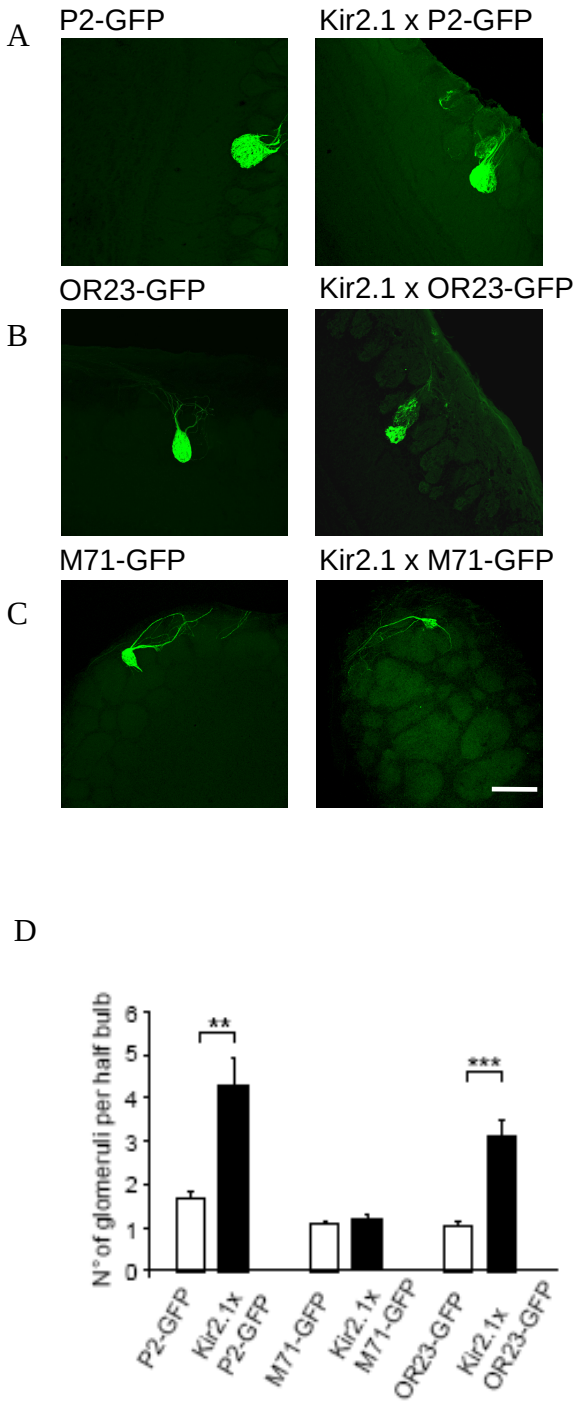



**Figure 2** Number of glomeruli in olfactory bulb of control and Kir2.1 mice. **A-C** examples of GPF-labelled glomeruli in horizontal sections of olfactory bulb (OB) of P2-GFP control mice (**A**, left), and Kir2.1 x P2-GFP mice (**A**, right), of OR23-GFP control mice (**B**, left) and Kir2.1 x OR-23 mice (**B**, right), of M71-GFP control mice (**C**, left) and Kir2.1 x M71-GFP mice (**C**, right). Bar=200 µm. D Summary of results. Data are presented as mean ± SEM

### 3.1.3 Localization of glomeruli in controls and Kir2.1 mice

To ascertain the location of the main glomeruli in control and Kir2.1 mice we measured the medio- lateral and the postero-anterior coordinates that identify the location of the medial and lateral M71-GFP glomeruli on the dorsal surface of the OB; the ventral-dorsal and postero-anterior coordinates of the medial P2-GFP glomeruli on the medial surface of the OB; the the ventral-dorsal and postero-anterior coordinates of the medial and the lateral OR23-GFP glomeruli on the medial and the lateral surface of the OB. The measurements were performed on whole-mount olfactory bulbs. We found that the locations of the main P2, OR23 and M71 glomeruli were superimposable in Kir2.1xP2-GFP, Kir2.1xOR-GFP and Kir2.1xM71-GFP and control mice, as shown in Fig.3 (P2-GFP control mice n=5, Kir2.1x P2-GFP mice n=4; t test control vs Kir2.1, medial glomerulus V-D p= 0,3, P-A p= 0,9; M71-GFP control mice n=4, Kir2.1x M71-GFP mice n=4, t test control vs Kir2.1, lateral glomerulus P-A p= 0,6, M-L p=0,07, medial glomerulus P-A p= 0,5, M-L p= 0,24; OR23-GFP control mice n=5, Kir2.1xOR23-GFP mice n=3, t test control vs Kir2.1, lateral glomerulus P-A p= 0,6, V-D p=0,4, medial glomerulus P-A p= 0,37, V-D p= 0,07).

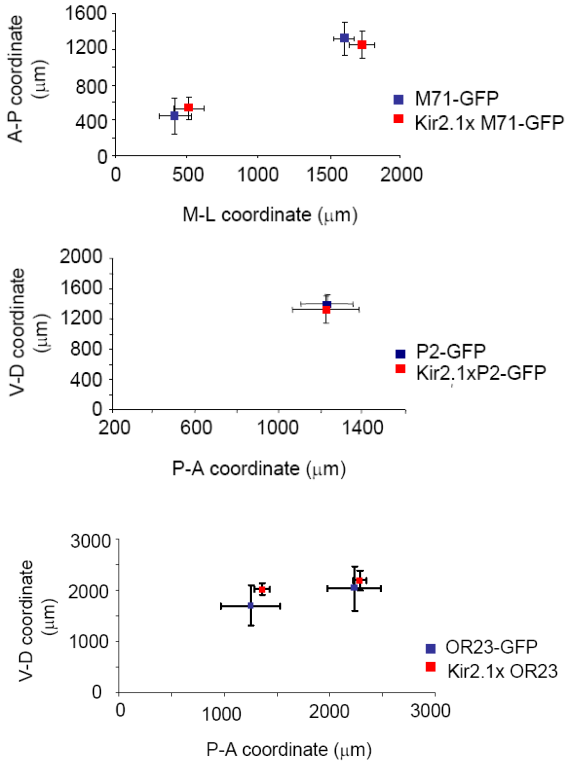
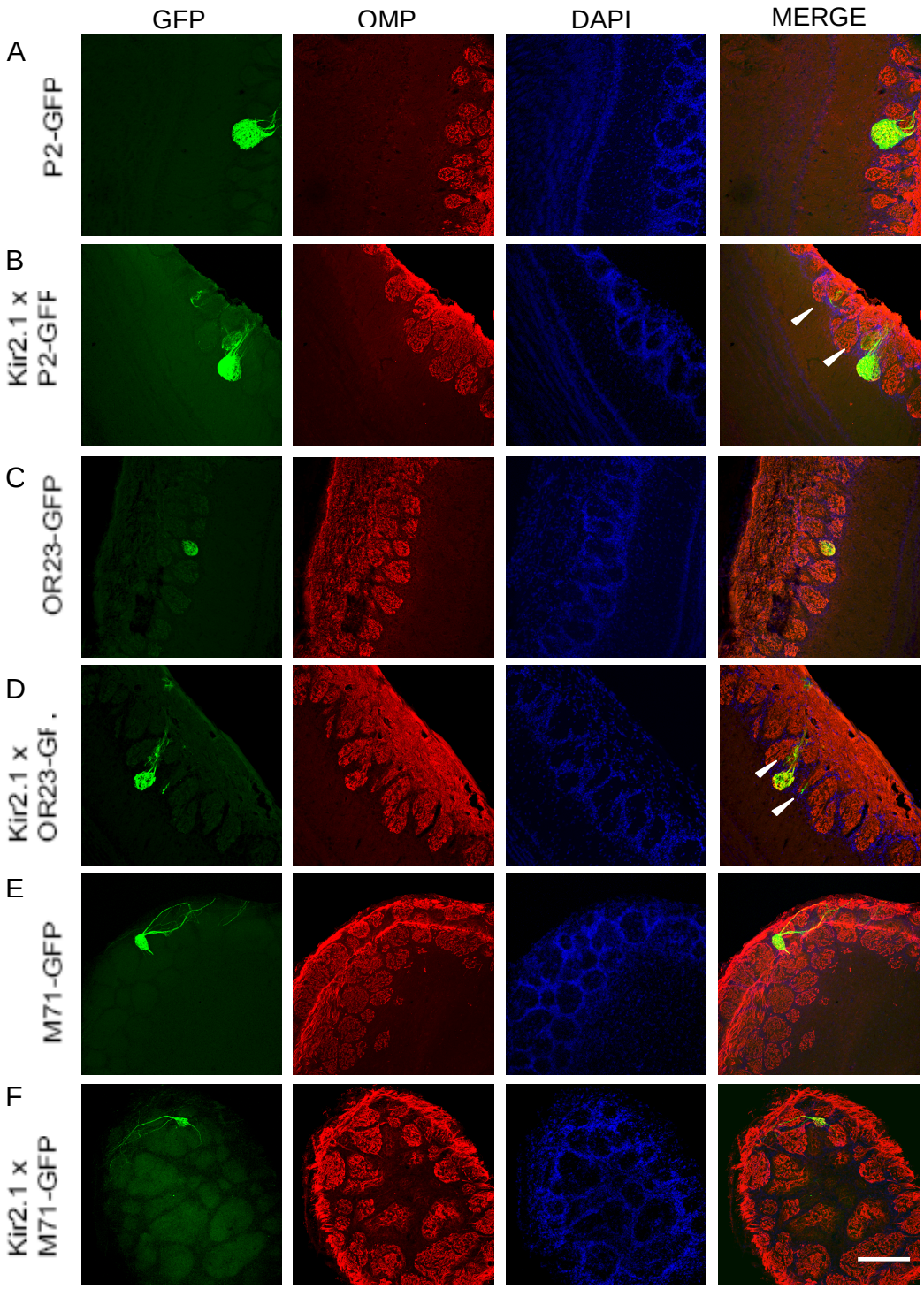


**Figure 3** Graphs of the coordinates that define the location of the main glomeruli in the OB of M71-GFP, Kir2.1x M71-GFP mice (top) of P2-GFP, Kir2.1 x P2-GFP mice (middle) and of OR23-GFP, Kir2.1xOR23-GFP mice (bottom). L= lateral, P= posterior, M= medial, A= anterior, V=ventral. Data are presented as mean ± SD.

### 3.1.4 Organization of glomeruli in control and Kir2.1 mice

A hallmark of mature glomeruli, that represents a key features in the organization of the olfactory bulb is that they are innervated exclusively by fibers expressing the same odorant receptor . To assess the organization of glomeruli, serial horizontal sections of the OB of Kir2.1xP2-GFP, Kir2.1xOR23-GFP and Kir2.1xM71-GFP mice were immunolabeled with antibodies against the olfactory marker protein (OMP), a protein expressed by all mature OSNs (Danciger et al, 1989). The supernumerary glomeruli contained fibers immunopositive for both OMP and GFP (i.e., axons expressing P2, OR23 or M71) along with axons positive only for OMP, but no for GFP (i.e. axons that express a different OR from P2, OR23 or M71). The main P2, OR23 and M71-glomeruli were composed exclusively by fibers positive for OMP and for GFP, i.e. P2, OR23 and M71 expressing neurons respectively (Figure 4). These data suggested that the increased number of P2, OR23 and M71 glomeruli can be accounted for by a greater number of heterogeneous glomeruli.


GFP
OMP
DAPI
MERGE
A
P2-GFP
B
Kir2.1 x
P2-GFP
C
OR23-GFP
D
Kir2.1 x
OR23-GF
E
M71-GFP
F
Kir2.1 x
M71-GFP

G

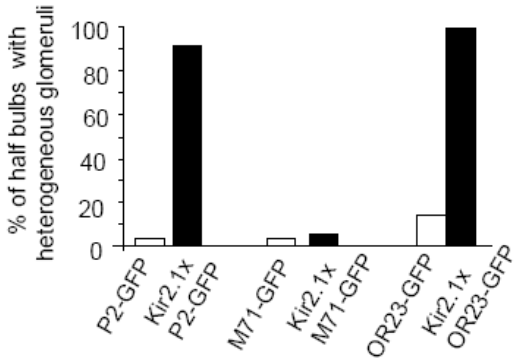
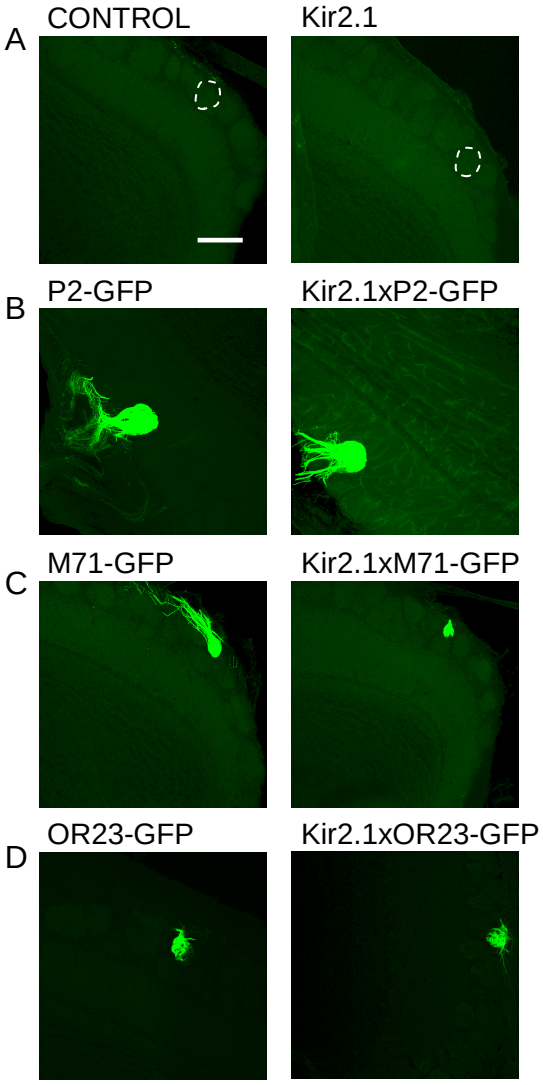



**Figure 4** Organization of glomeruli. Organization of P2, OR23 and M71 glomeruli was revealed by immunolabeling horizontal sections of OB with antibodies against the olfactory marker protein (OMP, in red). Green, GFP expressed in P2, OR23 and M71-axons. Blue, nuclei stained by the nuclear marker, DAPI. In **A** and **B**, P2-axons expressing both GFP and OMP coalesce to form homogeneous glomeruli, in P2-GFP and Kir2.1xP2-GFP mice. In **B**, P2 axons innervate also adjacent glomeruli (i.e. heterogeneous glomeruli, arrowheads) in Kir2.1xP2-GFP mice. In **C** and **D**, OR23-axons expressing both GFP and OMP coalesce to form homogeneous glomeruli, in OR23-GFP and Kir2.1xOR23-GFP mice. In **D**, OR23-axons innervate also adjacent glomeruli (i.e. heterogeneous glomeruli, arrowheads). In **E** and **F** M71 glomeruli are formed by OSN axons expressing both GFP and OMP (homogeneous glomeruli), in M71-GFP mice and in most Kir2.1xM71-GFP mice. Bar 200 µm. **G**, percentage of half bulb with heterogeneous glomeruli in P2-GFP mice (n=8) and in Kir2.1xP2-GFP mice (n=8), in M71-GFP mice (n=8) and Kir2.1xM71-GFP mice (n=8) and in OR23-GFP mice (n=3) and Kir2.1x OR23-GFP mice (n=5).

## 3.1.5 Size of glomeruli in controls and Kir2.1 mice

Alterations in the convergence of OSNs could affect also the size of the glomeruli. This could in turn affect the organization of the odour column. To verify this hypothesis the size of glomeruli in Kir 2.1 mice and in control mice was measured. The area of glomeruli was calculated outlining the profile of GFP-labelled glomeruli in P2-GFP, M71-GFP, OR23-GFP, Kir2.1 x P2-GFP, Kir2.1x M71-GFP and Kir2.1x OR23-GFP mice and outlining the profile of glomeruli in non-GFP- labelled mice. We found that the size of the glomeruli resulted superimposable in controls and Kir2.1 mice with the exception of M71-GFP glomeruli that were smaller in Kir2.1 mice respect controls (Figure 5, Control mice n=8, Kir2.1 mice n= 8, P2-GFP mice n=8, Kir2.1x P2-GFP

mice n=7, M71-GFP mice n=4, Kir2.1x M71-GFP mice n=7, OR23-GFP mice n= 6, Kir2.1xOR23-GFP mice n= 7; t test Control mice vs Kir2.1 mice, p=0,75, P2-GFP control mice vs Kir2.1xP2-GFP mice p= 0,76 M71-GFP control mice vs Kir2.1x M71-GFP mice p<0,001 ***, OR23-GFP control mice vs Kir2.1xOR23-GFP mice p=0,27).



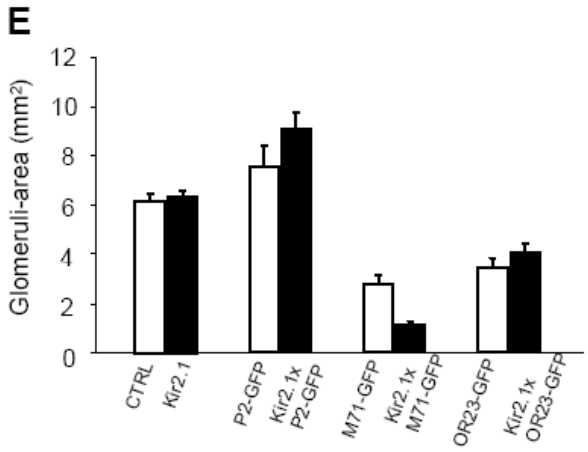
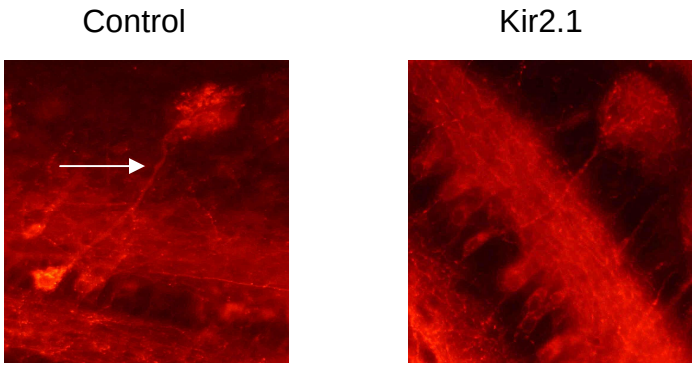



**Figure 5** Area of glomeruli. **A-D** examples of glomeruli in horizontal section of the OB in control (left) and Kir2.1 mice (right) Dashed circle outlines the profile of a single glomerulus. Bar=200 µm **E** Summary of results. Data are presented as mean ± SEM

## 3.2 Development of mitral cell apical dendrite in Kir2.1 mice

We asked whether the postsynaptic targets of the OSNs, the mitral cells (MCs), exhibited any effect related to their developmental maturation in Kir2.1 mice. Mature mitral cells extend a single apical dendrite into a single glomerulus to contribute to the organization of the odor column. This single apical dendrite remains after the withdrawal of other, supernumerary dendrites, a process that is completed in the first postnatal week (Lin et al., 2000). We found no difference in the developmental remodelling of the apical dendrite of MCs in Kir2.1 mice versus controls (from P6 to P8 mice). At this developmental stage, indeed, most MCs presented a single apical dendrites as in controls (Figure 6, MCs with single apical dendrites at P6 = 91,6% in controls (n=4), 91% in Kir2.1 mice (n=6); at P7 = 96,7% in controls (n=4) and 96,8% in Kir2.1 mice (n=5) ; at P8 = 98 %in controls (n=4) and 97% in Kir2.1 mice (n=6)).

A

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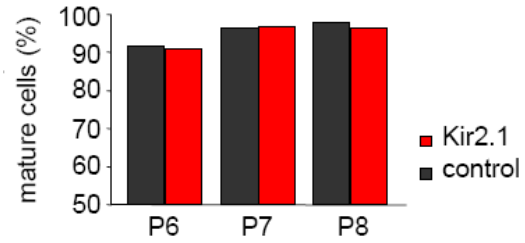


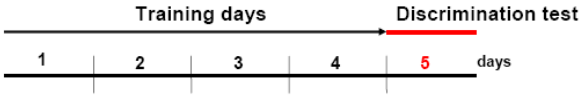

**Figure 6** Developmental refinement of the apical dendrite in mitral cell (MC). **A** examples of mitral cells, retrogradelly labelled by DiI, in horizontal sections of the OB, in control (left) and Kir2.1 (right) mice at postnatal day 7. In both cases a single apical dendrite (arrow) ends in a apical tuft within a single glomerulus. **B** Summary of the results. Bar = 100 µm.

## 3.3 Olfactory discrimination behaviour in Kir2.1 mice

The altered organization of the anatomical maps of the OB could affect the olfactory behaviour. To address this question we studied the ability of Kir2.1 mice to discriminate between two odors. We found that the altered connectivity for the sensory map was confined around the main glomeruli. We reasoned therefore that Kir2.1 mice could have difficulties in discriminating odorants that elicit very similar spatial maps of activated glomeruli, such as enantiomers. Enantiomers, mirror symmetric pairs of molecules that differ only in their optical activity, are represented by similar functional maps that differ for one or a few activated glomeruli located in a restricted area , in rodents. It has been shown that the spatial pattern of glomeruli activity provides

sufficient information to discriminate molecular shapes (Rubin et al., 2001; Kobayakawa et al., 2007; Clarin et al., 2010; Linster et al., 2001; Linster et al., 2002; Mori and Sakano, 2011; Murthy, 2011). To asses the discrimination capabilities of Kir2.1 mice, we performed an olfactory discrimination test (Schellink et al., 2001) using 4 different couples of odorants. Mice were trained for 4 days to associate either of the two related odors to a food reward (sugar). On the fifth day, the sugar was removed and the animal ability to discriminate between the two related odors, tested. The lengths of time the mouse spent investigating, digging and sniffing the odorants were measured (see methods for details). If animals spends most the time investigating the conditioned odor, this indicates that the animal is able to discriminate the two odors. We found that Kir2.1 mice were unable to discriminate between odors that elicit very similar spatial patterns of activation, such as the couples of enantiomers 2-heptanol (+) and (-), fenchone (+) and (-), carvone (+) and (-). However Kir2.1 mice retained the ability to distinguish between odorants that presented a more distinct spatial pattern of activated areas, such as 2-methylbutyric acid- cyclobutanecarboxylic acid (2MB-cB), (Figure7).

A

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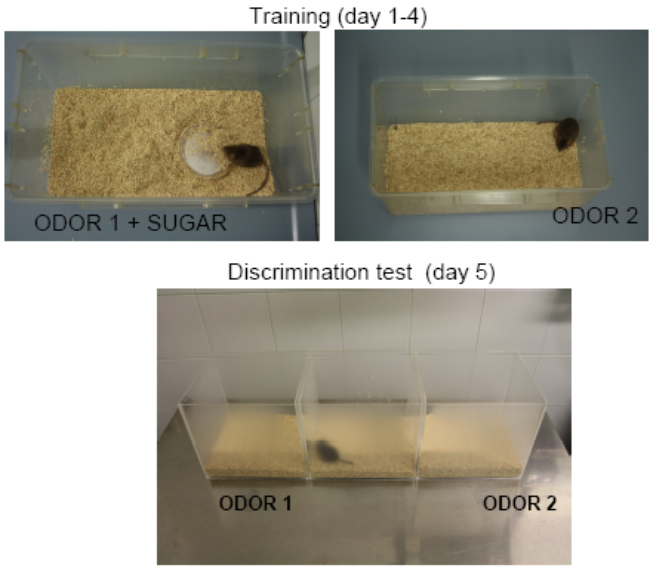

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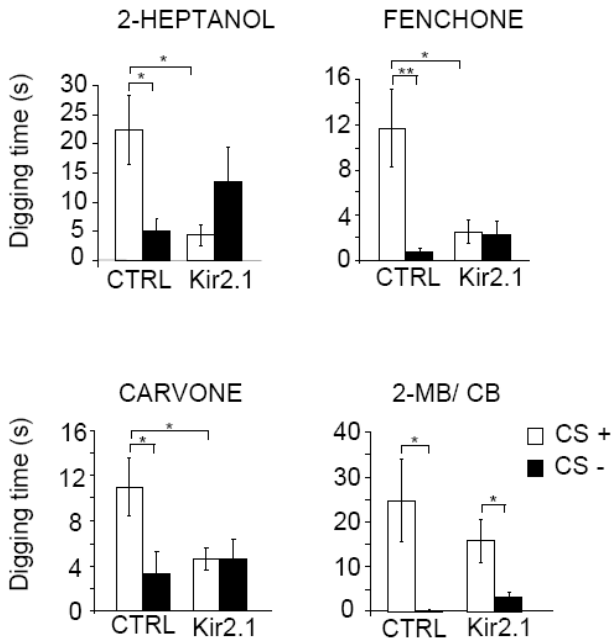


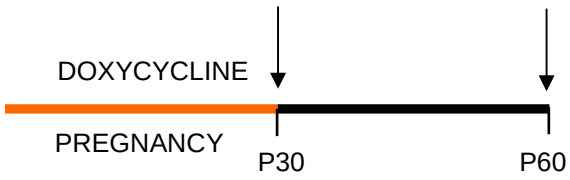

**Figure 7** Discrimination tests in control and Kir2.1 mice. **A** schematic of the behavioural paradigm. **B** top, Control and Kir2.1 mice were trained for 4 days to discriminate between the conditioned odor (odor1+sugar, Cs+) and the unconditioned odor (odor2, Cs-) on the fifth day (bottom) the animals were tested in a three chamber apparatus and the digging time for each odor (without sugar) of the related pair was scored. **C** digging time for each pair of odors tested. Bar =SEM *=p<0.005, ** 0.001<p<0.01.

## 3.4 Over-expression of Kir2.1 in adults disrupted the already refined connectivity

Spontaneous activity has always been thought to play a prominent role in early stages of development (Goodman et al., 1993; Huberman et al., 2008) Once the sensory systems become responsive to sensory stimuli, evoked activity contributes to the stabilization and further refinement of neuronal connections (Shatz and Katz, 1996; Hensch, 2004). Whether spontaneous activity can modulate synaptic connections in adult life remains largely unknown. To address this point we took advantage of the inducible nature of the Kir2.1 construct. In one group of experimental animals, the expression of the Kir2.1 channel was suppressed by doxycycline administration in the food, during gestation and in the first 30 day of postnatal life (P30) in Kir2.1 mice. In a second group of animals, at

P30 the administration of doxycycline was suspended, allowing the Kir2.1 channel to be expressed till P60 (Figure 8). In the first experimental design we immunolabeled serial sections of OB of Kir2.1xOR23-GFP and OR23-GFP mice with antibodies against the olfactory marker protein (OMP) to asses the organization of OR23 glomeruli. Both Kir2.1xOR23-GFP and OR23-GFP mice present homogeneous OR23 glomeruli (Figure 8Ca-b) We analyzed the organization of OR23 glomeruli at P60 in Kir2.1 mice that over expressed Kir2.1 channel from P30 to P60 and we observe additional heterogeneous glomeruli that coexist with the main OR23 glomerulus on the lateral and the medial side of the olfactory bulb (Figure 8Cc-d). These data indicate that overexpression of Kir2.1 in adults induced a regression in the organization of the glomeruli.

A

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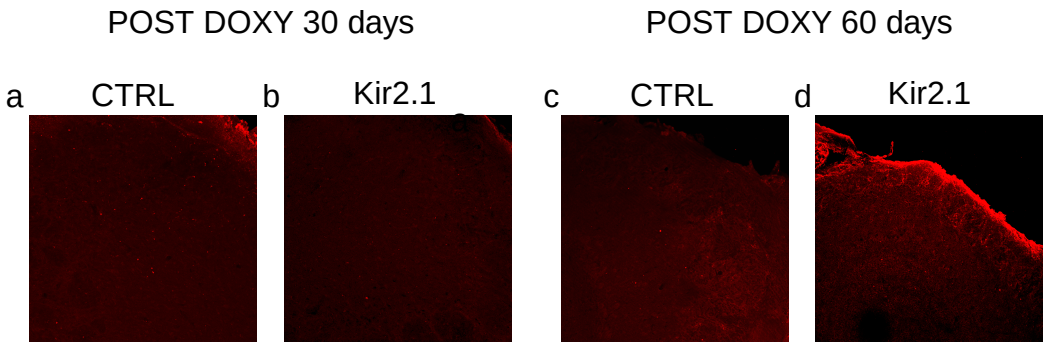

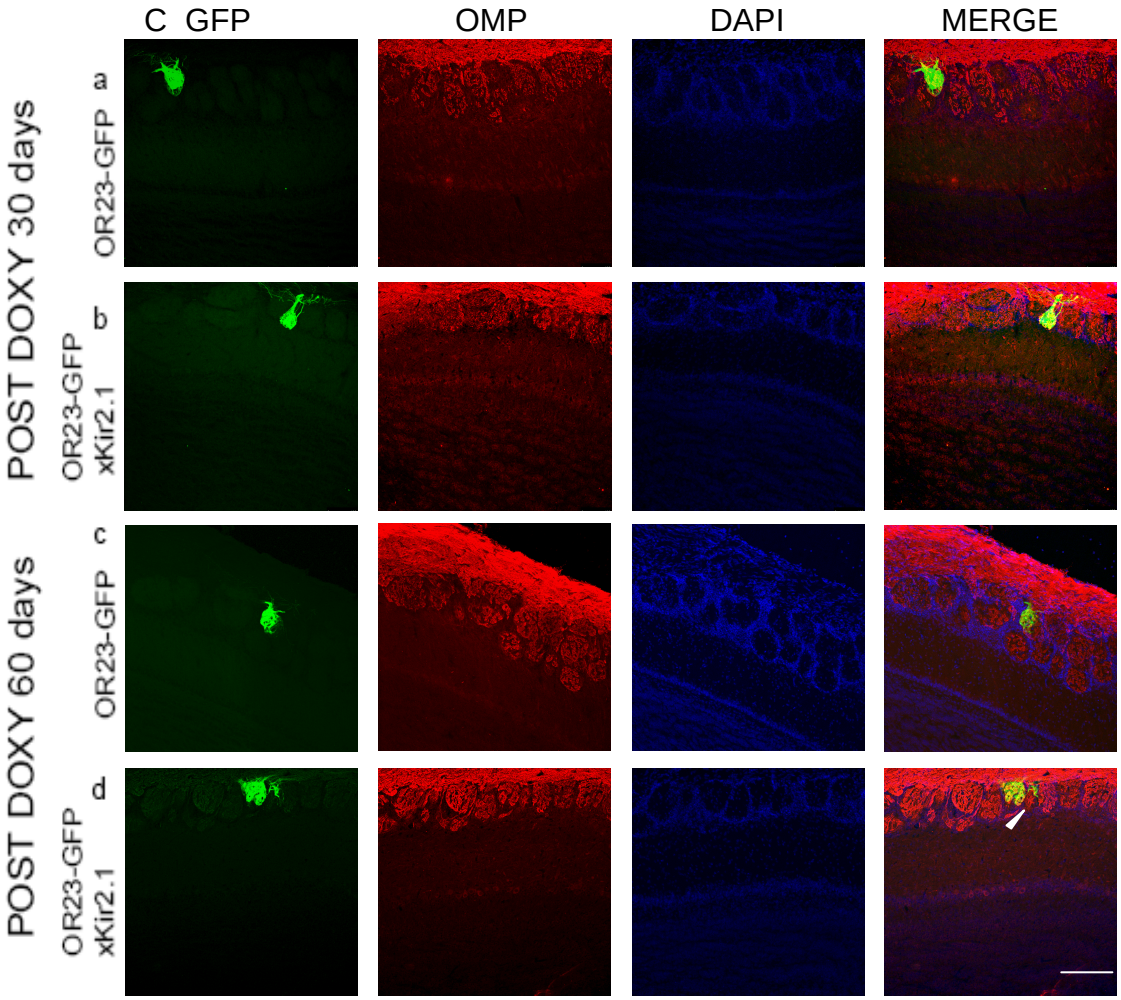
C GFP
OMP
DAPI
MERGE
POST DOXY 30 days
a
OR23-GFP
b
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OR23-GFP
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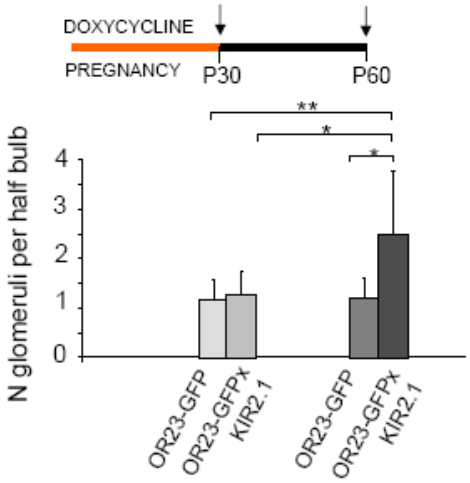
OR23-GFP
xKir2.1

D


DOXYCYCLINE
PREGNANCY
P30
P60
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N glomeruli per half bulb
4
3
2
1
0
OR23-GFP
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OR23-GFP
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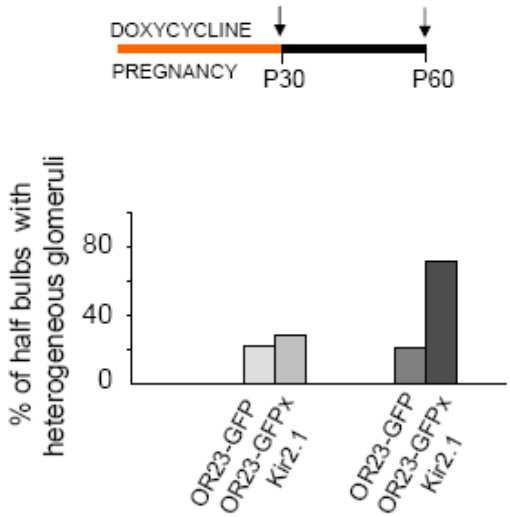



**Figure 8 A** schematic of the experimental strategy . Doxycycline was supplied with the food throughout the gestation and in pups till postnatal day 30. **B** The expression of Kir2.1 gene was revealed by immunolabeling horizontal sections of OB with antibodies against the βgal in controls and Kir2.1 mice, both treated with doxycycline till P30. In b, the βgal (i.e. the Kir2.1) is suppressed by the administration of doxycycline. In d, the βgal (i.e. the Kir2.1) is expressed again, due the suspension of doxycycline treatment from P30 to P60. **C** Organization of OR23 glomeruli was revealed by immunolabeling horizontal sections of OB with antibodies against the olfactory marker protein (OMP, in red). Green, GFP expressed in OR23-axons. Blue, nuclei stained by the nuclear marker, DAPI. In a and b, OR23-axons expressing both GFP and OMP coalesce to form homogeneous glomeruli, in OR-GFP and Kir2.1xOR-GFP mice treated with doxycycline from pregnancy to postnatal day 30. In c and d, OR23-axons expressing both GFP and OMP coalesce to form homogeneous glomeruli, in OR23-GFP and Kir2.1xOR23-GFP mice. In d, OR23-axons innervate also adjacent glomeruli (i.e. heterogeneous glomeruli, arrowheads), Bar 200 µm. **D**, number of glomeruli per half bulb (top) and percentage of half bulb with heterogeneous glomeruli (bottom) in OR-GFP mice (n=5) and in Kir2.1xOR-GFP mice (n=6) treated with doxycycline to P30, and in OR-GFP mice (n=3) and Kir2.1xOR-GFP mice (n=7) in which the supply of doxycycline was suspended from P30 to P60 to allow the expression of the Kir2.1 gene.

## 3.5 Neurogenesis in the olfactory bulb of controls and Kir2.1 mice

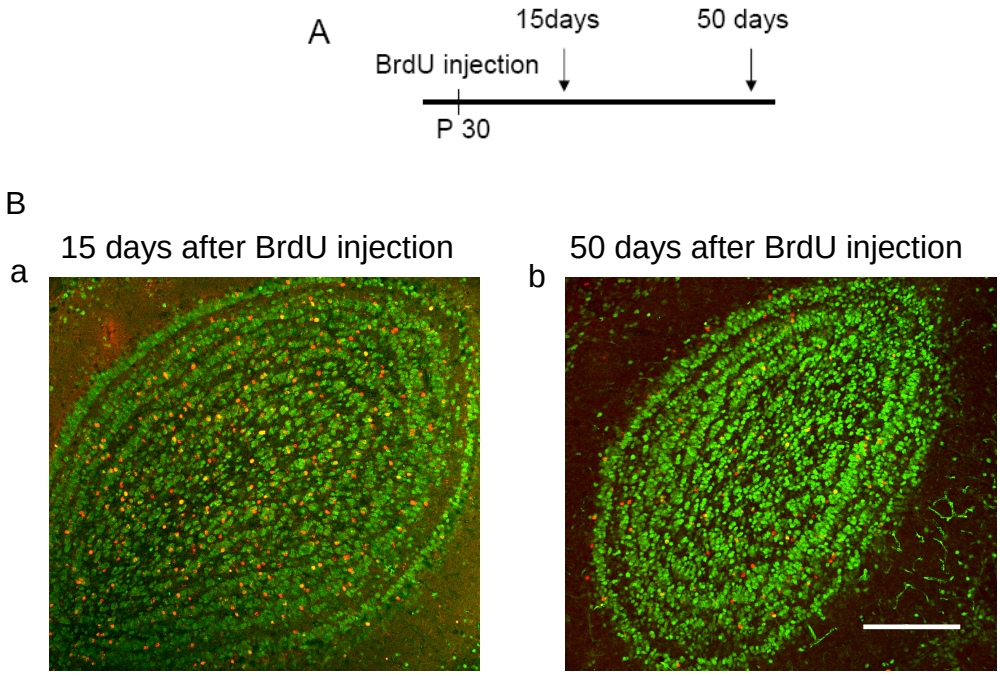
### 3.5.1. Migration and survival of newly generated granule cells

Granule cells are the main inhibitory interneurons of the olfactory bulb that continuously regenerate throughout the life of the individual. Previous studies

demonstrated that the odorant-evoked activity influence the maturation and the maintenance of the new born granule cell. Whether spontaneous activity can influence the migration and the maturation of the granule cells remains largely unknown.

To address this issue we studied the neurogenesis in Kir2.1 mice, in particular we focused our attention on two topics: the migration and the maturational process of the newly generated granule cells.

It is known that in the olfactory bulb, half of the newborn cells died between 15 and 45 days after their generation (Petreanu and Alvarez-Buylla, 2002). To evaluate the possible influence of the afferent spontaneous activity on the migration and on the wave of death between 15 and 45 days, we performed intraperitoneal injection of BrdU and we analyzed the number of newborn cells in coronal sections of the olfactory bulb in Kir2.1 and in controls mice at these two time points. We found no significant difference in the number and in the survival of granule cells at 15 and 45 day post injection between controls and Kir2.1 mice, which means that the migration is normal also in Kir2.1 mice. To determine the possible influence of spontaneous afferent activity on the fate of the newborn cells, we estimate the proportion of cells double labelled for BrdU and the neuronal marker NeuN. We found that both controls and Kir2.1 mice present a comparable percentage of neurons (Figure 9).



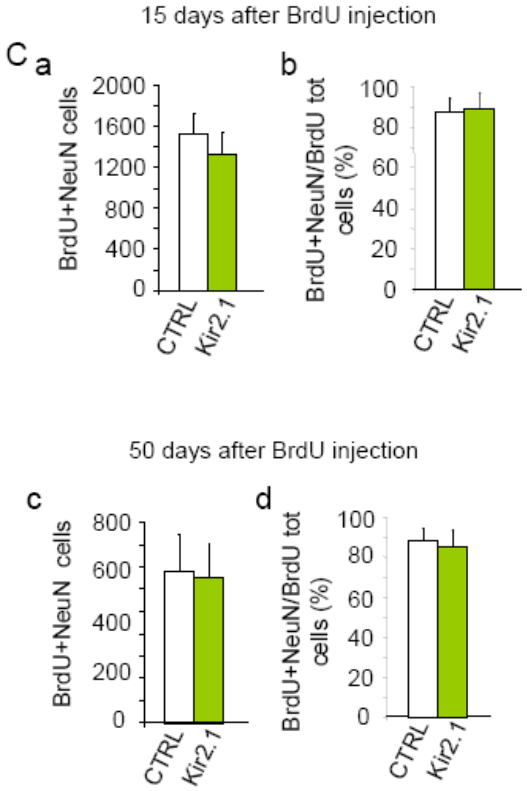
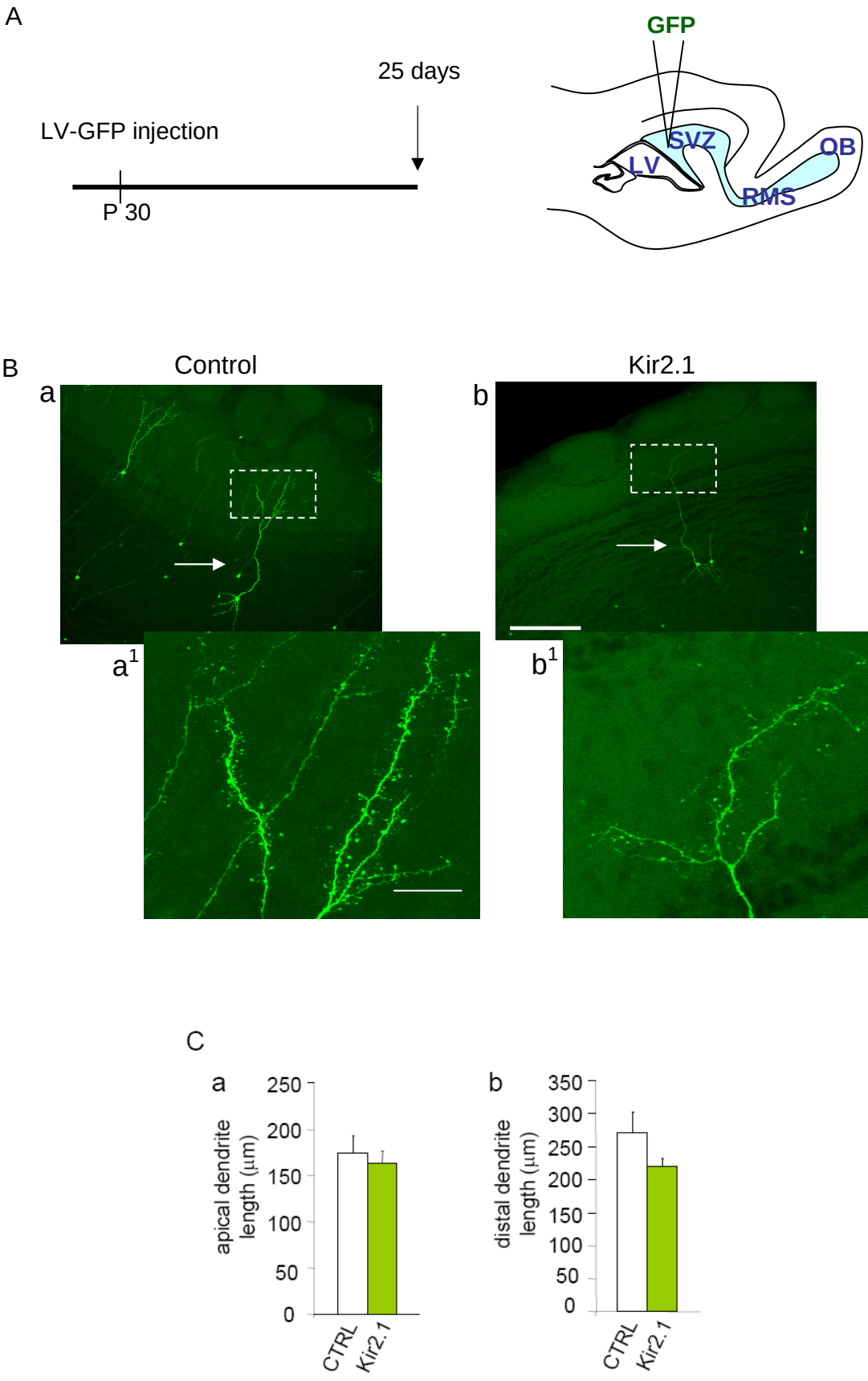



**Figure 9 A** Scheme of the experimental strategy. New born cells were analyzed 15 and 50 days after peritoneal injection of Brdu. **B** examples of coronal section of OB immunolabeld with antibodies against BrdU (red) and the neuronal marker neuN (green) 15 day (**a**) and 50 days after BrdU injection (**b**). **C** summary of results. Number of new granule cells in controls (n=4) and Kir2.1 mice(n=4) 15 days (**a**) and 50 days (**c**) after BrdU injections, and percentage of new born neuronal cells in controls and Kir2.1 mice days (**b**) and 50 days (**d**) after BrdU injections.

## 3.5.2. Maturational process of the newly generated granule cells

To assess whether the maturational process of the newly generated granule cells is affected by the lack of spontaneous afferent activity we performed injection of a viral vector encoding GFP in the subventricular zone in Kir2.1 and control mice. We analyze the injected cells cells in the olfactory bulb 25 days after injection. At this time point the migration and the process of maturation of the newly granule cells are completed. We observe that the length of the dendrites is similar in Kir2.1 mice and controls. However the filopodia-spine density was reduced in Kir2.1 mice respect to controls (Control mice (n=3) vs Kir2.1 mice (n =4) t test $p < 0.001$. , Figure 10).


A
25 days
LV-GFP injection
P 30
GFP
SVZ
LV
OB
RMS
B
Control
Kir2.1
a
b
a1
b1
C
a
apical dendrite length (µm)
250
200
150
100
50
0
CTRL
Kir2.1
b
distal dendrite length (µm)
350
300
250
200
150
100
50
0
CTRL
Kir2.1

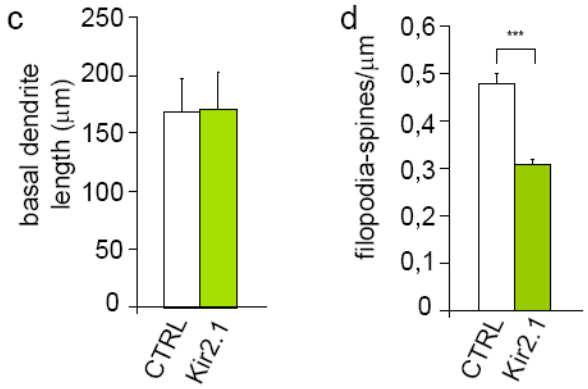



**Figure 10** Morphology of adult new born granule cells. **A** Scheme of the experimental strategy. Virus vector encoding GFP was injected in the subventricular zone (SVZ). The new born cells was analyzed after 25days post injection when they had complete the migration to the olfactory bulb and their maturation. **B** new born granule cells in coronal section of OB in control mice (**a**) and its higher magnification (**a**[1]) and new born granule cell in coronal section of the OB in Kir2.1 mice (**b**) and the higher magnification (**b**[1]). Bar 100 µm, and 20 µm in the higher mangnifications. **C** summary of results. Apical dendritic length (**a**), distal dendritic length (**b**) basal dendritic length (**c**) and filopodia-spines density (**d**) in controls and Kir2.1 mice

# 4. Discussion

In this work we analyzed circuit formation and function in the olfactory bulb of mice genetically modified to have reduced spontaneous activity in olfactory sensory neurons, the Kir2.1 mice (Yu et al.2004). These mice have very little afferent spontaneous activity due to the over-expression of the inward rectified potassium channel Kir2.1 in all olfactory sensory neurons. As a result of this genetic manipulation, all sensory neurons are hyperpolarized and their spontaneous firing is abolished.

We found that the size of the olfactory bulb, the location of the main M71, OR23 and P2 glomeruli and the size of most of the glomeruli are superimposible in control and in Kior2.1 mice . However, in Kir2.1 mice the sensory map was perturbed by the presence of additional heterogeneous glomeruli that coexist with the main homogeneous glomeruli. This type of glomeruli configuration is proper of early stages of development, when olfactory sensory neuron axons project to multiple glomeruli, most of which present a heterogeneous organization. The subsequent refinement process leads to a single homogeneous glomerulus on each side of the olfactory bulb.
From our data it emerged that the refinement process is hampered in Kir2.1 mice.
Although the mechanism underlying the neural circuit refinement remains to be clarified, different scenarios can be envisioned. 1) Disrupted spontaneous activity is likely to influence the levels of second messengers such as cyclic nucleotides and $Ca^{2+}$ that are known to play a critical role in axon targeting in several system (Song et al., 1997; Ming et al., 1997; Zheng and Poo, 2007) including the olfactory system (Maritan 2009, Pietrobon 2011 Sakano, 2010). 2) Spontaneous firing could facilitate the action of factors, such as neurotrophic factors, in the activity-dependent competition that operates on branch stability or formation (Thoenen, 1995; Bonhoeffer, 1996). It as been shown that the action of neurotrophins has to be coupled to afferent spontaneous activity to modulate neuronal plasticity in the developing neuronal cortex (Caleo et al., 1999). In addition, the action of trophic factors on the axon growth in ganglion cell is dramatically enhanced by activity (Goldberg et al., 2002). It is worth noting that the olfactory receptor determines not only odor-evoked activity but also specific pattern of spontaneous activity, in olfactory sensory neurons in Drosophila (Hallem et al., 2004) and also in mice (Reisert, 2010, Connelly 2013). Therefore, an activity based

competition could modulate the response of olfactory sensory neuron to trophic factors and /or guidance molecules present in the bulb, favouring the refinement, pruning and stabilization of axon arbors.

**Continuous neuronal plasticity in the olfactory bulb**

Sensory experience modulates the development of neuronal circuitry mostly within a defined period of time (the critical period) during which the brain is particularly plastic (Hensch, 2004). Beyond this critical period, the wiring of neuronal circuitry is hardly affected by changes in evoked activity. This is not the case for the olfactory system, that remains sensitive to manipulations of sensory experience also in adulthood (Cumming and Belluscio, 2010). The effect of the spontaneous activity on mature neuronal circuits has been scarcely examined. Indeed the role of spontaneous activity is generally thought to be confined to the early phase of development ( Shatz, 1996; McLaughlin et al., 2003; Spitzer, 2006; Huberman, 2008). Our data demonstrated that the OS differs significantly from this paradigm. We found the re-expression of Kir2.1 in adult animals induces a regression of the already formed olfactory sensory map in the olfactory bulb. Namely, animals manipulate to have the expression of the Kir2.1 channel only for four weeks in adulthood present additional heterogeneous glomeruli that coexist with the main homogeneous glomeruli. These data suggest not only the absence of a "critical period" but the requirement of proper spontaneous electrical activity for the maintenance of neuronal circuit in the olfactory bulb.

**Adult neurogenesis in Kir2.1 mice**

Previous studies demonstrated that evoked activity influence the adult neurogenesis in the olfactory system. Odorant deprivation reduced the survival of the newborn cells during the early wave of cell death between days 15 and 45 after neuronal birth. (Petreanu and Alvarez-Buylla, 2002) and alters their morphology (Saghatelyan et al., 2005). In contrast odor enriched environment increase the survival of the new born cells in the adult mice (Rochefort et al., 2002).

Analyzing the adult neurogenesis in mice with reduced afferent spontaneous activity, we found that the migration and survival of newborn granule cells was unaltered respect control mice. The fate, namely the number of newborn neurons (double labelled for BrdU and NeuN), was also unaltered in Kir2.1 mice respect to controls.

Analyzing the maturational process of adult born granule cells, we found that the length of dendrites was also unaltered in kir2.1 mice respect to controls, , however the filopodia-spine density that was reduced in Kir2.1 mice respect controls.

**Altered odor discrimination ability in Kir2.1**

The specificity of connection among neurons is essential to translate sensory experience into a meaningful neural code that is ultimately reflected in the animal behaviour. Analyzing the olfactory behaviour, we found that Kir2.1 mice were not able to discriminate odorants that elicited similar spatial maps of activated glomeruli but retained the ability to distinguish odorants that activated a more distinct spatial pattern of glomeruli. These results could be explained by the alterations we found in the anatomical and functional maps of the OB in Kir2.1 mice. The maps activated by the (-) or (+) element of each pair of the enantiomers we tested have been previously shown to be distinct by one or few glomeruli located in a restricted region (Rubin and Katz, 2001; Clarin e al., 2010; Linster et al., 2001; Linster et al., 2002) Moreover, that restricted region is superimposed on the area where we found additional heterogeneous glomeruli. Discrimination of odorants that are represented by glomeruli scattered in more distinct locations in the bulb was not significantly affected. Our data corroborate the view that spatial maps in the olfactory bulb carry sufficient information to discriminate different molecules (Rubin et Katz, 2001; Kobayakawa et al., 2007; Linster et al., 2001; Linster et al., 2002).

Overall, our data suggest that spontaneous afferent activity plays a permissive role in the refinement and in the maintenance of the topographic organization of the olfactory bulb, that in turn, could affect odor information processing and olfactory behaviour.

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