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Adult neurogenesis: regulation, heterogeneity and functions of adult born interneurons in the olfactory bulb

Thèse

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RÉSUMÉ

De nouveaux neurones sont continuellement ajoutés au bulbe olfactif adulte par un processus connu sous le nom de neurogénèse adulte. Dans ce processus, les précurseurs d'interneurones qui sont produits dans la zone sous-ventriculaire près du ventricule latéral migrent suivant un trajet préétabli, le courant de migration rostral, avant d'atteindre le bulbe olfactif. Une fois arrivé dans le bulbe olfactif les précurseurs arrivent à maturation, s'intègrent au réseau neuronal existant et jouent un rôle essentiel dans le comportement olfactif. Tout au long des plusieurs étapes de ce processus, une variété de facteurs moléculaires travaillent en tandem pour orchestrer correctement la production, la maturation, la survie et le guidage de ces nouveaux interneurones, afin qu'ils s'intègrent avec succès au bulbe olfactif adulte. Mes travaux de recherches démontrent que les neurones générés à l'âge adulte détiennent un rôle capital pour le fonctionnement normal du réseau bulbaire et du comportement olfactif. En utilisant des souris transgéniques dont l'expression d'un facteur moléculaire spécifique a été réduite, j'ai premièrement démontré que la neurogénèse post-natale et la neurogénèse adulte dépendent chacune de facteurs moléculaires distincts. J'ai ensuite démontré que les neurones de projections du bulbe olfactif, chez les souris ayant un niveau de neurogénèse adulte réduit, reçoivent une plus faible inhibition qui affecte la synchronisation de leur activité neuronale. Par conséquent, les souris sont incapables d'établir une mémoire à court-terme des odeurs.

Dans ma seconde étude, j'ai démenti la croyance stipulant que les cellules granulaires générées à l'âge adulte formaient une population homogène de cellules. J'ai pu décrire, aux niveaux moléculaire et électrophysiologique, différentes sous-populations d'interneurones générés à l'âge adulte dans la couche granulaire du bulbe olfactif, qui pourrait jouer des rôles distincts dans le décodage des odeurs. Finalement, dans ma dernière étude, j'ai démontré le rôle de l'activité sensorielle sur différentes sous-populations de neurones générés à l'âge adulte. Dans cette étude effectuée en équipe, nous avons prouvé que la dépravation

sensorielle affecte la spécification et/ou le maintien de certaines sous-populations de neurones générés à l'âge adulte dans le bulbe olfactif. Les résultats de ces trois études nous permettent donc de mettre de l'avant la façon à laquelle la neurogénèse adulte est orchestrée, le rôle des différents sous-types de neurones générés à l'âge adulte dans le bulbe olfactif et la manière par laquelle l'activité sensorielle affecte la spécification neuronale de cellules nouvellement générées.

ABSTRACT

New neurons are continuously being added to the adult olfactory bulb in a process known as adult neurogenesis. Interneuron progenitors produced from stem cells in the subventricular zone of the lateral ventricle migrate along a well establishes route, the rostral migratory stream to reach the olfactory bulb. Upon reaching the olfactory bulb they mature, integrate into the existing network and play an important role in odor behavior. All throughout this multi-step process various molecular cues work in tandem with each other to properly orchestrate the production, maturation, survival and guidance of these newborn interneurons to successfully integrate into the adult olfactory bulb. My studies show that adult generated interneurons are crucial for proper functioning of the bulbar network and odor behavior. By using mice that lack a specific molecular cue, I first showed that early postnatal and adult neurogenesis rely, at least partly, on different molecular cues. I then demonstrated that mice with impaired adult neurogenesis have reduced inhibition on their principle output neurons that affects their synchronous activity. This in turn leads to inability of mice to establish short-term odor memories.

In my next study I have shown that adult-born granule cells are not a homogeneous population of cells, as previously thought. I have described molecularly and electrophysiological different subpopulations of adult-born interneurons in the granule cell layer of the olfactory bulb that may play distinct roles in odor processing. Finally, in my last study, I explored the role of sensory activity on different sub-populations of adult-born neurons. In this collaborative study we showed that sensory deprivation affects fate determination and/or maintenance of particular sub-population of adult-born neurons. All these studies taken together allows us to further our understanding of how adult neurogenesis is orchestrated, the possible role of different sub-populations of adult-born neurons in the olfactory bulb and how sensory activity affects the fate of different sub-population of adult-born cells.

FOREWORD

The process of new neuron production is normally thought to occur only during development which ceases when the brain is fully developed. However in the 1960s Altman described a phenomenon in song birds where neurogenesis occurred ceaselessly throughout the bird's life span. This same phenomenon has seen to apply to mammals as well. Remarkably even in adult brains that have fully developed there are two neurogenic niches the subgranular zone and the subventricular zone that give rise to thousands of neurons daily to supply the hippocampus and the olfactory bulb respectively. A lot of effort and work has been put into furthering our understanding of several aspects of adult neurogenesis, the newly generated neurons and their functions which has yielded many studies demonstrating the complexity of adult neurogenesis. Even so our knowledge of adult neurogenesis is still incomplete, and hence I worked on adult neurogenesis in the olfactory bulb of mice under the guidance of Dr. Armen Saghatelyan to describe novel aspects of this process.

During my PhD I have concentrated my work on adult neurogenesis in the olfactory bulb of mice and tried to better understand the molecular cues important for orchestrating adult neurogenesis. My work also aims to better understand the functions of these newly generated interneurons, the existence of functionally distinct adult generated granule interneurons and the role of sensory activity in the maintenance of subpopulations of glomerular interneurons. My thesis consists of two published research article and a third work in progress, spanning chapters II-IV. In chapter II of my thesis I have presented a research article with work done under the guidance of and in the lab of Dr. Armen Saghatelyan. This work titled `**The extracellular matrix glycoprotein tenascin-R affects adult but not developmental neurogenesis in the olfactory bulb**` reveals that the extracellular matrix glycoprotein tenascin-R is involved exclusively in helping in detachment and guidance of radially migrating adult generated interneurons in the bulb. In this study we also show that adult generated granule interneurons are crucial for short

term olfactory memory, while other olfactory behavior remained unchanged. Thus, this work describes the first data demonstrating that adult neurogenesis is governed in part by different mechanisms when compared to those during development.

In chapter III of my thesis I describe the data obtained thus far in an ongoing investigation performed by the labs of Dr. Armen Saghatelyan in collaboration with Dr. Isabelle Caille (CNRS, Paris). This work entails the characterization of a CaMKIIα expressing subpopulation of adult generated inhibitory, granule interneurons unique to the olfactory bulb. My work in our lab is mainly to perform electrophysiological recordings and behavioral assessment of the role these neurons play in olfaction. We would like to determine if the inputs both excitatory and inhibitory that these cells receive are different from adult generated interneurons that do not express CaMKIIα. Following which we would like to selectively inhibit or activate adult generated granule interneurons expressing CaMKIIα during olfactory behavior to understand the role that these cells play in olfaction.

Finally in Chapter IV of my thesis, I present a second research article titled `**Role of sensory activity on chemo specific populations of interneurons in the adult olfactory bulb**` In this paper my contributions are as a second author. This work was also performed under the guidance of Dr Armen Saghatelyan in his lab. In this work we show that odorant driven activity from the periphery is crucial for the fate specification of new born dopaminergic glomerular interneurons and also for the survival and fate maintenance of preexisting glomerular interneurons also produced during adulthood.

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Last but definitely no less, my thanks to my parents and brother, there are no words that could convey my thanks to you, nonetheless, thank you for making all this possible for me. Without you none of this makes any sense.

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ABBREVIATIONS

ACSF:	Artificial cerebrospinal fluid
AD:	Alzheimer's disease
AMPA:	2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl) propanoic acid
Ang1:	Angiopoietin 1
ApoER2:	Apolipoprotein E receptor 2
AraC:	Cytosine-beta-D-arabinofuranoside
Arc:	Activity-regulated cytoskeleton associated protein
ARX:	Aristaless-related homeobox gene
BDNF:	Brain derived neurotrophic factor
BMI:	Bicucullin methiodide
BMP:	Bone morphogenic factor
Bral1:	Brain link protein1
BrdU:	5-bromo-2-deoxyuridine
CA:	Cornu Ammonis
CaMKIIα:	Calcium/calmodulin- dependant protein kinase II alpha
CB:	Calbindin
CBA:	Chicken beta actin
Cck:	Choelcystokinin
cfos:	Cellular oncogene response 1
CNTF:	Ciliary neurotrophic factor
CR:	Calretinin
CREB:	Cyclic adenosine monophosphate (cAMP) response element binding protein
Crtl1:	Cartilage link protein1
CSF:	Cerebrospinal fluid
CSPG:	Chondrotin sulphate proteoglycan
Dab-1:	Disabled 1
Dcx:	Doublecortin
DG:	Dentate gyrus
Dlx2:	Distal-less homeobox 2
ECM:	Extracellular matrix
EGF:	Epidermal growth factor
EGFR1/Zif68:	Early growth response 1
Enk:	Enkephalin
Eph:	Ephrine
EPL:	External plexiform layer
EPSC:	Excitatory post synaptic current
EPSP:	Excitatory post synaptic potential
ET:	External tufted cells
FGF:	Fibroblast growth factor
GABA:	Gamma aminobutyric acid
GAD:	Glutamic acid decarboxylase

GAGs:	Glycosaminoglycans
GCL:	Granule cell layer
GCs:	Granule cells
GDNF:	Glial-cell derived neurotrophic factor
GFAP:	Glial fibrillary associated protein
GFP:	Green fluorescent protein
GL:	Glomerular layer
HB-EGF:	Heparin-binding epidermal growth factor
HD:	Huntington`s disease
HSPG:	Heparin sulphate proteoglycan
HVC:	Higher vocal centre
IGF1:	Insulin like growth factor
IGFR:	Insulin-like growth factor receptor
IPL:	Internal plexiform layer
IPSC:	Inhibitory postsynaptic currents
IR-DIC:	Infrared differential interference contrast
Kyn:	Kyneurenic acid
LGE:	Lateral ganglionic eminence
LOT:	Lateral olfactory tract
LTP:	Long term potentiation
MAM:	methylazoxy methanol
MAPK:	Mitogen activated protein kinase
Mash1:	Mammalian achaete-Scute homolog
Math1:	Mammalian atonal homolog
MC:	Mitral cells
MOB:	Main olfactory bulb
MOE:	Main olfactory epithelium
Msi1:	Musashi 1
nAChRs:	Nicotinic acetylcholine receptors
NCAM:	Neural cell adhesion molecule
Neo1:	Neogenin 1
Neurog2:	Neurogenin 2
NMDA:	N-Methyl-D-Aspartate
Nrg:	Neuregulin
NSE:	Neuron specific enolase
NueN:	Neuronal nuclei
OB:	Olfactory bulb
OE:	Olfactory epithelium
Olig2:	Oligodendrocyte lineage transcription factor 2
ONL:	Olfactory nerve layer
ORN:	Olfactory receptor neurons
PACAP:	Pituitary adenylate cyclise-activating polypeptide.
Pax6:	Paired box gene 6
PD:	Parkinson`s disease
PFA:	Paraformaldehyde

PGs:	Periglomerular interneurons
PI3K:	Phosphotidylinositol 3 kinase
PK2:	Prokineticin 2
PNN:	Perineuronal net
PSA-NCAM:	Polysialylated neuronal cell adhesion molecule
PV:	Parvalbumin
RMS:	Rostral migratory stream
sAPP:	soluable amyloid precursor protein
SDF1:	Stromal cell derived factor 1
SGZ:	Subgranular zone
Shh:	Sonic hedgehog
Sox2	Sex determining region- Y box2
SVZ:	Subventricluar zone
TA:	Tenascin assembly domain
TBI:	Traumatic brain injury
TBR2:	T-box brain protein 2
TGF:	Transforming growth factor
TH:	Tyrosine hydroxylase
tMCAO:	transient middle cerebral artery occlusion
TNR:	Tenascin-R
TRH:	Tyrotrophin releasing hormone
TTX:	Tetradotoxin
Tuj1:	Neuron specific class III beta-tubulin
Unc:	Unco-ordinated like protein
VEGF:	Vascular endothelial growth factor
VIP:	Vasoactive intestinal peptide
VLDLR:	Very low density lipoprotein receptor
VNO:	Vomeronasal organ
Wnts:	Wingless-type MMTV integration site

1. INTRODUCTION.

The mammalian olfactory system has a remarkable capability to recognize and discriminate a wide range of odor molecules. This not only provides essential information for the animal`s survival but has also profound effects on its behavior. Odor information from the environment influences complex behavioral functions such as food localization, reproductive and maternal functions, recognition of co species, emotional states and social status. Interestingly, the olfactory bulb (OB), the first-order sensory relay for olfactory processing, retains the ability to acquire new interneurons throughout life (Luskin, 1993; Lois and Alvarez-Buylla, 1994). The main objectives of my thesis work was to understand some of the mechanisms governing adult and early postnatal neurogenesis, the role of sensory activity in the integration, survival and fate determination of newborn cells, as well their role in the OB network functioning and odor behavior. In the following subchapters of the Introduction, I will discuss the organization of the OB, the postnatal neurogenesis in various species with main emphasis on the adult OB neurogenesis in rodents as well as factors regulating postnatal neurogenesis.

1.1 The olfactory bulb development and structure:

Mammals can detect odors and pheromones via multiple chemosensory systems. The olfactory receptor neurons (ORNs) in the main olfactory epithelium (MOE) are specialized ciliated cells whose axons cross the basement membrane and enter the glomeruli to synapse with the primary dendrite of mitral cells in the main OB (Mombaerts, 2006). The other chemosensory system found in most mammals is the vomeronasal organ (VNO), situated in the nasal septum the VNO mainly processes pheromones (Brennan and Zufall, 2006). Like the ORNs of the MOE the VNO houses ciliated cells called vomeronasal sensory neurons (Dulac and Torello, 2003). The rest of the chapter is concentrated on the MOE and the OB as my work is on the interneurons of the MOB and the functions of the adult generated MOB interneurons.

The OB development starts at around embryonic day 12 (E12) and continues late into development by continuously adding vast numbers of interneurons roughly half of which survive, integrate and modulate the output neurons who are already established in the OB by E17. I will discuss in the following chapter the development and structure of the olfactory bulb that will then allow us to better understand adult neurogenesis in the OB.

1.2 Early development and establishment of the olfactory system:

Mammalian olfaction results from the passage of information in a structured and multi synaptic relays from the **periphery** olfactory epithelium via the OB to the olfactory cortices. *The process of olfaction in mice occurs in two distinct relays*, the primary relay involves the olfactory receptor neurons (ORNs) in the olfactory epithelium (OE) whose axons synapse on the primary dendrite of the mitral/tufted cells (M/T), the principle output neurons of the olfactory bulb. The second relay is carried out by the axons of the mitral/tufted cells that travel through the lateral olfactory tract (LOT) to enervate multiple cortical regions. Intriguingly both the ORNs of the epithelium and the interneurons of the OB are continuously being replaced but the processing of odors and passage of odor information to the higher olfactory cortices is left unhindered and efficient.

In humans it has been known that the free nerve endings of the ophthalmic and maxillary branches of the trigeminal nerve distributed throughout the nasal mucosa are also stimulated by most odorants that stimulate the ORNs (Tucker, 1971). Sensations derived from the trigeminal nerve are somatosensory in nature (Proctor, 1982). The trigeminal system maybe more involved in protective reflexes when compared to the olfactory system that functions to perform identification, recognition and in memorizing odors (Brand, 2006).

During embryonic rodent development the OE and OB formation are being dictated by completely different and unconnected programs. Following the differentiation of the ORNs in the OE their axons cross the basement membrane and navigate

towards the developing OB along with the olfactory ensheathing cells (Valverde et al., 1992). Simultaneous with the OE, the OB is developing from a predetermined part of the rostral telenchephalon. By E12.5 the OB was seen to be morphologically distinct from the surrounding tissues appearing as an evagination of the rostral part of the telencephalon. During this time the initial establishment of the central projection from the OB was already able to proceed independently of the ORN axons from the OE (Hinds, 1968; Lopez-Mascaraque et al., 1996; Sugisaki et al., 1996; Lopez-Mascaraque and de Castro, 2002; Inaki et al., 2004). While the OB is being defined and its first outputs are being established, a small, pioneering population of interneuron precursors are migrating to the OB form the lateral ganglionic eminence (LGE) which peaks between E18 and postnatal day 5 (P5) (Hinds, 1968; Tucker et al., 2006). The principal neurons of the OB, the M/Tcells are produced exclusively during embryonic development during a period spanning E11-E13. However these cells are being morphologically fine tuned even at adulthood (Blanchart et al., 2006).

As development advances further, the mechanisms governing the OB and OE genesis seem to become interrelated; mitral cell primary dendrite development for example is closely linked to the sensory activity from the ORN terminals or the presence of the sensory axons themselves. Sensory deprivation as early as postnatal day results in serious affects to the developing mitral cell primary dendrites (Matsutani and Yamamoto, 2000). The OE retains two populations of basal cells throughout life, the horizontal and globose basal cells that can act as stem cells and give rise to ORNs and the supporting sustentacular cells by rounds of unequal division.

1.3 Organization of the olfactory bulb:

The mammalian OB is a layered structure whose general layout was described in great detail as early as 1875. Camillo Golgi using a technique, to this day that bears his name, impregnated cells in the dog OB with silver and made detailed drawings of the cells and their positions in the distinct layers of the OB(**Fig. 1.1**)

(Shepherd et al., 2011). Other pioneering neuroanatomists like Cajal (1890, 1911) showed the same using Golgi's technique. Others like Van Gehuchten and Martin (1891) along with Calleja (1893), Kolliker (1896) and Blanes Viale (1897) performed detailed study and described and established the arrangements of the OB neurons (Allison, 1953). As described in Golgi's paper in 1875 and later translated by Shepherd et al. (2011) the first layer of the OB is the **olfactory nerve layer** ``*a thin outer layer of white-grayish color consisting of bundles of peripheral olfactory nerve fibers, which, issuing from the lamina cribrosa, enter into the parenchyma of the bulbs."*

Following the olfactory nerve layer (ONL) is the ***glomerular layer (GL)***. The glomeruli are spherical regions where the ORN terminals synapse with the primary dendrite of the principal output neurons and pass on olfactory information from the periphery at the first synapse of the relay. Glomeruli are seen to range in different sizes and shapes, in fish and amphibians they are small and measure between 20-40 microns in diameter, in small mammals like mice it ranges from 30-50 microns and in larger mammals like rabbits and cats the size has been seen to be 100-200 microns (Allison, 1953).

Interestingly each glomerulus receives axons from ORNs that uniformly express just one of the receptors from the whole possible pool and they then synapse on the primary dendrite of a few mitral/tufted cells forming the basis of a odor processing column (Ressler et al., 1994; Shepherd et al., 2004; Mori and Sakano, 2011). In the glomerular network the periglomerular (PGs) and short axon cells provide inter and intra glomerular inhibition and excitation at the mitral cell primary dendritic tuft. Short axon cells send long interglomerular axons and form excitatory synapses with the PGs of distant glomeruli in order to inhibit the principal cells targeting that glomerulus, setting up a centre surround inhibition (Aungst et al., 2003). Other studies have characterized the inhibition both feedback and feed forward in the glomerular network (McGann et al., 2005; Shao et al., 2012).

Following the glomerular layer is the ***external plexiform layer (EPL)***. The EPL represents the second level of odor processing where the lateral dendrites of principal output neurons make dendrodendritic synapses with the granule cells (GC). But the EPL has also been shown to have a diverse population of interneurons making synapses onto output neurons or other interneurons. Even though normally the EPL is thought to be a layer with few cell bodies, interneurons, astrocytes and tufted cells are routinely found here (Hamilton et al., 2005). Parvalbumin (PV) positive interneurons that form reciprocal dendrodendritic synapses with M/T cells, have cell bodies and dendrites in the EPL (Toida et al., 1994). Studies by Kosaka and Kosaka (2008, 2011) obtained similar results when they showed that the EPL has PV positive cells that was also (Glutamic acid decarboxylase) GAD positive, along with these some large short axon cells with axons primarily placed in the EPL were also seen (Kosaka and Kosaka, 2008, , 2011). It has been also shown that short axon cells located in the EPL form synapses with newly generated granule cells (Arenkiel et al., 2011). A population of calretinin (CR) positive cells was also documented among the PV interneurons in the EPL (Batista-Brito et al., 2008). Lepousez et al. (2010) described a somatostatin subpopulation of interneurons, 95% of whom are situated in the inner layer of the EPL having their dendritic fields exclusively in this region. These cells are shown to have morphology resembling the Van Gehuchten short axon cells and they were also seen to express GAD, CR and vasoactive intestinal peptide (VIP). These cells were also seen to form dendrodendritic synapses with mitral cells (Lepousez et al., 2010). The second group of output neurons, the tufted cells also located in the EPL and is considered as smaller versions of the large mitral cells (Allison 1953). But the studies of Cajal (1911) showed that the pattern of axon collaterals of these two classes of output neurons were very different and could also be thought to provide very different inputs to the GCs as that of the mitral cells (Shepherd, 1972).

The mitral cell layer is comprised of a thin layer of mitral cell somas present a few hundred microns below the glomeruli (200-400). These cells have a soma 15-30 microns in diameter having a single primary dendrite and branching secondary dendrites that are smooth and devoid of spines (Shepherd et al., 2004). Studies also showed that mitral cell axon collaterals stayed within the internal plexiform layer (IPL) and the granule cell layer (GCL) (Kishi et al., 1984; Orona et al., 1984). Both mitral and tufted cells send their axons to the olfactory cortex via the lateral olfactory tract (LOT), these two output cell populations have been shown to project to distinct olfactory cortical regions (Igarashi et al., 2012).

The core of the OB is comprised of the ***granule cell layer***. The GCL`s major cell population are the GCs (Shepherd et al., 2004). Price and Powell (1970a, 1970b) described in detail the GCs of the OB. These cells have a small, round cell body measuring 6-10 microns. Notably, these cells are quiet unique as they have no axon as was observed and described by Golgi (1885). However, these cells had primary dendrites that ascend into the EPL where they synapse with the output neurons and basal dendrites that are confined in the GCL. These cells were seen to have numerous gemmules or spines on their dendrites. GCs are GABAergic (Price and Powell, 1970b, 1970a), however, many other immunocytochemically distinct cells have been described from studies that used the Golgi labeling technique and immunocytochemical labeling. GCs in the OBs of Macaques express the calcium binding proteins CB, CR, PV and neurocalcin (Alonso et al., 2001). In addition to GCs, the GCL also contains two types of short axon cells; Blanes cells and Golgi cells (Schneider and Macrides, 1978). Blanes cells are seen as large stellate cells in the GCL under infrared differential interference contrast (IR-DIC) (Pressler and Strowbridge, 2006). These cells have been characterized as GABAergic and to be synapsing onto GCs (Pressler and Strowbridge, 2006). Yet another cell type was described in the GCL that showed elaborate protrusions close to the cell body, commonly seen in the hedgehog OB. These cells were

suggested to be inhibitory interneurons acting on GCs and PGs (Lopez-Mascaraque et al., 1986).

Figure 1.1: Structure of olfactory bulb

Olfactory bulb of the dog, impregnated by the Golgi method. Golgi. (1875). Shepherd et al., 2011.

1.4 The glomerular synapse and dendrodendritic synapse.

There are two main types of synapses made in the OB. The primary synapse in olfaction is made between the axon terminals of the ORNs onto two cell types, the mitral cell primary dendrites and a subclass of PG cells made within discrete compartments called glomeruli lining the periphery of the OB. The other being the reciprocal dendrodendritic synapses made between the apical dendrite of the GCs onto the lateral dendrites of mitral cells (**Fig. 1.2**).

1.4.1 Glomerular synapse:

Some of the earliest studies done on mitral cells of the turtle OB (Mori et al., 1981) documented that a single shock in the olfactory nerve bundle elicited a volley of responses that caused excitatory post synaptic potentials (EPSP) in the mitral cells that was closely followed, typically after just a few milliseconds, by hyperpolarizing inhibitory post synaptic currents (IPSC) (Mori et al., 1981). This antidromic inhibition was thought to be that of PGs and or GCs. Further experiments in turtles and rats showed that both N-methyl-D-aspartate (NMDA) and non-NMDA components are seen in the mitral cell responses to the ORN input (Nowycky et al., 1983; Ennis et al., 1996). The presence of prolonged NMDA activity at these synapses is thought to be a key factor in olfactory processing by facilitating integration and plasticity (Aroniadou-Anderjaska et al., 1997). The mitral cell excitatory post synaptic currents (EPSC) are enhanced by different mechanisms. Studies have shown that the auto receptors present on the mitral cells respond to glutamate produced and released by mitral cells themselves (Nicoll and Jahr, 1982). EPSC enhancement in the mitral cells could also be brought about by auto inhibition of PGs (Smith and Jahr, 2002).

Some of the salient features of this synapse are that the release probability at these synapses seems to be very high. And the ORN terminal can also be presynaptically controlled. The ORN terminals have D_2 receptor that in response to dopamine released from TH positive interneurons induces decrease in glutamate release (Ennis et al., 2001).

1.4.2 Dendrodendritic synapses:

Rall et al. (1966) were the first to describe the dendrodendritic synapses in the rabbit OB. Bringing together two sets of data; electron microscopy studies (Reese and Brightman) and theoretical analysis (Rall and Shepherd) they were able to describe a synapse where there seemed to be two synaptic contacts in each synapse between mitral and granule cells. As inferred by the morphological data, a single GC acts as a presynaptic and postsynaptic site with respect to the mitral cell they contact. They further postulated that at these synapses there is mitral-to-granule excitation and granule-to-mitral inhibition (Rall et al., 1966). These synapses could then provide a pathway for both lateral and self inhibition. Apart from inhibition these synapses are also important for setting the rhythmic, synchronous activity of the output neurons of the OB (Rall and Shepherd, 1968).

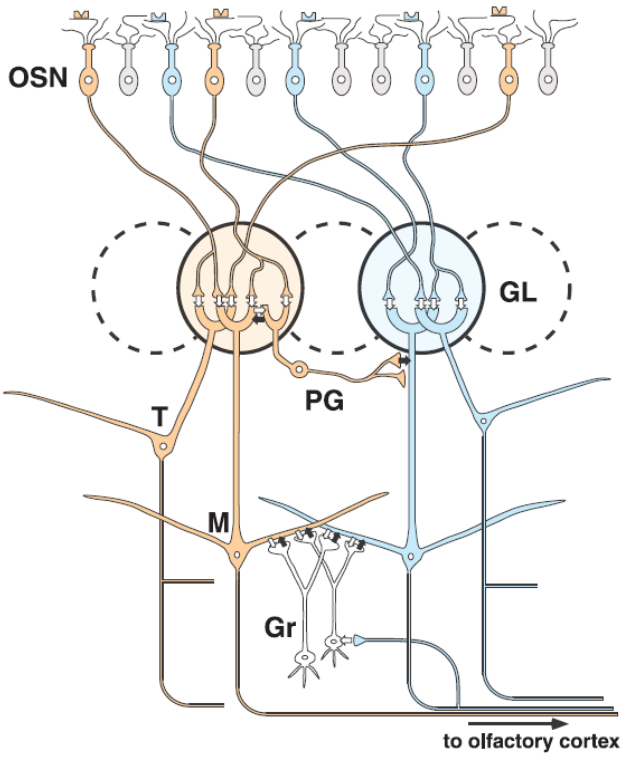

Figure 1.2: Basic circuit diagram summarizing the synaptic organization of the mammalian OB. Two glomerular modules (brown and blue) represent two different types of odorant receptors. Mitral cells (M) and tufted cells (T) are output neurons, and granule cells (GC) and periglomerular cells (PG) are local interneurons. OSN, olfactory sensory neuron; GL, glomerulus (Mori et al., 1999).

2. Postnatal Neurogenesis

The OB is one of two regions in the brain that receive and integrate neuronal precursors throughout the lifespan of animals. In the next subchapters I give a general overview of postnatal neurogenesis with major emphasis on the OB neurogenesis.

2.1 A brief historical overview of the study of neurogenesis:

Early neuroanatomists like Ramon Cajal thought the adult nervous system was fixed and immutable. However, we now know that even if neurogenesis is most active during pre-natal development it continues throughout life in certain regions of the brain of most vertebrates and invertebrates. The dentate gyrus (DG) of the hippocampus and the OB of the adult brain are the two regions that keep adding neurons throughout life as described in many animal species. In 1962 Altman while studying the lesioned adult rat brain combined intracranial injections of thymidine-H(3) along with lesions and saw that along with brain regions associated with the lesion some neurons and neuroblasts were also labeled in the cerebral cortex, which suggested that the adult rat brain maybe capable of producing new neurons (Altman, 1962). In further studies using the same thymidine-H(3) technique Altman showed that new neurons were being generated in the DG of the adult rat, cat and guinea pig hippocampus (Altman, 1962, , 1963; Altman and Das, 1967). In contrast however, was the data obtained from the rhesus macaque visual cortex (Rakic, 1974). Using titrated thymidine injections and radiographic evidence Rakic concluded that there was no neurogenesis occurring postnatally based on the fact that no labeled cells were detected in the neocortex. Later in 1985, Rakic performed further experiments on the rhesus macaque; monkeys were injected with doses of thymidine-H (3) and sacrificed from 3 days to 6 years after injection. In this study the results revealed no labeled neurons in the neocortex, thus the study concluded that all neurogenesis ceases during early postnatal days (Rakic, 1985). At the same time, however, it has been demonstrated that the hippocampus retained dividing cells in adult mice much like in young mice and the same was also true for the adult OB (Kaplan and Hinds, 1977; Kaplan and Bell, 1984). Thus these conflicting sets of data set up a debate on the existence or absence of adult neurogenesis.

A new frontier was established when studies on the song bird brain (Goldman and Nottebohm, 1983) showed the existence of adult born neurons in the higher vocal

area (HVA) of the telenchephalon. This study was seen as the first definite proof for adult neurogenesis and was widely accepted by the scientific community. In addition to the two well established niches of adult neurogenesis, studies have indicated that neurogenesis occurs in other regions of the adult brain as well, like the dorsal vagal complex of the brain stem, CA1 region of the hippocampus, the amygdala, neocortex, the substantia nigra and also the spinal cord (Gould et al., 1999; Rietze et al., 2000; Yamamoto et al., 2001; Bernier et al., 2002; Zhao et al., 2003a; Bauer et al., 2005). Most of these studies have however been contested (Horner and Gage, 2000; Frielingsdorf et al., 2004; Lie et al., 2005).

But the most important questions remain; why does the adult brain need new neurons? What could be the role of these new neurons in a mature circuit? Several theories have been suggested for the relationship between increased neurogenesis and learning, memory or improved cognition; new neurons may increase memory capacity, reduce interference between memories and could be crucial for placing time details onto memories. All of the above possibilities are under research. In the following chapters I will discuss the studies documenting and describing adult neurogenesis that occurs in various species of animals and also describe briefly human adult neurogenesis. After a brief subchapter on the adult neurogenesis in the hippocampus I will concentrate the rest of the introduction on OB neurogenesis, which is the main subject of my research and thesis.

2.2 Neurogenesis is widespread in various species:

Adult neurogenesis is widespread in vertebrates and also seen in many invertebrate species (Barker et al., 2011). In this subchapter I discuss some of the species where adult neurogenesis has been documented.

Invertebrate neurogenesis: Among invertebrates, many species have been reported to have adult neurogenesis like the ***decapods crustaceans*** including crayfish, lobsters and spiny lobsters (Sandeman et al., 1998; Harzsch et al., 1999;

Schmidt, 2001). In crayfish the neuronal precursors , has been shown to reside in a specialized niche in the ventral surface of the brain, like in adult vertebrates (Sullivan et al., 2007). Another study done with crayfish proposed a hematopoietic connection for the neural stem cells (Beltz et al., 2011). ***Hydrozoans*** were also shown to have production of new neurons throughout their lifecycle in the nerve net (Sakaguchi et al., 1996; Miljkovic-Licina et al., 2004). The mushroom bodies of ***several insects*** spanning a wide range of families (Holometabolous, Orthopteroid and Coleopteroid) have been shown to have new neurons throughout life (Bieber and Fuldner, 1979; Cayre et al., 1996). Cockroaches, crickets, moths and red flour beetles among many have been documented to have adult neurogenesis (Gu et al., 1999; Malaterre et al., 2002; Dufour and Gadenne, 2006; Zhao et al., 2008). It has been also shown that sensory activity is crucial for neurogenesis in adult crickets much like in mammals (Scotto-Lomassese et al., 2003). Similarly agonistic behavior was also seen to play a vital role in adult neurogenesis in crickets (Ghosal et al., 2009). Of interest is also the parallel that has been drawn between the productions of the Kenyon cells of the red flour beetle to the mushroom body neurogenesis in the fruit fly (Zhao et al., 2008).

Neurogenesis in adult vertebrate organisms: Several vertebrate species including fish, reptiles, mammals (including non human primates and marsupials), chiropterans and avian have been shown to have adult neurogenesis.

Fish are one of the many vertebrates that have constitutive adult neurogenesis. Several studies have shown that multiple regions of the fish brain receive and integrate new neurons through out life; the central/posterior pacemaker nucleus of the electric knife fish, OB of zebra fish and the whole of the brain in Austrolebias (Teleostean) are known to have adult neurogenesis (Zupanc and Zupanc, 1992; Byrd and Brunjes, 2001; Fernandez et al., 2011). Studies in ***reptiles*** like the Iberian wall lizard (Podarcis hispanica), the turtle and Moorish Gecko have shown that a continuous addition of cells are made in brain regions like the ependymal layer of the medial cortex, the OB, nucleus sphericus and the whole of the

telenchephalon (Lopez-Garcia et al., 1988; Garcia-Verdugo et al., 1989; Perez-Sanchez et al., 1989; Lopez-Garcia et al., 1990; Perez-Canellas and Garcia-Verdugo, 1996). In a study spanning eight wild caught ***Megachiropterans*** (Megabats/ fruit bats) species Chawana et al., (2013) showed that these bats have a very primate like rostral migratory stream (RMS) and new neurons produced in the sub ventricular zone (SVZ) travel via the RMS to reach the OB. However unlike the single stream of the well studied rodent RMS, the bat RMS is composed of the dorsal and ventral sub streams that merge before entering the OB. Cells were also observed to leave both limbs of the RMS to integrate in the rostral neocortex. Proliferating cells were also identified in the temporal horn of the SVZ which then migrates via a temporary RMS (similar to primate brains) to the rostral and caudal piriform cortex. Hippocampal neurogenesis was also observed in all species documented in this study (Chawana et al., 2013).

Avians have been part of the history of adult neurogenesis from as far back as the 1980 when Nottebohm showed adult neurogenesis in the brains of song birds (Nottebohm, 1989). Alvarez-Buylla and Kirn (1997) published their study on the adult song bird that showed that neural stem cells resided in the ventricular zone of the lateral ventricle and produced neurons that migrate long distances to populate the telencephalon. In song birds like the canary these newly generated cells are destined for the higher vocal centre (HVC) (Alvarez-Buylla, 1997), which was shown to be a unique phenomenon owing to the fact that only song bird brains had a HVC (Nottebohm, 2005). The newly generated cells in the HVC are produced in a seasonal manner and production is at its peak during the time of the year when canaries modify their song. Non song birds have also been shown to have adult neurogenesis. The neostriatum caudale and the hyperstriatum of the adult ring tail dove was shown to receive new neurons throughout life with the maximal number of cells being incorporated within the first year after hatching (Ling et al., 1997). This timing is crucial as these cells are implicated in the integration of sensory cues and for reproductive behavior (Ling et al., 1997).

Mammalians like the Hedgehogs and Moles (Laurasiatheria), Meadow Vole, Giant Otter Shrew (Tenrecidae), non human primates like Macaques and Marmosets, red foxes (Vulpes) and Grey squirrels are some of species investigated for adult neurogenesis. Marsupials like the Fat-tailed dunnart and Opossum also were shown to have neurogenesis during adulthood. Bartkowska et al. (2010) investigated adult neurogenesis in the DG and SVZ of Hedgehogs and Moles (Laurasiatheria), they showed that both granule cells and periglomerular cells are generated during adulthood. This work was also the first to show that Dcx positive fibers traverse the anterior commissure, the study goes on to postulate that these fibers maybe the axons of newly generated cells making interhemispheric connections between the two bulbs or piriform cortices (Bartkowska et al., 2010). Dcx positive cells were also seen in the striatum and piriform cortex. In the giant Otter Shrew (Tenrecidae) adult neurogenesis has been observed in both the SVZ and the DG. SVZ precursors were seen to migrate via the RMS to reach the OB where they differentiated into the granule and periglomerular interneurons. Newly generated neurons were also seen in the olfactory tubercle and the piriform cortex, similar to the results obtained in Moles and Hedgehogs the axons of newly generated neurons were seen to cross the anterior commissure (Patzke et al., 2013). Amrein et al. (2011) in a study spanning a vast number of species including Marmosets, Macaques, red foxes, showed that all these species retained the ability to add new neurons in the hippocampus throughout life albeit the process being age dependent (Amrein et al., 2011). Adult neurogenesis has been also demonstrated in the hippocampus of macaques, although severely reduced even in comparison to what happens in rodent brains as they age (Kornack and Rakic, 1999). The presence of adult neurogenesis in the grey squirrels was also shown and new born neurons were shown to be important for memory of the multiple locations where food was stored (Barker and Boonstra, 2005). Several other species of animals have also been studied and shown to have retained the ability to add new neurons during adulthood including mice and rats we use as model organisms for investigation adult neurogenesis.

2.3 Adult neurogenesis in the rodent hippocampus:

2.3.1 Stem cells in the adult hippocampus:

In the sub granular zone (SGZ) of the hippocampus a subset of astrocytes are thought to act as stem cells (Seri et al., 2001). These astrocytes have their cell bodies in the SGZ with radial processes going through the granule cell layer and short tangential processes extending along the border of the hilus and the granule cell layer. Upon division these stem cells produce immature neurons that migrate upwards radially to take their place in the hippocampal network. Another group studying the adult hippocampal stem cells described them as sex determining region-Y box2 (Sox2) positive that can give rise to both neurons and astrocytes (Suh et al., 2007). However, the stem cells seen in the rodent brain do not seem to have resemblances to stem cells that are involved in adult human hippocampal neurogenesis (Eriksson et al., 1998). Even though there is still not a complete understanding of the factors influencing the in vivo proliferation of hippocampal stem cells, mitogenic factors such as fibroblast growth factor (FGF) and epidermal growth factor (EGF) are thought to play pivotal roles (Lie et al., 2004).

2.3.2 Maturation and integration of interneurons in the granule cell layer:

In the SGZ of adult rodents, stem cells produce immature neurons via an intermediate progenitor. In contrast to the neuroblasts migrating long distances via the RMS to the OB, the newly generated neurons of the DG only need to migrate short distances to the inner granule cell layer. Once there the neurons extend long axonal processes along mossy fiber pathways and reach their targets in the hilus and CA3 pyramidal cell layer 4-10 days after they were born (Hastings and Gould, 1999; Zhao et al., 2006). The dendrites of these neurons however extend in the opposite direction and take 2 weeks to reach the molecular layer where they mature and increase in complexity over many months (van Praag et al., 2002; Faulkner et al., 2008).

As progenitors are being produced from nestin positive stem cells, interneurons (basket cells) release gama aminobutyric acid (GABA) tonically and phasically onto the progenitors. This ambient GABA has been shown to be the first activators of neural progenitors (Bhattacharyya et al., 2008). Further studies also showed that following activation of progenitors by the ambient GABA, these cells receive phasic GABAergic followed by glutamatergic inputs while they are maturing (Ge et al., 2006; Bhattacharyya et al., 2008). GABA initially brings about excitation in newborn neurons as they have high chloride content in their cytoplasm. As these cells mature the depolarization brought about by GABA becomes inhibitory and hyperpolarizing, and these changes are crucial for the synapse formation and dendritic development of these new neurons (Ge et al., 2006). After receiving GABAergic and glutametergic inputs the newly generated interneurons make mossy fiber synaptic outputs to the hilus and CA3 neurons. Studies done by using retroviral labeling and electron microscopy showed that mossy fiber en passant buttons of adult born GCs form initial synapses with the hilus and CA3 neurons within 2 weeks after birth (Faulkner et al., 2008; Toni et al., 2008) (**Fig. 1.3**).

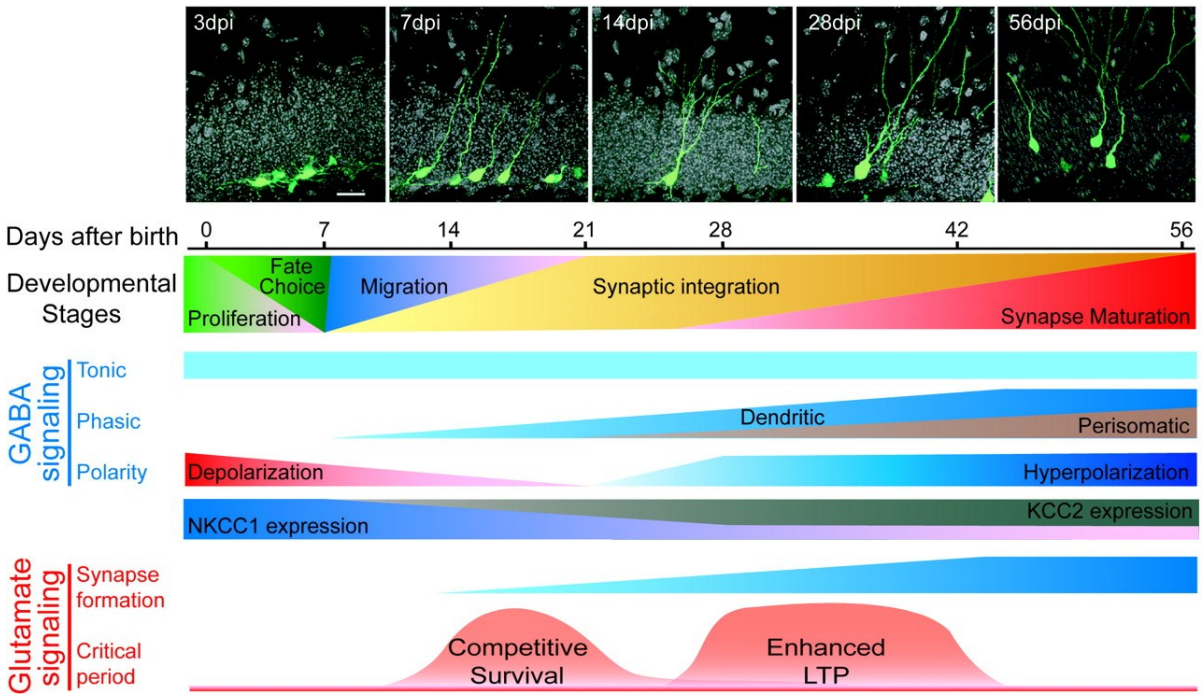

Figure 1.3: Synaptic integration of new granule cells in the dentate gyrus of the adult mouse hippocampus Shown is a schematic summary of the synaptic integration and maturation process of newborn dentate granule cells in the adult mouse hippocampus. The images shown in the top panel were adapted from Ge et al. (2006, 2007b) (Ge et al., 2008).

2.3.3 Functions of adult generated hippocampal interneurons:

Hippocampal neurogenesis is important for learning and memory. Physical activity has been shown to increase hippocampal neurogenesis resulting in improved memory formation (Van Praag et al., 1999). Conversely sleep deprivation, stress or diseases like Alzheimer's negatively affects neurogenesis (Gould et al., 1998; Jin et al., 2004; Mueller et al., 2011). An interesting link between hippocampal neurogenesis and antidepressants has also been made. Administration of antidepressants has also been shown to increase neurogenesis in the hippocampus (Malberg et al., 2000) and a reverse study (Santarelli et al., 2003) revealed that increased neurogenesis in the hippocampus is necessary to mediate

the effects of antidepressants. The disruption of hippocampal neurogenesis seems to affect certain types of memory and not others. By using the anti mitotic drug methylazoxy-methanol (MAM) to block hippocampal adult neurogenesis, studies have demonstrated that impairment was selectively seen in trace eye-blink conditioning and trace fear conditioning but not contextual fear conditioning and trace fear conditioning even though all of them are normally considered as memory dependent on the hippocampus (Shors et al., 2001). On the other end is the implication of hippocampal neurogenesis in pathology. Seizure induced neurogenesis is thought to actually contribute to epiletogenesis and also long term cognitive impairments (Jessberger et al., 2007).

2.4 OB neurogenesis in rodents:

Neurogenesis in the mouse OB happens at three distinct developmental stages or in three waves; *embryonic neurogenesis, perinatal neurogenesis and adult neurogenesis*. As discussed earlier embryonic neurogenesis begins while the OB is being delineated from surrounding tissue and cells begin to take their place in the primordial OB as the axons of the ORNs are being targeted to the developing OB. Perinatal neurogenesis peaks between postnatal day three and postnatal day seven (Lemasson et al., 2005) which then carries on during the whole of the animals lifespan constituting adult neurogenesis. Each of these waves is discussed in the following chapter.

2.4.1 Neurogenesis in the embryonic OB:

The OB is thought to develop from a pre-determined part of the telencephalon (Lopez-Mascaraque et al., 1996). The mitral/tufted cells of the OB are known to be produced between E11-E13; they then undergo refinement and pruning to attain their final morphology from E17 into adulthood (Blanchart et al., 2006). The interneurons of the mouse OB falls into two major groups the granule cells (GCs) that are placed in the granule cell layer in the core of the olfactory bulb and the

periglomerular neurons that are placed in the glomerular layer. Using thymidine-H3 radiographic studies of rat olfactory bulb neurogenesis it has been demonstrated that the vast majority of interneurons are produced nearly simultaneously during the first three postnatal weeks (Bayer, 1983). However studies have also identified a small but definite pioneering, molecularly diverse population of interneurons produced from the lateral ganglionic eminence as early as E12.5 (Toresson and Campbell, 2001; Tucker et al., 2006) or between E18 and P5 (Hinds, 1968) (**Fig. 1.4**). These early generated cells persist and are also thought to act as a framework for all the subsequent postnatal addition of interneurons (Bayer, 1983).

The OB interneuron precursors are located in very spatially defined regions of the LGE (Tucker et al., 2006). It has been also shown the presence of local population of interneuron precursors housed within the OB itself that gives rise to GABAergic and dopaminergic cells within the OB (Vergano-Vera et al., 2006). The LGE not only provides interneurons to the developing OB but also provides a pool of proliferating stem cells to the SVZ that persist on into adulthood (Lledo and Lazarini, 2007).

The precursors from the LGE are programmed to migrate in a very specific migratory pathway that leads to the developing OB (Tucker et al., 2006). Using organotypic culture assays of the developing telenchephalon LGE derived cells were seen to migrate out from the margins and following well organized streams till they reached the OB (Tucker et al., 2006). Cells derived from the LGE were able to migrate to the OB faithfully when they were transplanted into the adult SVZ (Wichterle et al., 1999). Two closely related homeobox genes Gsh1 and Gsh2 have been shown to be crucial for the developing OB and these genes were also implicated for the maintenance of the precursor pool (Toresson and Campbell, 2001).

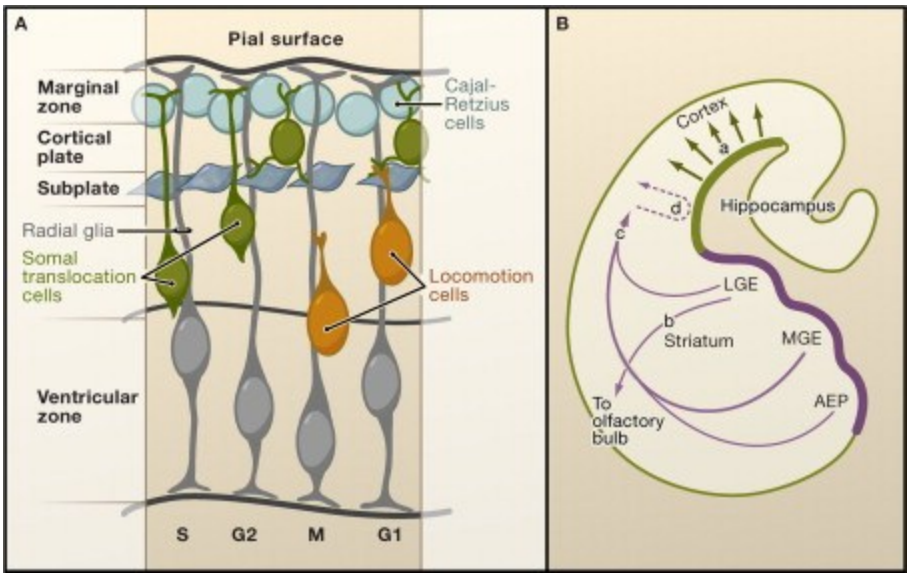

Figure 1.4: Lateral ganglionic eminence derived progenitors migrate to the embryonic OB (B) Interneurons expressing GABA originate from the subpallium structures, the LGE, MGE, and AEP, and migrate tangentially into the olfactory bulb (Ayala et al., 2007).

2.4.2 Perinatal Neurogenesis:

Very early in postnatal development radial glia from the ganglionic eminences retract their processes and establish a population of astrocytic stem cells that define the SVZ niche that lasts all through life (Wichterle et al., 1999; Wichterle et al., 2001; Merkle et al., 2004). During early postnatal neurogenesis the SVZ develops above the primary ventricular zone. The region gives rise to a large population of glia and neurons. This region was first described by Altman (1969). Unlike embryonic neurogenesis where multiple migratory routes bring neurons to the OB from the LGE, during early postnatal neurogenesis newly generated cells are confined to migrate within the RMS, thereby restricting these cells to only populate the OB (Conover and Notti, 2008).

During early postnatal neurogenesis in addition to the SVZ other regions like the RMS (Gritti et al., 2002; Pencea and Luskin, 2003) and the OB have also been implicated in being zones for production of new neurons (Lemasson et al., 2005). Early postnatal neurogenesis provide the bulb with newly generated GCs that vary from their adult generated counterparts in their origin, time to reach bulb, position and survival (Lemasson et al., 2005).

Perinatally generated GCs have been shown to have longer turnover periods, and owing to this crucial feature these cells are thought to be the building blocks of OB functioning (Lemasson et al., 2005). In pups early generated PGs have been shown to be implicated in learning maternal odor to promote feeding (Singh et al., 1976). These new bulbar interneurons are also involved in forming long-term memories that last long into an animal's life span, which could be the reason why the neonatal OB shows an increase in the number of cells subsequent to olfactory learning (Woo and Leon, 1991). Thus the functional attributes of perinataly produced cells varies from their adult counterparts.

2.4.3 Adult Neurogenesis:

The adult brain retains the ability to produce new interneurons throughout the life of an animal. Stem cells proliferate in the SVZ throughout the lateral wall of the lateral ventricle. These precursors to the OB interneurons then form chains that are positive for the polysialylated neural cell adhesion molecule and migrate tangentially till they reach the core of the OB via the well defined RMS (Doetsch and Alvarez-Buylla, 1996). In the OB they are then guided by specific molecular cues like the extracellular matrix molecule TNR (Saghatelyan et al., 2004) and reelin (Hack et al., 2002) to their final position where they integrate into the network and differentiate into molecularly varying subpopulations (Luskin, 1993; Batista-Brito et al., 2008) (**Fig. 1.5**). In the following sub-chapters I will discuss different processes of adult neurogenesis such as cell proliferation, migration, maturation,

survival and their functional role.

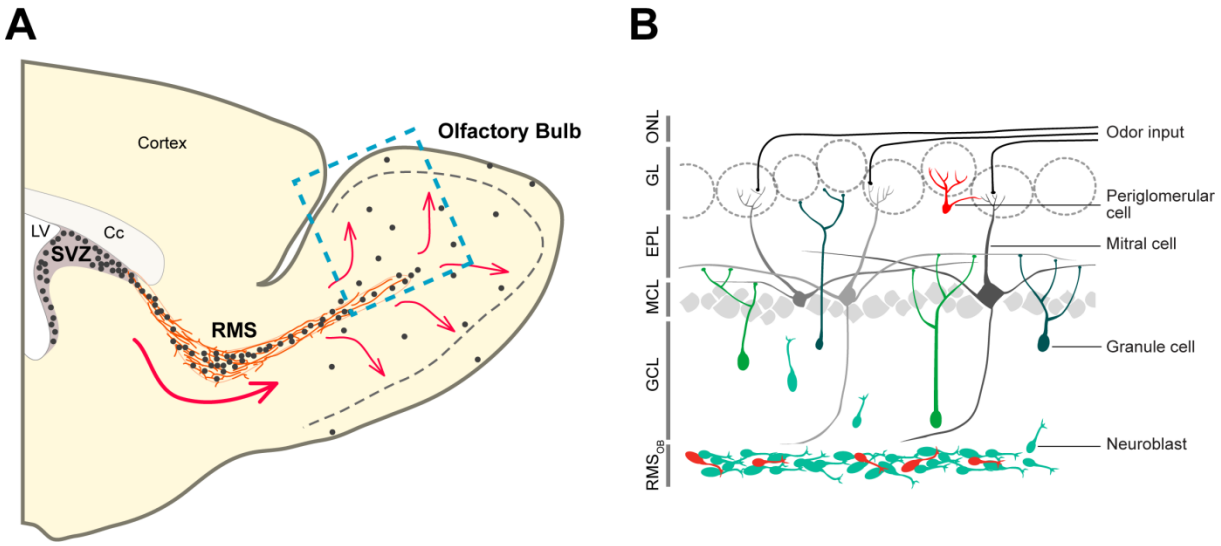

Figure 1.5: Adult generated cells are constantly arriving in the OB

(A) Illustration representing the adult OB neurogenesis. Adult-born cells migrate tangentially via the rostral migratory stream (RMS). In the OB, they migrate radially and integrate into the pre-existing neural network. LV: lateral ventricle; Cc: corpus callosum. (B) View of boxed area in A representing the laminar organization of the OB. Adult-born cells differentiate into the bulbar interneurons, granule and periglomerular cells, adapted from (Breton-Provencher and Saghatelyan, 2012).

2.4.3.1 Proliferation and production of precursors:

The SVZ develops from the residual progenitors of the LGE (Bayer et al., 1994). The SVZ along with the sub granular zone of the hippocampus are the only regions that maintain the ability for producing new neurons throughout adulthood. The work of Reynolds and Weiss (1992) was one of the first studies to show that the SVZ indeed houses stem cells (Reynolds and Weiss, 1992). Later it has been shown that a population of slowly dividing astrocytes expressing glial fibrillary acidic protein (GFAP) with radial glial properties act as the precursors in the SVZ (Doetsch et al., 1999; Alvarez-Buylla and Lim, 2004). These cells are known as the type B cells. These B cells which are multipotent give rise in turn to fast dividing immature, transit amplifying precursor cells that label positively for Dlx2, these cells

are known as the C type cells. Finally from these C type cells, neuroblast that are positive for PSA-NCAM are produced. These cells are also known as A type cells (Doetsch and Alvarez-Buylla, 1996) (**Fig. 1.6**).

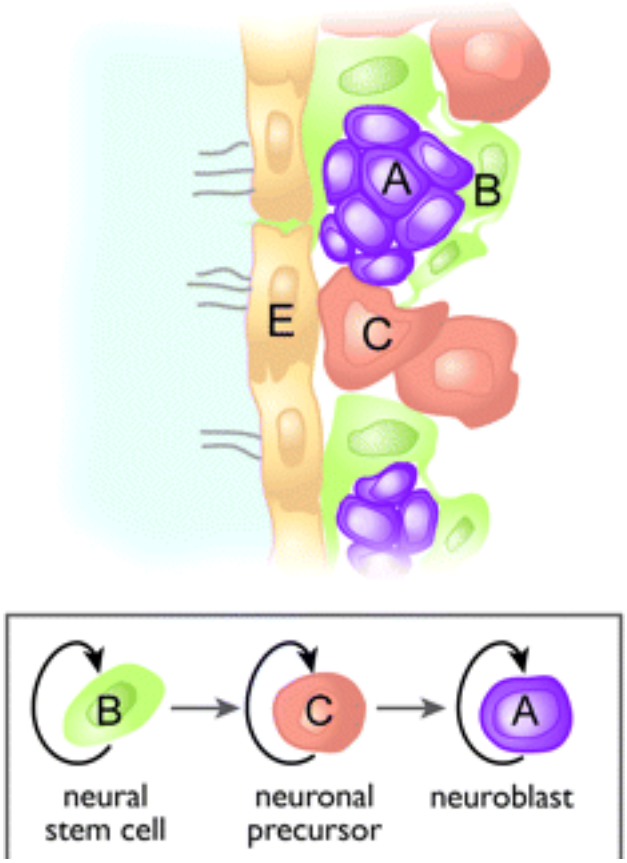

Figure 1.6: Proliferation of stem cells in the SVZ niche during adult neurogenesis

Adult neural stem cells are cells with the structural and molecular characteristics of astrocytes (called type B cells). The SVZ astrocytes, which express GFAP, divide to generate transit-amplifying cells (called type C cells), which in turn give rise to neuroblasts (type A cells) that migrate in the RMS to their final destination in the OB (Gheusi and Lledo, 2007).

2.4.3.2 Migration of precursors to the OB:

Stem cells of the SVZ produce in a continuous manner around 30,000-50,000 precursors migrating to the OB on a daily basis. These precursors then differentiate into the local interneurons (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Belluzzi et al., 2003; Carleton et al., 2003). In order to reach the OB, the SVZ derived precursors undertake tangential migration in chains by binding with each other aided by cell adhesion molecules like PSA-NCAM, integrins (Murase and Horwitz, 2002) and move down a astrocyte lined migratory trail called the RMS (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Wichterle et al., 1999; Kornack and Rakic, 2001). Repulsive and attractive factors like slits and ephrins (Conover et al., 2000; Nguyen-Ba-Charvet et al., 2004), receptor tyrosine kinases like ErbB4 (Anton et al., 2004), neurotransmitters like GABA (Bolteus and Bordey, 2004; Snapyan et al., 2009) and multiple growth factors like BDNF, VEGF, GDNF (Paratcha et al., 2006; Snapyan et al., 2009; Wittko et al., 2009) act to guide and control neuroblast migration. In addition, the neuroblasts use blood vessels that precisely outlined the RMS as a scaffold for migration (Snapyan et al., 2009) and repel astrocytic processes during their migration via slit-robo signaling (Kaneko et al., 2010).Once these cells reach the core of the OB they arrest their tangential migration break away from their chains and begin their second mode of migration, this time radially to integrate in the GCL and GL (Kriegstein and Alvarez-Buylla, 2009). Several factors like reelin, TNR, PK2 and IGF1 have been all been implicated in radial migration (Hack et al., 2002; Saghatelyan et al., 2004; Ng et al., 2005; Hurtado-Chong et al., 2009). These factors act as detachment, dispersion and guidance factors that lead to interneuron precursors migrating in chains to separate, turn and migrate in the GCL till they reach their designated site (**Fig. 1.7**). Apart from these factors a newly discovered class of radial glia residing in the GCL of adult OBs has also been implicated in radial migration (Emsley et al., 2012).

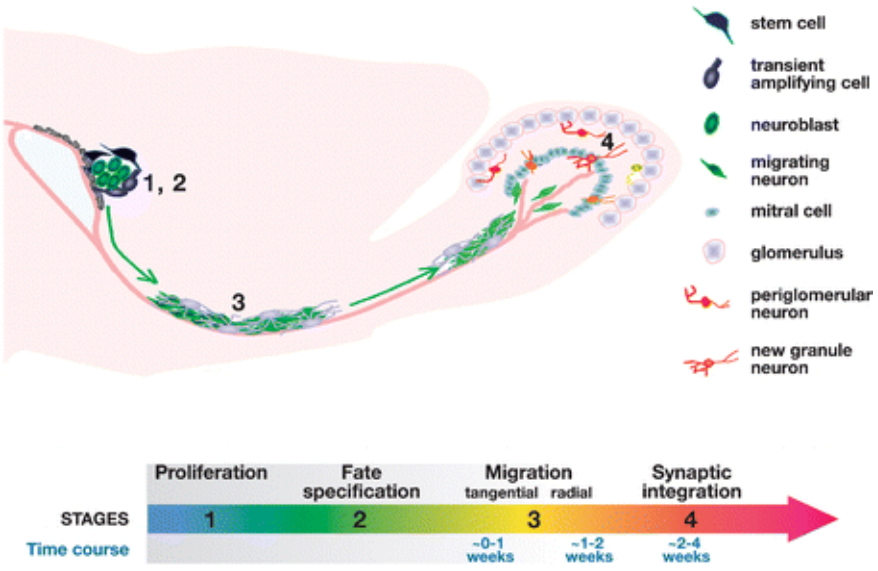

Figure 1.7: Generation of new interneurons in the olfactory bulb from neural stem cells in the sub ventricular zone (SVZ)

Adult neurogenesis in the SVZ/olfactory systems undergoes four developmental stages. Stage1. Proliferation: stem cells (*blue*) in the SVZ of the lateral ventricles give rise to transient amplifying cells (*light blue*). Stage2. Fate specification: transient amplifying cells differentiate into immature neurons (*green*). Adjacent ependymal cells (*gray*) of the lateral ventricle are essential for neuronal fate specification by providing inhibitors of gliogenesis. Stage3. Migration: Immature neurons (*green*) migrate with each other in chains through the rostral migratory stream (RMS) to the olfactory bulb. The migrating neurons are ensheathed by astrocytes. Once reaching the bulb, new neurons then migrate radially to the outer cell layers. Stage4. Synaptic integration: Immature neurons differentiate into either granule neurons (Gr, *orange*) or periglomerular neurons (PG, *red*) (Ming and Song, 2005).

2.4.3.3 Maturation and integration of newly generated interneurons:

The time it takes for tangentially migrating cells to reach the OB has been shown to be around 4 to 10 days in rodents (Luskin, 1993; Jones et al., 1994; Hu et al.,

1996; Winner et al., 2002). Once in the OB these cells need to mature and integrate into the existing network (**Fig. 1.8**), the vast majority of cells integrating are GCs with a considerably smaller number of PGs integrating in the GL. A distinguishing factor of GCs are that they are axon less cells that make synapses using their dendrites with principal cells dendrites giving rise to specialized dendrodendritic synapses. Granule cells though axon less have short basal dendrites that are confined within the GCL whereas their much longer, spiny apical dendrite is extended towards the EPL where they form dendrodendritic synapses with lateral dendrites of the mitral/tufted cells (Shepherd et al., 2004; Price and Powell, 1970).

Using viral vectors to label and follow neuronal precursors, several studies have been done on understanding the synaptic integration and maturation of the interneurons. The manner in which the process of maturation is achieved by adult generated neurons is starkly different from those of developing neurons in the fetal brain. During tangential migration, the neuroblasts in the RMS express $GABA_A$ receptors extrasynaptically followed by sequential expression of NMDA and AMPA receptors (Bolteus and Brodey, 2004; Carleton et al., 2003). The work of Panzanelli et al., (2009) who used lentiviral vectors to label neuroblasts in the RMS with eGFP was followed with electrophysiological characterization and immunohistochemical analysis showing that within a relatively short time of the first 24 hours post their arrival in the OB the new interneurons receive both GABAergic and glutamatergic inputs. The next step is for these cells to then extend their spiny apical dendrites towards the EPL during which time new inhibitory and excitatory synapses are continuously being established on the GC dendrite shafts. At around seven days post injection labeled newborn neurons were seen to exhibit mature morphology with spine rich dendrites in the EPL (Panzanelli et al., 2009).

Where Panzanelli et al, used lentiviruses introduced in the RMS other studies done by Carleton et al., (2003) and later Whitman and Greer, (2007) used retroviruses encoding GFP stereotaxically injected into the SVZ. Owing to the region where

precursors were labeled in these studies, the newly produced cells appeared in the OB at 8 to 10 days post injection. After receiving both types of inputs onto their dendrites, the GCs need to fire action potential and release neurotransmitters in order to integrate into the OB (Carleton et al., 2003).

The number of spines on the newly generated GCs increases up to 28 days post injection of viral vectors after which the superfluous numbers of spines are reduced between 28 and 58 days post injection (Whitman and Greer, 2007). In fact newborn GCs and PGs where shown to have spine reorganization even as long as 3 months after cells were labeled (Mizrahi, 2007). Interestingly, these newborn neurons receive synapses that display long-term potentiation for a few weeks after their birth that subsequently disappears as they age. Sensory activity is also known to be important for integration and maintenance which will be discussed later in the chapter.

nAChRs are also implicated in the integration via in the olfactory bulb interneurons (Changeux and Danchin, 1976; Mechawar et al., 2004). The early postnatal and adult generated periglomerular cells of OB, however, unlike the GCs have very similar maturational processes. The synapses made by ON terminals on the PGs born during early postnatal and adult periods show the same properties (Grubb et al., 2008).

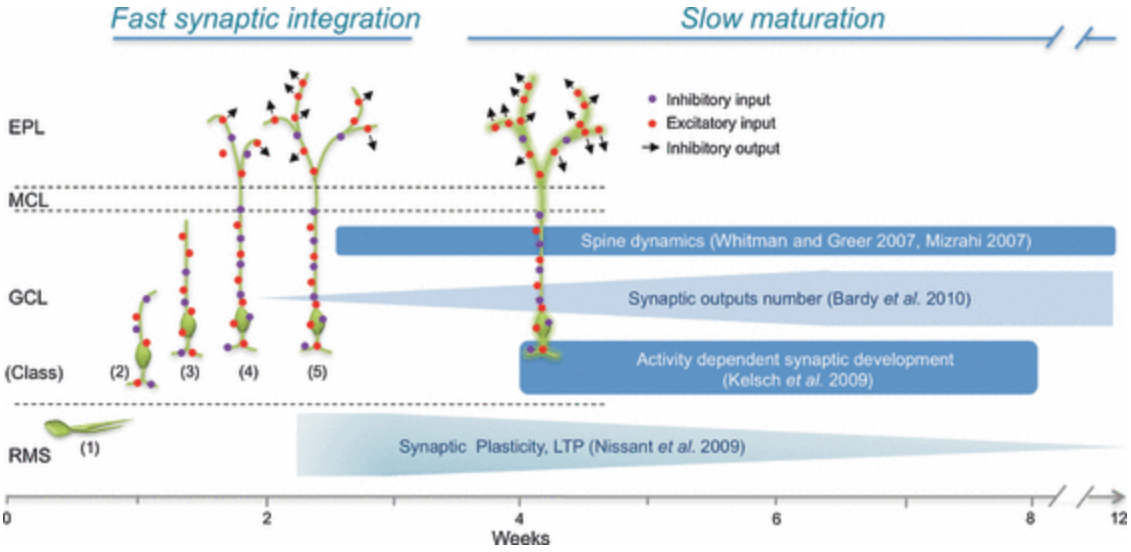

Figure 1.8: Integration and maturation of newborn neurons in the adult olfactory bulb-from synapses to function

Schema showing the fast synaptic integration and the slow long process of maturation of newborn interneurons in the adult bulb (Nissant and Pallotto, 2011).

2.4.3.4 Survival of newly generated interneurons:

Various studies have estimated the number of cells being integrated into the adult OB to be about 30,000-50,000 (Lois and Alvarez-Buylla, 1994) and as much as 80,000 (Kaplan et al., 1985). Even with this massive number of cells integrating into the network, about 50% of these cells do not survive and are efficiently cleared out of the system. The method of cell clearance appears to be programmed cell death, with the highest numbers of cells dying in the OB and in much lesser numbers in the RMS and SVZ (Biebl et al., 2000; Winner et al., 2002).

Cells that do survive, mature and integrate into the network and play crucial roles in odor processing in the OB. Survival of these cells can be attributed to a few factors; neurotransmitter systems like acetylcholine, glutamate and GABA seem to be implicated along with sensory activity.

2.4.3.5 Diversity of adult generated interneurons in the OB.

In 1970 Price and Powell described the granule cells of the OB after studying slices of rat brains using the Golgi method and electron microscopy. They described GCs as axon less cells with small somata with very little cytoplasm (Price and Powell, 1970). These cells were seen to extend spiny processes into the EPL and smaller processes in the deep GCL (Price and Powell, 1970). In 1971 a similar study was undertaken by Pinching and Powell that again used the Golgi labeling and electron microscopy to look more closely at the interneurons of the rat OB glomerulus, describing the morphologies of external tufted cells (ET), periglomerular and superficial short axon cells. In addition to the positioning and the morphology of interneurons Shipley and Ennis (1998) described the synaptic partners of interneurons, the dendrodendritic synapses that they form with output neurons, and neurotransmitters they employ (Shipley and Ennis, 1996). In a comprehensive review spanning their work and work of many others, Kosaka and Kosaka (2005) discussed the chemical heterogeneity of periglomerular cells. Studying the rat OB they showed that approximately half of all periglomerular neurons are either glutamic acid decarboxylase (GAD), calretinin (CR) or calbindin (CB) class cells, whereas the tyrosine hydroxylase (TH), thyrotropin releasing hormone (TRH) and enkephalin (Enk) class represent the subtypes of the earlier group (Kosaka and Kosaka, 2005). In mice they saw that most of the CB cells were also GABAergic (GAD+) (Kosaka and Kosaka, 2005).

The various subpopulations of adult generated OB interneurons can be characterized by properties like their location, synaptic connectivity, morphology, firing pattern and immunomarkers (Price and Powell, 1970b; Pinching and Powell, 1971; Shipley and Ennis, 1996; Crespo et al., 2001; McQuiston and Katz, 2001; Kosaka and Kosaka, 2005). Using inducible fate mapping experiments in Dlx1/2 precursors it has been shown that in the GL; TH class cells were produced at maximal rates during early embryogenesis and slowed after birth (Batista-Brito et al., 2008). In contrast is the production profile of CB class neurons where it peaks

during late embryogenesis and later wanes after birth (Batista-Brito et al., 2008). Finally CR neurons were produced in very small numbers during embryogenesis with their numbers increasing postnatally (Batista-Brito et al., 2008). In the EPL, PV cells were present only postnatally. In the GCL the three classes of cells followed were the 5T4+ granule cells placed in the mitral cell layer which were noted to be the only cells to have a steady state of production throughout life, while CR+ granule cells being acquired only postnatally and Blane`s cells during embryogenesis (Batista-Brito., 2008).

Whereas Batista-Brito et al. (2008) undertook a comprehensive study to follow the various subpopulations of interneurons of the OB over time, De Marchis, 2007 tried to explore the temporal specificity of GL interneuron production. Using another approach Merkle et al. and Hack et al. looked at progenitor domains of the SVZ or RMS in producing specific subpopulations of newborn cells (Hack et al., 2005; Merkle et al., 2007).

Apart from inhibitory interneurons it has been revealed that during adult neurogenesis the SVZ also gives rise to subpopulation of juxtaglomerular cells that were glutamatergic (Brill et al., 2009). Their origins lie in progenitors expressing Tbr2 and Neurog-2 normally associated with glutamatergic cells (Brill et al., 2009).

2.4.3.6 Regulation by sensory activity:

Cell fate determination, maturation and integration are tightly controlled by intrinsic factors like genes and transcription factors that dictate to the cell its identity and position in the network. Sensory activity is presumably very important for the integration, proper wiring and maturation of any newly generated cells. Little is actually known about how it actually dictates the integration, maturation or survival of newly generated cells. Nevertheless many studies document the positive and negative effect that sensory activity or the absence of it has on these processes. In adult mice sensory deprivation has a drastic effect on olfactory bulb ipsilateral to the occlusion (Maruniak et al., 1989). The size of the bulb ipsilateral to the

unilateral naris occlusion was 27% atrophied six months after occlusion (Maruniak et al., 1989). One of the reasons for this drastic deficit could be that unilateral nostril occlusion affected radial migration of neuroblasts in the GCL as a result of the down regulation of guidance cues as well as maturation of newborn interneurons (Saghatelyan et al., 2004; Saghatelyan et al., 2005). The survival of interneurons after naris occlusion seems to also be affected with an accompanying increase in cell death as seen by increased TUNEL or caspase-3 labeling in both mice and rats (Corotto et al., 1994; Fiske and Brunjes, 2001; Yamaguchi and Mori, 2005). Interestingly, similar results were obtained using anosmic mice (Petreanu and Alvarez-Buylla, 2002). Newborn cell loss was as much as 70% of BrdU+ cells spanning both GL and GCL 180 days after labeling and this loss of cells occurs faster in the GL when compared to the GCL (Mandairon et al., 2006).

Conversely odor enrichment increased the number of newborn interneurons integrating into the OB of adult mice without affecting proliferation of progenitors, and these mice also displayed improved odor memory (Rochefort et al., 2002). These changes however occur to be transient (Rochefort and Lledo, 2005). This study showed that the number of BrdU cells in the OB was elevated after odor enrichment, but it quickly returned to normal within 1 month (Rochefort and Lledo, 2005). The improvements in odor memory also seemed to last only for this short period after which it returned to that of non odor enriched mice (Rochefort and Lledo, 2005). Upon administration of discrimination tests to mice, the number of newborn interneurons was increased owing to prolonged survival (Alonso et al., 2006). Further analysis of the odor activation maps using Zif268 locations of the surviving cells was shown to be in regions activated by novel odors (Alonso et al., 2006). Thus not only sensory activation mediates learning, but it also controls their distribution by influencing their survival. Fate determination and maintenance of newly generated interneurons is also controlled by sensory activity. One of the best documented being the down regulation of tyrosine hydroxylase (TH) expression

after naris occlusion (Stone et al., 1990; Baker et al., 1993; Brunjes, 1994; Mandairon et al., 2006).

2.4.3.7 Functional role of new interneurons in the adult OB:

Two of the most important questions to be asked when studying adult neurogenesis are; *is adult neurogenesis a functionally relevant process?* And *what makes the OB and hippocampus special enough to maintain neurogenesis throughout an animal's lifespan?* All of the answers regarding neurogenesis will have to be answered in a relevant manner by addressing animal behavior.

The very first evidence that new neurons generated during adulthood are recruited into functional circuits was furnished by Paton and Nottebohm (1984). Adult canaries were given 3H-labeled thymidine and electrophysiological recordings were made a month later. Electrophysiological recordings in the labeled cells of nucleus hyperstriatum ventralis pars caudalis showed that labeled cells were responding to auditory stimulations. Thus showing for the first time that adult generated neurons are functionally integrated into the birds brain (Paton and Nottebohm, 1984). In the rodent, Altman suggested as early as 1967 that newly generated neurons in adult brains are central to learning and memory. Many studies in the hippocampus of rodents and humans have shown that an increase in neurogenesis brought about by exercise improved spatial memory, gives rise to LTP and amelioration of depression and Alzheimer's disease (van Praag et al., 1999; Dery et al., 2013; Lee et al., 2013; Marlatt et al., 2013). On the other hand when neurogenesis was affected by pharmacological manipulations, deficits were observed in specific types of memories like trace memory and fear responses while other types of memories were unaffected (Shors et al., 2001; Snyder et al., 2001; Shors, 2002). Additionally electrophysiological recordings made in slices from animals with reduced hippocampal neurogenesis demonstrated that LTP was also absent along with impaired neurogenesis (Snyder et al., 2001). In the olfactory bulb, it has been shown that odor enrichment up regulated neurogenesis and improved odor memory without affecting the hippocampal neurogenesis (Rochefort

et al., 2002). A study done on aged mice (24 months) showed that aged mice have reduced neurogenesis when compared to younger adults. Olfactory behavior done with these two groups of mice revealed that the aged mice were just as capable of discriminating different odors, but were deficient in comparison to the group of younger adults in differentiating similar odors (Enwere et al., 2004). This study also went on to show that neurogenesis was very important for fine odor discrimination and a reduction in neurogenesis resulting in fewer newly integrated neurons impaired the mouse`s ability to discriminate between similar odors (Enwere et al., 2004). Using neural cell adhesion molecule (NCAM) deficient mice where chain migration was affected, another study was also able to show that the reduced number of cells integrating in the OB due to disrupted RMS migration resulted in an impairment of odor discrimination in these mice (Gheusi et al., 2000).

The use of conditionally expressed diphtheria toxin under the nestin promoter to selectively eliminate only adult generated neurons in the hippocampus and OB, revealed severe deficits in the retention of spatial memory owing to affected hippocampal neurogenesis (Imayoshi et al., 2008). The authors surprisingly did not see any effect on odor memory tasks like simple discrimination and odor associated memory tasks (Imayoshi et al., 2008). The authors suggest that neurogenesis maybe important for the maintenance and organization of the adult OB, but do not exclude the possibility that adult neurogenesis is crucial for discrimination or learning of novel odors. Genetic modulation or pharmacological manipulations that affect adult neurogenesis, have, however shown that adult OBs with reduced adult generated interneurons suffer a significant reduction in the inhibition on principal neurons (Breton-Provencher et al., 2009). Reduction in inhibition results in affected synchronized activity of the output neurons (Breton-Provencher et al., 2009). In these mice administration of various olfactory behavioral tasks showed that short-term odor memory is significantly reduced while all other tasks as odor detection thresholds, discrimination and long-term memory were left unaffected (Breton-Provencher et al., 2009).

Neurogenesis in adult female mice has also been implicated in mate selection and maternal behavior. Neurogenesis is induced by both of these behaviors and is also used for consolidating mating and pup rearing (Shingo et al., 2003; Mak et al., 2007). Using dominant and subordinate male pheromones it has been shown that neurogenesis was triggered in both the hippocampus and OB aided by prolactin and luteinizing hormone only upon exposure to the dominant male pheromones. (Mak et al., 2007). Upon ablation of neurogenesis with the anti-mitotic drug cytosine-beta-D-arabinofuranoside (AraC), augmented neurogenesis in response to dominant male pheromones was lost and resulted in females mating with both subordinate and dominant males (Mak et al., 2007). During subsequent pregnancy, the neurogenesis is increased in females, and this increase in OB interneurons helps in odor discrimination which is a critical requirement for the recognition and rearing of the pups by their mothers (Shingo et al., 2003). Neurogenesis is also implicated in paternal-offspring recognition (Mak and Weiss, 2010). It has been shown that interaction of fathers with their pups increased neurogenesis in the hippocampus and OB of adult male mice, once again aided by prolactin which then allowed the fathers to consolidate the memory of their offspring (Mak and Weiss, 2010). Six weeks after birth of pups, fathers were exposed to and allowed to interact with their own pups or foreign pups. The results showed that fathers spent 41% more time investigating foreign pups in comparison to their own offspring indicating that they recognize their own offspring and are more intrigued by unfamiliar pups (Mak and Weiss, 2010). In contrast is the study by Feierstein et al., showing the importance of newly generated interneurons in social interaction. OB neurogenesis was disrupted in female mice by focal irradiation of the SVZ resulting in abnormal social interactions with males. However conspecific interactions with other females was unaffected. These results show that female mice with impaired neurogenesis could not recognize male odors having serious implications in choosing mating partners. However in this context of reduced neurogenesis the female mice did not exhibit any aberrant maternal behavior to their offspring,

neither did it affect the ability to discriminate their own offspring from others (Feierstein et al., 2010).

2.5 Adult neurogenesis in humans:

As early as (1934) Opalski suggested that regions similar to the rodent SVZ exist in the human as well. Other studies went on to postulate that this presumptive SVZ may harbor stem cells (Globus and Kuhlenbec, 1944). In 1998, BrdU along with other neuronal markers like NeuN, NSE and calbindin allowed to for the very first time to provide definitive proof that the human dentate gyrus retains the ability to generate neurons throughout life (Eriksson et al., 1998). However, to date the possibility of human OB neurogenesis is a topic of controversy. Questions such as *does the RMS such as seen in rodents exist in the human brain? And does the number of cells born steadily reduce with age just as in rodents?* are still being asked (Macklis, 2012).

Several groups of researchers have attempted to answer these pressing questions. The results have revealed multiple structural, organizational and functional differences between the human SVZ and those of rodents. The human SVZ houses astrocytic stem cells in a ribbon along the lateral ventricle separated by a hypocellular region (Sanai et al., 2004) which is quiet different from that of rodents. In another study the human RMS was also seen to be organized around a tubular extension of the lateral ventricle in order to reach the OB (Curtis et al., 2007). Most conflicting however seems to be the results regarding the presence or absence of cells migrating in chains via the RMS to the OB; Bernier et al. (2000) characterized the human SVZ and found that there were chains of neuroblasts (Tuj1+) in the SVZ (Bernier et al., 2000). However, other studies performed a few years later reported that there was no chain migration in the RMS, instead, cells were seen migrating individually or in pairs (Sanai et al., 2004; Wang et al., 2011). These studies also showed that even though not in chains the migrating neuroblasts did exhibit migratory morphology and like rodent neuroblasts expressed PSA-NCAM, Dcx, Pax6 and Olig2. Sanai and coauthors in a later study

published in 2011 showed that the infant human SVZ and RMS are populated with vast numbers of neuroblasts which is significantly reduced by the age of 18 months and disappear at adulthood. The other intriguing piece of data to be got from this study is that the neuroblasts migrating in infant human RMS are not only targeted to the OB but also to the prefrontal cortex. Finally, the presence or absence of adult generated cells in the OB is also being debated. Whereas some groups (Wang et al., 2011) reported a failure to find any newly generated neurons in the adult human OB, others using ^{14}C cell birth dating showed the presence of a limited number of cells in OB (Bergmann et al., 2012). Finally, Pagano et al not only showed the presence of neuroblasts and stem cells within the adult OB itself they also isolated and transplanted these stem cells in Parkinson`s disease animal model and showed functional recovery in their animals (Pagano et al., 2000).

Thus these studies have highlighted the similarities and differences between human adult neurogenesis and those of rodents. The understanding of which could better able us to design therapeutic strategies that can be tested in rodents and non human primates that can then be successfully adapted to human subjects. In the following subchapter I will discuss the cellular and molecular mechanisms controlling postnatal neurogenesis.

3. Factors and mechanisms involved in neurogenesis in the rodent brain:

Starting from embryonic brain development through out adulthood a vast number of factors are involved in the proper regulation of neurogenesis. For better understanding of the role, importance and functions of these molecular cues we can broadly classify them into intrinsic and extrinsic factors. *Intrinsic factors* are those that are programmed into a cell from its birth, they control crucial events throughout the lifetime of the cell. *Extrinsic factors* are those that are present in the environment of the cell, produced by other cells and released into the environment or anchored to the extracellular matrix surrounding cells. These cues can be growth factors, neurotransmitters or hormones. The extrinsic factors act along with

the intrinsic programme in series of complex bidirectional interactions to modulate the various steps of neurogenesis.

In postnatal brains the first step to becoming a new interneuron of the OB starts with proliferation, which is controlled by a multitude of both intrinsic and extrinsic factors like several transcriptional factors, neurotransmitters, hormones, growth factors (Ming and Song, 2005; Lledo et al., 2006; Ming and Song, 2011). After production the interneuronal progenitors undertake tangential migration in chains along the RMS and maintaining a constant interaction with the microenvironment. These interactions involve multiple receptor ligand complexes that regulate contact mediated attraction and repulsion of migrating cells (Lledo et al., 2006). Upon reaching the OB core, chain detachment and radial migration are initiated under the guidance of external factors like tenascin-R (TNR), reelin, prokineticin (PK2) and Insulin like growth factor 1(IGF1) (Hack et al., 2002; Saghatelyan et al., 2004; Ng et al., 2005; Hurtado-Chong et al., 2009). Following the detachment and radial migration of these progenitors they differentiate into the granule and periglomerular neurons which then integrate/mature into the network (Carleton et al., 2003; Ming and Song, 2005; Lledo et al., 2006; Ming and Song, 2011).

3.1. Proliferation:

Multipotent precursors give rise to both neurons and glia in the forebrain. As mentioned earlier the intrinsic programming of multipotent precursors in the SVZ niche includes several factors. Proliferation of SVZ progenitors can be also subject to modulation by microenvironment. A wide variety of molecular cues have profound effects on proliferation and production of neuronal precursors.

Transcription factors: Several transcription factors like retinoblastoma and its related factors necdin along with the E2F family are important cell cycle regulators governing proliferative activity (Lledo et al., 2006). In adult mice lacking E2F1 the level of proliferation of the progenitors is drastically lower (Yoshikawa, 2000;

Cooper-Kuhn et al., 2002). ***Guidance cues:*** Similar to the neogenin (neo1) and uncoordinated like protein (Unc), signaling pathways ephrins (Eph) and their receptors also control cell proliferation. In the SVZ EphB1-3 and EphA4 are expressed alongside their ligand Ephrin-B. Infusion of EphB2 or ephrin-B2 into the lateral ventricle has been shown to be disruptive to migrating neuroblasts; instead they seem to increase the stem cell proliferation thus favouring proliferation over migration and differentiation (Conover et al., 2000). Ephrin-A2 has also emerged as a major class of regulators molecules in the SVZ niche. Lack of this molecule in the SVZ precursors seems to affect their cell cycles (Conover et al., 2000). Similarly in the adult brain the interaction of Ephrin-A2 with its receptor EphA7 results in aberrant proliferation (Holmberg et al., 2005). ***Neurotransmitter:*** GABA signaling between astrocytes and neuroblasts is well known to control stem cell proliferation in a feed back loop (Liu et al., 2005). This interaction may be crucial therefore to maintain a balance between the amplification and the mobilization of progenitors (Liu et al., 2005). Glutamate while signalling via its metabotropic or NMDA receptors down regulates proliferation, whereas signaling via the AMPA class of receptors seems to up regulate proliferation (Bernabeu and Sharp, 2000; Bai et al., 2003; Yoshimizu and Chaki, 2004). Nitric oxide has also been implicated in slowing down proliferation in the SVZ, whereas serotonin and dopamine promotes proliferation (Baker et al., 2004; Banasr et al., 2004; Moreno-Lopez et al., 2004; O'Keeffe et al., 2009). ***Large secreted proteins***: like the secreted soluble form of amyloid precursor protein (sAPP) has also been shown to partake in control of proliferation in the SVZ. sAPP binds to progenitors expressing EGF in the SVZ, and sAPP infusion resulted in an increase proliferation of EGF responsive cells (Caille et al., 2004). On the other hand a blockage of sAPP secretion or down regulation of its synthesis decreased the proliferation of EGF responsive cells (Caille et al., 2004). Another study used the (3xTg-AD) mouse model for AD, mutant for beta-amyloid precursor, presenelin-1 and tau and noted that in both the DG and SVZ of these mice the numbers of proliferating cells, early lineage progenitors and neuroblasts at middle age was reduced. This then resulted in the

reduction of new neurons being integrated into the hippocampus and OB (Hamilton et al., 2010). The potent morphogen ***sonic hedgehog (shh)*** is also known to perform multivariate roles ranging from neuronal specification, oligodendrocyte precursor induction and proliferation and survival (McMahon et al., 2003). The ***cell adhesion molecule*** CD24 also takes part in controlling the proliferation of progenitors both in the SVZ and the SGZ. CD24 is a highly glycosylated molecule anchored onto a glycosylphosphatydyl inositol entity. In the SVZ of mice lacking this molecule a dramatic increase is observed in the number of fast and slow proliferating cells (Belvindrah et al., 2002). Several ***growth factors*** are also implicated in increasing proliferation like; ciliary neurotrophic factor (CNTF), heparin-binding epidermal growth factor (HB-EGF), fibroblast growth factor (FGF), insulin growth factor 1 (IGF1), transforming growth factor (TGF) and vesicular endothelial growth factor (VEGF) (Craig et al., 1996; Zigova et al., 1998; Jin et al., 2002; Emsley and Hagg, 2003; Zhu et al., 2003). ***Several hormones*** are also implicated in controlling proliferation like prolactin in both males and females (Shingo et al., 2003; Mak and Weiss, 2010). Thyroid hormone, pregnenolone and pituitary adenylate cyclase-activating polypeptide (PACAP) are also known to increase proliferation of progenitors (Mercer et al., 2004; Lemkine et al., 2005; Mayo et al., 2005).

3.1.1 Proliferation in the diseased brain:

Several brain pathologies induce the up regulation or down regulation of proliferation in the SVZ. Alzheimer's disease and schizophrenia have been shown to decrease proliferation in the hippocampus.

The SVZ of both rodents and humans have been studied extensively in Parkinson's disease (PD), Alzheimer's disease (AD) and Huntington's disease (HD). The mice suffering from HD have largely expanded SVZs with increased progenitor proliferation, which was also seen in the quinolinic acid rat model for HD (Tattersfield et al., 2004). Another study also showed that in the R6/2 mouse model

progenitor cells in the SVZ/RMS migrated towards the striatum (Batista et al., 2006). However, a study performed on the same mouse model showed that progenitors were recruited to the striatum without increased SVZ proliferation (McCollum et al., 2013). Interestingly R6/2 mice were shown to also have impaired olfactory interneuron integration in the OB (Kohl et al., 2010). In human patients suffering with HD the SVZ has been shown to have increased in thickness accompanied by increased progenitor proliferation, which can be attributed to the presence of various factors like neurotrophic factors, neurotransmitter receptors and proliferative factors (Curtis et al., 2003; Curtis et al., 2005).

PD has also been studied in rodents and humans. In the 6-OHDA mouse model for PD, the SVZ progenitor proliferation was seen to be reduced but progenitors were still being re-routed into the striatum (Baker et al., 2004; Freundlieb et al., 2006; Liu et al., 2006; Borta and Hoglinger, 2007; van den Berge et al., 2011). However, two studies done on human PD patients describe very different results. The first study reported that D2 receptor mediated innervations and signaling in the SVZ profoundly increased the proliferation in the SVZ and proliferation is drastically reduced in patients suffering from PD (Hoglinger et al., 2004). In the second study however, the authors describe the SVZ of PD patients to be similar to that seen in normal brains and no changes in proliferation was seen (Van Den Berg et al., 2011). Studies done in mouse models and in humans suffering from AD, however, provide more consistent results. Studies using the PS1deltaE9 mice lacking Presenilin 1 and the triple transgenic (3XTg-AD) mouse model, mutant for beta-amyloid precursor, presenelin-1 and tau have shown that progenitor proliferation in the SVZ is reduced (Rodriguez et al., 2009). These studies also show that the reduction in proliferation is worsened with age and that precursors exit prematurely towards a neuronal fate (Rodriguez et al., 2009; Veeraraghavalu et al., 2010). In line with rodent studies human patients of AD also have reduced proliferation of SVZ progenitors. Neuronal differentiation of cultured human and rodent progenitors was impaired by amyloid beta peptide (Aβ) that also promotes apoptosis (Haughey

et al., 2002). Proliferation of Musashi1 (Msi1) positive progenitors in the SVZ of human AD patients was also seen to be significantly reduced (Ziabreva et al., 2006). Impaired SVZ proliferation also affect olfactory discrimination in patients suffering from AD (Doty, 2012).

3.2.1 Factors involved in tangential migration in RMS:

Interneuron progenitors born in the SVZ must reach the OB in order to play their role in the bulbar network. In order for these newborn neurons to reach their final destination they need to migrate via a specialized route, the RMS. The migrating neuroblasts are under the stringent control of multiple factors that influence the directionality of their migration and also the speed at which they migrate. The migrating neuroblasts receive these crucial factors via contact information brought about by cell-cell adhesion or interactions with the extracellular matrix (ECM), blood vessels or the glial tube through which they migrate and chemoattractive/ chemorepulsive secreted cues (Ming and Song, 2005; Lledo et al., 2006). After reaching the OB, neuroblasts migrating in chains detach under the influence of specific guidance cues, followed by radial migration which again is controlled by a variety of cues. Both tangential migration and radial migration will be covered in this subchapter that will then allow us to deal in greater detail about the extracellular matrix of the OB and the tenascin family of proteins that is crucial to one of the studies further elucidated in my thesis.

Soon after production the precursor cells adhere to one another to form the chains that are characteristic of chain migration in the RMS. Owing to the fact that these cells are in such proximity to the ventricle, the effects of cerebrospinal fluid (CSF) on migration is crucial. The ependymal cells lining the ventricular cavity beat their cilia in a rhythmic fashion in order to create the chemorepulsive gradients so crucial to proper oriented migration (Sawamoto et al., 2006). Cell adhesion molecules like the neural cell adhesion molecules (NCAMs) have been implicated in migration of the neuronal progenitors to the OB (Bonfanti and Theodosis, 1994; Cremer et al., 1994). PSA-NCAM the polyscialated version of the NCAM is

expressed by proliferating and migrating cells in the RMS and also by subpopulations of granule and periglomerular cells in the MOB and AOB (Bonfanti and Theodosis, 1994). The lack of PSA-NCAM is shown to affect tangential migration in the RMS leading to cells accumulating near and around the lateral ventricle with a concomitant reduction in the size of the OB (Ono et al., 1994; Rousselot et al., 1995; Hu et al., 1996).

Cytoskeleton remodeling proteins like Stathmin and β-III tubulin also play important roles in tangential migration. Stathmin is a cytosolic phosphoprotein that is conserved among vertebrates; it has been implicated in the physiological regulation of microtubule dynamics (Belmont and Mitchison, 1996; Camoletto et al., 1997). Microtubule dynamics is crucial to many cellular processes like mitosis, cell migration and maintenance of cell shape. It must be noted that stathmin is expressed by all cells expressing PSA-NCAM in the RMS (Camoletto et al., 1997). The other cytoskeleton remodelling protein of interest is β-III tubulin that all neuroblasts in the RMS express. This class III tubulin may favour a decreased rate of microtubule polymerization, increased plasticity of microtubule assembly thus bringing about rapid changes in cell conformation (Menezes and Luskin, 1994; Peretto et al., 1997).

The extracellular matrix around these migrating cells also participates in the proper migration. The matrix metalloproteases (MMPs) are also expressed in the RMS and play a role in neuron migration (Bovetti et al., 2007). Integrins are also expressed in the RMS and have been thought to be important in making the RMS a migration-permissive zone (Emsley and Hagg, 2003). Integrins in the RMS interact with the laminins in the ECM and act to guide migrating neuroblasts (Emsley and Hagg, 2003). Disruption of this signaling is thought to disrupt the chain migration as integrins favour dispersion of the neuroblasts (Emsley and Hagg, 2003). This function of integrins on chain migration could be due to their association with laminins that act as potential ligands for certain integrins (Murase and Horwitz, 2002). Another factor affecting chain migration is A Disintergrin And

Metalloprotease (ADAM) which is also expressed in the RMS (Murase et al., 2008). In mice lacking ADAM the structure of the RMS is drastically altered along with severe perturbation in normal migration in the RMS like loss of directionality and lowered rate of migration (Murase et al., 2008).

Insulin-like growth factor 1 (IGF1) IGF1 has been described as a `departure` factor in the adult avian SVZ (Jiang et al., 1998). In vitro migration assay of adult SVZ derived progenitors has shown that IGF1 acts as an inducer of migration and directs migrating cells by both chemotaxis and chemokinesis (Maucksch et al., 2013). The same study showed the ectopic expression of IGF could reroute progenitors migrating in the RMS to the striatum (Maucksch et al., 2013). This factor is implicated in migration in the RMS by initiating exit of precursor cells from the SVZ and starting of migration in the RMS (Hurtado-Chong et al., 2009). In the absence of IGF1 these cells are seen to be stalled within the SVZ (Hurtado-Chong et al., 2009).

SLIT and Robo receptor pathway is important for tangential migration in the RMS. Slits 1 and 2 are expressed in the choroid plexus, septum and ventricular zone and are shown to repel the migrating chains towards the OB (Wu et al., 1999; Nguyen-Ba-Charvet et al., 2004). Slits achieve this by not only orienting cells for migration but by also acting on their leading processes and orienting them as well. In mice lacking slit1 the cells migrate abnormally in the RMS as they are seen to leave the RMS and enter the corpus collosum (Nguyen-Ba-Charvet et al., 2004). Intriguingly type A and type C cells in adult mice are also slit positive suggesting other roles than just repulsion for this cue (Wu et al., 1999; Nguyen-Ba-Charvet et al., 2004). To better understand the functions of Slit1 expressed on neuroblasts, time-lapse recordings of neuroblasts migrating in the RMS of Slit1 knockout mice derived organotypic cultures was performed. The results showed that not only was migration hampered, but also the morphology and arrangement of the astrocytic processes was significantly affected (Kaneko et al., 2010). These results suggested that Slit1 released from the neuroblasts is involved in the formation and

maintenance of the glial tube through which they migrate. The study also went on to show that this affect was mediated by the Slit/Robo interaction as the astrocytes forming the glial tube expressed high levels of Robo receptors. Thus the study concluded that the neuroblasts release Slits and clear a path for their own migration by repelling astrocytic processes (Kaneko et al., 2010). **Ephrins** are another group of chemotactic molecules that plays a role in migration of neuroblasts in the RMS. These receptor tyrosine kinase EphB1-3 and EphA4 and their transmembrane ligand ephrin B2/3 are expressed by cells in the SVZ (Conover et al., 2000). By employing electron microscopy the interaction between ephrin-B ligand and astrocytes of SVZ was established (Conover et al., 2000). Infusion of either EphB2 or ephrin-B2 into the ventricle showed that apart from affecting proliferation, migration of neuroblasts in the RMS was also perturbed (Conover et al., 2000). The other receptor tyrosine kinase of interest is ErbB4. ErbB4 ligands belonging to the neuregulin family; NRG1-NRG3 is expressed within the RMS or adjacent to the RMS (Anton et al., 2004). Mice deficient in ErbB4 were noted to have altered chain organization and subsequently migration was also altered (Anton et al., 2004). Apart from repulsive signals, attractive cues were also described to be involved in the migration of interneuron precursors in the RMS. A study reported that explants of OB had the ability to attract chains of neuroblasts migrating out from SVZ explants; the exact identity of this attractive cue was however not discovered by this study (Lin and Rao, 2003).

Structural features important in tangential migration: Migration of neuroblasts in the RMS is also crucially dependent on structural factors that affect their migration. The arrangement of GFAP positive astrocytes around the chains of migrating neuroblasts in the RMS forms a meshwork tunnel called the glial tube (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996; Peretto et al., 2005). This structure is suspected to have functions that are yet to be discovered. The vasculature of the RMS is the other structural factor crucial to the migration of neuroblasts (Snapyan et al., 2009; Whitman et al., 2009). The role of vasculature in

the migration of neuroblasts in the RMS has been examined and the significant roles they play have been uncovered. The blood vessels in the RMS was seen to be present at a higher density than in any other part of the brain (Snapyan et al., 2009; Whitman et al., 2009). In addition these vessels were oriented parallel to the RMS. The neuroblats migrating via the RMS was seen to be in close apposition to these vessels which was confirmed by electron microscopy (Whitman et al., 2009). This same phenomenon has also been reported of late to occur in adult zebra fish RMS (Kishimoto et al., 2011). Time-lapse video-imaging of cells migrating in the RMS shows cells migrating using the blood vessels as scaffolds (Snapyan et al., 2009). The blood vessels not only provide a structural support for neuroblasts migration, but they also secrete molecular cues required for cell migration. The endothelial cells of the vessels synthesize and release brain derived neurotrophic factor (BDNF) that binds to the p75NTR receptors expressed on the neuroblasts (Snapyan et al., 2009).

3.2.2 Radial migration:

At the end of tangential migrations, the cells enter the OB. In the OB these cells must undertake radial migration to integrate into the GCL and GL. In order to achieve this, the cells must detach from the migratory chains, make a 90° turn; take up radial mode of migration. These processes are controlled by factors that cause the arrest of migration, chain detachment and radial migration. In this subchapter I will discuss the signals and cues documented in literature to take part in the above mentioned processes.

On arrival in the OB, the neuroblasts must be initiated to migrate radially. At the junction of the RMS and OB the migrating neuroblasts must express certain proteins in order to enter the OB. One such factor is aristaless-related homeobox gene (ARX). In ARX mutant mice a large variety of defects were observed such as inability of the interneurons to enter the OB resulting in the backup of migrating

interneurons at the junction of the rostral part of the RMS and the OB (Yoshihara et al., 2005).

Transition of tangentially migrating neuroblasts to radially migrating ones are controlled by detachment factors and those that encourage radial migration like reelin, tenascin-R (TNR), prokineticin 2 (PK2) and insulin-like growth factor1 (IGF1) (Hack et al., 2002; Saghatelyan et al., 2004; Ng et al., 2005; Hurtado-Chong et al., 2009). **Reelin**, a large secreted glycoprotein produced in the mitral cell layer of the OB binds to its receptors apolipoprotein E receptor 2 (ApoER2) and very low density lipoprotein receptor (VLDLR) resulting in the phosphorylation of the adaptor protein disabled-1 (Dab1). These reactions are essential for its function as a detachment signal for the neuroblasts migrating in chains (Hack et al., 2002). **Tenascin-R (TNR)** an extracellular matrix glycoprotein of the OB has been shown to play a dual role in controlling the detachment and radial migration of neuroblasts in the adult OB (Saghatelyan et al., 2004). TNR expression has also shown to be activity dependent (Saghatelyan et al., 2004). The absence of this protein during adulthood reduces the number of new interneurons integrated in the adult OB by severely retarding their radial migration (Saghatelyan et al., 2004). **Prokineticin 2** (PK2) is another secreted protein that along with TNR and reelin acts as detachment signals for tangentially migrating neuroblasts. PK2 is a cysteine rich protein expressed in the GCL and GL of the OB. Where as its G-protein coupled receptor has been identified as (PKR2) expressed in the SVZ and the RMS. This factor has proven to be a chemoattractant for SVZ-RMS-OB cells in in vitro tests (Ng et al., 2005). Another guidance cue of interest is IGF1, highly produced during early development along with its receptor (IGFR); this growth factor is down regulated after birth (Hurtado-Chong et al., 2009). However, in the OB the level of this protein and its receptor is constantly maintained throughout life (Hurtado-Chong et al., 2009). The lack of this protein has been seen to disrupt the normal layering of the OB and also the normal migration of interneurons (Hurtado-Chong et al., 2009).

Apart from these secreted factors and ECM molecules, the mitral cells of the bulb and a newly described class of radial glia have also been shown to have some role in the radial migration and placement of interneurons in the adult OB (Valero et al., 2007; Emsley et al., 2012). Mitral cells in the Purkinje cell degenaration mutant mice (pcd/pcd mice) are degenerated and this loss affects the radial migration of interneurons in the OB of these mice along with mis- positioning of the new interneurons due to a lack of synaptic targets (Valero et al., 2007). Another cell type in the OB implicated in the radial migration and positioning of the newly born interneurons is a recently described class of radial glia. This new class of radial glia are seen spanning the GCL and the mitral cell layer, these cells have been characterised as radial glia distinct from astroglia, oligodendrocytes and tanycytes (Emsley et al., 2012). These cells have been described as being produced within the RMS of the OB during development and adulthood. Very much like what has been reported during tangential migration studies have shown that radial migration of neuroblasts in the OB can also take place in association with blood vessels (Bovetti et al., 2007; Snapyan et al., 2009).

3.2.3 Migration in the diseased brain:

Following brain damage like degenerative diseases, ischemic stroke and traumatic brain injury (TBI), the SVZ precursors normally migrating via the RMS to the OB can be re-routed to the site of injury. In order for these precursors to migrate to and undertake repair at the site of insult they are guided into these regions by cytokines and chemokines produced during normal inflammatory responses. In addition several growth factors and neurotrophic factors are also implicated in the repair (Christie and Turnley, 2013).

The vast majority of studies performed to understand the ectopic migration and differentiation of SVZ derived precursors in response to brain insult is normally performed in models of ischemic stroke. Several studies have been done in adult mice and rat models of transient middle cerebral artery occlusion (tMCAO) in order

to understand how these endogenous mechanisms of repair take place. Despite several adverse conditions in the adult brain for such repair to be undertaken; lack of manoeuvring space, paucity of signaling molecules and growth factors not withstanding some levels of repair have been documented in the adult brain. Studies have shown that in response to injury the SVZ niche increases progenitor proliferation and these progenitors migrate individually or in chains from the SVZ via the RMS or the lateral cortical stream to the site of injury where they integrate and replicate all the phenotypes of the neurons lost to injury (Arvidsson et al., 2002; Parent et al., 2002; Jin et al., 2003). Studies have also shown that angiogenesis and neurogenesis in the injured brain are causally linked. Immature neurons are seen to associate with the remodelling blood vessels and astrocytic processes and are thought to respond to vasculature produced stromal derived factor 1 (SDF1), angiopoietin 1 (Ang1) and BDNF (Ohab et al., 2006; Yamashita et al., 2006; Grade et al., 2013). PI3 kinases promote migration of neuroblasts from adult SVZ after stroke injury to the brain. This is thought to be achieved via the PI3K/Atk signal transduction pathway (Katakowski et al., 2003).

3.3 Maturation and Integration:

Newly generated interneuron precursors migrate tangentially to reach the OB where they undertake radial migration followed by differentiation in to PGs and GCs (Kishi, 1987; Luskin, 1993; De Marchis et al., 2001; Carleton et al., 2003). These cells then need to integrate in to the adult bulbar network where they play a key role in modulating the output neurons (Imayoshi et al., 2008; Breton-Provencher et al., 2009; David et al., 2013). The newly generated interneuron`s integration into the adult network is dictated by many factors like sensory activity and innervations from OB and cortical areas (Whitman and Greer, 2007; Bovetti et al., 2009; Panzanelli et al., 2009).

Fate determination of a progenitor to become a neuron is the first step. This process is carefully co-ordinated by many factors one of which is BMP. It has been shown that BMP signaling instructs adult neural progenitors to adopt a glial fate

(Lim et al., 2000). The presence of a BMP antagonist like noggin, locally secreted from SVZ epithelial cells, has been shown to be partially responsible for neural fate by suppressing the gliogenic BMP (Lim et al., 2000). In the hippocampus neuronal fate over glial fate is mediated by the presence of neurogenesin1 secreted from the SGZ astrocytes (Ueki et al., 2003). Another morphogen of interest is the Wingless-Type MMTV Integration Site Family (Wnts) members. In the hippocampus this morphogen has been shown to induce neurogenic fate both in vivo and in vitro (Lie et al., 2005). These extracellular signaling mechanisms act in part by interacting with cellular epigenetic mechanisms (Hsieh and Gage, 2004). Other factors influencing a neuronal fate include interactions of chromatin remodeling enzymes with neurogenic factors that is important for maintaining genomic stability and also the fate choice of adult neural progenitors by noncoding RNAs (Zhao et al., 2003b; Kuwabara et al., 2004).

Migrating precursors in the RMS are also influenced by ambient GABA. Tangentially migrating neuroblast precursors express extrasynaptic $GABA_A$ receptors before acquiring AMPA receptors, the NMDA receptors are expressed while the cell is migrating radially (Belluzzi et al., 2003; Carleton et al., 2003). The principal output neurons of the OB, the mitral cells innervate and provide functional input onto newly generated GCs as soon as ≈ 10 days after they are generated (Whitman and Greer, 2007). Using synaptic markers and electron microscopy it has been shown that GCs integrate into the reciprocal synapse circuitry in the EPL around 21 days after their birth. These inputs on the newly generated GC were thought to contribute to the differentiation, maturation and synaptic integration of these GCs even before spines are formed to make output synapses (Whitman and Greer, 2007). At this stage the GCs make dendrodenritic synapses with mitral cell lateral dendrites in the EPL using their dendritic shafts which might contribute to spine formation later (Panzanelli et al., 2009). It has been shown that GCs fire action potentials earlier than PGs after synaptic input has occurred (Belluzzi et al., 2003; Carleton et al., 2003). Several studies have shown the role of sensory

activity on the integration and fate maintenance of adult generated bulbar interneurons (Maruniak et al., 1989; Corotto et al., 1994; Rochefort et al., 2002; Saghatelyan et al., 2004; Alonso et al., 2006; Mandairon et al., 2006; Bastien-Dionne et al., 2010; Kato et al., 2012). Studies have shown that differentiation of all types of interneurons in the bulb occurs in a window of 40-75 days after they are generated (Bagley et al., 2007).

4 The extracellular matrix of the central nervous system:

The extracellular matrix (ECM) can be described as a complex network of polysaccharides such as glycosaminoglycans and proteins secreted by surrounding cells. The ECM which is normally produced by the local cells is released to the extracellular milieu and compacts around interneurons to give rise to the perineuronal nets (PNN). PNN was described more than a centaury ago by Camillo Golgi.

4.1 Structure of the EMC/PNN:

The ECM is composed of two major classes of molecules: the polysaccharides comprised from chains of glycosaminoglycans (GAGs) linked to proteins to give proteoglycans and fibrous proteins like collagen, elastin, fibronectin and laminins.
Apart from the above mentioned structural components, the EMC/PNN abound in condroitin sulfate proteoglycans (CSPGs) like aggrecan, brain link protein (Bral), brevican, neurocan, phosphocans and versican. Heparin sulfate proteoglycans (HSPGs) like agrin and several extracellular glycoproteins like reelin, netrin1, TNR/TNC, slit1/2, Sema3A, thrombospondins, leucine-rich glioma activated (LGl1) (Bruckner et al., 2006; Dityatev et al., 2010). Apart from cell- cell interactions, cells also constantly interact with the ECM via integrins heterodimers and proteins from the Ig-CAM family. In most brain regions the ECM is compacted into lattice like accumulations of proteoglycans around interneurons. These lattices are typically built of aggregated proteoglycans, hyaluronan and TNR along with the two link proteins cartilage link protein 1 (Crtl1) and brain link protein 1 (Bral1) typically seen

ensheathing the soma, the initial axon segments and the dendrites of the neurons (Bruckner et al., 1996; 2006; Vo et al., 2013). This specialised lattice that plays many crucial developmental, structural and functional roles is known as the PNN.

4.2 Functions of the EMC/PNN:

The ECM has been implicated in several functions like cell migration, storage of soluble growth factors, promotion of neurite outgrowth or the inhibition of neuritogenesis by formation of tissue boundaries. The formation of the PNN around the interneurons of the visual cortex for example has been identified as a key event in the closing of the critical period (Carulli et al., 2010). The PNN has also been implicated in neuroprotection, owing to the polyanionic character of the PNN. Because of highly charged chondrotin sulfate glycosaminoglycans and hyaluronan components it has been proposed that the PNN is involved in local ion homeostasis (Suttkus et al., 2012). The PNN could scavenge and bind redox-active ions, thereby reducing local oxidative potential (Suttkus et al., 2012). Studies have recorded that PNN ensheathed neurons have significant lower degeneration rate when compared to neurons without PNN (Suttkus et al., 2012). The PNN is also involved in the wound healing of the spinal cord (Kwok et al., 2011).

4.3 The tenascin family of extracellular matrix proteins:

The tenascins (TN) are a highly conserved family of large oligomeric glycoproteins found in the ECM. This family is comprised of five members (TN-C, TN-R, TN-W, TN-X and TN-Y) (Jones or Jones, 2000). TNC was the first of the family members to be discovered, and is one of the first proteins to be shown to have adhesion modulatory function by antagonising cell attachment with fibronectins. The tenascins are expressed during development and during adulthood in some cases, these proteins are also involved in pathology such as tissue damage and tumors (Hsia and Schwarzbauer, 2005).

The tenascins have several conserved features that are shared by all the family members only differing in number of each feature. These proteins all share amino-terminal heptad repeats, epidermal growth factor (EGF) like repeats, fibronectin type III domain repeats and carboxyl-terminal fibrinogen like globular domains. These proteins can occur as multimeric units; the heptad repeats lying in a highly conserved amino terminal of this protein allows individual subunits to assemble into trimers (**Fig. 1.9**). In some tenascins additional cysteine residues, the tenascin assembly (TA) domain allows the assembly of two such trimers to produce further hexamers. TNC is an example of a hexamer, with six arms. I will now discuss each of the tenascins briefly before describing TNR in detail in the next subchapter.

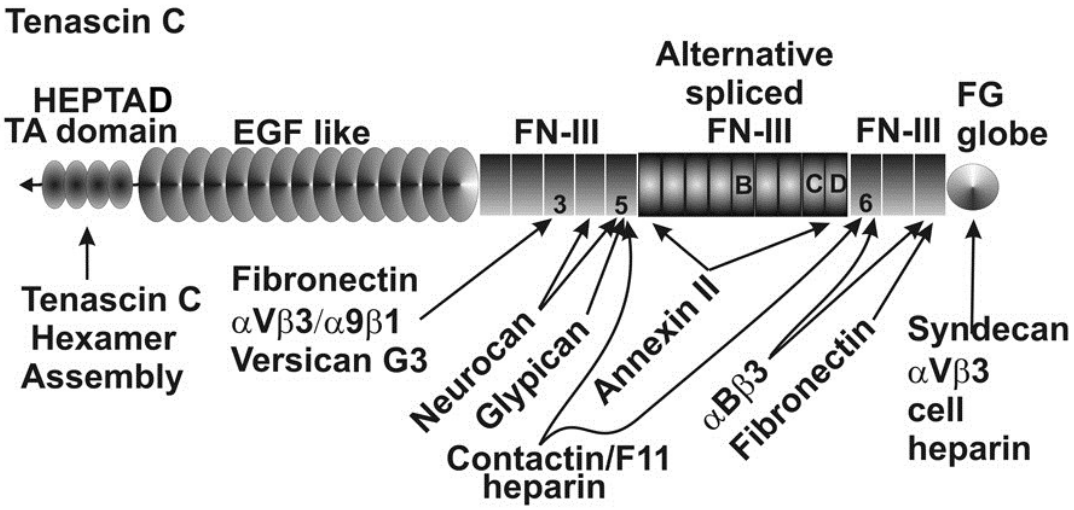

Figure 1.9: Tenascin-C domain structure

Tenascin-C domain structure, binding sites and alternative splicing. Heptad repeat hexamer assembly domain, EGF-like domains, type III fibronectin and fibrin globular domains are as indicated (Acott and Kelley, 2008).

4.3.1 Tenascin-C (TNC):

TNC is a hexamer normally ranging in weight from 200 to 300kDa. TNC also shows the most number of isomers and splice variants among the tenascins. Being the first of the family to be discovered, TNC remains the best investigated of the

tenascins. A large number of binding sites for a wide variety of interacting partners has been identified on TNC, including integrin cell surface receptors proteoglycans, cell adhesion molecules of the immunoglobulin (Ig) family along with those for annexin1 receptor protein and EMC components such as heparin, fibronectin and collagen. TNC functions to block the focal adhesion kinase and activate the downstream Rho signaling mediated by fibronectin and hence controls the extent of tissue remodeling in wound healing (Midwood and Schwarzbauer, 2002). TNC on the other hand stimulates Wnt and other growth promoting pathways. In cultures of tumor cells grown on fibronectin TNC was shown to down regulate the Wnt inhibitor Dickkopf 1, consequently Wnt signaling was enhanced through stabilization of β catenin (Ruiz et al., 2004). TNC is normally expressed during embryonic development and declining postnatally. However, during tissue remodeling in wound repair or during tumorigenesis its expression is up regulated (Orend, 2005).

4.3.2 Tenascin-R (TNR):

TNR is a protein with two isoforms at 160 and 180 KDa weight. Like TNC, TNR can also oligomerize forming trimers with two or three polypeptides. TNR is exclusively expressed in the CNS overlapping partially with the expression of TNC. Studies done on the expression of TNR has shown that this protein is expressed during the peak of myelination (Bartsch et al., 1993). In vitro studies have shown that this protein influences neural pattern formation through adhesive and non-adhesive effects in cell matrix interactions (Pesheva and Probstmeier, 2000). TNR will be addressed in greater detail in the next subchapter as it pertains to the first publication included in my thesis as chapter II.

4.3.3 Tenascin-X (TNX):

TNX is a large protein with a weight of 400 KDa expressed during development in many organs of the animal. During adulthood however it is not detected in the brain. TNX does not have the cysteine rich TA domain and thus does not form large hexamers. TNX has been directly linked to a human pathology. TNX

deficiency has been associated with a variant of a heritable connective tissue disorder known as Ehler-Danlos syndrome associated with fibrillar collagen defects (Schalkwijk et al., 2001).

4.3.4 Tenascin-W (TNW):

This protein was first discovered in zebra fish (Weber et al., 1998). TNW has been associated with osteogenesis; it has been shown to be involved in osteoblast maturation/ mineralization (Mikura et al., 2009). TNW has been described as a good marker for most human solid tumors (Brellier et al., 2012).

4.3.5 Tenascin-Y (TNY):

TNY was identified in chickens. In contrast to all the above mentioned tenascins this comparatively newly discovered protein had an additional domain rich in serines and prolines interrupting the fibrinogen domains (Hagios et al., 1996). It has been seen to be expressed in several tissues of chickens especially the connective tissue of skeletal muscles. This protein has been described to be analogous to mammalian TNX (Hagios et al., 1999).

4.4 Functions of the tenascins:

Several distinct functions have been shown for the tenascins owing to the various family members, the tissues of expression and the time of expression. The lack of TNC was shown to result in subtle changes in the animal's behavior and also in the process of wound healing (Mickie and Tucker, 1999). Organ formation was also affected in TNC deficient mice. In TNC deficient mouse foetuses the lungs failed to develop properly due to reduced airway branching (Roth-Kleiner et al., 2004). TNX as mentioned earlier is one of the tenascins that has a direct implication in a human disease. Ehler-Danlos syndrome results from the lack of TNX (Burch et al., 1997). The syndrome is a group of inherited disorders that affect connective tissues that result from affected collagen fibrillogenesis (Mao and Bristow, 2001). Patients suffering from this syndrome also have abnormally elastic fibers (Zweers

et al., 2004). The functions of TNR will be discussed in detail in the next subchapter.

4.5 Tenascin R (TNR):

4.5.1 Structure of TNR:

TNR is a typical member of the tenascin family with cysteine rich amino terminal regions, epidermal growth factors-like domains, fibronectin type III homologous repeats and a domain homologous to fibrinogen (**Fig. 1.10**) (Jones and Jones, 2000). TNR is expressed as two isoforms with 160KDa and 180KDa weight respectively due to the alternate spicing of the sixth fibronectin type III repeat. TNR subunits have been described to have typically 4.5 EGF-like repeats and 8-9 fibronectin type IIII repeats (Hsia and Schwarzbauer, 2005).

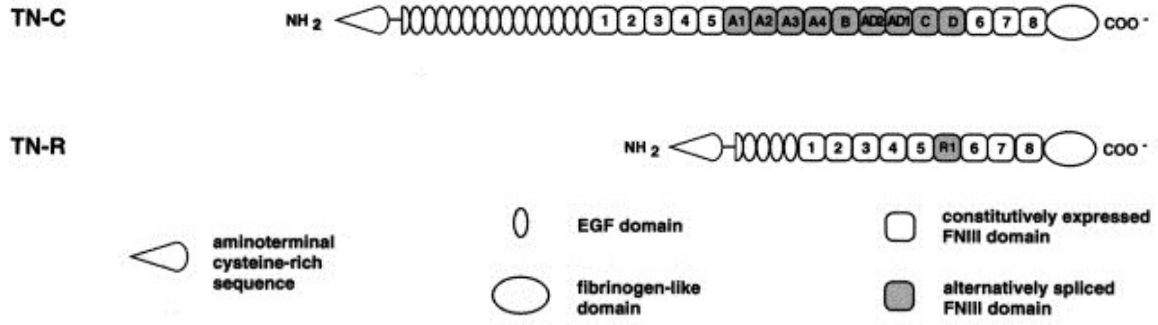

Figure 1.10: Tenascin-R structure

Tenascin-R in comparison with Tenascin-C. Adapted from (Joester and Faissner, 2001).

4.5.2 Expression of TNR:

Unlike the other members of the tenascin family, TNR expression is exclusively restricted to the CNS. TNR expression was examined using in situ hybridisation and was shown to be present in the cerebellum oligodendrocytes from postnatal day 7-14 and also in the hippocampal interneurons (Jones and Jones, 2000; Fuss

et al., 1993). In the OB, TNR expression was also exclusive to postnatal stages of development peaking at around one month after birth (David et al., 2013).

TNR is a member of the perineuronal net along with hyaluronan, phosphacan and chondroitin sulphate proteoglycans surrounding most but not all interneurons of the CNS. Studies in TNR knockout mice showed severe disruption of the molecular scaffolding of the ECM components in the perineuronal net (Bruckner et al., 2000). A recently published study documents for the first time the spatiotemporal distribution of TNR in the developing and adult human cerebral cortex (El Ayachi et al., 2011). This study showed that the basal telenchephalon, the cortical plate, the striatum, the thalamus and the cortex expresses TNR during varying times during gestation. This expression persists and intensifies with age. This study also suggests a role for TNR in corticogenesis (El Ayachi et al., 2011).

4.5.3 Functions of TNR:

TNR is a multivariant protein with a wide variety of roles. TNR has been shown to bind to the beta2 subunit of sodium channels which acts like a cell adhesion molecule (CAM) (Srinivasan et al., 1998). This crucial binding was shown to be important for the localization of sodium channels in the axon initial segment and at the nodes of Ranvier (Ffrench-Constant et al., 1986; Bartsch et al., 1993). In mice deficient for TNR the action potential conduction velocity in optic nerve axons is severely impaired (Srinivasan et al., 1998; Xiao et al., 1999; Weber et al., 1999). In the hippocampus, TNR is implicated in modulation of long term potentiation (LTP) and certain types of learning (Saghatelyan et al., 2001; Morellini et al., 2010). In mice lacking TNR, N-methyl-D-aspartate (NMDA) mediated LTP was impaired which was accompanied by an increased basal excitatory synaptic transmission onto the CA1 neurons due to altered inhibition (Saghatelyan et al., 2001). The reduction in perisomatic inhibition was attributed to the lack of HNK1 carbohydrate which is normally present on the TNR backbone and mediates the modulation of perisomatic inhibition and synaptic activity (Saghatelyan et al., 2000; Saghatelyan et al., 2001). TNR was shown to play an important role in the regulation of the

number and structure of perisomatic inhibitory synapses (Nikonenko et al., 2003). An in vitro study done on primary cultures of hippocampal neurons and astrocytes from TNR deficient animals showed that the synapse formation and stabilization was severely impaired 3 weeks into the culture; and electrophysiological recordings made from these neurons showed that the frequency of inhibitory and excitatory postsynaptic currents (IPSCs and EPSCs) was reduced (Geissler et al., 2013). Attempts at a rescue reported that even if TNR (-/-) neurons were co cultured with wild type astrocytes they still exhibited same deficiencies (Geissler et al., 2013). This study then concluded the presence of the TNR gene itself seemed to be necessary for the proper functioning of these neurons (Geissler et al., 2013). Another study concentrating on the role of TNR on the dentate gyrus reported that in mice lacking TNR the ratio of inhibitory to excitatory neurons was increased (Morellini et al., 2010). On the level of behaviour, adult mice that are TNR (-/-) showed faster reversal learning, improved working memory and an enhanced reactivity to novelty in comparison to their wild-type littermates (Morellini et al., 2010).

4.5.3.1 TNR in proliferation and migration of neural progenitors:

TNR also plays a role in proliferation and migration of SVZ derived neural progenitor cells. Neurospheres derived from mouse SVZ were treated in vitro with purified EGFL and FN6-8 domains of TNR and the results showed that these domains were able to inhibit the migration of cells from the neurospheres (Huang et al., 2009). Furthermore these two domains affected the distribution of cells migrating out of the neurosphere by inhibiting the neuron migration (Huang et al., 2009). However, another study showed a different function for these two domains on SVZ progenitors, their indirect function via microglia was shown to have a beneficial effect on proliferation and differentiation of progenitor cells (Liao et al., 2008). These effects of the EGFL and FN6-8 domains of TNR on the proliferation and differentiation have been attributed to the production and release of factors like BDNF and TGF-β from microglia (Liao et al., 2008). In addition FN6-8 was also

capable of inducing the production of NGF (Liao et al., 2008). Another role has also been suggested for the FN6-8 and EGFL domains of TNR. TNR has been shown to have a neuroprotective role on primary cultures of cortical neurons (Liao et al., 2008). However, it must be taken into consideration that all of the above mentioned studies used isolated domains of TNR and not the full length protein.

4.5.3.2 Functions of TNR in the OB:

TNR has been shown to be a detachment signal and a guidance cue for the radially migrating cells in the OB (Saghatelyan et al., 2004). TNR expression was also shown to be activity dependent (Saghatelyan et al., 2004). The lack of TNR in adult mice was shown not to affect proliferation or tangential migration of precursors in the RMS. However the radial migration of the precursor neurons was severely impaired which resulted in a significant reduction in the number of adult generated interneurons integrated into the bulb (Saghatelyan et al., 2004).

4.5.4 TNR in disease:

TNR has both beneficial and a detrimental role in diseases and in their treatments. In spinal cord injuries for example, TNR expression is up regulated in lesioned areas preventing repair and axonal regrowth (Becker et al., 2000; Becker et al., 2003). A study performed on promoting axonal regeneration and functional recovery in rats with spinal cord injury administered TNR polyclonal antibody to injured rats as a form of passive immunization. Upon administration of this high titre, highly specific antibody directly into the lesioned spinal cord, a functional recovery in rats was seen (You et al., 2012). This beneficial effect was brought about by the decrease in RhoA activation as a result of the TNR antibody injections (You et al., 2012). TNR is also implicated in hyperexcitability during kindling and mice deficient in TNR had a retarded progression of kindling (Hoffmann et al., 2009). In mice whose striatum was lesioned with qunolinic acid as a model to study Huntington's disease an implantation of cells expressing TNR produced beneficial outcomes (Hargus et al., 2008). Taking into account that TNR can reroute cells from the SVZ (Saghatelyan et al., 2004), the authors grafted embryonic stem cells

over expressing TNR which led to the production of GABAergic internerurons, 1 and 2 months after transplantation (Hargus et al., 2008). TNR has also been implicated in human diseases. Homozygous deletion of TNR in humans results in a global delay of development, transient choreoathetosis and opisthotonic posturing, all indicating intellectual disability (ID) (Dufrense et al., 2012). Thus TNR is shown to be implicated in human cognition and in the development of the CNS (Dufrense et al., 2012). TNR has also been shown to be implicated in astrocytomas and gangliomas. The expression of 3' untranslated region of TNR and TNR itself was over expressed in these kinds of tumor (El Ayachi et al., 2010).

5. RATIONAL AND OBJECTIVES OF RESEARCH:

Neurogenesis in the OB is controlled by a huge variety of extrinsic and intrinsic factors that govern the proliferation, migration, maturation, integration and survival of newly generated interneurons (Lledo et al., 2006). Despite continuous progress in understanding neurogenesis, it is completely unknown whether the same molecular mechanisms control OB neurogenesis during early postnatal development and during adulthood. Among the adult-born neurons, the periglomerular interneurons are a molecularly varied population with cells expressing GAD, CR, CB, TH, THR, Enk (Kosaka and Kosaka, 2005). However, the granule interneurons of the GCL are considered as a uniform population and until now it is not clear if adult-born GCs represent a heterogeneous population of cells that can play distinct role in odor behaviour.

In addition, while it has been demonstrated that sensory activity plays a key role in adult neurogenesis (Maruniak et al., 1989; Saghatelyan et al., 2004; Rochefort et al., 2002; Alonso et al., 2006), our knowledge on the impact of sensory activity on different subpopulations of new neurons needs to be deepened.

Taking all of this into consideration it becomes clear that it is imperative to better understand adult neurogenesis. In order to further our understanding of adult neurogenesis my work was aimed at asking questions like; ***are the mechanisms governing early postnatal/perinatal neurogenesis and adult neurogenesis different? Are all granule interneurons produced during adult neurogenesis the same and do they all perform the same function? How does sensory activity affect the various subpopulations of glomerular interneurons?*** I will discuss the results of each of these questions in chapters II to IV.

In chapter II I present my work that hypothesizes that ***the mechanisms governing adult neurogenesis is different from those governing perinatal neurogenesis.*** Using morphological, functional and behavioral analysis I aimed to show that adult neurogenesis is governed by different molecular cues and that the granule interneurons produced during adulthood play specific role in the odor behavior. In particular, I demonstrated that extracellular matrix glycoprotein, tenascin-R (TNR) controls radial migration and new synapse formation/maintenance in the adult, but not early postnatal OB, and adult-born neurons play an important role in the short-term memory.

Having shown that neurogenesis during perinatal and adulthood are governed by distinct molecular cues, I address my next question, ***are all granule interneurons produced during adult neurogenesis the same and do they all perform the same function?*** In chapter III I discuss an ongoing work that aims to show that there are molecularly distinct subpopulations of granule interneurons that could possibly perform different functional roles in the adult mouse OB. I show that adult-

born GCs can be subdivided to at least CamKIIa+ and CamKIIa- cells that play distinct function in the OB network.

In chapter IV, I describe a study done in collaboration that aims to understand the effect of sensory deprivation on the molecularly distinct subpopulation of glomerular interneurons. Using sensory deprivation and looking at each of the subpopulations, we show that sensory deprivation selectively affects certain subpopulations in comparison with the others. The full implications of this are yet to be described.

Thus by bringing together morphological, functional and behavioral studies I have tried to better understand the many aspects of adult neurogenesis spanning the guidance cues, functions of adult generated interneurons and the role that sensory activity plays on the fate acquisition and maintenance of these adult generated interneurons.

Chapter II:

The extracellular matrix glycoprotein Tenascin-R affects adult but not developmental neurogenesis in the olfactory bulb (2013) *The Journal of Neuroscience,* June 19. 33(25): 10324-10339.

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Dr. Armen Saghatelyan

This work was performed under the guidance of Dr. Armen Saghatelyan. All of the work included in this publication was performed by me except time-lapse imaging experiments in the adult animals performed by Armen Saghatelyan. Dr. Melitta Schachner provided TNR deficient animals.

RÉSUMÉ

Les précurseurs neuronaux, produits dans la zone su ventriculaire tout au long de la vie d'un animal, migrent tangentiellement en suivant le courant de migration rostrale et, une fois arrivée dans le bulbe olfactif (MOB), tourne pour entamer leur migration radiale afin de finalement atteindre les différentes couches du bulbe, où ils se différentieront en interneurones. Malgré les efforts du passé pour mieux comprendre la neurogénèse adulte, il est encore indéterminé si les mécanismes moléculaires qui contrôlent la neurogénèse bulbaire durant le développement post-natal précoce sont les mêmes qu'à l'âge adulte. Dans la présente étude, nous démontrons que la glycoprotéine contenue dans la matrice extracellulaire, la tenascin-R (TNR), est produite dans la couche granulaire du MOB et que son expression augmente durant le développement post-natal. L'imagerie en temps réel et l'analyse morphologique ont révélé que l'absence de TNR réduit la migration radiaire des précurseurs neuronaux dans le bulbe adulte, mais pas dans celui en développement. Un manque de TNR réduit aussi la formation des épines dendritiques sur les nouveaux neurones bulbaires de la souris adulte. Afin de mieux comprendre les conséquences fonctionnelles de l'absence de TNR, nous avons conduit une analyse électrophysiologique et comportementale comparant les souris jeunes aux souris adultes. Les enregistrements électrophysiologiques ont montré que les cellules mitrales, les cellules cibles des interneurones nouvellement générés, reçoivent une activité inhibitrice réduite autant au niveau de l'activité spontanée que l'activité induite et ce, seulement dans les souris adultes déficientes en TNR. De plus, la synchronisation de l'activité électrique des cellules mitrales est réduite dans le MOB des souris adultes déficientes en TNR. Les études comportementales ont révélé que le nombre réduit d'interneurones dans le MOB des souris adultes déficientes en TNR altère la mémoire à court terme des odeurs. Finalement, nos résultats indiquent que la TNR module la neurogénèse adulte et non la neurogénèse développementale dans le MOB et, ainsi, démontre

le fait que la régulation de la neurogénèse bulbaire varie au fur et à mesure que la vie d'un animal avance.

ABSTRACT

Neuronal precursors produced in the sub ventricular zone (SVZ) throughout an animal's life migrate tangentially along the rostral migratory stream (RMS) and, once in the olfactory bulb (OB), turn to migrate radially to the bulbar layers where they differentiate into interneurons. Despite extensive investigations, it has remained largely unknown whether the same molecular mechanisms control OB neurogenesis during early postnatal development and in adulthood. In this study, we show that the extracellular matrix glycoprotein tenascin-R (TNR) is produced in the granule cell layer of the OB and that its expression increases during postnatal development. Time-lapse video-imaging and morphological analyses revealed that a lack of TNR decreases the radial migration of neuronal precursors in the adult, but not in the developing OB. A lack of TNR also reduces spine development of newborn neurons in adult mice. To understand the functional consequences of a lack of TNR, we performed electrophysiological and behavioral studies on young and adult mice. Electrophysiological recordings showed that mitral cells, the target cells of newly generated interneurons, receive reduced spontaneous and evoked inhibitory activity in adult, but not young TNR knock-out mice. Moreover, the synchronized activity of mitral cells was decreased in the OB of adult TNR knock-out mice. Behavioral studies revealed that the lower numbers of newborn interneurons in the adult OB induce alterations in short-term odor memory. Our results indicate that TNR modulates adult but not developmental neurogenesis in the OB and also highlight that the regulation of OB neurogenesis can vary during an animal's lifetime.

2.1. INTRODUCTION.

In the rodent forebrain, olfactory bulb (OB) interneurons are continuously replenished throughout an animal's lifetime. While some bulbar interneurons are produced during embryonic development (Wichterle et al., 2001; Vergano-Vera et al., 2006), the majority of interneuronal production peaks during the perinatal period and continues throughout adulthood (Altman, 1969; Lemasson et al., 2005; Batista-Brito et al., 2008). In the postnatal period, stem cells in the sub ventricular zone (SVZ) of the lateral ventricle give rise to transit amplifying cells, which in turn produce neuroblasts (Kriegstein and Alvarez-Buylla, 2009). The neuroblasts migrate tangentially in the rostral migratory stream (RMS) and, when they reach the core of the OB, turn to migrate radially toward the granule cell layer (GCL) and glomerular layer (GL) of the OB (Kriegstein and Alvarez-Buylla, 2009). These cells mature in the OB, form functional synapses with principal neurons, and modulate some but not all odor-associated behaviors (Imayoshi et al., 2008; Breton-Provencher et al., 2009; Lazarini and Lledo, 2011; Mandairon et al., 2011; Breton-Provencher and Saghatelyan, 2012).

Both extrinsic and intrinsic mechanisms have been shown to control and modulate the various stages of postnatal neurogenesis. A multitude of molecular cues and signaling pathways have been identified and implicated in the orchestration of neurogenesis (Lledo et al., 2006). However, it is not clear whether these molecular cues and signaling pathways are equally involved in regulating both perinatal and adult OB neurogenesis or whether some of the processes governing postnatal neurogenesis are regulated in different ways during distinct periods of an animal's life. Importantly, structural and functional differences between bulbar interneurons born at different periods during an animal's life have been observed (Lemasson et al., 2005; Saghatelyan et al., 2005; Kelsch et al., 2008; Nissant et al., 2009;

Breton-Provencher and Saghatelyan, 2012). For example, the bulbar interneurons born during the perinatal period are predominantly located in the superficial GCL, whereas those born during adulthood are preferentially located in the deep GCL (Lemasson et al., 2005; Imayoshi et al., 2008). It is thus conceivable that the mechanisms controlling the targeting of newborn interneurons during different postnatal periods are distinct.

The extracellular matrix (ECM) glycoprotein tenascin-R (TNR) affects the arrival of newborn interneurons in the adult OB (Saghatelyan et al., 2004), and in some regions of the nervous system (e.g., the retina) TNR expression begins postnatally at 1 week, peaks at 2-3 weeks, and then remains stable throughout adulthood (Bartsch et al., 1993). We thus hypothesized that TNR might specifically regulate adult, but not perinatal, OB neurogenesis. We used morphological, time-lapse video-imaging and electrophysiological approaches to show that a lack of TNR reduces the radial migration of neuroblasts in the OB, decreases the spine density of newborn neurons, and diminishes the inhibition and synchronized activity of mitral cells in the adult but not perinatal OB. Using a behavioral approach we show that these alterations lead to a reduced short-term memory in adult, but not young mice. The combined observations indicate that the mechanisms controlling postnatal neurogenesis are distinct at different periods during an animal's lifetime.

2.2. MATERIALS AND METHODS.

2.2.1 Animals

Four-, 7-, 15-, 25-30-, and 60 to 120-day-old tenascin-R (TNR) wild-type (+/+) and TNR knock-out (-/-) mice (Weber et al., 1999) were obtained from the breeding of TNR heterozygous animals backcrossed to C57BL/6 mice for at least 10 generations. All experiments were approved by the Université Laval animal protection committee. The mice were kept on a 12-h light/dark cycle at a constant temperature (22°C) with food and water *ad libitum*.

2.2.2 Immunohistochemistry and BrdU labeling

Immunohistochemistry and BrdU labeling were performed as described (Snapyan et al., 2009; Bozoyan et al., 2012). Briefly, the mice were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA). The brains were post-fixed in 4% PFA overnight at 4°C,and 40-µm-thick free-floating vibratome (VT 1000S, Leica) sections were incubated with a mouse anti-TNR (1:50; R&D Systems) monoclonal antibody and then with an AlexaFluor conjugated anti-mouse 488 secondary antibody (1:1000; Life Technologies).

The DNA synthesis marker 5-bromo-2'-deoxyuridine (BrdU; Sigma) was dissolved in sterile 0.9% NaCl and 0.4N NaOH. Ten-day-old (P10) mice were injected intraperitoneally with 25 mg/kg of the BrdU solution. The adult mice were injected with 50 mg/kg of BrdU. To study the proliferation of neuronal precursors during the perinatal period, P10 TNR +/+ and TNR -/- mice received a single BrdU injection and were sacrificed 1 h later. To study the arrival of neuroblasts in the OB, P10 mice were given two BrdU injections with an interval of 2 h and sacrificed 5 days later. To quantify the number of newborn interneurons integrating into the OB of TNR +/+ and TNR -/- mice, two BrdU pulses with an interval of 2 h were administered at P10 or at P60, and the mice were sacrificed 21 days later, allowing the labeled cells in the SVZ to migrate to the OB and integrate into the GCL and GL.

Serial coronal sections from the anterior tip of the OB to the SVZ were BrdU-immunostained as described previously (Snapyan et al., 2009). Briefly, the sections were treated with 2N HCl for 40min at 37˚C to denature the DNA. They were then incubated overnight with a rat anti-BrdU monoclonal antibody (1:500; Serotec) at 4˚C in 0.2% Triton-X supplemented with 4% BSA, then with secondary anti-rat biotinylated antibody (1:500, Thermo Scientific) for 3 h at room temperature and finally with avidin-biotin (ABC kit, Vectastain Elite, Vector Laboratories). Labeling was revealed using 0.05% diaminobenzidine in the presence of H_2O_2. The sections were mounted, dried, dehydrated in a series of alcohol baths, and

counterstained with cresyl violet to measure the areas of the SVZ, RMS, RMS_{OB}, GCL, and GL. Sections were analyzed using an upright BX51 microscope equipped with a motorized stage (Olympus).

2.2.3 Western blot analysis

OBs from P4, P7, P15, P30, and P60 mice were collected and homogenized in lysis buffer (50mM HCl, 1mM EDTA, 1mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium β-glycerophosphate, 0.1% 2-mercaptoethanol, and 1% Triton X-100, pH 7.5) containing Protease Inhibitor Cocktail Set III (Calbiochem). The homogenates were sonicated and then centrifuged at 13000*g* at 4°C for 20 min to remove insoluble material. The protein concentration in each supernatant was quantified, and 60 µg of protein was separated on NuPage 4-12% Bis-Tris gels (Life Technologies), and the bands were transferred to a nitrocellulose membrane (Amersham, GE Healthcare). The membrane was cut horizontally into two parts at approximately 60 kDa. The upper part was incubated with the mouse anti-TNR antibody (1:100), and the lower was incubated with a mouse anti-actin antibody (1:3000; Cedarlane). The membranes were then incubated with an anti-mouse HRP-conjugated secondary antibody, and the bands were revealed using chemiluminescence enhancer ECL (Amersham, GE Healthcare). Two bands (180 kDa and 160 kDa) corresponding to the two isoforms of TNR were detected.TNR expression was normalized to that of actin in the corresponding sample.

2.2.4 Stereotaxic injections

To study neuroblast migration and maturation, GFP-encoding lentiviruses or retroviruses were injected into the rostral migratory stream (RMS) of P10 and P60 TNR +/+ and TNR -/- mice at the following coordinates (in mm with respect to the bregma): for P10 anterior-posterior 2.05; medial-lateral 0.65, and dorsal-ventral 2.7; and for P60, anterior-posterior 2.55; medial-lateral 0.82, and dorsal-ventral

3.15. To study dendritic arborization and spine density of newborn cells in both genotypes, a GFP-expressing lentivirus was injected into the RMS, and the mice were sacrificed 21 days later. To explore if possible changes in the spine density in TNR -/- mice results from the direct effect of TNR on the spine development of newborn cells or are a consequence of affected migration, a GFP-expressing lentivirus was injected into the RMS of TNR+/+ mice and 10 days later anti-TNR antibodies (100µg/ml, 500 nl per injection site; R&D Systems) were injected into the OB at the following coordinates (in mm with respect to the bregma): anterior-posterior 4.70; medial-lateral 1.5, and dorsal-ventral 0.45; and anterior-posterior 5.3; medial-lateral 1.2, and dorsal-ventral 0.6. The mice were perfused following an additional 4 days and the spine density of newborn granule cells was analyzed as described below in morphological analysis. To perform time-lapse video-imaging of tangential and radial migration in the OB of TNR +/+ and TNR -/- mice, GFP-encoding retroviruses were injected into the RMS, and horizontal 250-µm-thick slices were prepared 7 days later as described below in slice preparation and time-lapse video-imaging.

2.2.5 Morphological analysis

To analyze the dendritic arborization and spine density of newborn cells, mice of both genotypes were deeply anesthetized and were perfused transcardially with 4% PFA. The brains were then postfixed overnight in 4% PFA at 4°C. The OBs were embedded in a block of 4% agar, and horizontal 100-µm-thick slices were prepared. The slices were mounted and coverslipped with fluorescence mounting medium (Dako). Images of GFP-labeled granule cells were acquired using a 60x oil emersion objective on a FV1000 confocal microscope equipped with argon 488 nm, helium–neon 543 nm, and helium–neon 633 nm lasers (Olympus). For analysis of spine density of GFP labeled newborn cells, part of the distal dendrites projecting toward the external plexiform layer was randomly chosen and images were reacquired using a 60x oil emersion objective with a 3x zoom. Images were analyzed manually using Fluoview software (Olympus) to measure the lengths of

the primary and secondary dendrites. Spine density was calculated by counting the number of spines on a given length of dendrite.

In the TNR +/+ mice that received injections of mouse anti-TNR antibodies in the adult OB, 10 days after infection of neuroblasts in the RMS with GFP-encoding lentivirus, the animals were perfused transcardially and the sections were incubated with anti-mouse Alexa568 secondary antibodies. The spine density of newborn granule cells was calculated in the injection sites of anti-TNR antibody (near injection site; NIS) and compared to spine density in the regions located far away from the injection site (FIS). In control experiments Alexa568 secondary antibody was injected instead of anti-TNR antibody.

2.2.6 Slice preparation and time-lapse video-imaging

Acute horizontal 250-µm-thick slices were prepared for electrophysiological recordings and time-lapse video-imaging of cell migration in the OB of TNR +/+ and TNR -/- mice. The adult mice were first perfused transcardiacally with ice-cold sucrose-based artificial cerebrospinal fluid (ACSF) containing (in mM) 250 sucrose, 3 KCl, 0.5 $CaCl_2$, 3 $MgCl_2$, 25 $NaHCO_3$, 1.25 $NaHPO_4$, and 10 glucose. The brains were rapidly removed and immersed in the solution used for the transcardiac perfusion. Horizontal slices of the OBs were obtained using a vibratome. After a 30-min recovery period at 32°C, the slices were placed in the recording chamber and were continuously perfused with oxygenated ACSF containing (in mM) 124 NaCl, 3 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$, 1.25 $NaHPO_4$, and 10 glucose at a rate of 1.5-2 ml/min (bubbled with 95% O_2/5% CO_2; pH≈7.4). For time-lapse video-imaging of cell migration, multiple *z*-stacks images (at least 6-10 *z*-sections at 3-5 µm intervals) were acquired every 30 s for at least 2 h using a BX61WI up-right microscope (Olympus) equipped with CCD camera (CoolSnap HQ2). Cell migration was analyzed manually using ImagePro software (Media Cybernetics). The total displacement during 1 h of cell migration, the speed of migration, and the percentage of the stationary phase with respect to the total migration time were calculated. For analysis of the stationary periods, only those phases that were

intercepted by two migratory periods were used. Time-lapse video-images of the RMS of the OB (RMS_{OB}) and GCL were acquired to track tangential migration of neuroblasts in the RMS_{OB} and radial migration in the RMS_{OB} and GCL.

2.2.7 Patch-clamp recordings

For electrophysiological recordings we prepared acute slices from young P25-P30 and adult P60-P120 mice. We choose P25-P30 since bulbar interneurons that are produced during the first two postnatal weeks require 2-3 weeks to arrive and integrate into the bulbar network. Acute horizontal 250-µm-thick vibratome slices from the OBs were prepared for patch clamp recordings as described above. Recordings were made using a Multiclamp 700A amplifier (Molecular Devices). Patch electrodes with resistances ranging from 2.5-4 MΩ were filled with an intracellular solution containing (in mM) 135 CsCl, 10 HEPES, 0.2 EGTA, 2 ATP, 0.3 GTP, and 10 glucose (pH≈7.2). Spontaneous inhibitory currents (sIPSCs) were isolated by bath application of 5 mM kynurenic acid (Kyn) to block glutamatergic activity. Miniature inhibitory currents (mIPSCs) were isolated by applying 1 µM tetrodotoxin (TTX) to block voltage sensitive sodium channels in the presence of 5 mM Kyn. Magnesium-free ACSF containing 1 µM TTX was used to record evoked dendrodendritic currents. The mitral cells were depolarized to 0 mV with voltage steps of varying durations, and dendrodendritic currents were recorded from the same mitral cells. The recordings were first performed under control conditions, i.e., in the presence of Mg-free ACSF containing 1□M TTX. A $GABA_A$ receptor antagonist (50 µM bicuculline methochloride, BMI) was then applied together with 1 µM TTX, and evoked currents were recorded again. The traces representing dendrodendritic inhibitory (DDI) responses were obtained by subtracting the traces recorded in the presence of BMI (Mg-free ACSF with TTX and BMI) from those recorded under control conditions (Mg-free ACSF with TTX).

2.2.8 Local field potential recordings

To record local field potentials (LFP), the electrodes were filled with 2M NaCl. The slices were prepared using the protocol for the patch-clamp recordings and were maintained at 35ºC throughout the recordings. The olfactory nerve layer was stimulated with a glass microelectrode in the range of 150-250 µA using an A360 stimulus isolator (World Precision Instruments). The recordings were made using a Multiclamp 700B amplifier (Molecular Devices). LFP recordings were made in the mitral cell layer in the absence and presence of the $GABA_A$ receptor antagonist gabazine (20 µM). Only the recordings showing a significant reduction in the oscillation index (see below) in the presence of gabazine were kept for analysis. To analyze LFP oscillations, we used a custom-tailored program written in Matlab (The Mathworks Inc.) to calculate the frequency spectrum and autocorrelation using a fast-Fourier transform algorithm. The oscillation index, a measure of oscillation power, was calculated as follows:

$$Oscillation\ Index\ = \frac{\int_{20}^{80} DFT_{pre}(f)df}{\int_{20}^{80} DFT_{post}(f)df},$$

Where $DFT_{pre}(f)$ and $DFT_{post}(f)$ represent discrete Fourier transforms evaluated over a 200-ms window taken before and after the stimulus, respectively. This formula represents the sum of the oscillation amplitudes recorded at all frequencies between 20 and 80 Hz after the stimulus was normalized to the frequency amplitudes (from the same range) recorded before the stimulus. Similar results were obtained when the oscillation index was defined as the second peak height of the normalized autocorrelation function.

2.2.9 Behavior

Olfactory behavior was assessed on age-matched male mice at P25-P30, and the same cohort of mice was retested using the same olfactory tests at 3-4 months of age. Each mouse was individually housed and provided with food and water *ad libitum*. The mice were maintained in a 12-h inverted light/dark cycle and initially subjected to a 5-day habituation period to familiarize the mice with the test

environment. During the habituation period, the home cages were used as testing chambers where the feeding grid was replaced by a clean grid from which Pasteur pipettes containing the filter paper inserts were suspended. The filter paper inserts were saturated with test odor (diluted in mineral oil) or mineral oil alone (control).
Odor detection threshold: Upon completion of the 5-day habituation period, which allowed the mice to familiarize themselves with the test procedures, the odor detection thresholds were assessed. The mice were presented with two pipettes for 5 min, one containing a filter paper insert saturated with mineral oil (control) and the other with octanol (test odor) diluted in mineral oil to different concentrations (10^{-7}, 10^{-5}, 10^{-4} and 10^{-3}). Each concentration was tested during separate sessions in ascending order. The time spent investigating the two pipettes was recorded. The mice were considered to be able to detect the test odor if the time spent investigating the test pipette was longer than the time spent investigating the control pipette.

2.2.9.1 Odor discrimination:

The olfactory discrimination task is a habituation/dishabituation test. During the habituation protocol, which consisted of 3 successive 5-min trials with a 15-min interval between each exposure, the mice were presented with heptanol (Hept) diluted 10^{-3}. During the successive exposures, the total investigation time progressively decreased as the mice became habituated to the odor. The final exposure represented the dishabituation protocol, where a novel odor (limonene, Lim) was presented mixed with heptanol at varying percentages (Hab: 100% 1-month-old mice and (Hab: 100% Hept, Dishab: 100% Lim; 30% Hept/70% Lim; 42% Hept/58% Lim; 46% Hept/54% Lim; 49% Hept/51% Lim; and 58% Hept/42% Lim) for 4-month-old mice. The mice were considered to be able to discriminate the novel odor from the odor they were habituated to if the investigation time during the dishabituation exposure was longer than the investigation time recorded during the third and final habituation phase.

2.2.9.2 Olfactory short-term memory:

After a 4-day habituation period, reverse light/dark cycle individually housed mice were exposed to the same odor during two 5-min sessions separated by a 30, 60, 90, or 120-min interval. The pipette containing the odor was inserted randomly in the cage grid to avoid a spatial recognition effect. Individually housed mice were presented with the same odor during two 5-min sessions separated by a 30, 60, 90, or 120 min interval between the 2 presentations. The odors used were (+) terpinen-4-ol, limonene (-), decanal, and valeric acid for the 1-month-old mice and (+) terpinen-4-ol, limonene (-), carvone (-), and valeric acid for the 4-month-old mice. All odors were purchased from Sigma and were diluted 10^{-3}. The mice were considered to remember the odor if there was a significant decrease in the time taken to investigate the second odor compared to the first odor.

2.2.10 Statistical analysis

Results are expressed as means ± SEM. Statistical significance was determined using Student's *t*-test (*$p < 0.05$ and **$p < 0.01$).

2.3. RESULTS.

2.3.1 Tenascin-R is expressed during late perinatal development in the OB:

To establish the spatio-temporal expression profile of TNR in the OB, we performed immunohistochemical and quantitative Western blot analyses. TNR immunofluorescence performed on coronal OB sections from P4, P7, P14, P30, and P60 mice revealed that TNR expression becomes detectable at P7 in the granule cell layer (GCL) around the rostral migratory stream in the olfactory bulb ($RMS_{OB)}$ (**Fig. 2.1a**). Expression increased during the second to third postnatal weeks and peaked at P30 (**Fig. 2.1a**). At P30, TNR was homogeneously expressed in the GCL and glomerular layer (GL), but not in the RMS_{OB}. This spatial pattern was maintained throughout life.

To quantify TNR levels at different development stages, we performed quantitative Western blot analyses using protein samples extracted from the OBs of P4, P7, P15, P30, and P60 mice. Two bands (160 and 180 kDa) representing the two isoforms of TNR were detected at all ages (**Fig. 2.1b**). The Western blot results revealed that TNR expression is low at P4-P7, is increased at P15, peaks at P30, and is subsequently maintained at this level throughout adulthood (**Fig. 2.1c**), which is in agreement with the immunofluorescence results.

2.3.2 Lack of TNR does not affect perinatal neurogenesis in the OB:

We previously demonstrated that the absence of TNR leads to a decrease in the number of interneurons in the adult OB, without affecting the proliferation and tangential migration of neuroblasts (Saghatelyan et al., 2004). However, TNR expression is low during the first and second postnatal weeks when the majority of bulbar interneurons arrive in the OB (Altman, 1969; Lemasson et al., 2005; Batista-Brito et al., 2008). We thus asked if TNR plays a role in the bulbar neurogenesis at

these early postnatal ages. We thus investigated whether a lack of TNR during the early postnatal period would affect the number of cells that integrate into the OB. We injected BrdU into P10 TNR +/+ and TNR -/- mice and analyzed cell proliferation, migration, and survival in the SVZ-OB pathway (**Fig. 2.2a**). To determine whether a lack of TNR affects cell proliferation, a single BrdU pulse was administered to P10 mice which were sacrificed 1 h later. Immunolabeling for BrdU revealed dense BrdU+ profiles in the SVZ and at the outer borders of the RMS (**Fig. 2.2b**), as reported previously (Pencea and Luskin, 2003). No difference in BrdU+ cell density between the genotypes was observed (3754.5 ± 352.0 cells/mm^2 for TNR +/+ and 3827.3 ± 248.7 cells/mm^2 for TNR -/- mice, n=3 mice per group) (**Fig. 2.2d**). To determine whether a lack of TNR affects the tangential migration of neuroblasts at early postnatal ages, two BrdU pulses were given to P10 mice, which were sacrificed 5 days later when most of the BrdU+ cells had reached the RMS_{OB} (**Fig. 2.2c**). The quantification of the density of the BrdU+ profiles in the RMS_{OB} did not reveal any difference between genotypes (3281.4 ± 366.8 cells/mm^2 for TNR +/+ mice, and 3814.1 ± 526.8 cells/mm^2 for TNR -/- mice; n=5 and n=4 mice per group, respectively) (**Fig. 2.2d**). We then quantified the number of BrdU+ cells in the GCL and GL produced at P10 TNR +/+ and TNR -/- mice 21 days after the injection of two BrdU pulses. Twenty-one days were required for newborn cells produced in the SVZ and RMS to reach the OB and integrate into the bulbar network (**Fig. 2.2e**). Similarly, the densities of newly integrated cells in the GCL and GL of TNR -/- OBs were undistinguishable from those of their TNR +/+ littermates (for the GCL: 811.5 ± 109.4 cells/mm^2 for TNR +/+ mice, and 698.9 ± 57.7 cells/mm^2 for TNR -/- mice; for the GL: 747.3 ± 133.4 cells/mm^2 for TNR +/+ mice, and 655.3 ± 43.5 cells/mm^2 for TNR -/- mice; n=4 mice per group) (**Fig. 2.2f**). These results indicated that a lack of TNR does not affect cell proliferation in the SVZ and RMS, migration in the RMS and OB, or integration into the OB during early postnatal periods. These results are consistent with the levels of TNR expression in the OB (**Fig. 2.1**) and sharply contrast with the role of TNR during adult OB neurogenesis (Saghatelyan et al., 2004). During

adulthood, lack of TNR leads to the decreased number of BrdU+ cells in the OB (for the GCL 21 days after BrdU injection: 309.5 ± 12.0 cells/mm^2 for TNR +/+ mice, and 211.6 ± 21.9 cells/mm^2 for TNR -/- mice, $p < 0.05$ and see also (Saghatelyan et al., 2004)) because of accumulation of neuroblasts in the RMS_{OB} without affecting cell proliferation and tangential migration in the SVZ and RMS (Saghatelyan et al., 2004). While the accumulation of neuroblasts in the RMS_{OB} has been attributed to defects in radial migration (Saghatelyan et al., 2004), cell migration in the OBs of TNR +/+ and TNR -/- mice has not been monitored. In addition, radial migration is a multistep process, which includes arrest of tangential migration, initiation of radial migration in the RMS_{OB}, and maintenance of radial migration in the GCL of the OB. We used time-lapse video-imaging of cell migration in acute horizontal slices of the forebrains of both genotypes and at different developmental ages to determine which of these processes is affected by a lack of TNR in the adult but not early postnatal OB.

2.3.3 Radial migration of neuroblasts is impaired in the RMS_{OB} and GCL of adult TNR -/- mice:

While the time-lapse imaging of tangentially migrating neuroblasts in the RMS has been routinely studied (Murase and Horwitz, 2002; Bolteus and Bordey, 2004; Kim et al., 2009; Snapyan et al., 2009), little is known about the dynamics of radially migrating neuroblasts in the RMS_{OB} and GCL of the OB at different developmental ages. In addition, it is not clear how the tangential migration of neuronal precursors in the RMS_{OB} is modified when cells turn 90° and initiate radial migration. To address these issues and understand how a lack of TNR affects the migration of neuronal precursors in the adult but not early postnatal OB, we performed time-lapse video-imaging of GFP-labeled neuroblasts in acute horizontal slices over a time period of 2 h (**Figs. 2.3,4**). The cells were then subdivided into three groups depending whether they migrated tangentially in the RMS_{OB} (**Figs. 2.3a, 4a**), radially in the RMS_{OB} (**Figs. 2.3b, 4b**), or radially in the GCL (**Figs. 2.3c, 4c**).

We first performed time-lapse video-imaging of cell migration in slices prepared from P15-P16 TNR +/+ and TNR -/- mice that received injection of GFP-encoding retrovirus in the RMS at P10. Tangentially (30.6 ± 4.0 μm for TNR +/+ mice, n=30 cells, and 30.2 ± 2.3 μm for TNR -/- mice, n=53 cells) and radially (22.4 ± 1.7 μm for TNR +/+ mice, n=25 cells, and 25.3 ± 1.5 μm for TNR -/- mice, n=39 cells) migrating cells in the RMS_{OB}, as well as radially migrating cells in GCL (23.1 ± 2.2 μm for TNR +/+ mice, n=26 cells, and 23.2 ± 1.2 μm for TNR -/- mice, n=43 cells) migrated the same distance during a 1h of recording in both genotypes (**Figs. 2.3a-d**). Similarly no changes in the speed of migration of tangentially (80.9 ± 4.7 μm for TNR +/+ mice, n=30 cells, and 76.4 ± 2.0 μm for TNR -/- mice, n=53 cells) and radially (80.0 ± 2.5 μm for TNR +/+ mice, n=25 cells, and 75.6 ± 1.5 μm for TNR -/- mice, n=39 cells) migrating neuroblasts in the RMS_{OB} as well as radially migrating cells in the GCL (78.8 ± 2.5 μm for TNR +/+ mice, n=26 cells, and 76.4 ± 2.0 μm for TNR -/- mice, n=43 cells) was observed **(Fig. 2.3e)**. Neuroblast migration in the SVZ-OB pathway is saltatory, with the migratory phases interrupted by the stationary periods. We thus calculated the amount of time that neuroblasts spent in the stationary phase and again did not detect any changes for neuroblasts migrating tangentially (61.6 ± 5.6 % for TNR +/+ mice, n=30 cells, and 61.1 ± 2.8 % for TNR -/- mice, n=53 cells) and radially (72.2 ± 2.4 % for TNR +/+ mice, n=25 cells, and 67.5 ± 1.9 % for TNR -/- mice, n=39 cells) in the RMS_{OB} as well as radially in the GCL (68.8 ± 3.6 % for TNR +/+ mice, n=26 cells, and 69.0 ± 1.7 % for TNR -/- mice, n=43 cells) **(Fig. 2.3f)**. These data are in line with our BrdU analysis **(Fig. 2.2)** and show that migration of neuronal precursors in the early postnatal TNR -/- is undistinguishable from their wild-type counterparts.

We then performed time-lapse video-imaging of cell migration in the adult TNR +/+ and -/- mice **(Fig. 2.4)**. Cells migrating tangentially in the RMS_{OB} of adult TNR +/+ and TNR -/- mice migrated the same distance during a 1h recording (37.9 ± 3.1 □m for TNR +/+ mice, n=57 cells, and 39.4 ± 3.7 □m for TNR -/- mice, n=36 cells) (**Fig. 2.4d**). There was no difference in the speed of migration (91.0 ± 5.0 □m for TNR

+/+ mice, n=57 cells, and 100.4 ± 7.1 □m for TNR -/- mice, n=36 cells) (**Fig. 2.4e**) or the percentage of time the cells spent in the stationary phase during migration (59.2 ± 2.6% for TNR +/+ mice, n=57 cells, and 61.1 ± 2.6% for TNR -/- mice, n=36 cells) (**Fig. 2.4f**).

We next examined the radial migration of neuroblasts in the RMS_{OB} and GCL and found that the radial migration of neuroblasts in the RMS_{OB} and the GCL of adult TNR -/- mice is indeed affected. Cells migrating radially in the RMS_{OB} of these TNR-/- mice stayed for a significantly longer time period in the stationary phase than those of their TNR +/+ littermates (61.9 ± 1.8% for TNR +/+ mice, n=12 cells, and 74.4 ± 3.2% for TNR -/- mice, n=7 cells; $p < 0.05$) (**Fig. 2.4f**). Given this, the overall distance travelled by radially migrating neuroblasts in the RMS_{OB} of TNR -/- mice was also lower (35.2 ± 1.9 □m for TNR +/+ mice, n=12 cells, and 24.8 ± 3.3 μm for TNR -/- mice, n=7 cells; $p < 0.05$) (**Fig. 2.4d**). However, the speed of migration was unaffected (93.2 ± 3.8 μm/h for TNR +/+ mice, n=12 cells, and 96.9 ± 7.3 μm/h for TNR -/- mice, n=7 cells) (**Fig. 2.4e**). Once the cells had exited the RMS_{OB}, they maintained their radial migration to their final destination in the GCL and GL. As in the RMS_{OB}, the radial migration of neuroblasts in the GCL was significantly altered by a lack of TNR. The overall distance travelled by radially migrating neuroblasts in the GCL during a 1h recording (35.6 ± 3.1 μm for TNR +/+ mice, n=27 cells, and 23.4 ± 3.5 μm for TNR -/- mice, n=12 cells; $p < 0.05$) (**Fig. 2.4d**) and the percentage of time spent by the neuroblasts in the stationary phase (63.1 ± 3.1% for TNR +/+ mice, n=27 cells, and 75.0 ± 3.1% for TNR -/- mice, n=12 cells; $p < 0.05$) (**Fig. 2.4f**) were significantly affected in the TNR -/- mice. However, the speed of migration was unaffected (100.9 ± 3.9 μm/h for TNR +/+ mice, n=27 cells, and 93.8 ± 7.1 μm/h for TNR -/- mice, n=12 cells) (**Fig. 2.4e**). Thus, using time-lapse monitoring of neuroblasts migrating in the OB, we were able to directly show that a lack of TNR induces alterations in radial migration in the RMS_{OB} and GCL but not in tangential migration in the RMS_{OB}. A lack of TNR impaired the ability of cells to leave the RMS_{OB} and enter the GCL, leading to the accumulation

of neuroblasts in the core of the OB, as suggested previously (Saghatelyan et al., 2004). Our results also demonstrate that migration in the adult RMS_{OB} and GCL is more efficient than in early developmental stages, as we have previously shown for the RMS (Bozoyan et al., 2012).

2.3.4 Lack of TNR decreases the spine density of newborn granule cells in the adult OB:

Lack of TNR in the hippocampus results in structural alterations in perisomatic inhibitory synapses (Nikonenko et al., 2003). We thus investigated whether the lack of TNR in the OB also causes changes in the number of spines on newborn cells. We first measured the lengths of the primary and secondary dendrites of granule cells born at P10. GFP-encoding lentiviruses were injected into the RMS of P10 TNR +/+ and TNR -/- mice, and the labeled cells were analyzed 21 days later (**Fig. 2.5a**). Our analysis did not reveal any differences in the length of the primary (128.8 ± 22.3 µm in TNR +/+ mice, n=80 cells from 3 mice, and 104.8 ± 4.0 µm in TNR -/- mice; n=70 cells from 3 mice) and secondary (381.7 ± 2.7 µm in TNR +/+ mice; n=80 cells from 3 mice, and 362.5 ± 9.3 µm in TNR -/- mice; n=70 cells from 3 mice) (**Fig. 2.5c)** dendrites of newborn granule cells in both genotypes. Also, no differences in the spine density of interneurons born at P10 was observed (0.44 ± 0.02 spines/µm in TNR +/+ mice; n=80 cells from 3 mice, and 0.45 ± 0.01 spines/µm in TNR -/- mice; n=68 cells from 3 mice) (**Fig. 2.5d**).

We next measured the lengths of the primary and secondary dendrites and the spine density of newborn granule cells in adult TNR +/+ and TNR -/- mice. The neuroblasts were labeled by stereotaxic injection of GFP-encoding lentiviruses into the RMS of P60 TNR +/+ and TNR -/- mice, and dendritic arborization and spine density were analyzed 21 days later. Virally labeled cells were found throughout the GCL and GL of the OBs of both genotypes (**Fig. 2.5b**). No differences were observed in the lengths of primary (109.8 ± 6.4 µm in TNR +/+ mice, n=46 cells from 4 mice, and 122.5 ± 3.8 µm in TNR -/- mice; n=49 cells from 3 mice) and secondary (566.4 ± 40.9 µm in TNR +/+ mice; n=46 cells from 4 mice, and 526.2 ±

21.3 µm in TNR -/- mice; n=49 cells from 3 mice) (**Fig. 2.5c)** dendrites of newborn granule cells in both genotypes. While the lengths of the dendrites of newborn cells were not altered by a lack of TNR, the marked difference in spine density between the two genotypes was observed (0.453± 0.03 spines/µm in TNR +/+ mice; n=49 cells from 4 mice, and 0.355 ± 0.015 spines/µm in TNR -/- mice; n=43 cells from 3 mice; $p < 0.05$) (**Fig. 2.5d**).

The decreased spine density of newborn granule cells in the adult TNR -/- mice can be caused either by defective radial migration or by direct role of TNR in the spine formation and/or stabilization. To address these issues we decided to block the TNR function in the adult OB, after the cessation of radial migration of neuroblasts. To this end, we labeled neuronal precursors by stereotaxic injection of GFP-encoding lentiviruses into the RMS of P90 TNR +/+ mice and 10 days later when radial migration is largely completed injected anti-TNR or control antibodies into the OB. The animals were sacrificed after additional 4 days and the spine density of newborn granule cells was calculated at the sites of antibodies injections (near injection site; NIS) as well as at remote locations (far from the injection site; FIS) **(Fig. 2.6a)**. Our experiments reveal that injection of anti-TNR (FIS: 0.42 ± 0.02 and NIS: 0.32 ± 0.01, n= 24 and 23 cells respectively, $p < 0.01$), but not control (FIS: 0.38 ± 0.002 and NIS: 0.39 ± 0.01, n= 50 and 38 cells, respectively) antibodies reduced the spine density of newborn granule cells **(Fig. II.6b,c)** and suggest that TNR plays a direct role in the formation and/or stabilization of spines of newborn neurons.

Altogether, our data show that a lack of TNR not only affects the radial migration of neuroblasts in the adult OB, but also alters the spine development of newborn granule cells. These defects are manifested in the adult but not in the early postnatal OB, likely due to the late expression of TNR. We thus expected that TNR -/- mice are a valuable tool for studying the relative contributions of neurons born during perinatal development and adulthood. Given this notion, we undertook electrophysiological and behavioral studies to assess the functioning of the bulbar

circuitry and odor behavior in young, P25-P30 (most bulbar interneurons are produced during the first two postnatal weeks) and adult P90-P120 mice.

2.3.5 Lack of TNR reduces the inhibition of principal output neurons in the adult OB:

OB neurogenesis results in the continuous renewal of interneurons that form dendrodendritic reciprocal synapses with mitral/tufted cells, the principle output neurons of the OB (Price and Powell, 1970a). In these synapses, GABA release can be triggered by the activation of glutamatergic receptors on granule cell spines (Saghatelyan et al., 2005; Boyd et al., 2012). In addition, spontaneous granule-to-mitral cell GABAergic inhibition also occurs in the absence of glutamatergic receptor activation through action potential-dependent and -independent GABA release (Castillo et al., 1999). To investigate the functional consequences resulting from the reduced number of interneurons that integrate into the OBs of adult TNR -/- mice and the significantly lower spine density on interneurons that manage to integrate, we performed patch-clamp recordings from mitral cells in horizontal slices prepared from young and adult mice of both genotypes.

We first recorded spontaneous postsynaptic currents (sPSC) in P25-P30 mice in the presence of ACSF. Kynurenic acid (Kyn, 5mM) was then bath-applied to block glutamatergic transmission, allowing to record spontaneous inhibitory postsynaptic currents (sIPSCs). Lastly, we applied 1 µM TTX together with Kyn to abolish voltage-dependent sodium currents to allow recording of miniature spontaneous postsynaptic currents (mIPSCs). In P25-P30 TNR +/+ and TNR -/- mice, the amplitude (sPSCs: 41.3±3.9 pA vs. 56.6±5.8 pA, n=8 and 10 cells, respectively; sIPSCs: 36.4 ±4.2 pA vs. 42.3±4.8 pA, n=8 and 10 cells, respectively; mIPSCs: 27.6 ±3.0 pA vs. 26.7 ±2.5 pA, n=8 and 10 cells, respectively) and frequency (sPSCs: 20.6±1.9 Hz vs. 17.1 ±3 Hz, n=8 and 10 cells, respectively; sIPSCs: 4.4 ±0.7 Hz vs. 5.1 ±0.9 Hz, n=8 and 10 cells, respectively; and mIPSCs: 2.5 ± 0.4 Hz vs. 2.7 ± 0.2 Hz, n=8 and 10 cells, respectively) of the sPSCs, sIPSCs and

mIPSCs were unaffected (**Fig. 2.7a,b**). There were also no changes in the rise time and decay time of mIPSCs (data not shown). We thus concluded that at P25-P30, both genotypes exhibit the same level of inhibition in their bulbar circuitry, which is in agreement with the lack of morphological deficits observed during this developmental period (**Figs. 2.2, 2.3, 2.5**). Interestingly, however, when the same experiments were performed with mitral cells from adult TNR +/+ and TNR -/- mice, significant differences in the frequency were noted (sPSCs: 12.4±1.9 Hz vs. 8.3 ±1.5 Hz, n=12 and 13 cells, respectively; sIPSCs 2.8 ± 0.4 Hz vs. 1.4 ± 0.3 Hz, n=12 and 13 cells, respectively, $p < 0.05$; mIPSCs 0.8 ± 0.1 Hz vs. 0.4 ± 0.1 Hz, n=7 and 10 cells, respectively, $p < 0.05$) (**Fig. 2.7c,d**). There were no differences in the amplitude (sPSCs: 41.1±8.5 pA vs. 43.1±8.9 pA, n=12 and 13 cells, respectively; sIPSCs: 32.0 ± 6.4 pA vs. 33.8 ± 8.4 pA, n=12 and 13 cells, respectively; mIPSCs: 37.2 ± 9.2 pA vs. 34.2 ± 9.36 pA, n=7 and 10 cells, respectively) (**Fig. 2.7c,d**) or kinetics (data not shown) of the same events. The reduced frequency but not amplitude of sIPSCs and mIPSCs in adult TNR -/- mice compared to their TNR +/+ littermates was consistent with the histological results showing reduced radial migration and spine density of newborn cells in the adult OB.

We next recorded evoked dendrodendritic inhibitory currents from young and adult mice. Mitral cells were recorded under the whole-cell configuration and held at –60mV. The cells were then depolarized to 0mV for varying periods using voltage steps ranging in duration from 10 to 50ms. The dendrodendritic inhibitory currents (DDI) were obtained by subtracting traces recorded in the presence of 50 µM BMI and 1 µM TTX from control traces recorded in the presence of 1 µM TTX alone. At P25-P30, both the amplitude and the charge of DDIs were indistinguishable between genotypes (**Fig. 2.7e,f**). In contrast, DDIs recorded from mitral cells of adult, P60-P120 TNR -/- mice exhibited a reduction in the amplitude of inhibitory events in response to 50ms, 25ms, and 10ms depolarization steps (50ms: 70.6 ± 4.8 in TNR +/+ mice, and 48.8 ± 4.7 in TNR -/- mice, n=12 and 8 cells, respectively,

$p < 0.05$; 25ms: 60.8 ± 6.8 in TNR +/+ mice, and 38.8 ± 2.8 in TNR -/- mice, n=12 and 8 cells, respectively, $p < 0.05$; 10ms: 42.5 ± 4.5 in TNR +/+ mice, and 25.4 ± 4.3 in TNR -/- mice, n=12 and 7 cells, respectively; $p < 0.05$) (**Fig. 2.7g,h**). We also observed a reduction in the charge (**Fig. 2.7g,h**), but not the kinetic (data not shown), of the inhibitory event in response to the depolarization step. These results clearly indicated that there was a reduction in inhibition in the OB of adult TNR -/- mice compared to their littermates. These functional deficits can be explained by the fact that the number of interneurons integrating into the OB circuitry as well as the spine density of these newborn cells is significantly reduced in adult TNR -/- mice.

2.3.6 Reduced inhibition of mitral cells in adult TNR -/- mice alters their synchronous activity:

It is well established that inhibition caused by interneurons in the OB plays a major role in the synchronous activity of output neurons, which is crucial to relaying information to the piriform cortex (Lagier et al., 2004; Lagier et al., 2007). To investigate the effects of reduced inhibition on the synchronous activity of output neurons, local field potentials (LFP) were recorded from the mitral cell layer in acute slices from P25-P30 and P60-P120 mice. The olfactory nerve layer was briefly stimulated at 150-250μA to elicit LFP oscillations in the gamma range (20-80 Hz) (**Fig. 2.8a,c**). We calculated the oscillatory index as the strength of the oscillations, which represents the integral of the oscillations taken at a given time point. No differences in the oscillatory index were observed between genotypes at P25-P30 (**Fig. 2.8a,b**). Importantly, however, the oscillatory index was significantly reduced in the adult, P60-P120 TNR -/- mice compared to their TNR +/+ littermates (**Fig. 2.8c,d**). A reduction in the oscillatory index was observed over the initial 400-ms recording period (0-200ms: 4.7 ± 0.5 in TNR +/+ mice, and 3.2 ± 0.2 in TNR -/- mice, n=13 and 14 slices, respectively, $p < 0.05$; 200-400ms: 3.4 ± 0.3 in TNR +/+ mice, and 2.7 ± 0.2 in TNR -/- mice, n=13 and 14 slices, respectively, $p < 0.05$). These results indicate that the decrease in the number of interneurons and the

reduction in the spine density of those that had integrated into the adult TNR -/- OB affected the overall inhibition of the mitral cells, which in turn might affect the strength of the LFP oscillations.

2.3.7 Lack of TNR impairs some forms of olfactory behavior in adult but not young mice:

It has been suggested that newborn neurons play an important role in some but not all odor-associated behaviors (Imayoshi et al., 2008; Breton-Provencher et al., 2009; Lazarini and Lledo, 2011; Mandairon et al., 2011; Breton-Provencher and Saghatelyan, 2012). Since our histological and electrophysiological studies revealed specific alterations in adult but not young TNR -/- mice, we investigated how young and adult mice perform in different odor behavior tasks which were performed in sequence on the same mice at P25-P30 and P110-P120. We first determined the odor concentration threshold at which the mice were able to detect an odor. Individually housed mice were exposed to an ascending series of octanol concentrations (diluted 10^{-7}, 10^{-5}, 10^{-4}, and 10^{-3}). The times taken by each mouse to investigate the test odor and the control (mineral oil) were recorded. At P25-P30, both genotypes were able to detect the odor at dilutions of 10^{-4} and 10^{-3} (**Fig. 2.9a**). Adult P110-P120 TNR +/+ and -/- mice were able to detect the odor at a dilution of 10^{-3} (**Fig. 2.9a**).

We next tested the odor discriminative ability of young P25-P30 and adult P110-P120 knock-out mice. The mice were first habituated to heptanol (Hept) and then tested for their ability to discriminate between two discrete odors (limonene and Hept) as well as a mixture of different proportions of limonene (Lim) and Hept. In this habituation-dishabituation test, the mice were habituated to Hept diluted 10^{-3} using three consecutive 5-min exposures with 15-min intervals between each exposure. The investigation time during each consecutive exposure (habituation) decreased progressively. For the final presentation, the mice were presented with a novel odor (Lim) alone or mixed with Hept at different percentages. Both at P25-

P30 and P110-P120 the two genotypes increased their investigation times during the fourth exposure (dishabituation), indicating that they were able to discriminate Lim from Hept at all the concentrations tested, except for the mixture in which the concentration of the novel odor was lower than that of the odor used for habituation, (Lim/Hept: 48:52%) (**Fig. 2.9b**). Since adult TNR -/- mice have a much lower number of newborn neurons (Saghatelyan et al., 2004), and since neurogenesis-impaired animals have defects in discriminating the complex mixture of odors (Enwere et al., 2004), we also tested adult mice at other Lim/Hept ratios, but observed no differences in the odor discriminative ability between genotypes, regardless of the odor ratios (**Fig. 2.9b**).

Since changes in the number of newborn neurons affect short-term odor memory (Rochefort et al., 2002; Breton-Provencher et al., 2009; Breton-Provencher and Saghatelyan, 2012) we tested the spontaneous short-term memory of young and adult mice by initially presenting them with an odor for 5 min followed by the same odor at 30-, 60-, 90-,or 120- min intervals (**Fig. 2.10a**). We considered that the mice remembered a given odor if the odor investigation time during the second exposure was less that 50% of the investigation time during the first and second exposures. If the time was equal to or greater than 50%, we considered that the mice did not remember the odor. The mice were tested using one time interval per day, with a new odor for every time interval. At P25-P30, both genotypes remembered the odor presented to them at each time interval, except for the longest one (120 min). No difference between the genotypes was observed (**Fig. 2.10b**). Interestingly, however, at P110-P120, while the TNR +/+ mice remembered the odor presented to them at all time intervals (T30: 28.5 ± 3.5%; T60: 27.7 ± 1.9%; T90: 33.3 ± 2.0%; and T120: 32.6 ± 4.7%, n=10 mice), the TNR -/- mice displayed significant alterations at all time intervals tested (**Fig. 2.10c**). The TNR -/- mice were unable to remember the second presentation of the odor after 60, 90, and 120 min (T60: 45.1 ± 4%; T90: 39.0 ± 4.1%; and T120: 49.2 ± 3.5%, n=11 mice). Even after 30 min, TNR -/- mice spent more time investigating the odor

during the second presentation than their TNR +/+ littermates (28.5 ± 3.5% vs. 37.8 ± 2.8% for TNR +/+ and TNR -/- mice, n=13 and 11 mice, respectively; $p < 0.05$) (**Fig. 2.10c**). These results indicate that reduced neurogenesis resulting in impaired bulbar function selectively affects the spontaneous short-term memory of adult mice.

2.4. DISCUSSION.

The present study reveals that the mechanisms guiding radial migration of neuronal precursors in perinatal and adult OBs rely on different molecular pathways. We show that TNR is expressed relatively late during bulbar development and that it reduces radial migration and spine development of newborn granule cells in the adult but not perinatal OB. Reduction in the number of newborn neurons profoundly affect the functioning of the bulbar neuronal circuitry by decreasing the overall level of inhibition received by mitral cells, which alters their synchronized activity. This, in turn, induces changes in specific types of odor behavior in adult but not young TNR -/- mice. Our novel results highlight for the first time that molecular mechanisms regulating various processes in postnatal neurogenesis are distinct at different periods of an animal's lifetime.

2.4.1 Olfactory bulb neurogenesis in perinatal and adult OBs:

It is thought that adult OB neurogenesis is a continuum from developmental neurogenesis and that it relies on mechanisms that also operate during embryonic and early postnatal development. It is noteworthy, however, that the SVZ-OB pathway undergoes marked structural modifications during postnatal development (Law et al., 1999; Peretto et al., 2005; Bozoyan et al., 2012) and important morphological and functional differences exist between newborn neurons produced at different periods of postnatal development (Lemasson et al., 2005; Imayoshi et al., 2008; Kelsch et al., 2008; Breton-Provencher and Saghatelyan, 2012). Particularly pertinent to the present study are observations of differential targeting of newborn neurons produced during neonatal development and adulthood (Lemasson et al., 2005; Imayoshi et al., 2008). Newborn neurons produced during the early postnatal period are predominantly localized in the superficial GCL, whereas the percentage of adult-born neurons is higher in the deep GCL (Lemasson et al., 2005). In addition, genetic ablation of adult neurogenesis results

in the elimination of granule cells in the deep, but not superficial, GCL (Imayoshi et al., 2008). These observations indicate that the molecular mechanisms controlling the targeting of newborn neurons in neonatal and adult OBs may be different. In line with this study are our results showing that the expression of TNR in the OB peaks at P30, remains at this level into adulthood and that TNR plays an important role in guiding newborn neurons in the adult OB but not in the perinatal OB. While the mechanisms controlling radial migration in the perinatal OB remain to be elucidated, it has been previously shown that embryonic OB harbors radial glia that extends processes from the ventricle to the OB surface (Puche and Shipley, 2001). During development the majority of neuronal precursors in the neocortex, hippocampus and cerebellum use glial processes for their migration (Hatten, 2002) and it is likely that in the developing OB, neuroblasts also migrate along radial glia processes to reach their final destination. Interestingly, while it has been recently demonstrated that radial glia-like cells are present and might modulate the neuronal migration in the adult OB (Emsley et al., 2012), their number is much higher in the developing OB as compared to adulthood (Puche and Shipley, 2001; Emsley et al., 2012). Thus, radial glia could provide an important structural support for migrating neuroblasts and the higher number may account for radial migration in the developing OB. We propose that drastic reduction in the number of radial glia-like cells in the adulthood (Puche and Shipley, 2001; Emsley et al., 2012) combined with expression of specific molecular cues such as TNR underlies changes in mechanisms of radial migration during postnatal development. This could explain the different pattern and dynamic of radially migrating neuroblasts at different periods of an animal's lifetime. Altogether, our results provide the first evidence for the existence of distinct molecular pathways controlling radial migration of neuroblasts during different periods of postnatal bulbar neurogenesis.

2.4.2 Role of TNR in adult OB neurogenesis:

Using time-lapse video-imaging, we demonstrated that a lack of TNR decreases

the radial migration of neuroblasts in the adult OB, which is consistent with the accumulation of BrdU+ cells in the RMS_{OB} of TNR -/- animals (Saghatelyan et al., 2004). Interestingly, a lack of TNR affected radial migration in the RMS_{OB} and GCL, but not tangential migration in the RMS_{OB}, indicating that TNR is not a “stop” signal for tangentially migrating neuroblasts, but rather favors the initiation of radial migration in the OB. A lack of TNR resulted in longer stationary phases of radially migrating neuroblasts. It has been suggested that TNR can be a ‘conducive’ molecule (Lochter et al., 1995) and our data are in line with the facilitating effect of TNR on neurite outgrowth and growth cone dynamics of DRG neurons (Taylor et al., 1993). In addition, TNR affected the spine density of newborn GCs in the adult OB. In the hippocampus, TNR is localized at the edges of perisomatic inhibitory synapses (Saghatelyan et al., 2001) and TNR deficiency leads to structural alterations and a reduction in the number of perisomatic inhibitory synapses (Nikonenko et al., 2003).

Defects in the radial migration and spine development of newborn interneurons in the adult OB compromises the functioning of the bulbar neuronal network. Both spontaneous and evoked inhibitory currents received by mitral cells in TNR -/- mice were affected. In line with reduced number of interneurons and spine density, and thus reduced number of GABA release sites, the frequency of sIPSCs and mIPSCs was decreased. On the other hand, no changes in the amplitude and kinetics of sIPSCs and mIPSCs were observed, indicating that the functioning of postsynaptic $GABA_A$ receptors is not affected by the lack of TNR. This contrasts with the results obtained in the hippocampus, where a lack of TNR also affects the amplitude of postsynaptic $GABA_A$ receptor currents (Saghatelyan et al., 2001). This effect has, however, been attributed to the role of the HNK-1 carbohydrate moiety on the TNR protein backbone (Saghatelyan et al., 2000; Saghatelyan et al., 2003). Thus, expression of HNK-1 in the OB will be interesting to analyze. Evoked DDIs recorded from mitral cells were also affected in the adult TNR -/- mice. It has been shown that the inhibition received by mitral cells is required to synchronize their

activity (Bathellier et al., 2006; Lagier et al., 2007; Breton-Provencher et al., 2009). Experimental and theoretical approaches have revealed that decreased DDI amplitudes underlie changes in the oscillatory activity of mitral cells (Lagier et al., 2007). Our results showing that a decrease in the inhibitory drive of mitral cells in the adult TNR -/- mice resulted in reduced synchronized activity of these principal neurons is in agreement with this.

The synchronized activity of mitral cells is thought to be involved in odor information processing (Laurent et al., 2001; Saghatelyan et al., 2003; Lagier et al., 2007; Breton-Provencher et al., 2009) and newborn bulbar interneurons play an important role in this process (Lledo et al., 2006; Mandairon et al., 2011; Breton-Provencher and Saghatelyan, 2012). Our behavior experiments are also in line with these observations since adult TNR deficient mice showing compromised adult neurogenesis and changes in the functioning of the bulbar network, performed poorly in specific odor behavior tasks. In terms of spontaneous odor tasks, it has been shown that adult-born neurons are involved in the discrimination of perceptually similar odors and short-term odor memory (Rochefort et al., 2002; Moreno-Lopez et al., 2004; Breton-Provencher et al., 2009). Using two dissimilar odors at different dilutions, we were unable to detect any differences in the odor discrimination between TNR +/+ and TNR -/- mice, unlike observations showing that aged mice with reduced neurogenesis perform poorly in fine odor discrimination tasks (Enwere et al., 2004). It should be mentioned, however, that Enwere et al. used an associative odor discrimination paradigm where mice are trained to associate an odor with a water reward (Enwere et al., 2004), which may not be comparable to the test used in the present study. It is conceivable that, depending on the nature of the odor discrimination test (spontaneous vs. associative), the involvement of adult-born neurons may be different. In line with this are observations that the involvement of newborn neurons in operant vs. non-operant odor conditioning olfactory tests is different (Mandairon et al., 2011). With regard to spontaneous short-term odor memory it has been shown that environmental enrichment that increases the number of newborn neurons in the

adult OB improves short-term odor memory (Rochefort et al., 2002), whereas pharmacological ablation of adult-born neurons reduces short-term odor memory (Breton-Provencher et al., 2009). Our observation that spontaneous short-term memory is reduced in adult TNR -/- mice is in agreement with these reports. While several behavioral alterations such as increased anxiety and decreased motor coordination have been reported for TNR -/- mice (Freitag et al., 2003), we believe that these changes are unlikely to account for the modifications in odor behavior observed in the present study. First, we used the same experimental paradigm in terms of spatial, motor, and contextual characteristics for the odor discrimination, odor detection, and short-term odor memory tests, and observed modifications exclusively in short-term odor memory tasks. In addition, we used the same cohort of mice at P25-P30 and P110-P120 and observed changes in odor behavior only in the mice at P110-P120, whereas alterations in anxiety and motor behavior appeared already in mice at 3 weeks of age. Thus our study in addition of identifying the molecular cue that differently regulates radial migration and spine development of newborn cells in the perinatal and adult OB, also support the notion that that newborn neurons in the adult OB are involved in some, but not all, odor behavior tasks (Breton-Provencher et al., 2009; Breton-Provencher and Saghatelyan, 2012).

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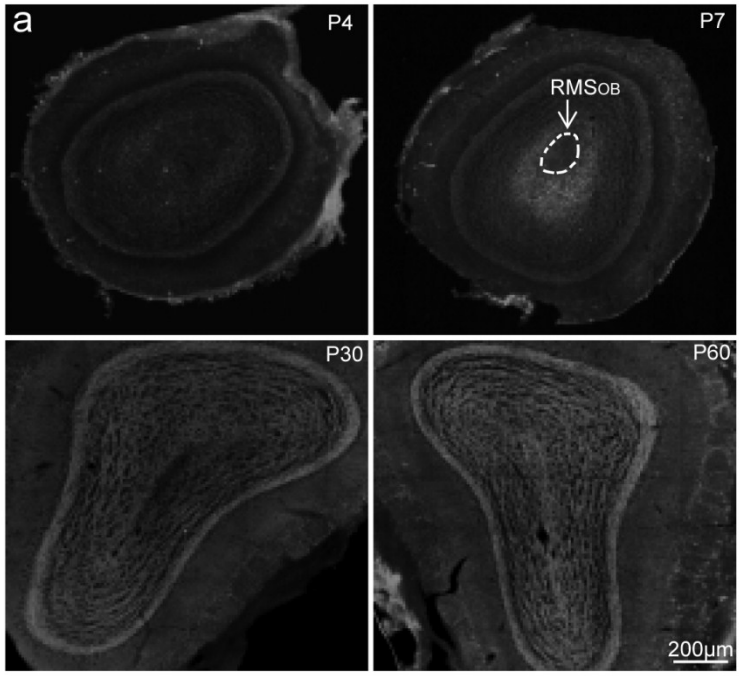
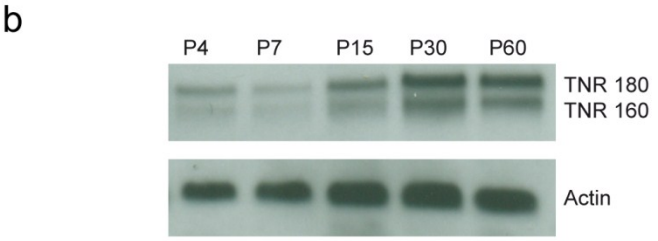

a
P4
P7
RMSOB
P30
P60
200μm


b
P4
P7
P15
P30
P60
TNR 180
TNR 160
Actin

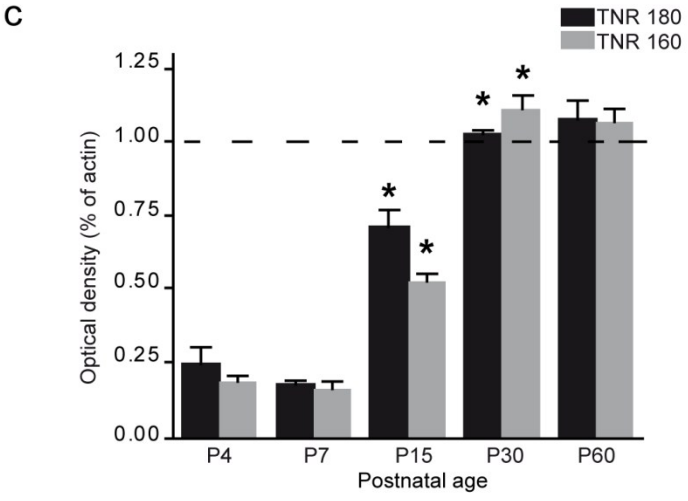

c
TNR 180
TNR 160
Optical density (% of actin)
1.25
1.00
0.75
0.50
0.25
0.00
P4
P7
P15
P30
P60
Postnatal age

Figure 11: Expression of TNR in the postnatal OB

a. Immunolabeling of TNR in coronal sections of the OBs of TNR +/+ mice from the early perinatal period to adulthood. TNR expression appears immediately around the RMS_{OB} at P7. **b**. Western blot analysis of OB samples from P4, P7, P15, P30, and P60 mice showing the late expression of TNR and the significant increase in both of its isoforms (160 and 180 kDa) with age. Actin was used as a loading control. **c**. Quantification of the expression of TNR in the OB. The optical density of the bands representing the two isoforms of TNR were normalized to the actin band from the same sample and were then plotted versus age to shown changes in the expression of the protein during postnatal development. Values are expressed as means ± SEM (n=3 OB extracts from TNR +/+ and -/- mice at each age).*$p < 0.05$ with Students t-test.

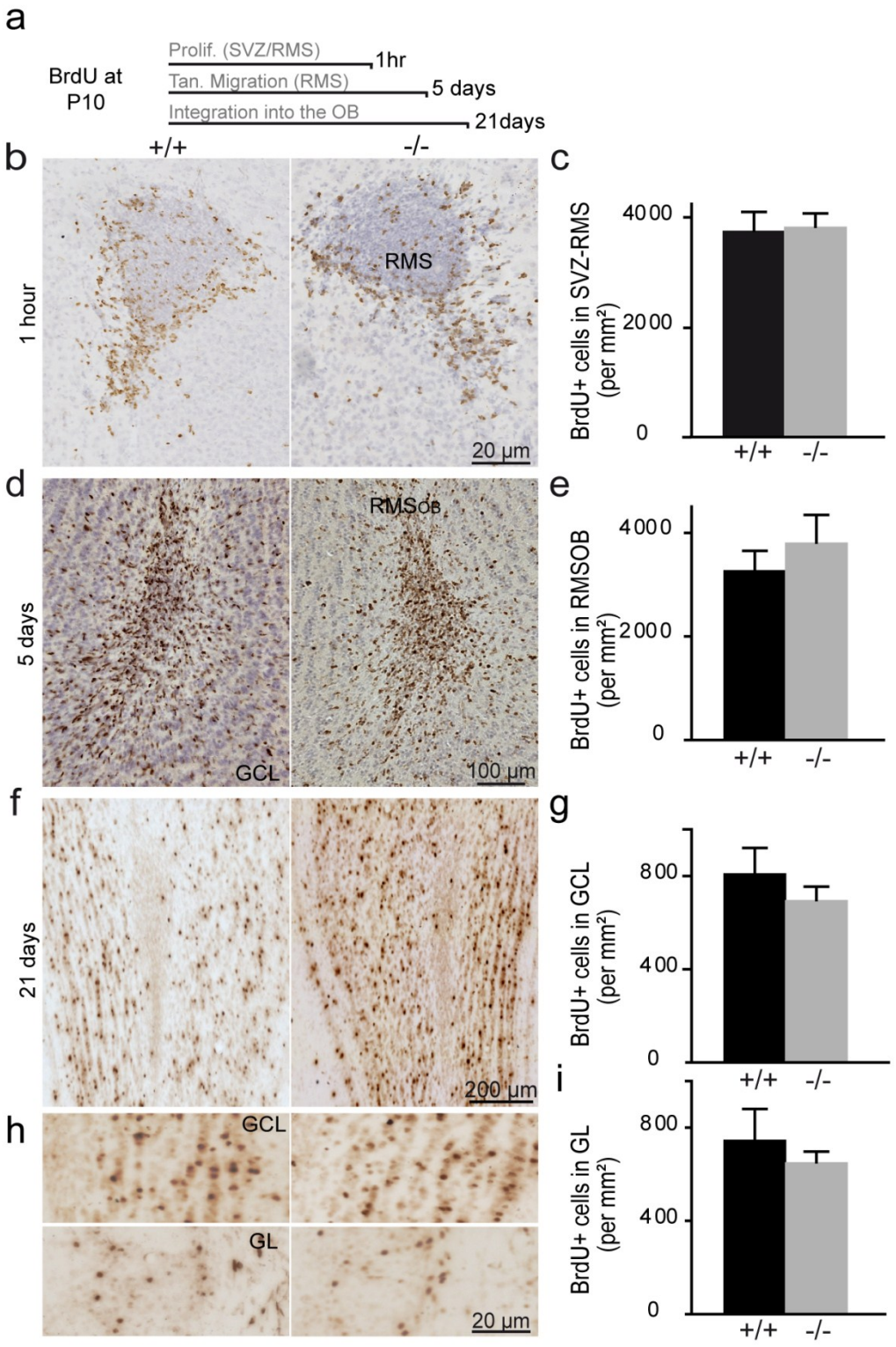

a
BrdU at P10
Prolif. (SVZ/RMS)
1hr
Tan. Migration (RMS)
5 days
Integration into the OB
21days
+/+
-/-
b
1 hour
RMS
20 µm
c
BrdU+ cells in SVZ-RMS (per mm²)
4000
2000
0
+/+
-/-
d
5 days
RMSOB
GCL
100 µm
e
BrdU+ cells in RMSOB (per mm²)
4000
2000
0
+/+
-/-
f
21 days
200 µm
g
BrdU+ cells in GCL (per mm²)
800
400
0
+/+
-/-
h
GCL
GL
20 µm
i
BrdU+ cells in GL (per mm²)
800
400
0
+/+
-/-

Figure 2.2: A lack of TNR does not affect the proliferation, migration, or integration of newborn interneurons into the perinatal OB

a. Schematic diagram showing the BrdU administration protocol and the time of sacrifice used to study the proliferation, migration, and integration of newborn interneurons in P10 pups. **b.** Similar numbers of BrdU+ cells can be seen in coronal sections of the SVZ-RMS from P10 TNR +/+ and -/- mice injected 1 h before being sacrificed.**c.** Immunolabeling showing BrdU+ cells in the RMS_{OB} of TNR +/+ and -/- mice. Two BrdU pulses were given at P10, and the mice were sacrificed 5 days later. **d.** Quantification of the density of BrdU+ cells in the SVZ-RMS and RMS_{OB} of P10 TNR +/+ and -/- mice, 1 h and 5 days after BrdU injection. No difference between the two genotypes was observed. Values are expressed as means ± SEM.**e.** Immunolabeling showing BrdU+ cells in coronal sections of OBs from TNR +/+ and -/- mice. Two BrdU pulses were given at P10, and the animals were sacrificed 21 days later. Lower panels show the magnified regions of GCL and GL in TNR +/+ and -/- mice.**f.** Quantification of the density of BrdU+ cells in the GCL and GL of TNR +/+ and -/- mice. Values are expressed as means ± SEM.

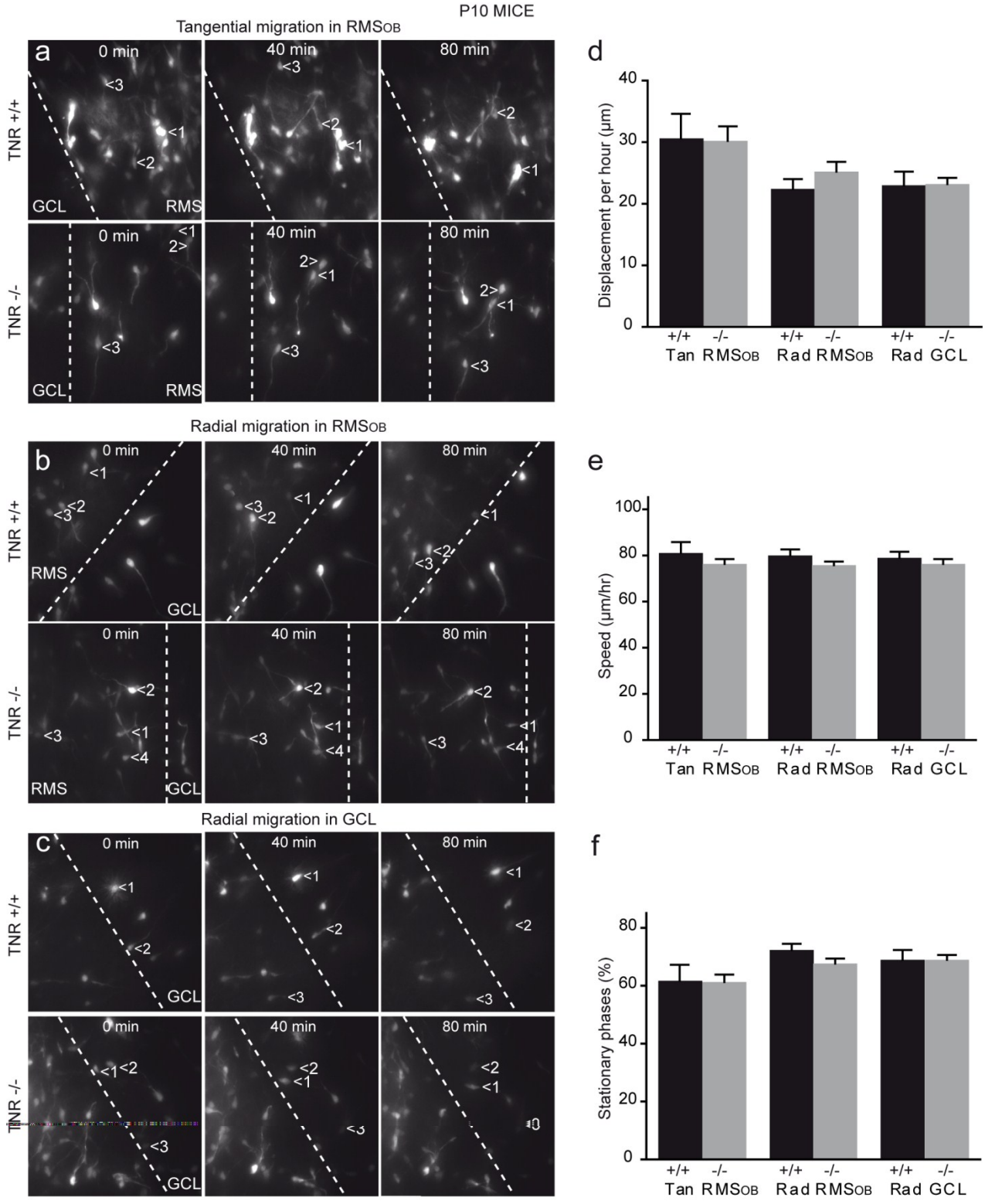

P10 MICE
Tangential migration in RMSOB
a
0 min
40 min
80 min
TNR +/+
TNR -/-
GCL
RMS
Radial migration in RMSOB
b
Radial migration in GCL
c
d
Displacement per hour (µm)
e
Speed (µm/hr)
f
Stationary phases (%)
+/+ -/-
Tan RMSOB
Rad RMSOB
Rad GCL

Figure 12.3: A lack of TNR does not affect tangential and radial migration in the developing OB

a-c. Snapshots of time-lapse video-imaging experiments showing tangentially (a) and radially migrating neuroblasts in the RMS_{OB} (b) and GCL (c) of P15-P16 TNR +/+ and -/- mice. Times are indicated in minutes and white dashed line indicates the border of RMS_{OB}.**d-f.** Quantification of total displacement during a 1-h recording (d), speed of migration (e), and percentage of time spent in the stationary phase (f) of neuroblasts migrating in acute OB slices prepared from P15-P16 TNR +/+ and -/- mice. Values are expressed as means ± SEM.

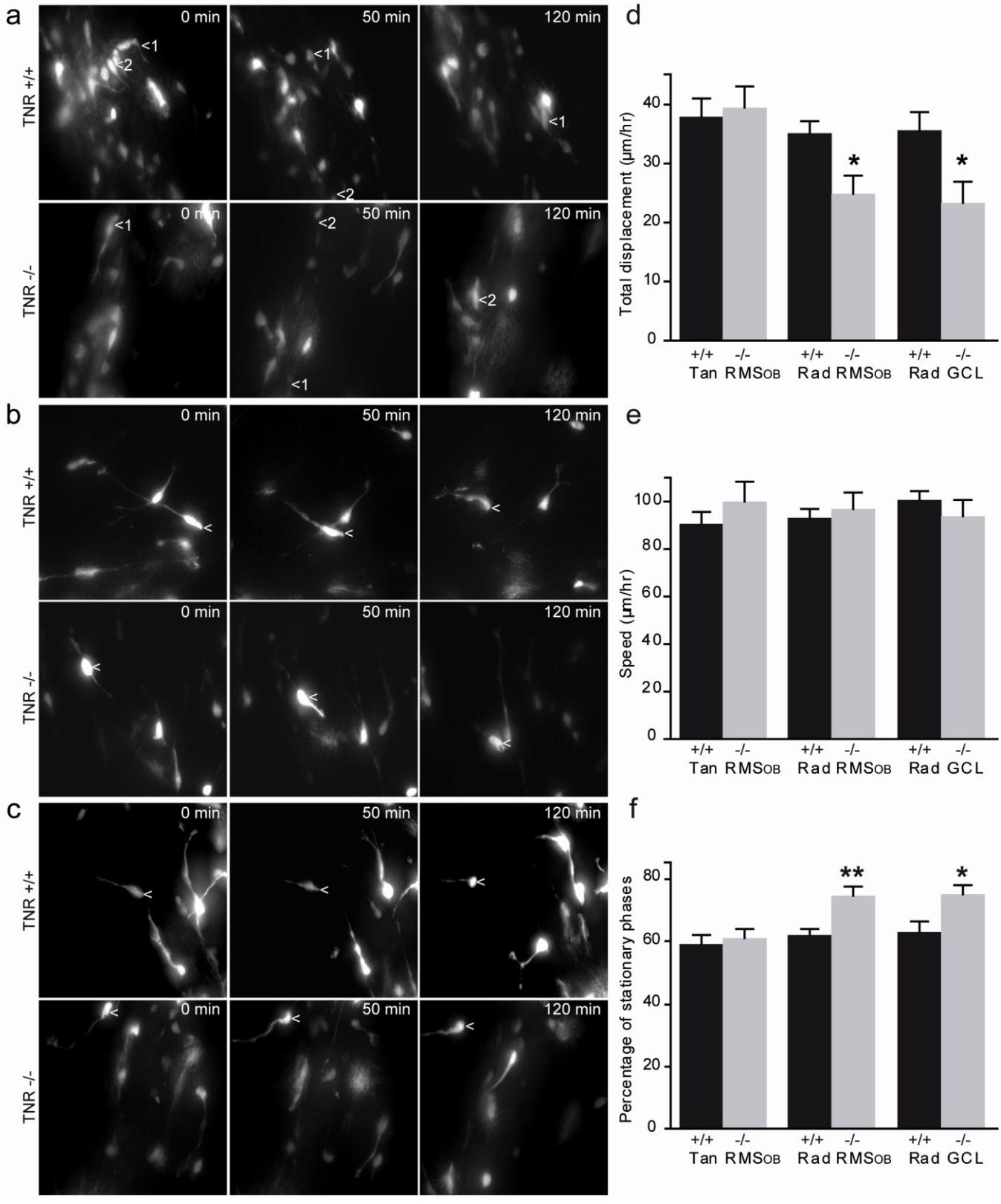

a
0 min
50 min
120 min
TNR +/+
TNR -/-
<1
<2
b
c
d
Total displacement (μm/hr)
e
Speed (μm/hr)
f
Percentage of stationary phases
*
**
+/+ -/-
Tan RMSOB
Rad RMSOB
Rad GCL

Figure 2.4: Affected radial migration of neuroblasts in the RMSOB and GCL of adult TNR -/- mice.

a-c. Snapshots of time-lapse video-imaging experiments showing tangentially (**a**) and radially migrating neuroblasts in the RMS_{OB} (**b**) and GCL (**c**) of TNR +/+ and -/- mice. Times are indicated in minutes in the upper right corner and white dashed line indicates the border of RMS_{OB}. **d-f**. Quantification of total displacement during a 1-h recording (**d**), speed of migration (**e**), and percentage of time spent in the stationary phase (**f**) of neuroblasts migrating in acute OB slices prepared from adult TNR +/+ and -/- mice. Note the significant decrease in the total displacement time and the increase in the percentage of time spent in the stationary phase by neuroblasts migrating radially in the OBs of adult TNR -/- mice. Values are expressed as means ± SEM. *p< 0.05 and *p< 0.01with Students t-test.

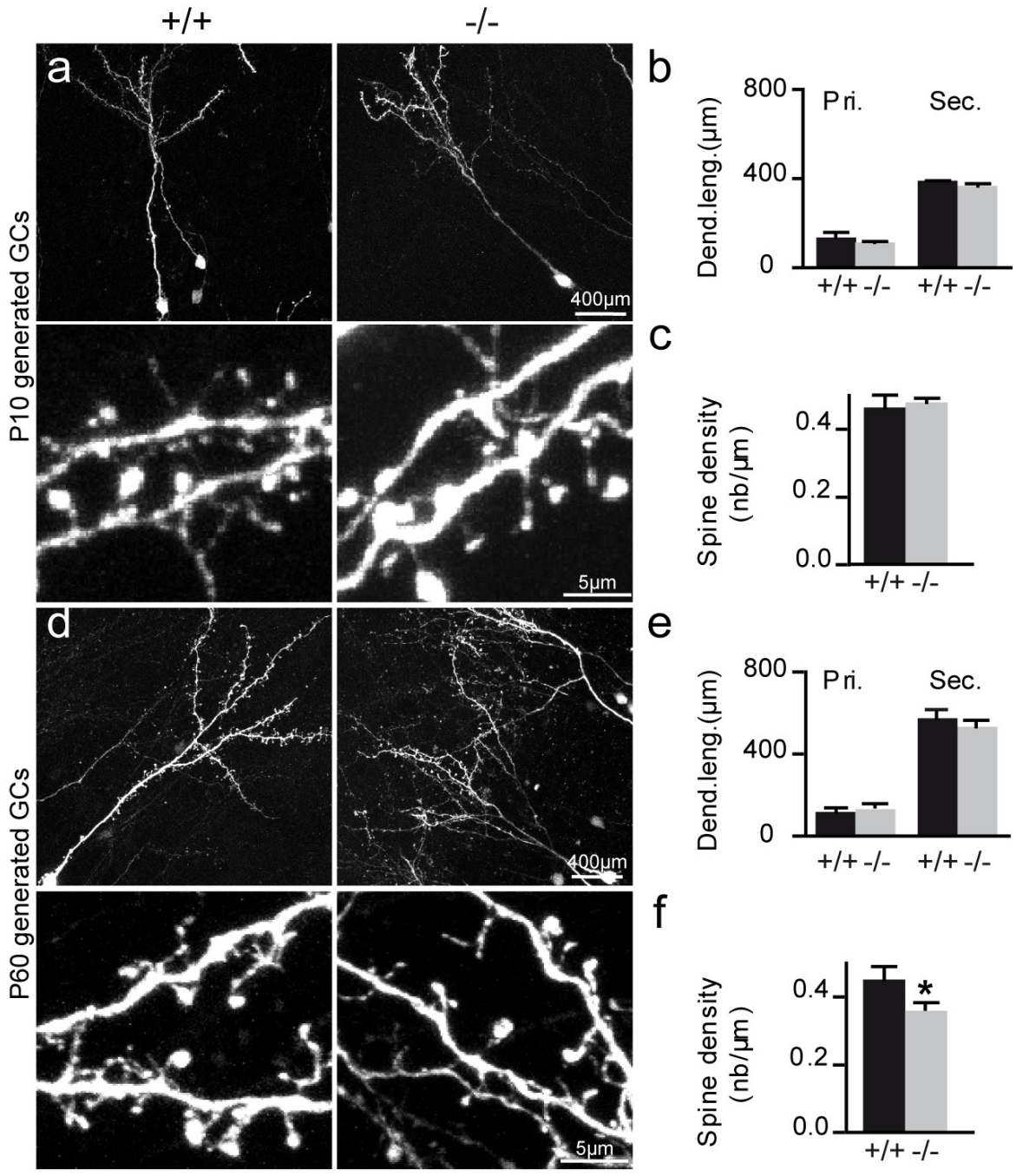

+/+
-/-
a
b
P10 generated GCs
400µm
5µm
c
Dend.leng.(µm)
800
400
0
Pri.
Sec.
+/+ -/-
+/+ -/-
Spine density (nb/µm)
0.4
0.2
0.0
+/+ -/-
d
e
P60 generated GCs
400µm
5µm
f
*

Figure 2.5: Decreased spine density of newborn interneurons integrating into the adult bulbar circuits of TNR -/- mice

a. Top: Confocal images of granule cells expressing a GFP encoded by a lentivirus injected into the RMS of P10 TNR +/+ and -/- mice 21 days prior to processing. Bottom: Magnified regions showing the dendrites and individual spines on granule cells in TNR +/+ and -/- mice. **b.** Top: Confocal images of granule cells expressing a GFP encoded by a lentivirus injected into the RMS of adult TNR +/+ and -/- mice 21 days prior to processing. Bottom: Magnified regions delineated in the top panels showing the dendrites and individual spines on granule cells in TNR +/+ and -/- mice. **c.** No differences in the lengths of the primary and secondary dendrites were observed in the granule cells of TNR +/+ and -/- mice at different developmental ages. Values are expressed as means ± SEM. **d.** A significant decrease in spine density can be seen in granule cells derived from adult, but not early postnatal, TNR -/- mice compared to their +/+ counterparts. Values are expressed as means ± SEM. *$p < 0.05$ with Students t-test.

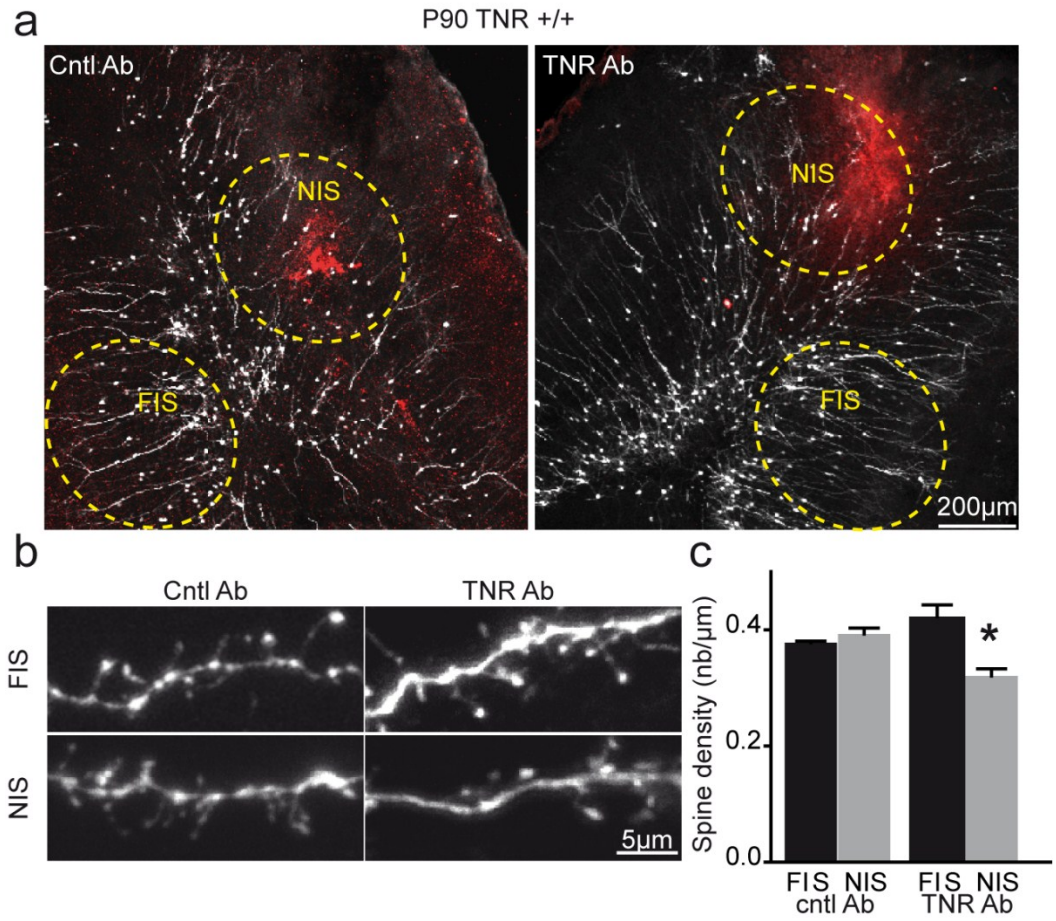


Figure 2.6: TNR plays a direct role in the spine development of newborn granule cells

a. Low magnification images of granule cells expressing a GFP (white) encoded by a lentivirus injected into the RMS of P10 TNR +/+ mice, 10 days before injection of control (Ctrl, left panel) and anti-TNR (right panel) antibodies directly into the OB. The spine density of newborn granule cells was calculated at the sites of antibodies injection (NIS; red) and was compared to the spine density at remote sites (FIS). Magnified regions showing the dendrites and individual spines on granule cells. **b.** Confocal images of granule cells expressing a GFP showing the spines in the FIS and NIS in TNR +/+ mice injected with control (Ctrl, left panel) and anti-TNR (right panel) antibodies. Note, low number of spines in the regions injected with TNR antibodies. **c.** A significant reduction in spine density can be observed for granule cells present in anti-TNR antibody injected bulbar regions

(NIS, TNR Ab). Values are expressed as means ± SEM. *p< 0.05 with Students t-test.

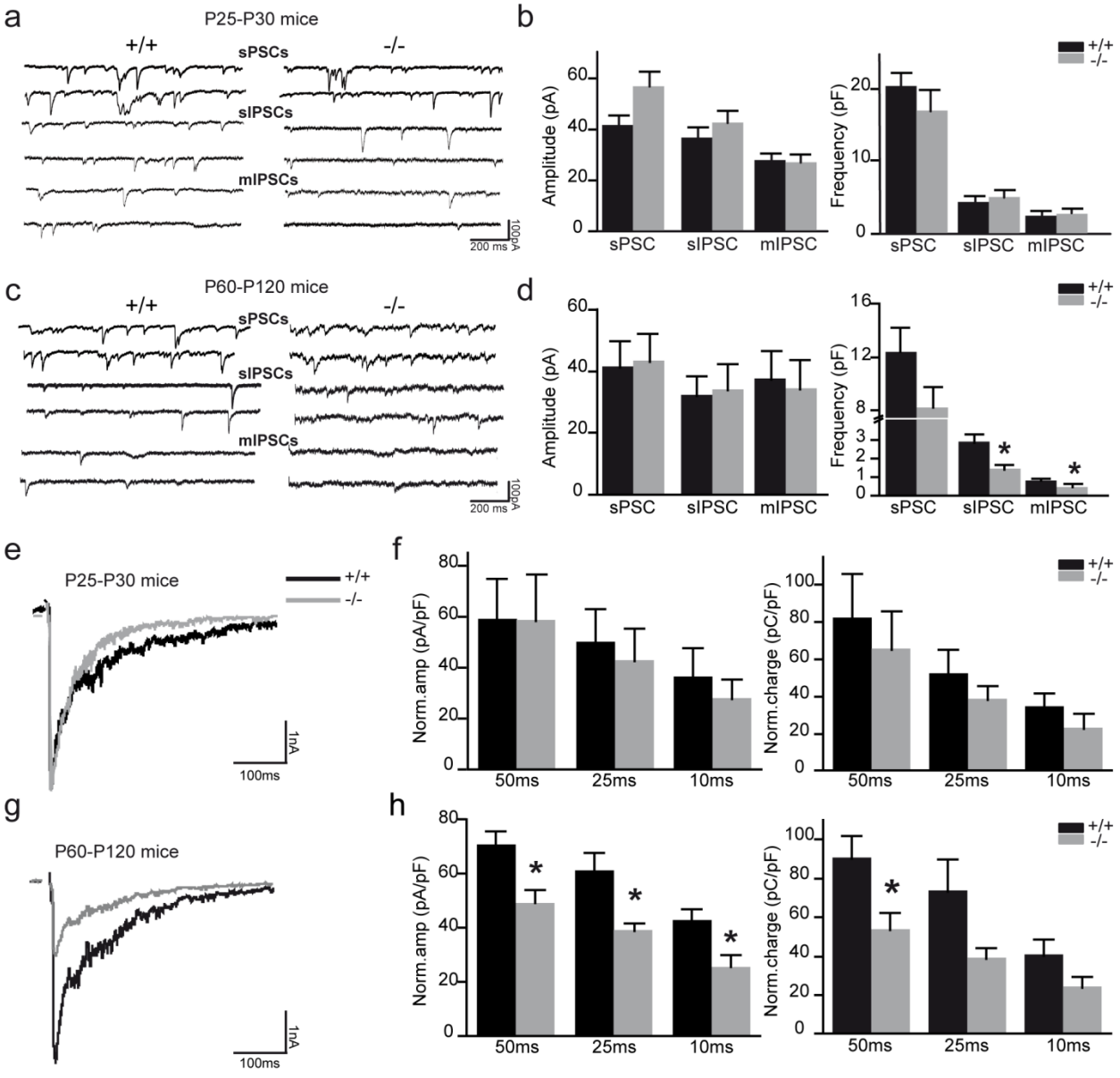

a
P25-P30 mice
+/+
-/-
sPSCs
sIPSCs
mIPSCs
100pA
200 ms
b
Amplitude (pA)
Frequency (pF)
+/+
-/-
sPSC
sIPSC
mIPSC
c
P60-P120 mice
d
e
P25-P30 mice
1nA
100ms
f
Norm.amp (pA/pF)
Norm.charge (pC/pF)
50ms
25ms
10ms
g
P60-P120 mice
h

Figure 2.7: Mitral cell inhibition is reduced in adult but not young TNR -/- mice

a. Electrophysiological recordings showing spontaneous sPSCs, sIPSCs, and mIPSCs in the mitral cells of P25-P30 TNR +/+ and -/- mice.**b.** Quantification of the amplitude and frequency of sPSCs, sIPSCs, and mIPSCs did not reveal any differences between P25-P30 TNR +/+ and -/- mice. Values are expressed as means ± SEM.**c.** Electrophysiological recordings showing spontaneous sPSCs, sIPSCs, and mIPSCs in the mitral cells of P60-P120 TNR +/+ and -/- mice.

d. A significant decrease can be seen in the frequency but not the amplitude of sIPSCs and mIPSCs in mitral cells recorded from P60-P120 TNR -/- mice compared to their +/+ counterparts. Values are expressed as means ± SEM. *p< 0.05 with Student t-test. **e,g**. Dendrodendritic inhibition (DDI) recorded in mitral cells in TNR +/+ and -/- mice in response to a 50-ms depolarization step at P25-P30 and P60-P120. **f.** No differences can be seen in the charge or amplitude of DDIs normalized to mitral cell capacitance recorded in P25-P30 TNR +/+ and -/- mice for any duration of the depolarization steps used to induce DDI. Values are expressed as means ± SEM. **h.** The amplitude and charge of DDIs in P60-P120 TNR -/- mice are lower than in their +/+ counterparts. Values are expressed as means ± SEM. *p< 0.05 with Student t-test.

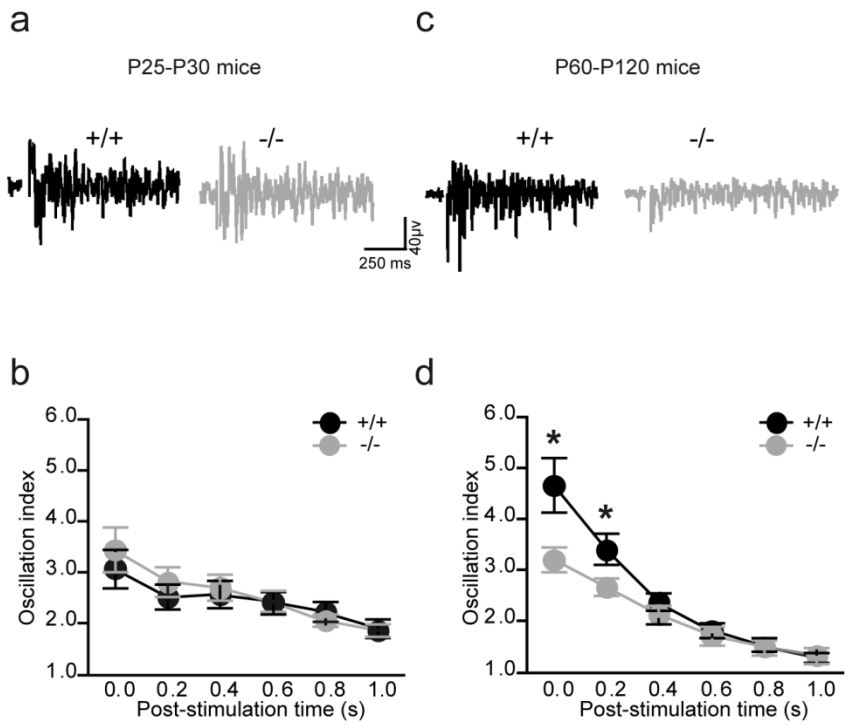
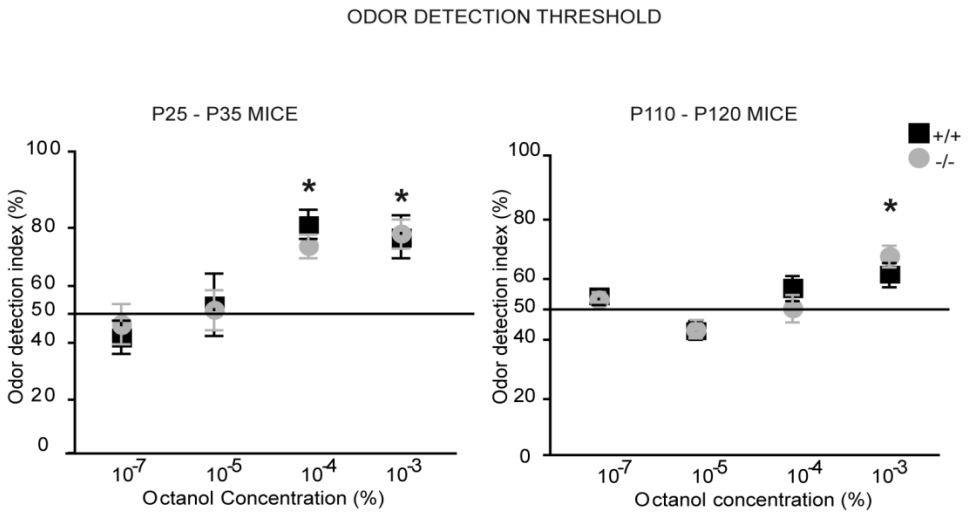


Figure 2.8: Synchronized oscillatory activity is reduced in adult TNR -/- mice

a, c. Local field potential (LFP) recordings from the mitral cell layer of acute slices derived from P25-P30 and P60-P120 TNR +/+ and -/- mice. **b,d.** The oscillation index was calculated by comparing the oscillations in a 200-ms window of the pre-stimulus baseline to the post-stimulus response. The oscillation index decreased significantly over the initial 400-ms post-stimulation period in adult (**d**) but not young (**b**) TNR -/- mice compared to their +/+ counterparts, indicating that the synchronized oscillatory activity of mitral cells is lower in TNR -/- mice than their +/+ counterparts. Values are expressed as means ± SEM. *p< 0.05 with Student t-test.

a


ODOR DETECTION THRESHOLD
P25 - P35 MICE
P110 - P120 MICE
+/+
-/-
Odor detection index (%)
Octanol Concentration (%)
Octanol concentration (%)

b

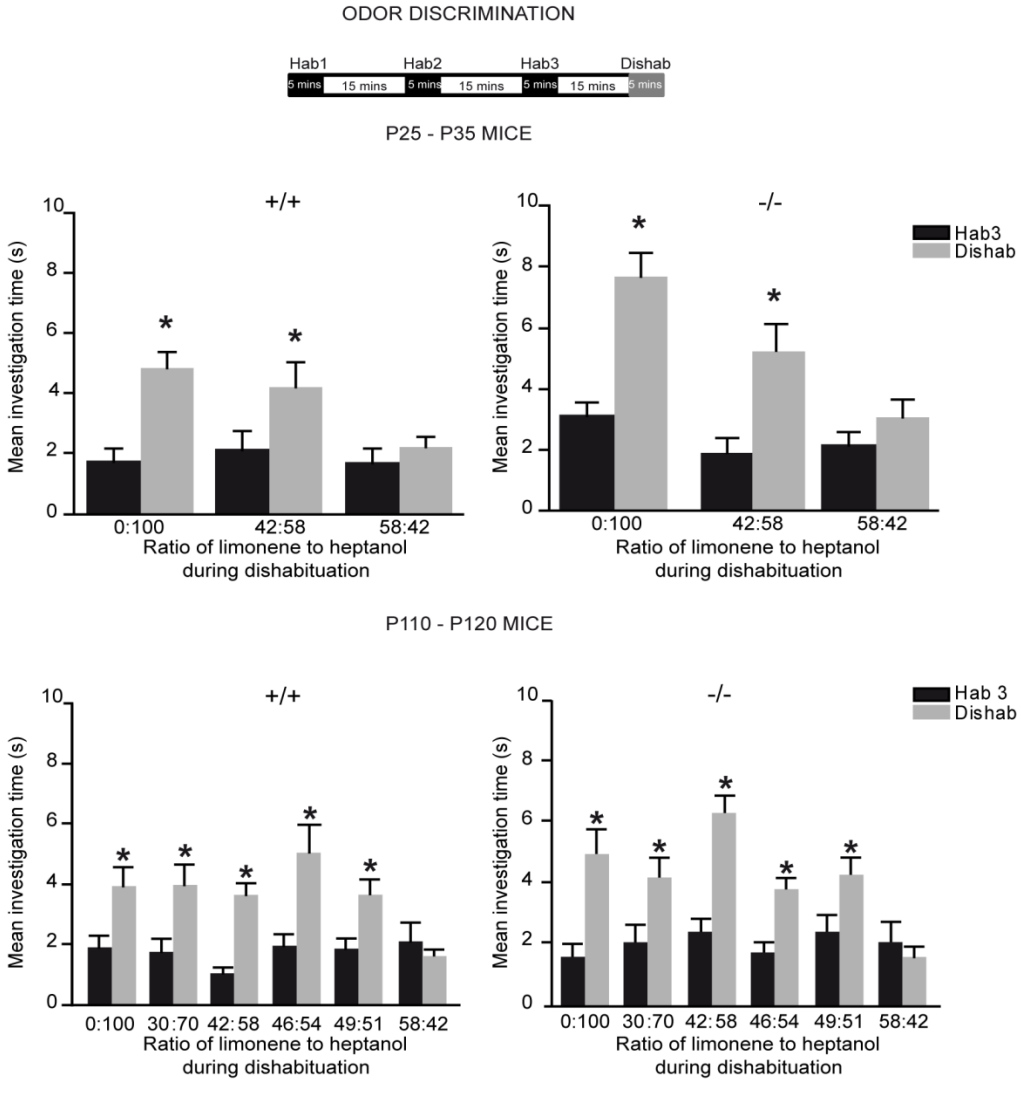

ODOR DISCRIMINATION
Hab1
Hab2
Hab3
Dishab
5 mins
15 mins
P25 - P35 MICE
+/+
-/-
Hab3
Dishab
Mean investigation time (s)
0:100
42:58
58:42
Ratio of limonene to heptanol during dishabituation
P110 - P120 MICE
Hab 3
30:70
46:54
49:51

Figure 2.9: Odor detection and discrimination is unaltered in adult TNR -/- mice

a. Odor detection thresholds in P25-P30 and P110-P120 TNR +/+ and -/- mice. Normalized values are expressed as the mean ratio of the time taken to investigate an odor at a given dilution (10^{-7}, 10^{-5}, 10^{-4}, and 10^{-3}) and the total investigation time (a combination of the time spent investigating the odor and the time spent investigating the control pipette containing mineral oil). A significant increase above the 50% threshold indicated that animals are able to detect an odor. Note that the odor detection threshold of P25-P30 and P110-P120 TNR +/+ and -/- mice are undistinguishable. Values are expressed as means ± SEM. *$p < 0.05$ with Student t-test. **b.** Top: Experimental design showing the habituation-dishabituation protocol for the odor discrimination test. Bottom: Comparison of the time taken to investigate the odor during the dishabituation (grey) and final habituation (black) exposures. During the dishabituation exposure, a novel odor (limonene) was presented at varying ratios with the habituation (heptanol) odor. Note that the abilities of P25-P30 and P110-P120 TNR +/+ and -/- mice to discriminate between the mixtures of odors are undistinguishable. Values are expressed as means ± SEM. *$p < 0.05$ with Student t-test.

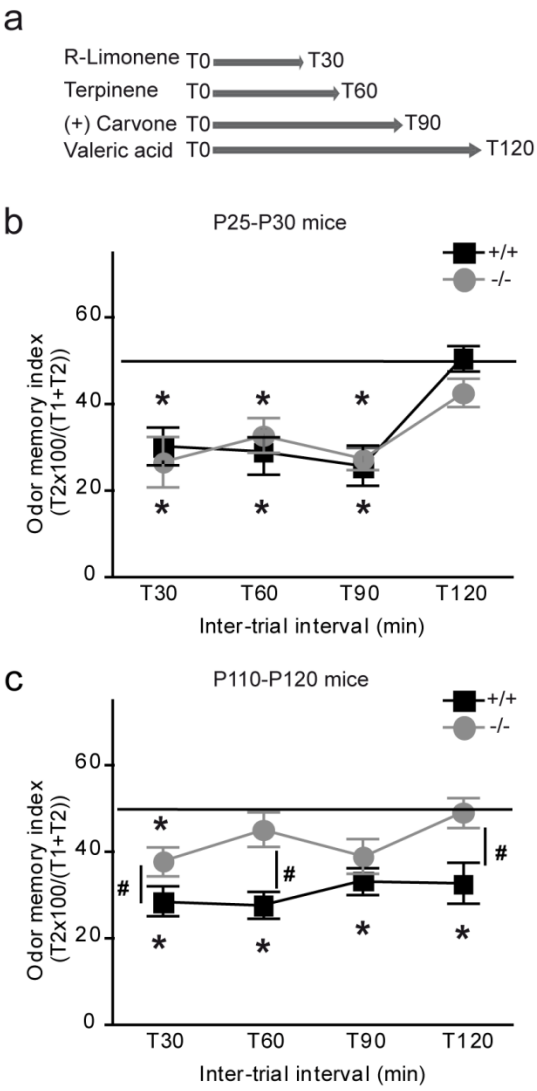

a
R-Limonene T0 T30
Terpinene T0 T60
(+) Carvone T0 T90
Valeric acid T0 T120
b
P25-P30 mice
+/+
-/-
Odor memory index (T2x100/(T1+T2))
0
20
40
60
T30
T60
T90
T120
Inter-trial interval (min)
c
P110-P120 mice
+/+
-/-
Odor memory index (T2x100/(T1+T2))
0
20
40
60
T30
T60
T90
T120
Inter-trial interval (min)

Figure 2.10: The short-term odor memory of adult TNR -/- mice is affected

a. Experimental design showing the odors and time intervals used to evaluate the short-term odor memory of P25-P30 and P110-P120 TNR +/+ and -/- mice. **b.** The odor memory index was calculated as a percentage of the time taken by the mice to investigate the odor at the second exposure compared to the combined time spent investigating the odor during first and second exposures. Note that the P25-P30 TNR +/+ and the -/- mice exhibit the same ability to remember the test odor at any interval other than the longest (120 min). Values are expressed as means ± SEM.*$p < 0.05$ with Student t-test. **c**. The same cohort of mice tested at P110-P120 of age showed that adult TNR -/- mice do not remember the test odor tested at any interval other than the shortest (30 min), whereas the adult TNR +/+ mice remembered the test odor at all the intervals, including the longest (120 min). Values are expressed as means ± SEM.*$p < 0.05$ and #$p < 0.05$ with Student t-tests. * indicates a significant difference between the first and second exposures, whereas # indicates a significant difference between genotypes.

Chapter III:

CaMKIIα expressing granule intereneurons represent a functionally distinct subpopulation of adult generated OB interneurons.

Linda Suzanne David
Dr. Armen Saghatelyan

This work which is still in progress is being done in collaboration between the labs of Dr Armen Saghatelyan, University Laval, Quebec, Canada and Dr. Isabelle Caille, Pierre and Marie Curie University, Paris, France. Dr Simona Grebado in the laboratory of Dr. Caille is in charge of morphological part, as well as characterization of immediate early gene expression in different population of adult-born neurons following odor stimulation. I performed all electrophysiological experiments. It is also planned to performed behavioural experiments.

3.1 INTRODUCTION.

The olfactory bulb (OB) is one of two regions in the adult brain that retain the ability to acquire new interneurons throughout an animal's life. The OB interneurons are, in part, generated during adulthood from neural stem cells in the sub ventricular zone (SVZ) bordering the lateral ventricle (Alvarez-Buylla and Garcia-Verdugo, 2002; Breton-Provencher and Saghatelyan, 2012). Neuronal precursors travel in chains toward the OB along the blood vessels in the rostral migratory stream (RMS) (Snapyan et al., 2009). Once in the OB, neuronal precursors differentiate

into periglomerular (5%) and GCs (95%) (Batista-Brito et al., 2008), mature (Petreanu and Alvarez-Buylla, 2002), acquire the electrophysiological properties of fully developed neurons (Belluzzi et al., 2003; Carleton et al., 2003), and integrate into bulbar network by forming output dendro-dendritic synapses with the principal cells (Whitman and Greer, 2007). Interestingly, while neuronal diversity among adult-born periglomerular cells is widely recognized (Batista-Brito et al., 2008), it is still though that adult-born GCs represent a homogenous population. Adult-born calretinin expressing GCs is the rare population of adult-born cells that was described for GCs (Batista-Brito et al., 2008). In this chapter I demonstrate that adult-born GCs can be sub-divided to distinct populations based on the expression of CamKIIa.

CaM kinases (CaMK) are a family of serine/threonine kinases. CaMKIIα is one of the most abundant proteins in the brain and is a major component of the postsynaptic density of neurons where it plays an important role in synaptic function. CaMKII is a multifunctional protein that is regulated by intracellular calcium levels.

CaMKII is known to exist as 28 isoforms due to the α, β, γ and δ genes that code for this protein. The structure of the holoenzyme was seen to be made up of 12 kinase functional domains, connected to the core as two clusters of six. Each of these subunits contains a catalytic, an autoregulatory and an association domain (Hudmon and Schulman, 2002). CaMKII is kept relatively inactive by the auto inhibitory domain, binding of Ca2+/calmodulin eliminates the inhibition and allows the kinase to phosphorylate its substrate and also itself (Schulman and Hanson, 1993).

Several functions have been attributed to CaMKII. Analysis of autophosphorylation /dephosphorylation indicates that this kinase could be implicated in long term memory storage (Lisman et al., 2002). Studies have shown that a block of the persistent activation of CaMKII blocks long term potentiation (LTP), experience-

dependent plasticity and memory (Lisman et al., 2002). The establishment of LTP has been shown to be due to the CaMKII/NMDAR complex formation. Once formed the CaMKII/NMDAR complex, serves as a tag, which leads to a binding cascade involving densin, delta-catenin, and N-cadherin to maintain the LTP (Sanhueza and Lisman, 2013). Electrophysiological studies in hippocampal slices have shown that H7 membrane permeable kinase inhibitors decrease excitatory synaptic responses phosphorylation of the presynaptic protein synapsin1, required for sustained synaptic transmission at mammalian synapses (Waxham et al., 1993). CaMKII has also been known to mediate the effect of most hormones, neurotransmitters and growth factors, carbohydrate metabolism, cell cycle and gene expression (Schulman, 1993; Schulman and Hanson, 1993).

This chapter is dedicated to describing my yet unpublished study demonstrating that adult-born neurons can be sub-divided to CamKIIα positive GCs and those that are CamKIIα negative; and that these two subpopulations may play different functions in the OB network functioning and odor behaviour.

3.2. MATERIALS AND METHODS.

3.2.1 Animals

C57/ Bl6 wild-type (Charles River) adult mice were used in all experiments. All experimental protocols were approved by the Université Laval animal protection committee. The mice were kept on a 12 h light/dark cycle at a constant temperature (22°C) with food and water available *ad libitum*.

3.2.2 Vectors used and stereotaxic injections

Adeno associated viral vectors were used to label newly generated adult granule neurons. Viral vectors were made at the *«Molecular Tools Platform"* at the Institute universitaire en santé mentale de Quebec. Two adeno associated viral vectors expressing green fluorescent protein (GFP) were used in this study. As a control we used vectors expressing GFP under the chicken beta actin (CBA) promoter that

labeled newly born interneurons irrespective of their molecular characteristics. In order to specifically label the newly generated interneuron subpopulation that express CaMKIIα we used a second vector expressing GFP under the CaMKIIα promoter. The efficiency of infection of these vectors was tested before use.

Adult C57/ Bl6 mice were stereotaxically injected with either the control or CaMKIIα specific vector directly in the RMS. The coordinates for the RMS with respect to bregma are anterior–posterior 2.55, medial–lateral 0.82, and dorsal–ventral 3.15, respectively. The mice were then allowed to recover and returned to their home cages. The experiments were carried out using these mice 2-3 weeks after injection.

3.2.3 Acute live slice preparation

Acute, horizontal, 250-µm-thick slices were prepared for electrophysiological recordings from mice injected with either the control or the CaMKIIα vector two weeks earlier. The adult mice were anesthetized with ketamine/xylazine, perfused transcardially with ice-cold sucrose-based artificial CSF (ACSF) containing the following (in mM): 250 sucrose, 3 KCl, 0.5 $CaCl_2$, 3 $MgCl_2$, 25 $NaHCO_3$, 1.25 $NaHPO_4$, and 10 glucose. The brains were rapidly removed and immersed in the solution used for the transcardiac perfusion. Horizontal slices of the OBs were obtained using a vibratome. After a 30min recovery period at 32°C, the slices were placed in the recording chamber and were continuously perfused with oxygenated ACSF containing the following (in mM): 124 NaCl, 3 KCl, 2 $CaCl_2$, 1.3 $MgCl_2$, 25 $NaHCO_3$, 1.25 $NaHPO_4$, and 10 glucose at a rate of 1.5–2 ml/min (bubbled with 95% O_2/5% CO_2; pH ≈7.4).

3.2.4 Electrophysiological recordings

Acute horizontal 250-µm-thick vibratome slices (Thermo scientific) were prepared for patch-clamp recordings as described above. Recordings were made using a Multiclamp 700A amplifier (Molecular Devices). Patch electrodes with resistances ranging from 7 to 9 MΩ were filled with a CsCl based solution in order to record

inhibitory currents from GFP labeled granule interneurons. The intracellular solution contained the following (in mM): 135 CsCl, 10 HEPES, 0.2 EGTA, 2 ATP, 0.3 GTP, and 10 glucose, pH≈7.2. Spontaneous IPSCs (sIPSCs) were isolated by bath application of 5 mM kynurenic acid (Kyn) to block glutamatergic activity. Miniature IPSCs (mIPSCs) were isolated by applying 1µM tetrodotoxin (TTX) to block voltage-sensitive sodium channels in the presence of 5 mM Kyn. For recording EPSCs the patch electrodes were filled with a K-Methylsulfate based solution containing the following (in mM): 130 K- Methylsulfate, 10 HEPES, 6 Kcl, 2 ATP, 0.4 GTP, 10 Na-Phosphocreatine and 2 ascorbate, pH≈7.2. Spontaneous EPSCs (sEPSCs) were isolated by bath application of 50 µM Biccuculine methiodide (BMI) to block $GABA_A$ receptors. Miniature EPSCs (mEPSCs) were isolated by applying 1µM tetrodotoxin (TTX) to block voltage-sensitive sodium channels in the presence of 50 µM BMI.

Both intracellular solutions also contained biocytin at a concentration of 2.5 mM for post-Hoc identification of patched GCs.

3.2.5 BrdU labeling and CaMKIIα immunohistochemistry

In order to be able to assess the ratio of newborn interneurons expressing CaMKIIα, we performed bromodeoxyuridine (BrdU) labeling of newborn GCs. Adult C57/ Bl6 wild-type mice were injected with DNA synthesis marker BrdU. BrdU (Sigma) was dissolved in sterile 0.9% NaCl. The mice were administered with 50 mg/kg BrdU and sacrificed at varying times from 5 days post injection up to 8 weeks post injection. The mice were deeply anesthetized and transcardiacally perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA). The brains were post-fixed in 4% PFA overnight at 4°C, and 40-µm-thick free-floating slices were made on a vibratome (VT 1000S; Leica). The sections were treated with 2N HCl for 40 min at 37°C to denature the DNA. They were then incubated overnight with a rat anti-BrdU monoclonal antibody (1:500; Serotec) at 4°C in 0.2% Triton-X supplemented with 4% bovine serum albumin (BSA), followed by anti-rat Alexa488 secondary antibody (Life Technologies).

Immunohistohemistry for CamKIIα was performed as described before (David et al., 2013). Horizontal slices of 250 µm used for electrophysiological recordings and post fixed over night with 4% PFA or sagittal slices of 40 µm thicknesses were permiabilized with 0.5% and 0.2% triton respectively supplemented with 4% BSA. Following permiablization the slices were incubated for two nights at 4° with anti-CaMKIIα (1:500; Affinity Bioreagents) monoclonal antibody and then with an Alexa Fluor-conjugated anti-mouse 647 secondary antibody (1:1000; Life Technologies).

3.2.6 Revelation of intracellular marker biocytin labeled cells

In order to reveal the morphology and to identify the GCs used in electrophysiological recordings the biocytin was introduced into them via the recording intracellular solution. After through washing, sections were permiabilized with 0.5% triton solution supplemented with 4% BSA. Following permiabilization the slices were incubated with avidin-biotin complex (ABC) and finally incubated with rhodamine (tetramethylrhodamine isothiocyanate) [rhodamine(TRITC)]-conjugated streptavidin antibody (Jackson ImmunoResearch) at a concentration of 1:200 for 3 hours at room temperature to reveal the biocytin filled granule interneurons. These steps allowed us to reveal the granule interneuron from which recordings were made. Co-localization of biocytin with GFP and further with CaMKIIα allowed us to select granule interneurons that were positive for CaMKIIα and comparison was made with the GCs not expressing CaMKIIα.

3.2.7 Statistical analysis

Results are expressed as means ± SEM. Statistical significance was determined using the Student's t test (*$p < 0.05$ and ***$p < 0.005$).

3.3 RESULTS.

3.3.1 Approximately half of all GCs express CaMKIIα in the adult bulb:

Unlike in other brain regions CaMKIIα in the OB is expressed by GABAergic granule interneurons, whereas GABAergic periglomerular interneurons do not

express it (Zou et al., 2002). In the above mentioned study they show that granule interneurons express CaMKIIα but not all of them do so, as they observed cells that were negative for CaMKIIα. Thus, we performed immunolabeling in OB slices of C57/ Bl6 wild-type adult mice to determine the extent of CaMKIIα expression in the GCs. By combining immunolabeling for CaMKIIα along with DAPI we were able to determine that about half of all granule neurons present in the GCL are CaMKIIα positive while the other half are negative for CaMKIIα (**Fig. 3.1)**. We also failed to detect any mitral cells that were positive for CaMKIIα. Thus we concluded that half of all granule neurons of the GCL are positive for CaMKIIα, next we wanted to check the ratio of CaMKIIα expression in adult generated granule interneurons.

3.3.2 Half of all adult generated GCs express CaMKIIα:

In order to study the ratio of newly generated granule interneurons that express CaMKIIα, we labeled newly generated interneurons with bromodeoxyuridine (BrdU) and performed immunolabeling for CaMKIIα (**Fig. 3.2a)**. Adult C57Bl/6 wild-type mice were administered BrdU intraperitonially and were sacrificed at varying times after administration starting from post injection day five (5 days) till eight weeks post injection. Free floating slices of OBs were immunolabeled with BrdU and CaMKIIα, and the results revealed that from as early as 12 days post injection half of all newly generated BrdU+ granule interneurons were also expressing CaMKIIα (**Fig. 3.2b)**. This expression of CaMKIIα by the newly generated granule interneurons was not a transient phenomenon as the expression was maintained even at two months after the cells were generated (**Fig. 3.2b)**. Thus we concluded that half of all adult generated granule interneurons integrating in the OB express CaMKIIα. This expression of CaMKIIα persists in these cells suggesting that adult generated GCs can be subdivided to CaMKIIα expressing and CamKIIα negative cells. In order to access the functional characteristics of these two subpopulations of adult generated GCs we used a viral vector based approach to label newly generated GCs expressing CaMKIIα and compare them to CaMKIIα negative counterparts.

3.3.3 Viral vectors specifically infect and label cells expressing CaMKIIα:

To distinguish between adult generated GCs that express CaMKIIα and those that do not express it, we used adeno associated viral vectors (scAAV) that drive the expression of GFP from a CaMKIIα promoter or from a chicken beta actin promoter (CBA) as a control. In order to assess the efficiency of these vectors we injected C57/ Bl6 wild-type mice with one of these two vectors in the RMS. After allowing the mice to recover for a period of two weeks, the time required for the newly generated progenitors to reach the OB and differentiate into interneurons, the mice were sacrificed and OB slices were prepared. Free floating slices in which we were able to detect GFP positive cells in the GCL were immunolabeled for CaMKIIα; and co labeling of GFP and CaMKIIα was determined using confocal microscopy. In the group of mice injected with the scAAV expressing GFP from the CaMKIIα promoter we recorded that (90.2± 4.1; n=3 mice) of all GFP labeled GCs colabled with CaMKIIα (**Fig. 3.3a, b)**. In control animals injected with scAAV-CBA-GFP only 53.2±3.2% of GFP+ cells were CamKIIa+ as expected because of the use of a ubiquitous promoter that targets all subpopulations of adult-born GCs and that CamKIIa+ population constitutes 50% of all adult-born GCs (**Fig 3.2)**. Thus we confirmed that the scAAV vector expressing GFP from the CaMKIIα promoter was highly specific and can be used for further characterization of these cells.

3.3.4 CaMKIIα positive adult generated GCs experience lesser inhibition when compared to CaMKIIα negative GCs:

Adult generated granule interneurons receive inhibitory and excitatory inputs very shortly after they arrive in the OB (Nissant et al., 2009; Panzanelli et al., 2009). In order to investigate if CaMKIIα expressing adult generated granule interneurons receive different inputs when compared to CaMKIIα negative adult generated cells, we recorded IPSCs from virally labled cells. Our results showed that the frequency of spontaneous inhibitory post synaptic currents (sIPSCs) and miniature inhibitory post synaptic currents (mIPSCs) recorded from CamKIIα granule interneurons was the same as that recorded from those negative for CaMKIIα (**sIPSCs:** 1.15± 0.144

Hz CaMKIIα neg n=11 vs 1.4± 0.073 Hz CaMKIIα pos n=9; **mIPSCs:** 0.97± 0.205 Hz CaMKIIα neg n=10 vs 1.018± 0.089 Hz CaMKIIα pos n=8; **Fig. 3.4c**). The amplitude of sIPSCs and mIPSCs however was significantly larger in CaMKIIα negative cells when compared to CaMKIIα positive cells (**sIPSCs:** 48.273± 5.235 pA CaMKIIα neg n=11 vs 33.108± 2.930 pA CaMKIIα pos n=9, $p< 0.05$; **mIPSCs:** 27.645± 2.1 pA CaMKIIα neg n=10 vs 21.731± 1.5 pA CaMKIIα pos n=8, $p< 0.05$; **Fig. 3.4c**). We also observed that CaMKIIα negative cells displayed significantly higher rise time and decay time of mIPSCs as well as significantly larger charge in these mIPSCs when compared to events recorded from CaMKIIα positive cells (**mIPSC Rt:** 4.9± 0.062 ms CaMKIIα neg n=10 vs 4.6± 0.103 ms CaMKIIα pos n=8, $p< 0.05$; **mIPSC Dt:** 55.5± 1.730 ms CaMKIIα neg n=10 vs 42.4± 2.420 ms CaMKIIα pos n=8, $p< 0.0005$; **mIPSC charge:** 734.8± 63.185 CaMKIIα neg n=10 vs 557.3± 28.631 CaMKIIα pos n=8, $p< 0.05$; **Fig. 3.4e**). Taking into account that the granule interneurons of both subgroups show responses of different amplitude and kinetics this may suggest that the granule interneurons of the two subpopulations have $GABA_A$ receptors with different composition. Overall these data suggest that CamKIIa expressing adult-born interneurons receive smaller inhibition as compared to cells not expressing this enzyme.

3.3.5 CaMKIIα positive adult generated GCs experience similar excitation as CaMKIIα negative adult generated GCs:

In order to study the excitatory input that GCs expressing CaMKIIα and those that don't express it receive, we made spontaneous EPSC recordings from both groups. Our results show that the excitatory input received by these cells is the same. The amplitude and frequency of sEPSCs and mEPSCs showed no significant difference between the two groups. The kinetics of the events was also similar. Amplitudes of both sEPSCs and mEPSCs between the two groups were not significantly different (**sEPSCs:** 8.9± 0.9pA CaMKIIα neg n=8 vs 10.3± 0.9 pA CaMKIIα pos n=10; **mEPSCs:** 7.9± 0.7 pA CaMKIIα neg n=8 vs 8.8± 0.8 pA CaMKIIα pos n=10). The frequency of sEPSCs and mEPSCs were also not

significantly different from each other (**sEPSCs:** 0.6± 0.2 Hz CaMKIIα neg n=8 vs 0.9± 0.2 Hz CaMKIIα pos n=10; **mEPSCs:** 0.2± 0.1 Hz CaMKIIα neg n=8 vs 0.45± 0.1 Hz CaMKIIα pos n=10). The kinetics of these events was also identical between the two groups. These results point to the fact that the excitatory inputs onto the CaMKIIα positive and negative cells are identical.

Altogether our data suggest that the CaMKIIα expressing adult generated GCs have different electrophysiological characteristics. These two subpopulations of GCs differ in the amplitude, kinetics and charge of the inhibitory events resulting in lesser inhibition in the adult generated CaMKIIα expressing GCs when compared to those that do not express CaMKIIα. These data then allows us to suggest that CaMKIIα expressing adult generated GCs experience lesser inhibition when compared to their CaMKIIα counterparts. This crucial difference could then allow them to be more prone to be activated by olfactory stimulation. Ultimately these differences could have behavioural implications which we are yet to explore.

3.4 DISCUSSION.

3.4.1 CaMKIIα expressing subpopulation of adult generated GCs receives reduced inhibition:

Studies have already shown that in the mammalian forebrain the expression of CaMKIIα protein is restricted to glutamatergic synapses where this kinase is seen abundantly in the postsynaptic density (Kennedy et al., 1983; Benson et al., 1992; Jones et al., 1994). The OB is the only structure in the brain that has been documented to have expression of CaMKIIα protein in the GABAergic interneurons (Zou et al., 2002; Neant-Fery et al., 2012). A varied list of functions has been attributed to CaMKIIα in multiple regions of the brain. In the hippocampus CaMKIIα has been implicated in the formation and maintenance of LTP (Malenka et al., 1989; Malinow et al., 1989; Silva et al., 1992). Mice lacking CaMKIIα have also been reported to have impaired spatial learning and memory associated with the hippocampus (Silva et al., 1992; Giese et al., 1998).

The function of CaMKIIα proteins in the GABAergic granule interneurons of the mammalian olfactory bulb is however not yet fully understood. The granule interneurons of the mouse OB express CaMKIIα (Zou et al., 2002). The mRNA of CaMKIIα is localized in the dendrites of GCs owing to its 3ÙTR which acts as a cis-acting signal for dendritic localization (Mayford et al., 1996). Another study has demonstrated that this localization of mRNA in the dendrites is regulated by olfactory activity (Neant-Fery et al., 2012). In response to sensory activity local translation of CaMKIIα mRNA can then be achieved. In mice that are mutant for CaMKIIα, lacking functional 3'UTR, the dendritic localization of CaMKIIα mRNA is drastically reduced resulting in impaired associative learning abilities (Neant-Fery et al., 2012). This study points to a fundamental role for CaMKIIα local translation in olfactory plasticity.

Newly born GCs receive inhibitory and excitatory inputs within a few days of birth (Panzanelli et al., 2009). The inhibitory inputs onto the GCs can be locally driven by Blane's cells (Pressler and Strowbridge, 2006) or from cortical feedback loops (Boyd et al., 2012; Markopoulos et al., 2012). Direct cortical synapses on the GCs or a di-synaptic inhibition via the Blane's cells disinhibits the mitral neurons by inhibiting the GCs (Pressler and Strowbridge, 2006; Boyd et al., 2012; Markopoulos et al., 2012). In our study we looked at the inhibitory inputs that the adult generated CaMKIIα expressing granule interneurons receive when compared to those that do not express CaMKIIα. Our data shows that the frequency of inhibitory activity in these two subpopulations does not vary; strongly indicating that the number of inhibitory synapses made onto these cells is the same. However, the amplitude of sIPSCs and mIPSCs along with the charge of mIPSCs was seen to be significantly lower in the interneurons expressing CaMKIIα when compared to those that do not express it. The rise time and decay time of the mIPSCs were also significantly different among the groups. These data all taken together may imply that CaMKIIα expressing adult generated GCs have GABA receptors with different

subunits. This difference can be directly or indirectly linked to the presence of CaMKII in the dendrites of cells expressing this protein.

3.4.2 Possible implications of CaMKIIα expressing subpopulation of adult generated GCs in odor behavior:

In line with our electrophysiological recordings are the data obtained by our collaborators on CaMKIIα positive cells expressing the immediate early gene cfos. Immunolabeling for cfos in OB slices obtained form mice expressing tomato red protein under the CaMKIIα promoter showed that 93± 2.8 % of cfos positive cells were also CaMKIIα positive. Taking our electrophysiology data and the cfos data together we could say that adult generated granule neurons that express CaMKIIα experience lesser inhibition and more likely to respond to sensory activity. As shown by others, inhibition provided by the GCs is crucial for the synchronous activity of mitral cells (Rall and Shepherd, 1968; Kashiwadani et al., 1999; Davison et al., 2003; Bathellier et al., 2006; Schoppa, 2006). Disruption of inhibition and synchronous activity resulted in impairments in short-term odor memory of mice without affecting other olfactory behaviors (Breton-Provencher et al., 2009; David et al., 2013). Thus, it could be possible that the subpopulation of adult generated GCs expressing CaMKIIα respond faster or with greater strength to sensory activity. This could then be the basis to explore the possibly different contributions that these two subpopulations of cells make to odor behavior of mice.

Another possible function for adult generated CaMKIIα expressing granule interneurons could be odor discrimination between similar odors. A recently published study has shown that adult generated interneurons when activated precisely during odor exposure improves the odor discrimination between similar odors and also memory (Alonso et al., 2012). This study opens a possibility that CaMKIIα expressing granule interneurons due to the lower inhibition are quicker to respond to incoming sensory activity, thus playing an important role in discrimination of very similar odors. These possibilities are yet to be explored.

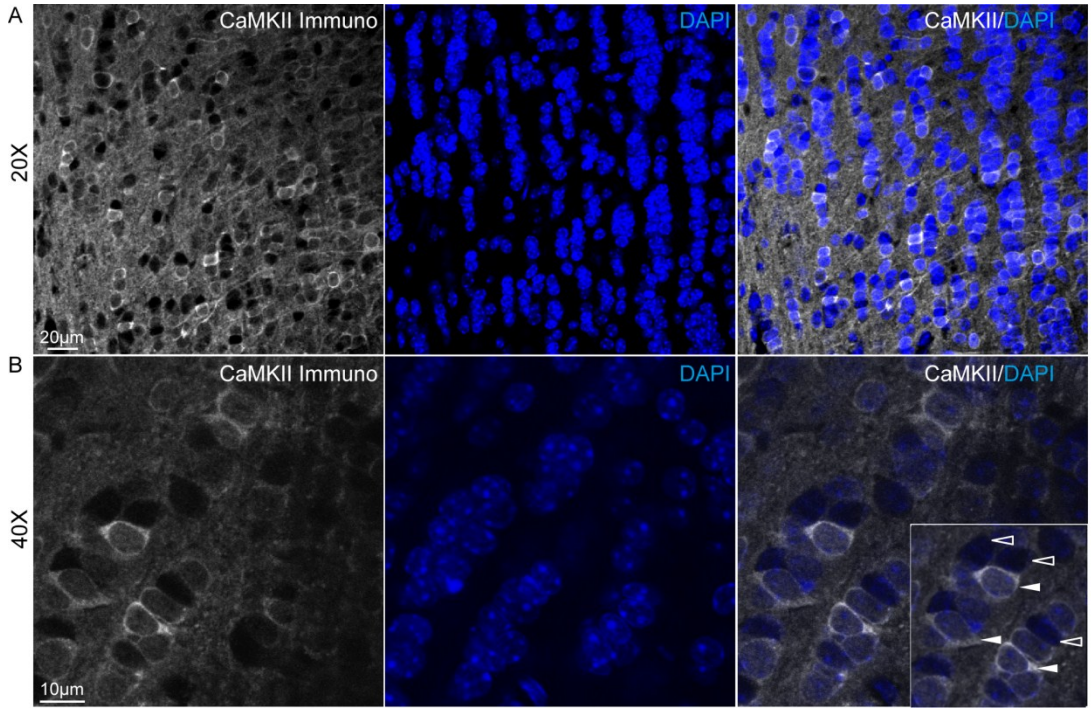


Figure 3.1: Approximately half of all GCs express CaMKIIα in the adult bulb
Confocal images taken in the GCL at two different magnifications in panels **A** and **B**. CamKIIα immunohistochemistry was coupled with DAPI labeling. The inlay of the last micrograph in panel B shows GCs positive for CaMKIIα (filled arrowhead) and those negative for CaMKIIα (open arrowheads).

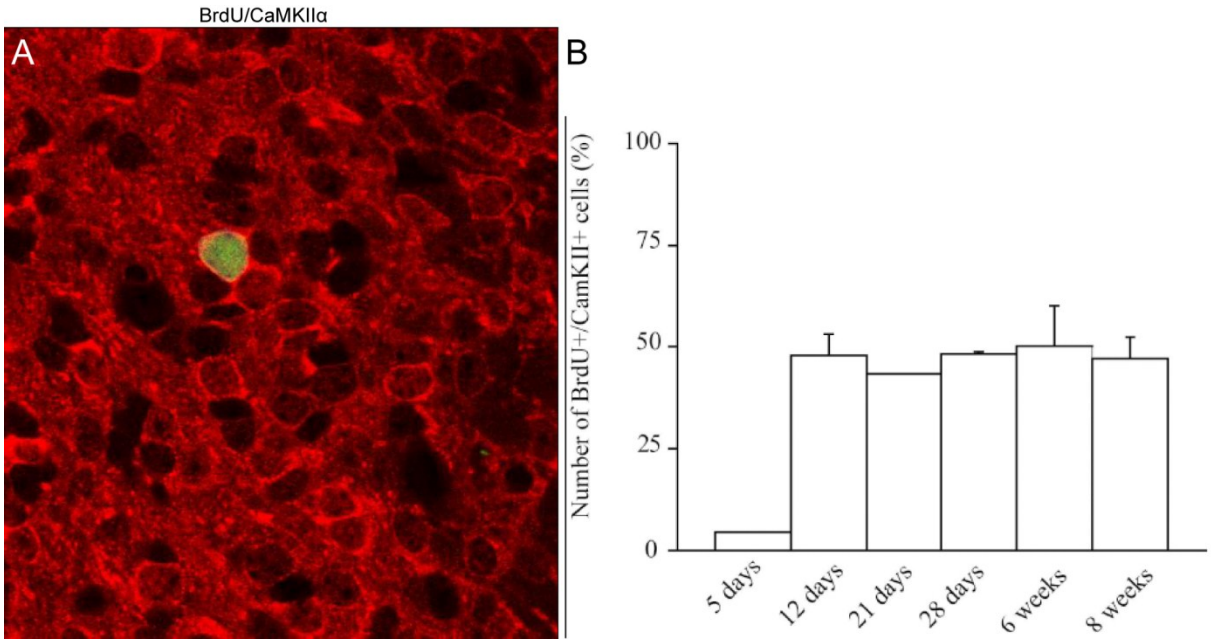


Figure 3.2: Half of all adult generated GCs express CaMKIIα

A BrdU and CaMKIIα immunohistochemistry was performed and BrdU positive cells that were also CaMKIIα positive were counted. **B** Quantification showing that starting from 12 days post BrdU administration onwards half of all adult generated cells were also CaMKIIα positive.

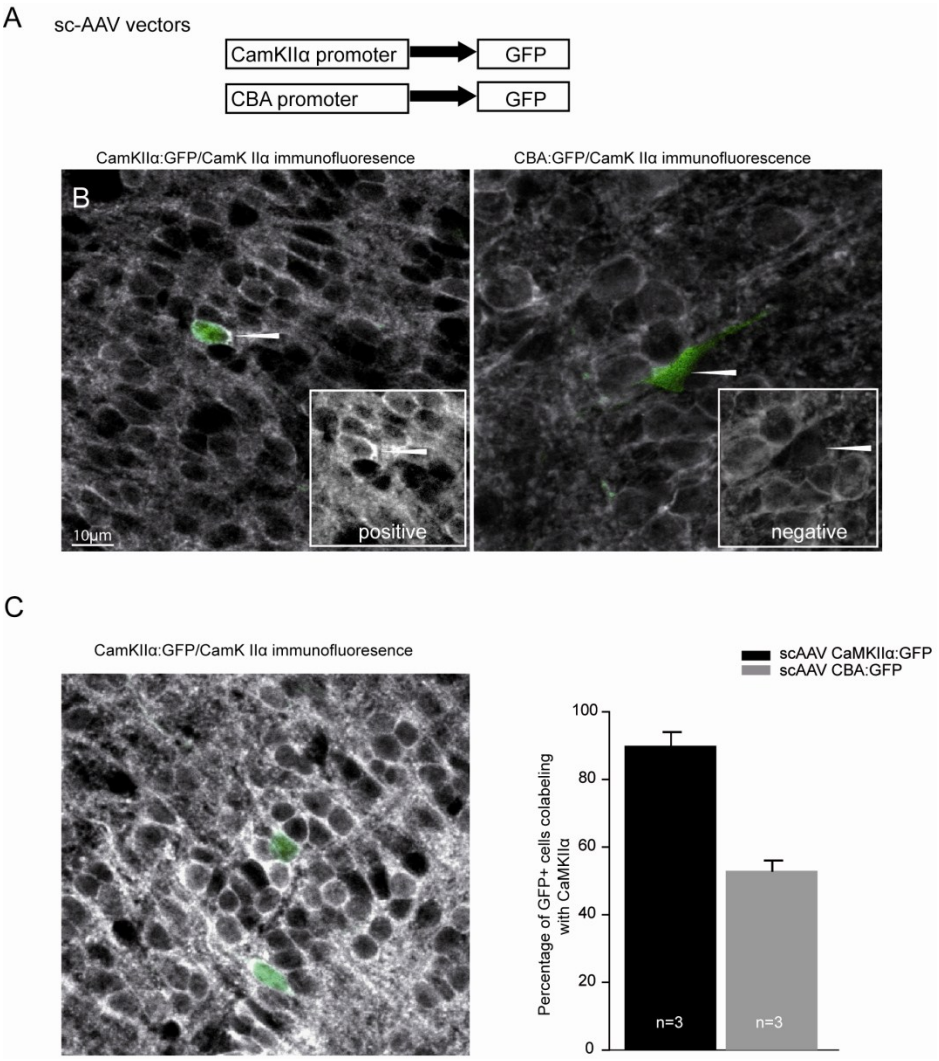


Figure 3.3: Viral vectors specifically infect and label cells expressing CaMKIIα

Two weeks after injection of the viral in the RMS, GFP positive cells were identified to be either CaMKIIα positive or negative (**B). C** Quantification of GFP positive cells colocalizing with CaMKIIα was done in slices form animals injected with either CaMKIIα:GFP or CBA:GFP, n=3 mice each group.

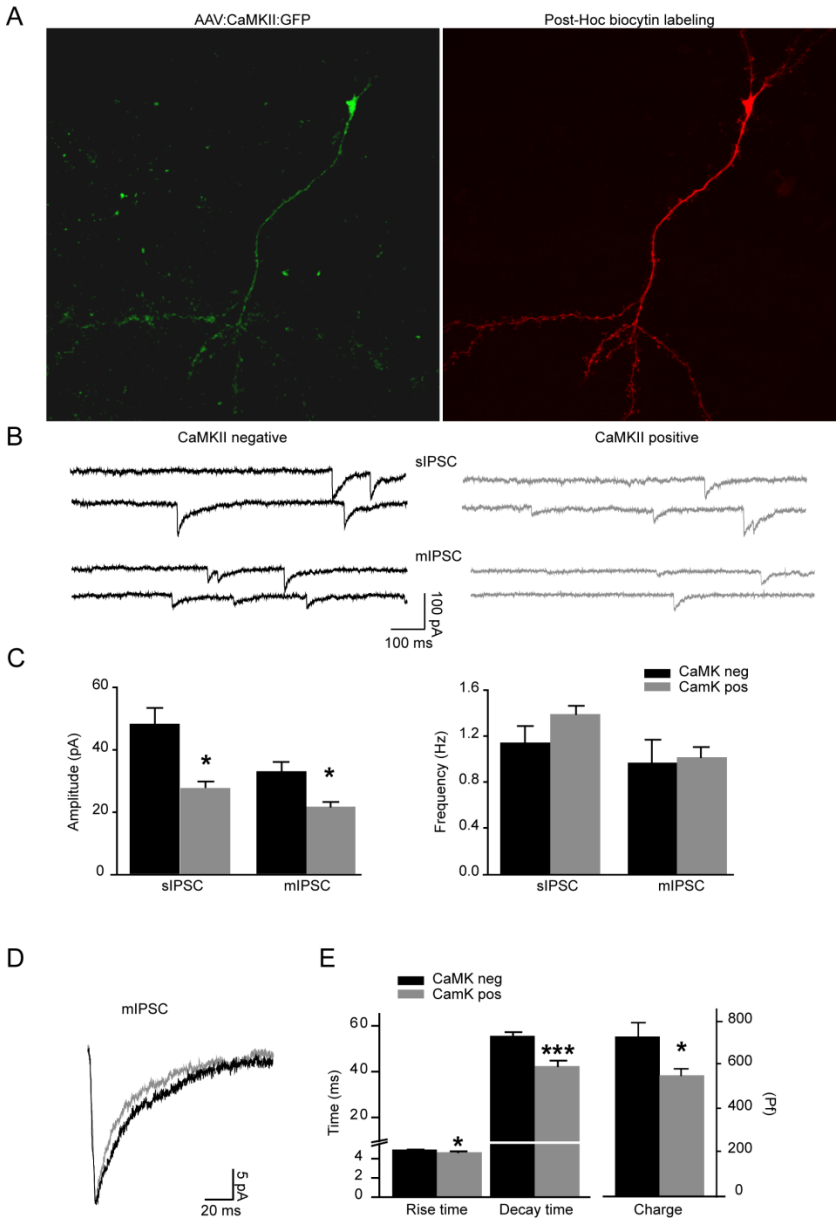

A
AAV:CaMKII:GFP
Post-Hoc biocytin labeling
B
CaMKII negative
CaMKII positive
sIPSC
mIPSC
100 pA
100 ms
C
Amplitude (pA)
60
40
20
0
*
*
sIPSC
mIPSC
Frequency (Hz)
1.6
1.2
0.8
0.4
0.0
CaMK neg
CamK pos
sIPSC
mIPSC
D
mIPSC
5 pA
20 ms
E
CaMK neg
CamK pos
Time (ms)
60
40
20
4
2
0
*
***
*
800
600
400
200
0
(pF)
Rise time
Decay time
Charge

Figure 3.4: CaMKIIα positive adult generated GCs experience lesser inhibition when compared to CaMKIIα negative GCs:

A Two weeks after injection with either CaMKIIα: GFP or CBA: GFP, GFP+ cells were patched in the GCL and filled with biocytin for post-hoc analysis. **B** Sample traces of sIPSCs and mIPSCs obtained from CaMKIIα positive and negative GCs**. C** Amplitude of sIPSCs and mIPSCs were significantly lower in CaMKIIα positive cells whereas frequency was unchanged**. D** Kinetics and charge of mIPSCs from CaMKIIα positive GCs was also significantly different from CaMKIIα negative GCs, $p< 0.05^{*}$, 0.0005^{***}**.**

Chapter IV:

Role of sensory activity on chemospecific populations of interneurons in the adult olfactory bulb (2010) *The Journal of Comparitive Neurology*, 518: 1847-1861.

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This work was performed in the laboratory of Dr. Armen Saghatelyan and under the guidance of Drs. Andre Parent and Armen Saghatelyan. Pierre-Olivier Bastien-Dionne performed all the immunohistochemisty and part of olfactory sensory deprivation. Part of experiments entailing olfactory deprivation, expression of Pax6 and western blot analysis were performed by me.

RÉSUMÉ

Le bulbe olfactif (MOB) possède la capacité remarquable de renouveler sa population d'interneurones tout au long de la vie d'un animal. Les précurseurs neuronaux qui donnent naissances aux interneurones bulbaires nouvellement générés sont situés dans la zone sous-ventriculaire et migrent sur une longue distance avant d'atteindre le MOB. Arrivés dans le bulbe adulte, ces précurseurs neuronaux se différencient en plusieurs sous-types neuronaux, incluant les interneurones GABAergiques situés dans la couche granulaire et les différents types de neurones de la couche glomérulaire du bulbe comme les interneurones GABAergiques et dopaminergiques ainsi que d'autre sous-types neuronaux exprimant la calrétinine et la calbindine. Malgré que le rôle de l'activité sensorielle dans l'intégration et la survie des cellules bulbaires nouvellement générées soit bien établi, le rôle de l'activité olfactive dans la spécification, la survie et le maintien des différents phénotypes produits par la neurogénèse dans le bulbe demeure inconnu. La présente étude démontre que la dépravation sensorielle diminue non seulement le nombre de cellules nouvellement générées dans le MOB, mais aussi réduit la densité des cellules granulaires et périglomérulaires générées avant l'occlusion de la narine des souris adultes. L'étude démontre aussi que l'activité sensorielle possède une influence importante sur le développement et l'expression des cellules possédant un phénotype dopaminergique et non un phénotype GABAergique, calrétininergigue ou calbindinergique. Nos données révèlent l'importance de l'activité sensorielle induite par les odeurs dans la survie à la fois des neurones générés à l'âge adulte pendant et avant la modulation de l'activité olfactive et suggèrent que les phénotypes neuronaux ainsi générés sont affectés différemment par la dépravation sensorielle.

ABSTRACT

The olfactory bulb (OB) retains a remarkable capacity to renew its interneuronal populations throughout the life-span of animals. Neuronal precursors giving rise to the bulbar interneurons are generated in the subventricular zone and have to migrate long distances before reaching the OB. In the adult OB these neuronal precursors differentiate into distinct neuronal types, including GABAergic cells located in the granule cell layer and a diverse set of neurons in the glomerular layer comprising GABAergic and dopaminergic interneurons, as well as other neuronal subtypes expressing calretinin and calbindin. While the role of sensory activity in the integration and/or survival of newly generated cells in the olfactory system is well established, very little is known about how odorant-induced activity affects fate specification of newborn cells as well as survival and fate maintenance of pre-existing neuronal populations generated in adulthood. The present study demonstrates that sensory deprivation diminishes not only the number of newborn cells in the OB, but also reduces the density of granule and periglomerular cells generated before nostril occlusion. It also shows that sensory activity has an important influence on the development and expression of dopaminergic, but not GABAergic, calretinin or calbindin phenotypes. Our data reveal that odorant-induced activity is important for the survival of both newborn and pre-existing OB interneurons generated at adulthood and suggests that these chemospecific populations are differentially affected by sensory deprivation.

4.1. INTRODUCTION

The olfactory system is characterized by constantly renewing cell populations both in the sensory epithelium and in the olfactory bulb (OB). The olfactory sensory neurons (OSN) located in the olfactory epithelium are in direct contact with the environment and, therefore, are subjected to various chemical and physical injuries. To compensate for the loss of mature OSNs that have a typical life-span of 30 to 60 days (Murray and Calof, 1999), the olfactory epithelium regenerates its neuronal population throughout the life of animals. Neuronal precursors are derived from the stem cells located in the basal compartment of the epithelium that via slow and rapid phases of mitosis give rise to the OSNs (Leung et al., 2007). The mature OSNs, expressing olfactory marker protein (OMP), send their axons toward the OB where they make synaptic contacts with the mitral cells, the principal output neurons in the OB, in the specialized compartment called glomeruli (Firestein, 2001; Mombaerts, 2006).

Interestingly, the OB also renews its interneuronal populations, the granule and periglomerular cells. Neuronal precursors for these interneurons are generated from neural stem cells in the subventricular zone (SVZ) bordering the lateral ventricle (Doetsch et al., 1999). The stem cells give rise to the fast transit-amplifying neural progenitors that further divide and give rise to neuroblasts (Doetsch et al., 1999; Alvarez-Buylla and Garcia-Verdugo, 2002). The neuroblasts migrate a long distance first tangentially in the rostral migratory stream (RMS) and then radially in the OB where they populate the granule cell layer (GCL) or glomerular layer (GL) (Alvarez-Buylla and Garcia-Verdugo, 2002; Lledo and Saghatelyan, 2005). Recently, it has been also shown that neuronal precursors labeled in the SVZ during early postnatal life can give rise to the interneurons in the external plexiform layer (EPL) (Yang, 2008). Upon their arrival to the OB newborn neurons undergo important maturational changes, develop dendrites and establish dendro-dendritic synapses with the principal output cells in the OB (Petreanu and Alvarez-Buylla, 2002; Belluzzi et al., 2003; Carleton et al., 2003). Granule cells

(GC) are a GABAergic population of interneurons that can be subdivided into deep, superficial and calretinin+ cells (Lemasson et al., 2005; Kelsch et al., 2007; Merkle et al., 2007), whereas periglomerular cells (PGC) can be dopaminergic and/or GABAergic, and can also express calbindin, calretinin or parvalbumin (Kosaka et al., 1998; Bagley et al., 2007; Whitman and Greer, 2007). It has been recently shown that these chemospecific interneuronal populations are constantly renewed in the OB (Bagley et al., 2007; Whitman and Greer, 2007). The fate of PGC is controlled by different transcription factors, including Sp8, which is important for calretinin-positive (+) PGCs (Waclaw et al., 2006), Dlx1/2 and Pax6 for dopaminergic fate determination (Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005; Brill et al., 2008) and Dlx for calbindin fate acquisition (Allen et al., 2007).

While all these reports provided strong evidence for the role of intrinsic factors in regulating fate determination of newborn cells in the OB, the influence of extrinsic signals, such as odorant-induced activity, on the integration, fate determination and maintenance of these cells is not fully understood. In adults, sensory deprivation via unilateral nostril occlusion results in 27% atrophy of the ipsilateral bulb after six months of occlusion (Maruniak et al., 1989). This effect is at least partially due to the reduced radial migration of adult neuronal precursors into the OB (Saghatelyan et al., 2004), as well as to their decreased survival in the bulbar circuitry (Corotto et al., 1994; Fiske and Brunjes, 2001; Petreanu and Alvarez-Buylla, 2002; Yamaguchi and Mori, 2005; Mandairon et al., 2006). Conversely, environmental enrichment increases the number of newly integrated GCs in the OB without affecting the rate of proliferation (Rochefort et al., 2002). It is yet not clear, however, how odor-induced activity affects the survival and fate maintenance of cells generated at adulthood and already differentiated into the particular neuronal subtype in the OB at the moment of sensory deprivation. Additionally, the role of sensory activity in the fate determination of newborn cells remains sparse and except the well-documented down regulation of tyrosine hydroxylase (TH) expression following

nostril occlusion (Stone et al., 1991; Baker et al., 1993; Brunjes, 1994; Mandairon et al., 2006), little is known on how odor-induced activity influences the acquisition and maintenance of calbindin+, calretinin+, and/or GAD+ phenotypes.

For this purpose, we designed a series of experiments that allows discriminating the effect of unilateral nostril occlusion on both the survival and the fate determination of newborn neurons arriving in the odor-deprived OB, as well as on the survival and fate maintenance of neurons generated at adulthood and already differentiated into the particular neuronal phenotype before sensory deprivation. We report that sensory activity is important not only for the integration and/or survival of cells born after the sensory deprivation, but also for the survival of cells that are already present in OB neuronal network. In addition, we demonstrate that odorant-induced activity is essential for the development and expression of dopaminergic, but not GABAergic, calretinin+ or calbindin+ phenotypes. Our data reveals the role of sensory activity in the survival of not only newborn but also pre-existing interneuronal populations in the OB and suggests that acquisition and maintenance of chemospecific phenotype by different PG cells can be differentially affected by the odor-induced activity.

4.2. MATERIAL AND METHODS:

4.2.1 Animals

Adult male C56BL/6 (Charles River) or GAD67-GFP (Δneo) (Tamamaki et al., 2003) mice of 2-to-3-months of age were used for these experiments. Animals were kept on a 12-hour light–dark cycle at constant temperature (22° C) with food and water *ad libitum*. The edges of the one nostril were sutured prior to heat occlusion under ketamine/xylazine anesthesia. Nostrils were checked every day to assure that they were well occluded. The work was performed in accordance with the *Canadian Guide for the Care and Use of Laboratory Animals* and all surgical and animal care procedures were approved by the Institutional Animal Care Committee of Laval University.

4.2.2 Bromodeoxyuridine (BrdU) injections and treatments

All mice were injected intraperitoneally with DNA replication marker, BrdU (Sigma, St. Louis, MO; 50 mg/kg in saline). To investigate the role of sensory input in the integration/survival and fate determination of newborn neurons, sensory deprivation was performed 21 days before BrdU injection (four injections every two hours). By contrast, to access the implication of odor-induced activity in the survival and fate maintenance of neurons already present in the OB neuronal network, BrdU (four injections every two hours) was injected either 21 or 60 days before sensory deprivation. To assess the proliferation of neuronal precursors, a pulse of BrdU was given to 21 days odor-deprived animals one hour before their perfusion. To evaluate the migration of neuronal precursors, BrdU-labeled profiles, with BrdU (2 injection spaced by 2 hours) being injected 5 days before the sacrifice of animals, were counted in the RMS and SVZ of 21 days odor-deprived animals.

4.2.3 Tissue preparation

At different time points after BrdU administration, animals were anesthetized with an overdose of pentobarbital and perfused transcardiacally with 0.9% NaCl followed by perfusion with paraformaldehyde (PFA; 4% in phosphate buffer saline

(PBS), pH 7.4). The brains were removed and postfixed in the same fixative overnight at 4° C. To distinguish occluded bulbs, the brains were embedded in a gelatine block (30% ovalbumine, 0.5% gelatine, 1.25% glutaraldehyde, in PBS pH 6-7) and a small cut was made on the gelatine block at the level of occluded bulb. Serial coronal sections (40 µm) were cut using a vibratome (Leica VT1000S).

4.2.4 BrdU immunocytochemistry

Immunolabeling was performed as described elsewhere (Saghatelyan et al., 2004). Briefly, sections were incubated in 0.2% Triton followed by incubation in 2% HCl for 40 min at 37° C to denature DNA. The slices were then extensively washed in PBS three times, 10 minutes each and were incubated overnight at 4° C in rat anti-BrdU antibody (Serotech, Raleigh, NC; catalog #MCA2060; clone#BU1/75; 1:200) raised against synthetic BrdU. The specificity of immunostaining was verified on the control sections derived from the animals not injected with BrdU. The incubation with primary antibodies was followed by a 3 hour incubation at room temperature with biotinylated anti–rat IgG antibodies (Pierce, Rockford, IL; catalog #31830; lot #JF1147794; 1:200) and 1 hour incubation in the avidin–biotin complex (ABC) (Pierce). To reveal BrdU staining, the sections were reacted in 0.05% 3, 3-diaminobenzidine tetrahydrochloride (DAB) and 0.027% H_2O_2 in Tris–HCl buffer 1 M, pH 7.6, dehydrated in graded ethanol baths, mounted and coverslipped in DePeX (VWR).

4.2.5 Immunohistochemistry

All immunohistochemical procedures were performed on 40 µm thick sections embedded in a gelatine block and containing control and occluded bulbs in the same section. For double– and triple–labeling immunofluorescence sections were pre-treated with 0.2% Triton and 4% BSA for 2 hours followed by overnight incubation in the same solution containing the primary antibodies. For calbindin D-28K immunostaining a polyclonal rabbit IgG antibody (Millipore, Billerica, MA; catalog #AB1778; lot #0603024559; 1:1000) raised against recombinant calbindin

was used. This antibody shows no cross-reactivity to calretinin by Western blot (manufacturer's datasheet) and produces specific staining in the OB in the pattern reported elsewhere (Bagley et al., 2007; De Marchis et al., 2007; Whitman and Greer, 2007). For calretinin immunostaining a polyclonal goat IgG antibody (Millipore, Billerica, MA; catalog #AB1550; lot #0603024584; 1:1000) raised against purified guinea pig calretinin was used. This antibody recognized a specific band of about 31 kDa in the extracts of mouse brain (data not shown; (Bubser et al., 2000) and produces specific staining in the OB in the pattern reported elsewhere (Bagley et al., 2007; De Marchis et al., 2007; Whitman and Greer, 2007). For Pax6 immunostaining a polyclonal rabbit IgG antibody (Covance, Princeton, NJ; catalog #PRB-278P, lot #14942001; 1:3000) raised against the peptide (QVPGSEPDMSQYWPRLQ) derived from the C-terminus of the mouse Pax6 protein was used. This antibody is effective in immunoblotting and immunohistochemistry (manufacturer's datasheet) and produces specific staining in the OB in the pattern reported elsewhere (Hack et al., 2005). For TH (tyrosine hydroxylase) immunostaining a monoclonal mouse IgG antibody (ImmunoStar, Hudson, WI; catalog #22941; lot #635001; 1:1000) raised against TH purified from rat PC12 cells was used. This antibody is believed to have wide species cross-reactivity (manufacturer's datasheet) and produces specific staining in the OB in the pattern reported elsewhere (Hack et al., 2005; Bagley et al., 2007; De Marchis et al., 2007; Whitman and Greer, 2007). Secondary antibodies were used as follows: goat anti-rabbit Alexa Fluor 488, 568, 633, goat anti-mouse Alexa Fluor 488, 546, 633, donkey anti-mouse Alexa Fluor 488, donkey anti-goat Alexa Fluor 568, goat anti-rat Alexa Fluor 568 and donkey anti-rat Alexa Fluor 488, 594, all from Invitrogen (Burlington, ON) at a dilution of 1:1000. Slices were then mounted on glass slides and coverslipped in Dako fluorescent mounting media (Dako, Carpinteria, CA).

4.2.6 Image acquisition and data analysis

Fluorescent images were captured with a FluoView 1000 confocal microscope (Olympus, Center Valley, PA) equipped with lasers Ar 488, HeNe1 543 and HeNe2 633. Images were acquired in the sequential mode with the 1 µm z step and analyzed with Fluoview 6.0 software (Olympus). The percentage of double immunostained cells were obtained by analyzing 3D images in the *x–z* and *y–z* orthogonal projections. To analyze the density and total number of BrdU, TH and Pax6 immunopositive cells, images were captured by a BX-51 microscope (Olympus) equipped with a motorized stage (Prior; MediaCybernetics, Bethesda, MD) that allows tile acquisition of entire OB section with 20x and 40x objectives. Immunostained cells were quantified in every third 40 µm thick coronal section of the OB starting from the tip of the OB and ending with the apparition of the accessory olfactory bulb. To assess the density of newborn neurons in the OB, the numbers of BrdU+ profiles were related to the surface of granule cell (including mitral cell and internal plexiform layers) and glomerular layers. Analysis of BrdU density and Pax6 density was made with Image-Pro Plus 6.0 software (MediaCybernetics), using an automatic counting method in which immunopositive cells were identified according to the intensity and nuclear size thresholds. For this, intensity and nuclear size thresholds were set manually using a segmentation tools algorithm in the Image-Pro Plus 6.0 software. First, the intensity threshold range was set manually on the slices in order to pick up immunopositive cells and avoid background immunolabeling. Then, nuclear size threshold was applied. Since slices derived from control and odor-deprived OBs were present in the same gelatin-embedded sections, and thus were treated simultaneously for all immunohistochemical procedures and image acquisition, we used the same intensity and nuclear size settings for the slices of the same animal. In total 14-15 sections per OB were used to determine the mean surface density of BrdU+ and Pax6+ cells. The densities of immunopositive profiles per OB were then averaged across the animals.

To derive total numbers of BrdU- and Pax6-labeled cells, a cell splitting correction factor based on the Abercrombie method (Guillery and Herrup, 1997) was used. The total number of BrdU cells was calculated using the formula $T = (N \times V)/(t + D)$, where T is the total number of cells, N is the number granule cell nuclei per unit area, V is the volume of the granule cell layer, t is the section thickness and D is the average diameter of BrdU-labeled nuclei. No difference in cell diameter was found between control and occluded bulbs (2.4 ± 0.07 µm for ctrl and 2.3 ± 0.6 µm for occl, n = 400-500 cells per animal, n = 3 animals, $p > 0.05$). The volumes of the granule and periglomerular cell layers were calculated with the equation:

$$\sum_{i=0}^{n} A_i d$$

Where A is the area of the i-th section, d is the distance between analyzed sections, and n is the total number of measured sections. All photomicrographs were acquired and stored using the Fluoview 6.0 (Olympus) and Image pro 6.0 (MediaCybernetetics) software. Final manipulations for the construction of figures were done using Abode Photoshop and Adobe Illustrator software.

4.2.7 Western blot analysis

21 days after the sensory deprivation, deeply anesthetized animals were transcardially perfused with modified oxygenated artificial cerebrospinal fluid (ACSF) containing in mM 250 sucrose, 3 KCl, 0.5 $CaCl_2$, 3 $MgCl_2$, 25 $NaHCO_3$, 1.25 $NaHPO_4$ and 10 glucose. Horizontal slices (250 µm thick) of the OB were obtained using a vibratome (VT1000S, Leica) and the glomerular layer of the control and odor-deprived bulbs were extracted under dissecting microscope. The extracts were homogenized in 50 mM HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 1mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 10 mM sodium beta-glycerophosphate, 0.1% 2-mercaptoethanol and 1% Triton X-100 lysis buffer containing Protease Inhibitor Cocktail Set III (Calbiochem). The homogenates were sonicated and centrifuged at 13,000g at 4° C for 20 minutes to remove insoluble fraction. Protein samples (60 µg) were separated on NuPage 4-12 % Bis-Tris Gel (Invitrogen) and transferred to nitrocellulose membranes

(Amersham Bioscience). Pax6 and actin immunoreactive bands were detected using mouse monoclonal antibodies raised against the N-terminal 206 amino acids of Pax6 of human origin (Santa Cruz Biotechnology, Santa Cruz, CA; catalog #sc-32766, lot #10105; 1:1000) and mouse monoclonal IgG antibodies raised against chicken gizzard muscle actin (Cedarlane Laboratories, Burlington, ON; catalog #CLT9001, lot #0125; 1:2000), respectively. According to the manufacturer's description Pax6 monoclonal antibody recognizes two bands one at about 47 kDa and a slightly lower band at about 34 kDa, whereas actin antibody recognized a single specific band at 42 kDa in the extract of the mouse brain. The same bands were found in our experiments using the extract of glomerular layer of the adult mouse OB. Secondary antibody (goat anti-rabbit HRP 1:5000; Millipore and goat anti-mouse HRP 1:1000; Bio-Rad) and a chemiluminescence substrate mixture (ECL, Amersham Biosciences) were then used to detect the bands. The expression level of Pax6 in control and occluded OBs were normalized to the level of actin. One slice per control and odor-deprived OBs was used for TH immunohistochemistry to ensure the efficiency of nostril occlusion.

4.2.8 PCR analysis

The glomerular layer for the control and occluded OBs was extracted as for Western blot analysis. Immediately after tissue extraction, the total RNA was isolated using RNeasy Micro Kit (Qiagen), according to the manufacturer instructions. First strand cDNA synthesis reaction was performed with RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas Life science) with oligo(dT) primers. Obtained cDNA were amplified using following primers: for Pax6 5′-TAGCGAAAAGCAACAGATGG-3′ and 5′- CAGCTGAAGTCGCATCTGAG-3′; and for b-actin 5′- CACCACTTTCTACAATGAGC-3′ and 5′-CGGTCAGGATCTTCATGAGG-3′.

4.2.9 Statistical analysis

Data are presented as mean ± SEM. Statistical significance was tested by using paired Student's *t*-test (with *$p < 0.05$ and **$p < 0.01$).

4.3. RESULTS:

4.3.1 Diminution of TH expression following nostril occlusion:

It is well established that sensory deprivation results in a drastic reduction in the TH immunoreactivity (Baker et al., 1984; Baker et al., 1993; Mandairon et al., 2006). We, therefore, tested the effectiveness of the nostril occlusion by performing TH immunostaining. As expected, sensory deprivation induces a significant decrease in the TH immunoreactivity in the glomerular layer of the occluded OB (Occl) as compared to the control (Ctrl), contralateral OB (**Fig. 4.1a,c**). Interestingly, the intensity of TH immunoreactivity was similarly reduced 21 and 42 days after sensory deprivation (67.0 ± 2.5% of reduction 21 days after sensory deprivation, n = 7 animals, $p < 0.001$; and 65.7 ± 6.9% of reduction 42 days after sensory deprivation, n = 8 animals, $p < 0.001$; **Fig. 4.1b,d**). These data suggest that increasing the time of odor deprivation does not cause a further decrease in the TH expression.

4.3.2 Sensory deprivation decreased the survival of OB interneurons generated before and after the occlusion:

To investigate the role of sensory activity on the fate of interneurons born before or after sensory deprivation, we created two groups of animals. The first group had its OB occluded 21 days before the BrdU injection (**Fig. 4.2**), whereas the second group was injected with BrdU 21 days prior to occlusion (**Fig. 4.3**). The animals were then sacrificed after an additional 21 days.

In agreement with previous reports (Fiske and Brunjes, 2001; Saghatelyan et al., 2005; Yamaguchi and Mori, 2005; Mandairon et al., 2006), the number of GCs and PGCs cells born 21 days after sensory deprivation and analyzed 21 days later (i.e. 42 days of nostril occlusion) was reduced (**Fig. 4.2a**). Since sensory deprivation also decreased the mean surface of both the granule cell layer (GCL) and the glomerular layer (GL) (for GCL: 0.88 ± 0.02 mm^2 for ctrl and 0.80 ± 0.04 mm^2 for

occl, n = 13-15 slices per animal, n = 5 animals, $p < 0.05$; for GL: 0.56 ± 0.01 mm^2 for ctrl and 0.48 ± 0.01 mm^2 for occl, n = 13-15 slices per animal, n = 5 animals, $p < 0.05$; **Fig. 4.2**) we calculated the density of BrdU+ cells to compare control and occluded sides**.** Our results show that the mean surface density of cells generated after sensory deprivation in the OB was lower in the occluded side, in both the GCL and GL (for GCL: 268.4 ± 18.0 cells/mm^2 for ctrl and 210.2 ± 18.7 cells/mm^2 for occl, n = 7 animals, $p < 0.001$; for GL: 34.6 ± 3.2 cells/mm^2 for ctrl and 27.1 ± 3.2 cells/mm^2 for occl, n = 7 animals, $p < 0.05$; **Fig. 4.2c-f**). The reduction was even more pronounced when we estimated the total number of cells in the control and occluded bulbs. The total number of cells in both the GCL and the GL was significantly reduced (for GCL: 10695.8 ± 822.5 cells for ctrl and 7709.9 ± 801.9 cells for occl, n = 7 animals, $p < 0.001$; for GL: 880.6 ± 88.2 cells for ctrl and 591.0 ± 77.7 cells for occl, n = 7 animals, $p < 0.001$). Since neither proliferation of neuronal precursors (assessed by the counting of BrdU+ cells in the SVZ and RMS one hour after BrdU injection) nor their tangential migration (assessed by the counting of BrdU+ cells in the SVZ and RMS 5 days after BrdU injection) were affected by sensory deprivation (data not shown) we concluded that the reduced number of newborn cells in the OB likely results from the affected survival. These data are in agreement with numerous previous reports showing reduced survival of newly generated cells in the odor deprived OB (Frazier-Cierpial and Brunjes, 1989; Fiske and Brunjes, 2001; Saghatelyan et al., 2005; Yamaguchi and Mori, 2005; Mandairon et al., 2006). We next tested the role of sensory activity on the population of interneurons that are already present in the OB operational network. Sensory deprivation for 21 days reduced the mean surface of the GCL and the GL (for GCL: 0.98 ± 0.09 mm^2 for ctrl, 0.80 ± 0.06 mm^2 for occl, n = 13-15 slices per animal, n = 5 animals, $p = 0.0527$; for GL: 0.57 ± 0.04 mm^2 for ctrl, 0.49 ± 0.05 mm^2 for occl, n = 13-15 slices per animal, n = 5 animals, $p < 0.05$; **Fig. 4.3a-c**). Interestingly, odor deprivation for 3 weeks induced similar reduction in the number of BrdU+ cells generated 21 days before occlusion **(Fig. 4.3a-f)**. The mean surface density of BrdU+ cells labeled 21 days before nostril occlusion was reduced (for

GCL: 287.8 ± 33.0 cells/mm^2 for ctrl and 208.5 ± 21.1 cells/mm^2 for occl, n = 5 animals, $p < 0.05$; for GL: 27.1 ± 1.3 cells/mm^2 for ctrl, 22.7 ± 2.3 cells/mm^2 for occl, n = 5 animals, $p < 0.05$; **Fig. 4.3d,f**). The total number of cells in both the GCL and GL was also significantly reduced (for GCL: 12189.1 ± 732.8 cells for ctrl and 7445.9 ± 822.8 cells for occl, n = 5 animals, $p < 0.001$; for GL: 704.5 ± 65.8 cells for ctrl and 500.6 ± 72.9 cells for occl, n = 5 animals, $p < 0.001$).

These results suggest that sensory activity is a major determinant for the survival of not only newborn cells generated after sensory deprivation, but also for cells that are already present in the OB operational network before nostril occlusion.

4.3.3 Effect of sensory deprivation on different populations of periglomerular cells:

We next decided to investigate how sensory activity affects the fate determination and maintenance of newborn PGCs. We concentrated our analysis on newborn PGCs that are known to acquired either dopaminergic (TH+) and/or GABAergic (GAD67+) or calbindin and calretinin phenotypes (Kosaka et al., 1995; Kosaka et al., 1998; Bagley et al., 2007; Kosaka and Kosaka, 2007; Whitman and Greer, 2007). To investigate how sensory activity affects fate determination of these different populations of PGCs, we co-labeled BrdU+ cells generated after nostril occlusion with the antibodies directed against calbindin (**Fig. 4.4a1-b3,c**) and calretinin (**Fig. 4.4e1-f3,g**). To detect GABAergic cells we used transgenic mice expressing GFP under the endogenous glutamate decarboxylase (GAD) 67 promoter (Tamamaki et al., 2003) (**Fig. 4.4i1-j3,k**). All calbindin+, calretinin+ and TH+ PG cells were reported to express GFP in GAD67-GFP (Δneo) mice (Lagier et al., 2007). No differences in the proportion of newborn cells expressing these markers was detected between control and occluded bulbs (for calbindin 1.1 ± 0.6 % in ctrl and 1.0 ± 0.2 % in occl bulbs, n = 492 and 335 cells in ctrl and occl bulbs, respectively; n = 3 animals; **Fig. 4.4d**; for calretinin 25.1 ± 1.6 % in ctrl and 27.2 ± 3.4 % in occl bulbs, n = 238 and 146 cells in ctrl and occl bulbs, respectively; n = 4

animals; **Fig. 4.4h**; and for GAD67 84.2 ± 2.3 % in ctrl and 82.5 ± 5.1 % in occl bulbs, n = 414 and 257 cells in ctrl and occl bulbs, respectively; n = 4 animals; **Fig. 4.4l**). These results suggest that sensory activity is not important for the fate determination of these populations of PGCs. We next examined whether odor-induced activity is involved in the fate maintenance of GABAergic, calretinin+ and calbindin+ PGCs. Double labeling of BrdU+ cells born 21 days before sensory deprivation together with above-mentioned markers revealed that odor-induced activity does not influence the fate maintenance of these chemospecific populations of cells. In fact, the proportion of BrdU+ cells co-labeled either with calbindin, calretinin or GAD67 was similar in the occluded side as compared to the control OB (for calbindin: 6.0 ± 1.8 % for ctrl and 4.7 ± 1.7 % for occl, n = 347 and 235 cells in ctrl and occl bulbs, respectively; n = 5 animals; **Fig. 4.4d**; for calretinin 28.2 ± 7.6 % for ctrl and 34.2 ± 5.8 % for occl, n = 186 and 159 cells in ctrl and occl bulbs, respectively; n = 3 animals; **Fig. 4.4h**; and for GAD67: 86.1 ± 3.3 % for ctrl and 79.1 ± 3.2 % for occl, n = 278 and 216 cells in ctrl and occl bulbs, respectively; n = 4 animals; **Fig. 4.4l**).

Unlike other subtypes of PGCs, dopaminergic neurons were differently affected by sensory deprivation. Virtually no BrdU+ PGCs born after nostril occlusion were found to be TH positive (14.6 ± 4.1 % for ctrl and 0.0 % for occl, n = 253 and 145 cells in ctrl and occl bulbs, respectively; n = 4 animals; **Fig. 4.5a-d**). Interestingly, however, we also observed a drastic decreases in the proportion of BrdU+/TH+ cells born 21 days before sensory deprivation (30.9 ± 5.2 % for ctrl, 0.7 ± 0.7 for occl, n = 156 and 103 cells in ctrl and occl bulbs, respectively; n = 4 animals; **Fig. 4.5d**). These results suggest that sensory activity is not only important for the acquisition of dopaminergic fate of newborn PGCs as previously reported (Stone et al., 1991; Baker et al., 1993; Brunjes, 1994; Mandairon et al., 2006), but also for the maintenance of this phenotype in the cells that have already differentiated to the TH+ phenotype.

It has been previously reported that subpopulation of GABAergic PGCs and all dopaminergic bulbar interneurons express Pax6 (Dellovade et al., 1998) and that this transcription factor is involved in the acquisition of dopaminergic phenotype (Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005; Brill et al., 2008). We therefore, investigated how sensory activity affects the number of Pax6+ cells in the GL of the OB. Interestingly, 21 days of sensory deprivation increased the mean surface density of Pax6+ cells ($3.2 \pm 0.2 \times 10^5$ cells/mm^3 for ctrl and $4.2 \pm 0.2 \times 10^5$ cells/mm^3 for occl, n = 4 animals, $p < 0.05$; **Fig. 4.6a1,a2 and b**). The same difference was observed at the level of the number of Pax6+ cells in the occluded side when compared to the control side ($5.2 \pm 0.3 \times 10^7$ cells for ctrl and $6.5 \pm 0.9 \times 10^7$ cells for occl, n = 4 animals, $p < 0.05$). However, this increase was transient since the density ($3.2 \pm 0.3 \times 10^5$ cells/mm^3 for ctrl and $3.1 \pm 0.1 \times 10^5$ cells/mm^3 for occl, n = 4, $p > 0.05$: **Fig. 4.6a3,a4 and b**) as well as the number ($5.1 \pm 1.0 \times 10^7$ cells for ctrl and $4.2 \pm 0.4 \times 10^7$ cells for occl, n = 4 animals, $p < 0.05$) of Pax6+ cells returned to normal level 42 days after odor deprivation. Importantly, immunoblot analysis of the Pax6 expression in the GL of the occluded mice revealed a 45.0 ± 21.3 % increase in the amount of this transcriptional factor 21 days following sensory deprivation (n = 6 animals, $p < 0.05$; **Fig. 4.6c,d**). The increase in the Pax6 expression was also observed in the PCR analysis of the GL extracts (74.6 ± 2.4 % increase in the occluded as compared to the control bulb, n = 3 animals, $p < 0.001$; **Fig. 4.6c,d**). These results indicate that sensory deprivation triggers Pax6 expression at the transcriptional level.

Since the OB responds to the decreased TH expression by increasing the number of Pax6+ cells, we found it relevant to investigate if this increase was mediated by PGCs already present at the time of occlusion or by newly arriving cells. For this purpose, we quantified BrdU+ cells born before and after sensory deprivation that were also Pax6+. Surprisingly, we found that the proportion of cells expressing Pax6 and born after the occlusion was decreased (37.9 ± 4.6 % for ctrl and 22.8 ± 1.8 % for occl, n = 4 animals, $p < 0.05$; **Fig. 4.7a-d**), whereas the proportion of cells

that express Pax6 and born before the olfactory deprivation was increased (**Fig. 4.7d**). The population of cells born 21 days before sensory deprivation already displayed a tendency towards increased percentage of Pax6+/BrdU+ in the occluded as compared to the control bulbs (44.7 ± 4.9 % for ctrl, 52.8 ± 4.5 % for occl, n = 4 animals, $p > 0.05$; **Fig. 4.7d**). This difference further increases when cells born 60 days before sensory deprivation were analyzed. Among 185 and 247 BrdU+ cells analyzed in the control and occluded bulbs, respectively, 56.3 ± 4.5 % were Pax6+ in control OB whereas 70.1 ± 2.1 % were immunopositive for Pax6 in the odor-deprived OB ($p < 0.05$; **Fig. 4.7d**). These data suggest that the transient increase in the number of Pax6+ cells is likely mediated by the expression of this transcriptional factor in the PGCs born 60 days before sensory deprivation.

4.4. DISCUSSION:

In the present study we show that sensory activity plays a multifarious role in the acquisition and fate maintenance of different chemospecific populations of newly generated PGCs in the OB. Sensory deprivation altered the number of dopaminergic neurons, while calbindin+, calretinin+ and GABAergic populations of newborn cells remained largely unaffected. Interestingly, the number of cells expressing Pax6, a transcription factor involved in the dopaminergic fate acquisition, was transiently increased in the GL of the odor-deprived OB. We also found that odor-induced activity is essential for the survival of not only newborn, but also pre-existing populations of GC and PG cells generated at adulthood.

4.4.1 The role of sensory activity on the survival of newborn bulbar interneurons:

Previous reports have demonstrated that odor deprivation reduces radial migration of neuroblasts (Saghatelyan et al., 2004), decreases survival of newborn interneurons in the OB (Frazier-Cierpial and Brunjes, 1989; Brunjes, 1994; Corotto et al., 1994; Fiske and Brunjes, 2001; Saghatelyan et al., 2005; Yamaguchi and Mori, 2005; Mandairon et al., 2006) and affects the dendritic morphology and spine density of newborn OB interneurons (Saghatelyan et al., 2005). In contrast, an odor-enriched environment increases the total number of newborn granule cells without affecting the proliferation of neuronal precursors in the SVZ (Rochefort et al., 2002). Our data suggests that sensory activity is not only important for the survival and/or integration of newly arriving GCs and PGCs, but also for the survival of cells that are already present in the operational bulbar network at the moment of sensory deprivation. It has been previously shown that most of the newborn cells integrate into the bulbar network and acquire full electrophysiological and morphological characteristics 21 days following their generation in the SVZ (Petreanu and Alvarez-Buylla, 2002; Belluzzi et al., 2003; Carleton et al., 2003). However, recently it has been highlighted that at this stage newborn neurons

overproduce dendritic spines and that the final stabilization of synapses ends at about 50-60 days following precursors' generation (Mizrahi, 2007; Whitman and Greer, 2007). It is thus conceivable that 21 days-old neurons present in the OB neuronal circuitry remain sensitive to the level of sensory input and might be more vulnerable as compared to the fully integrated newborn cells. Indeed, it seems that sensory deprivation has no effect on the survival of older GC cells (Yamaguchi and Mori, 2005). The concept of the existence of a "critical period" for the survival of newborn GC cells was proposed on the basis of these data (Yamaguchi and Mori, 2005), and our results are in agreement with this view.

4.4.2 The role of sensory activity on chemospecific populations of newborn interneurons in the OB:

Following their arrival at the OB, neuronal precursors have to mature and differentiate into particular neuronal phenotype. In rodents' OB, PGCs differentiate into either GABAergic, dopaminergic, calbindin and calretinin interneurons (Kosaka et al., 1995; Kosaka et al., 1998; Bagley et al., 2007; Kosaka and Kosaka, 2007; Whitman and Greer, 2007). Recently, it has been demonstrated that all these chemospecific populations of PGCs can be produced during adulthood (Bagley et al., 2007; De Marchis et al., 2007; Whitman and Greer, 2007; Batista-Brito et al., 2008) with the aid of the "molecular code" specific for each subtype of interneurons (Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005; Waclaw et al., 2006; Allen et al., 2007; Brill et al., 2008). However, it is likely that determination and maintenance of chemospecific phenotypes is controlled not only by genetic factors but also by environmental stimuli. Sensory activity is a major environmental factor that can affect the determination and/or fate maintenance of PGCs. Indeed, it is well documented that sensory deprivation drastically reduces TH immunoreactivity in the odor-deprived OB (Baker et al., 1984; Stone et al., 1991; Baker et al., 1993; Saino-Saito et al., 2004; Mandairon et al., 2006). However, these studies do not specify whether sensory activity downregulates TH expression in the newly

generated neurons or in the cells that have already been integrated to the operational network. In addition, the role of sensory activity in the fate determination and maintenance of GABAergic, calbindin and calretinin phenotypes remained unclear (Stone et al., 1991; Arenkiel et al., 2011). Our results demonstrate that odor-induced activity is essential for the development and expression of dopaminergic phenotype, but not for GABAergic, calretinin or calbindin fates. It is likely, that reduced TH immunolabeling results from the decreased enzyme expression rather than from the death of dopaminergic cells. In agreement with this hypothesis are our data showing that the proportion of GAD67+ cells is not altered following sensory deprivation, which would have been expected if dopaminergic cells, that co-express GAD67, were dying. In addition, it has been demonstrated that re-opening of the nostril results in the re-expression of TH (Baker et al., 1984). Furthermore, DOPA decarboxylase, the second enzyme in the dopamine biosynthesis pathway, does not appears to be downregulated in the same fashion as TH (Baker et al., 1984).

Our results showing the pattern of expression of Pax6 following sensory deprivation is particularly interesting. Twenty-one days of odor deprivation increases the number of Pax6+ cells in the glomerular layer of the OB. Our data highlighted some of the mechanisms by which sensory activity regulates Pax6 expression. We have shown that nostril occlusion induces upregulation of Pax6 expression at the transcriptional level. Pax6 is a key molecular determinant of the dopaminergic fate (Dellovade et al., 1998; Hack et al., 2005; Kohwi et al., 2005; Brill et al., 2008) and upregulation in the number of cells expressing this transcriptional factor can be interpreted as a compensatory response of the OB to the decreased expression of TH. It has been previously demonstrated that the OB might respond to the changing environmental conditions with compensatory mechanisms helping to maintain the normal functioning of the bulbar neuronal network (Leon, 1998; Saghatelyan et al., 2005; Waggener and Coppola, 2007; McCollum et al., 2013). Notably, the reduced expression of TH following sensory

deprivation is accompanied by an increase in the density of D2 receptors on the olfactory nerve terminals (McCollum et al., 2013). It is, thus, conceivable that the OB tries to respond to the decreased level of TH expression not only by increasing the density of D2 receptors (McCollum et al., 2013), but also by increasing the number of cells expressing a key molecular signal (Pax6) involved in the determination of dopaminergic phenotype. Intriguingly, however, newly generated cells do not appear to be a major element in this Pax6 increase at OB level. In fact, lower number of newly arriving neurons in the odor-deprived OB was found to express Pax6 as compared to the control OB. These data suggest that older populations of PGCs that were already present in the bulbar network at the moment of sensory deprivation start to express Pax6. Our investigation of cells that were born 21 and 60 days before sensory deprivation has revealed a noticeable increase in the number of BrdU+/Pax6+ cells in the occluded bulb. It should be noted, however, that the increase in the number of Pax6+ in the odor-deprived OB was transient, since their density returns to normal levels 42 days following sensory deprivation. These data are in line with the recently reported expression pattern of Pax6 after sensory deprivation (Cave and Baker, 2009). This effect is most likely due to the reduced proportion of newly generated cells that express Pax6. Therefore, on the one hand, sensory deprivation appears to lower the number of newly arriving Pax6+ cells in the glomerular layer and, on the other hand, increases its expression in the population of PGCs that were already present in the bulbar network at the moment of nostril occlusion. The reason(s) for such differential regulation of Pax6 expression in the OB is not clear and future studies aiming at investigating the functional proprieties of these two populations of dopaminergic cells need to be performed.

Despite such wide and dynamic changes in the dopaminergic cells, no other population of PGCs was affected by sensory deprivation. These data are at odds with a previously published report showing 30% reduction in the number of calbindin+ cells following sensory deprivation in the developing rat OB (Arenkiel et

al., 2011). One of the reasons for this discrepancy may be related to species (rat *vs.* mouse) and/or to age (developing *vs.* adult) differences. Important disparities exists between mice and rats regarding the fate specifications of PGCs in the OB (Kosaka et al., 1995; Kosaka and Kosaka, 2007). In the rat OB, only TH+ PGCs co-express GABA, whereas calbindin+ and calretinin+ neurons form distinct neuronal populations (Kosaka et al., 1988; Kosaka et al., 1995; Kosaka and Kosaka, 2007). In the mouse OB, all dopaminergic, calbindin+ and the majority of calretinin+ cells are also GABAergic (Kosaka and Kosaka, 2007). It is therefore conceivable that the impact of sensory activity in rats and mice OB may also differ. In addition, it has been shown that calbindin+ PGCs are preferentially generated during early postnatal life (De Marchis et al., 2007; Batista-Brito et al., 2008). Indeed, our data reveal that relatively few newborn calbindin+ PGCs were present in the adult mouse OB. The total number of BrdU+/calbindin+ neurons detected in the glomerular layer represents only 1% and 5% of the entire pool of cells at 21 and 42 days after BrdU injection. It is, thus, possible that nostril occlusion in the developing rat OB, at the peak of calbindin+ neurons differentiation, induces substantial changes in this population of cells (Arenkiel et al., 2011) that remained largely unaffected by odor deprivation during adulthood.

Why among all the chemospecific populations of PGCs only dopaminergic neurons are affected by sensory deprivation? One of the reasons for such specific regulation of dopaminergic cells by level of the sensory activity might be related to the compartmental organization of these neurons. It has been shown that dopaminergic PGCs, which are actually classified as type 1 PG neurons, are the only population of cells that receive input from the olfactory nerve (Kosaka and Kosaka, 2007). By contrast, other populations of PGCs, classified as type 2 neurons, send their dendrites to the intraglomerular zones that are not contacted by the sensory neuron's terminals (Kosaka and Kosaka, 2007). Therefore the dopaminergic population of PGCs that are in direct contact with the sensory neurons might be more sensitive to the degree of sensory processing that occurred

at the OB level. Intriguingly, dopaminergic PGCs are the only population of chemospecific neurons in the GL that was shown to be affected by behaviorally relevant stimuli. The social odor perception after mating is associated with the increased level of TH expression in the PGCs of the OB (Serguera et al., 2008). Increase in the dopaminergic transmission upholds presynaptic inhibition of sensory neurons and hampers neuronal activation in the OB leading thus to the modulation of social odors perception detrimental to pregnancy (Serguera et al., 2008).

Altogether, our data highlights the role of odor-induced activity in the integration and/or survival of newborn GCs and PGCs. Our findings also indicate that odor-induced activity acts upon bulbar newborn cells that were generated before nostril occlusion and influences the fate determination and maintenance of different populations of newly generated PGCs.

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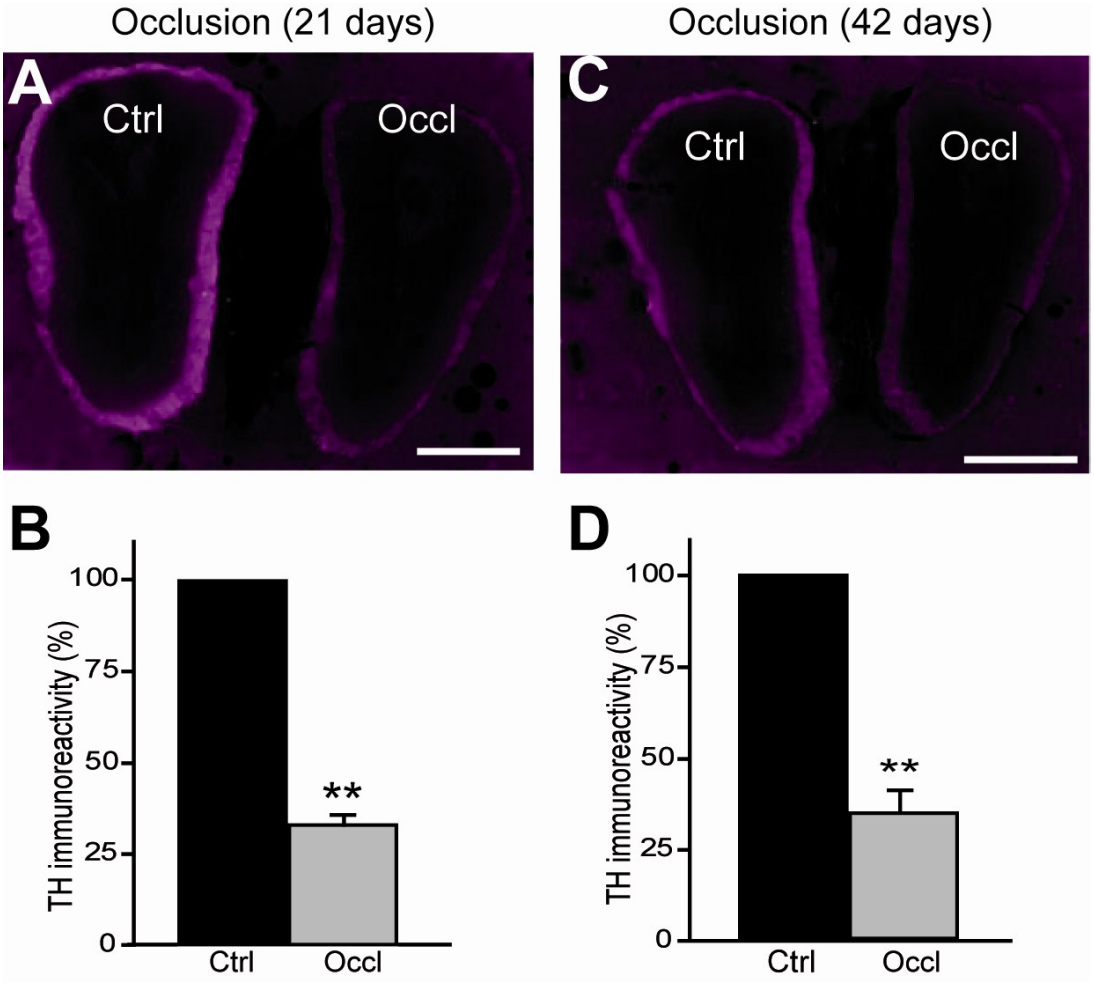


Figure 4.1: Decreased expression of TH in the odor-deprived bulb.

Photomicrographs of coronal sections showing TH immunoreactivity (red) in control (left) and occluded (right) OB, 21 (**A**) and 42 days (**C**) following nostril closure. There is a decrease in the expression of TH in the odor-deprived bulb at both 21 (**B**) and 42 days (**D**) after the initiation of sensory deprivation. **p < 0.001 with a Student's t-test. Ctrl, control side; Occl, occluded side. Scale bar: 1 mm.

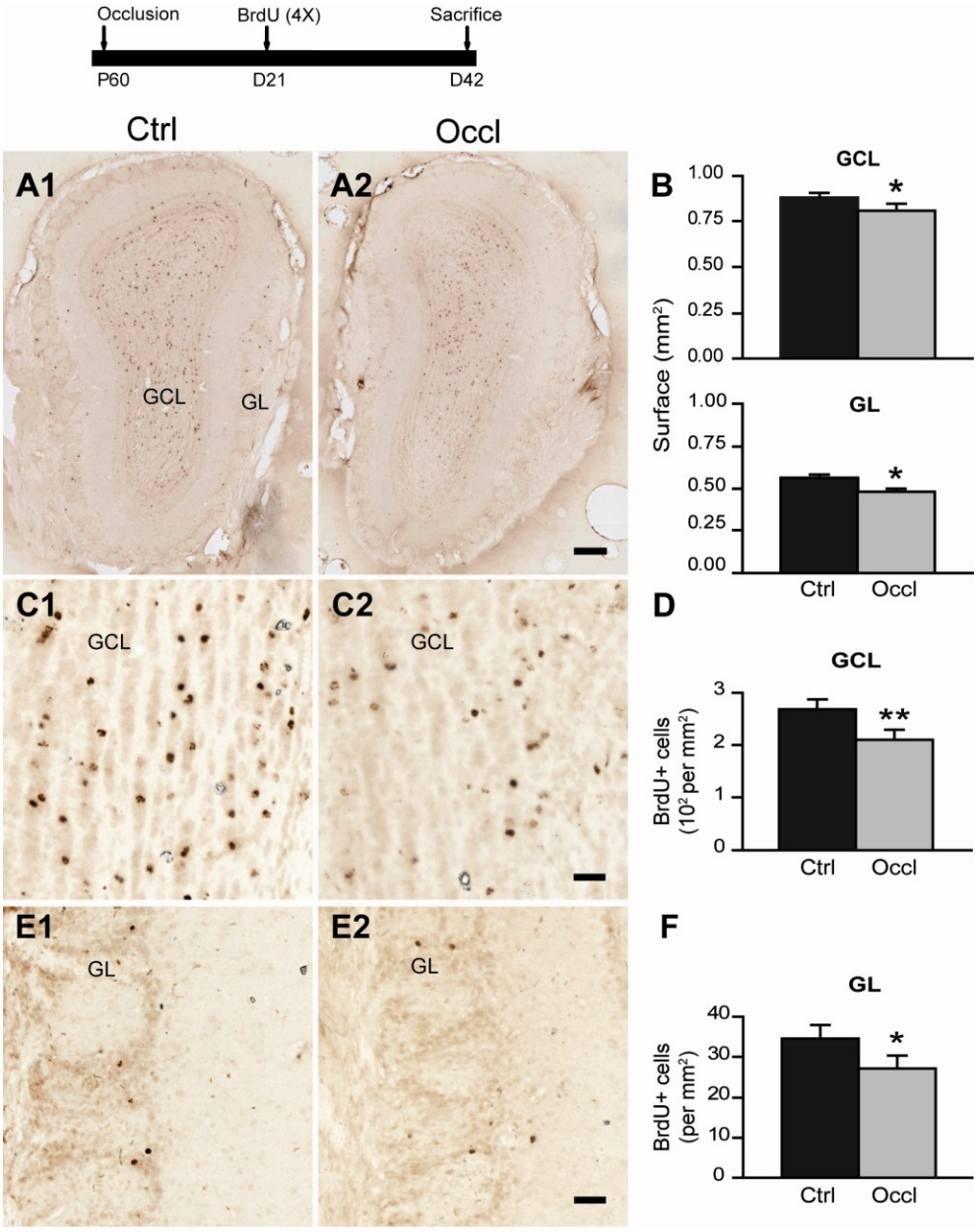
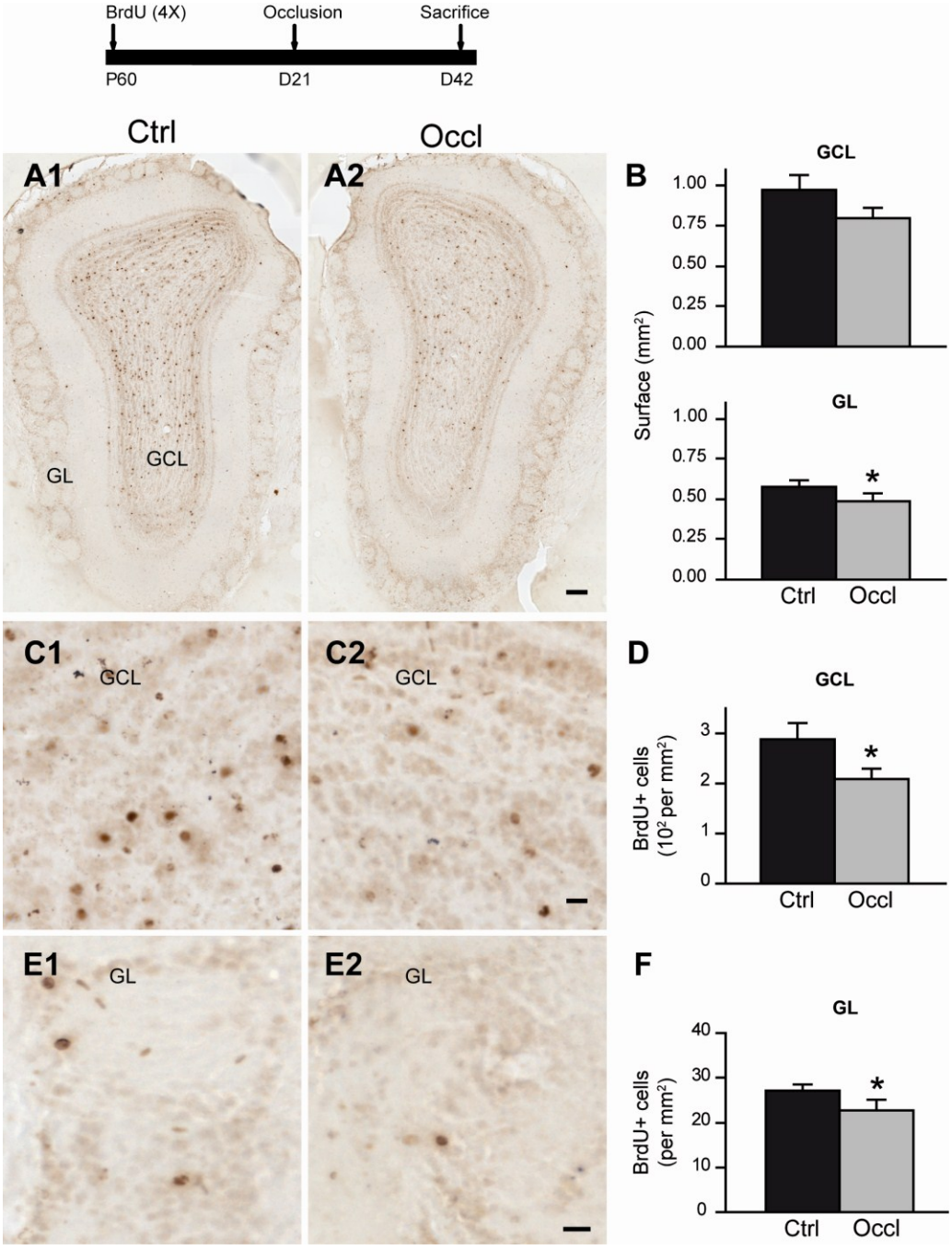

Occlusion
BrdU (4X)
Sacrifice
P60
D21
D42
Ctrl
Occl
A1
A2
GCL
GL
B
GCL
GL
Surface (mm²)
1.00
0.75
0.50
0.25
0.00
Ctrl
Occl
C1
C2
D
BrdU+ cells (10² per mm²)
3
2
1
0
E1
E2
F
BrdU+ cells (per mm²)
40
30
20
10

Figure 4.2: Effect of odor-induced activity on the integration and/or survival of newborn bulbar interneurons.

(**A**) Photomicrographs of coronal sections of control (**A1**) and occluded (**A2**) sides of the OB showing BrdU immunoreactivity. The experimental design is explained above the photomicrographs. (**B**) Histograms showing the decrease in the surface of the granule cell (GCL) and glomerular (GL) layers that occurred on the occluded side, as seen in coronal sections. *$p < 0.05$ with a Student's t-test. (**C, E**) High magnification photomicrographs of BrdU+ profiles in GCL (**C1-C2**) and GL (**E1-E2**) in control (**C1,E1**) and occluded (**C2,E2**) bulbs. (**D, F**) Histograms depicting the density of BrdU+ cells in the GCL (**D**) and GL (**F**) of the OB under control conditions (Ctrl) and following sensory deprivation (Occl). **$p < 0.001$ with a Student's t-test. Scale bars: 500 µm for **A** and 40 µm for **C** and **E**.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BrdU+ cells
(10² per mm²)
3
2
1
0
E1
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BrdU+ cells
(per mm²)
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10
0

Figure 4.3: Sensory deprivation affects the survival of newborn interneurons generated before nostril closure.

(**A**) Photomicrographs of coronal sections of control (**A1**) and occluded (**A2**) sides of the OB showing BrdU immunoreactivity. The experimental design is explained above the photomicrographs. (**B**) Histograms showing the decrease in the surface of the granule cell (GCL) and glomerular (GL) layers that occurred on the occluded side, as seen in coronal sections. *$p < 0.05$ with a Student's t-test. (**C, E**) High magnification photomicrographs of BrdU+ profiles in GCL (**C1,C2**) and GL (**E1,E2**) in control (**C1,E1**) and occluded (**C2,E2**) bulbs. (**D, F**) Histograms depicting the density of BrdU+ cells in the GCL (**D**) and GL (**F**) of the OB under control conditions (Ctrl) and following sensory deprivation (Occl). **$p < 0.001$ with a Student's t-test. Scale bars: 500 µm for **A** and 40 µm for **C** and **E**.

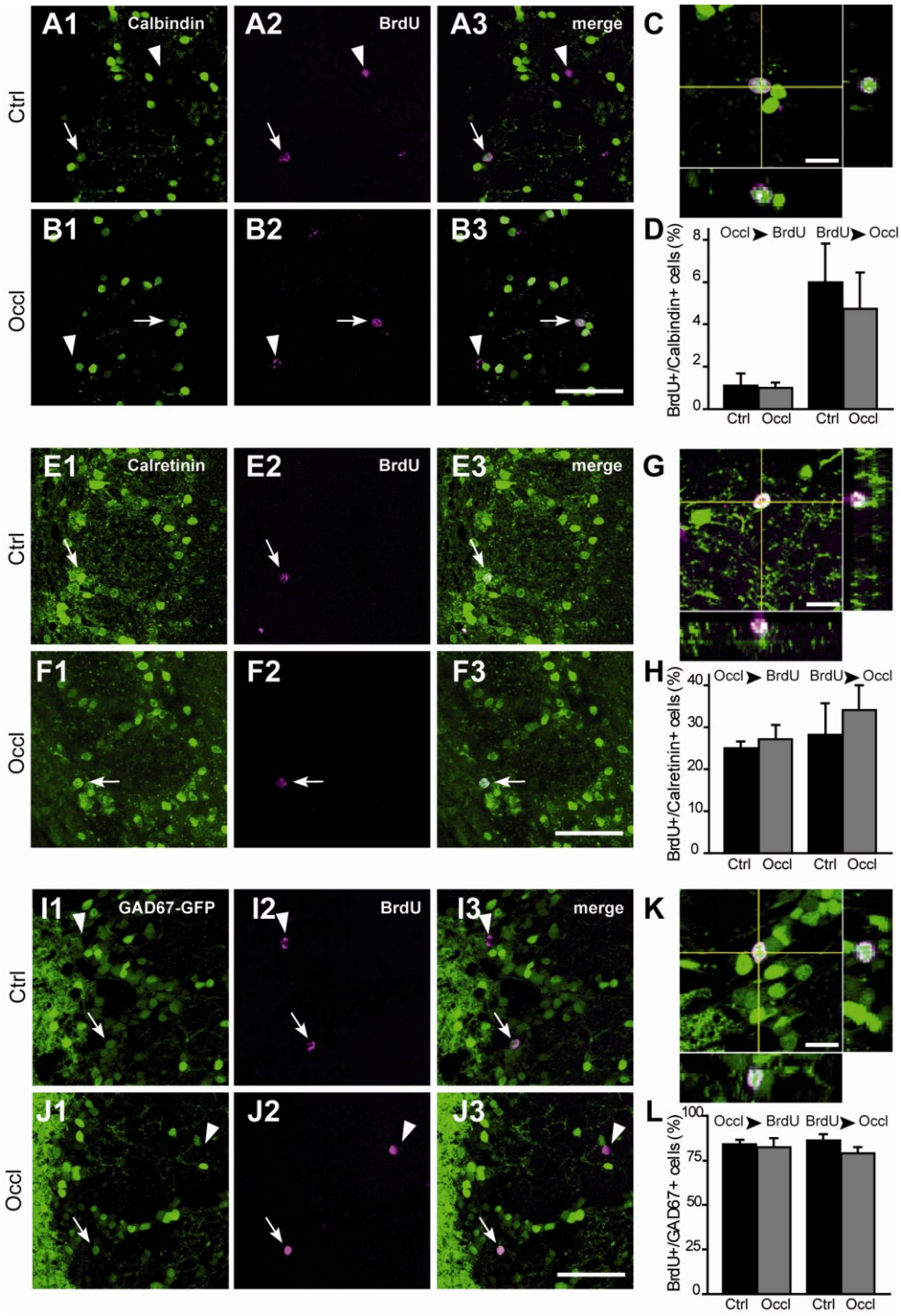

A1
Calbindin
A2
BrdU
A3
merge
C
Ctrl
B1
B2
B3
Occl
D
Occl ➤ BrdU
BrdU ➤ Occl
BrdU+/Calbindin+ cells (%)
8
6
4
2
0
Ctrl
Occl
Ctrl
Occl
E1
Calretinin
E2
BrdU
E3
merge
G
Ctrl
F1
F2
F3
Occl
H
Occl ➤ BrdU
BrdU ➤ Occl
BrdU+/Calretinin+ cells (%)
40
30
20
10
0
Ctrl
Occl
Ctrl
Occl
I1
GAD67-GFP
I2
BrdU
I3
merge
K
Ctrl
J1
J2
J3
Occl
L
Occl ➤ BrdU
BrdU ➤ Occl
BrdU+/GAD67+ cells (%)
100
75
50
25
0
Ctrl
Occl
Ctrl
Occl

Figure 4.4: Expression of calbindin, calretinin and GAD67 are maintained in periglomerular cells generated before or after sensory deprivation.

Photomicrographs of the control (**A, E, I**) and occluded (**B, F, J**) sides of the glomerular layer of the OB showing calbindin (**A1, B1**; in green); calretinin (**E1, F1**; in green) and GAD67 (**I1, J1**, in green) and BrdU (**A2, B2, E2, F2, I2, J2**, in magenta) immunolabelings. (**C, G, K**) Confocal 3D reconstruction of BrdU+ cells (magenta) stained for the calbindin (**C**), calretinin (**G**) and GAD67 (**K**) (green). Reconstructed orthogonal projections are presented as viewed in the *x*–*z* (bottom) and *y*–*z* (right) planes. (**D, H, L**) Histograms summarizing the percentage of periglomerular cells (PGC) expressing calbindin (**D**), calretinin (**H**) and GAD67 (**L**) born 21 days before (BrdU►Occl) or after (Occl►BrdU) occlusion. Note the gradual increase in the percentage of BrdU+/calbindin+ cells over time. Arrows point to double labeled cells, whereas arrowheads indicate cells stained only with BrdU. Scale bars: 50 µm for **B, F, J** and 10 µm for **C, G** and **K**.

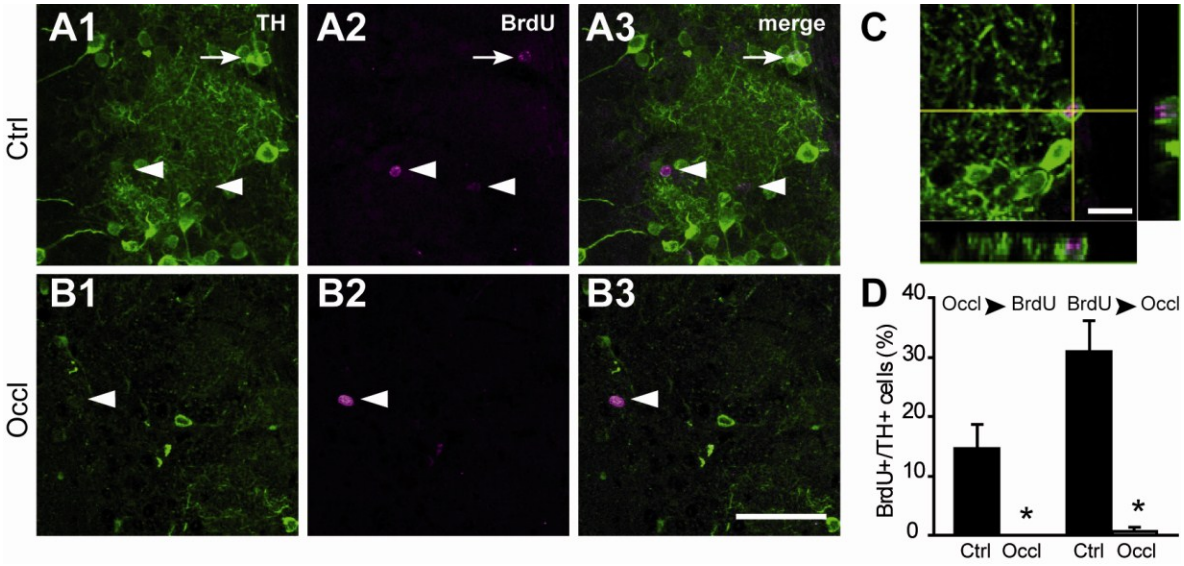


Figure 4.5: Olfactory deprivation reduces the number of TH+ cells born before or after sensory deprivation.

(**A, B**) Photomicrographs of sections immunostained for both TH (**A1, B1**; green) and BrdU (**A2, B2;** magenta) in control (**A1-A3**) and occluded (**B1-B3**) glomerular layer. (**C**) Confocal 3D reconstruction of BrdU+ cells (magenta) stained for the TH (green). Reconstructed orthogonal projections are presented as viewed in the *x*–*z* (bottom) and *y*–*z* (right) planes. (**D**) Note, reduced expression of TH by the newborn cells born before (BrdU►Occl) or after (Occl►BrdU) sensory deprivation. Arrows point to double labeled cells, whereas arrowheads indicate cells stained only with BrdU. *$p < 0.05$ with a Student's t-test. Scale bars: 50 µm for **A, B** and 10 µm for **C**.

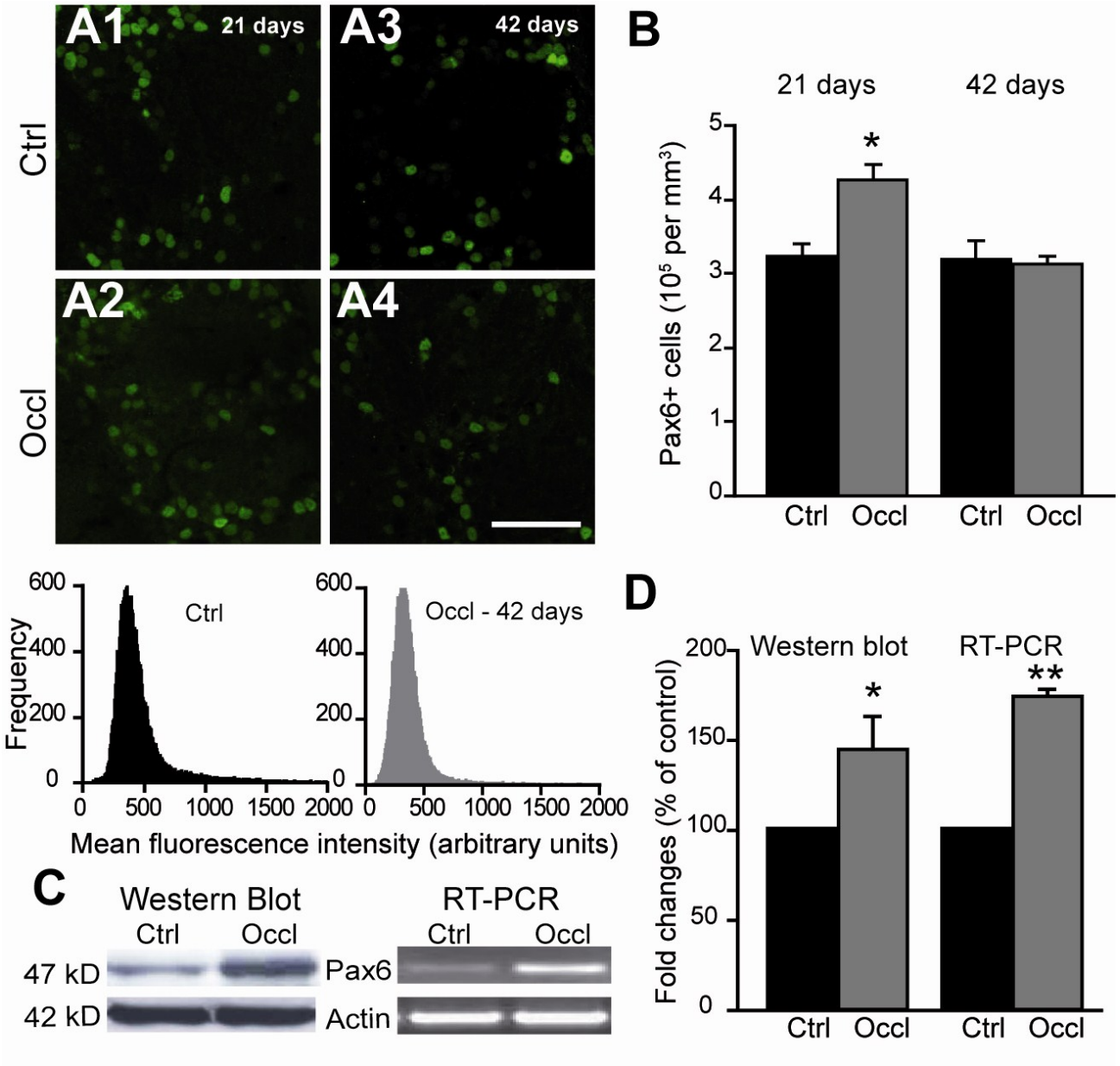
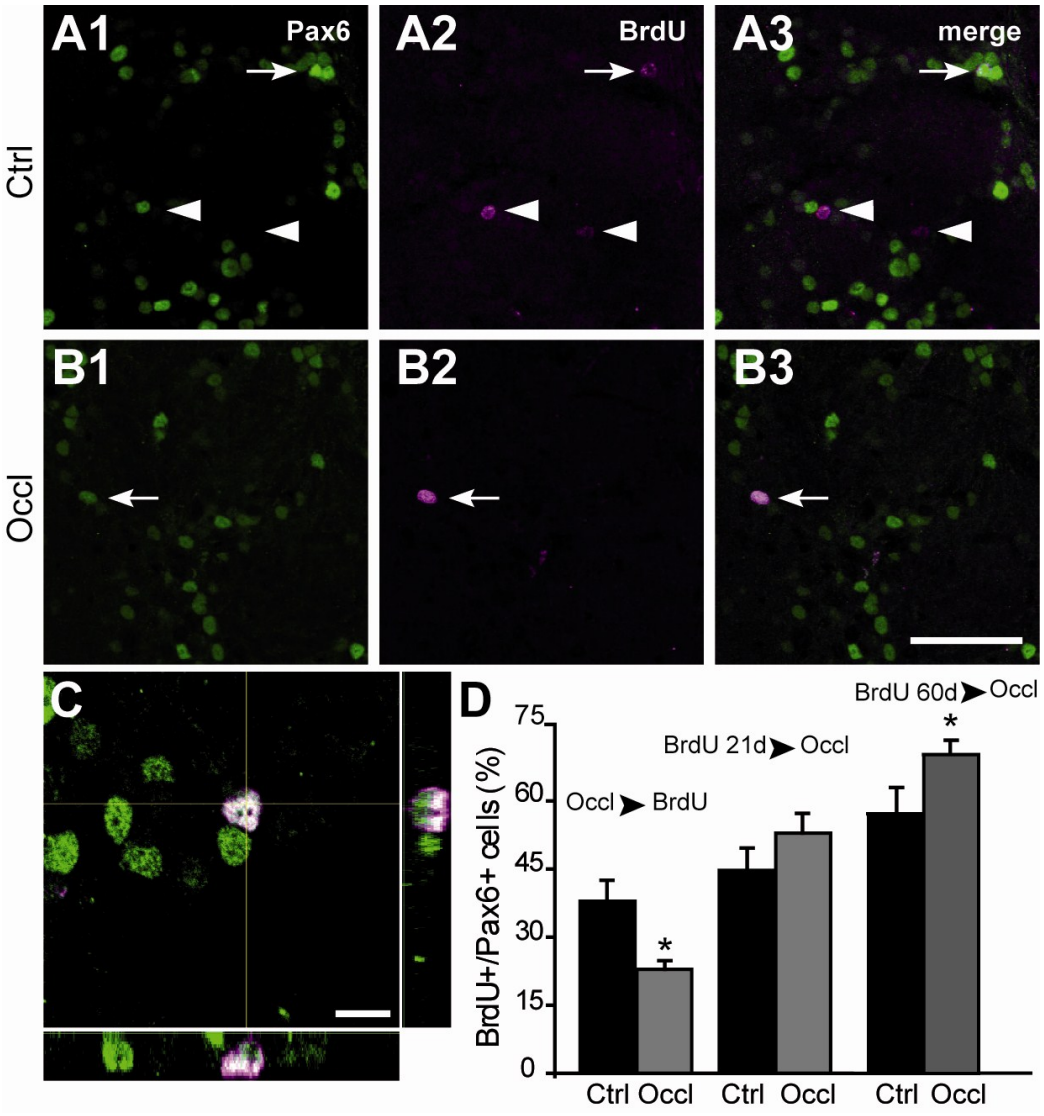

A1
21 days
A3
42 days
Ctrl
A2
A4
Occl
B
21 days
42 days
Pax6+ cells (10⁵ per mm³)
Ctrl
Occl
Ctrl
Occl
Ctrl
Occl - 42 days
Frequency
Mean fluorescence intensity (arbitrary units)
C
Western Blot
RT-PCR
Ctrl
Occl
Ctrl
Occl
47 kD
Pax6
42 kD
Actin
D
Western blot
RT-PCR
Fold changes (% of control)
Ctrl
Occl
Ctrl
Occl

Figure 4.6: Expression of Pax6 after sensory deprivation.

(**A**) Photomicrographs of Pax6+ cells in control (**A1, A3**) and occluded (**A2, A4**) bulbs, 21 (**A1, A2;** left panel) and 42 (**A3, A4;** right panel) days following sensory deprivation. Lower panels demonstrate the mean fluorescence intensity in the sections with Pax6 immunostaining 42 days following sensory deprivation. Note, similar fluorescence intensity between control and occluded sections. The same type of analysis performed on 21 days occluded mice showed increase in the mean fluorescent intensity due to the increased number of Pax6+ cells in this group of animals (data not shown). (**B**) The density of Pax6+ cells in the GL after 21 or 42 days of occlusion is illustrated here in the form of histograms. *$p < 0.05$ with a Student's t-test. (**C**) Western blot and RT-PCR analysis of Pax6 expression in the GL of the occluded mice 21 days following sensory deprivation. (**D**) The histograms show the quantification of Pax6 expression 21 days after nostril occlusion. The level of expression of Pax6 in the control and occluded bulbs were normalized to the level of actin expression. The data are expressed as the percentage of increase in the occluded as compared to the control (taken as 100 %) bulbs. *$p < 0.05$ and **$p < 0.001$ with a paired Student's t-test. Scale bars: 50 µm for **A.**


A1
Pax6
A2
BrdU
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merge
Ctrl
B1
B2
B3
Occl
C
D
BrdU 60d ➤ Occl
BrdU 21d ➤ Occl
Occl ➤ BrdU
BrdU+/Pax6+ cells (%)
75
60
45
30
15
0
*
*
Ctrl Occl Ctrl Occl Ctrl Occl

Figure 4.7: Differential regulation of Pax6 expression in cells born before or after sensory deprivation.

(**A, B**) Double-immunostaining for Pax6 (**A1, B1;** green) and BrdU (**A2, B2;** magenta) in control (**A1-A3**) and occluded (**B1-B3**) OB. (**C**) Confocal 3D reconstruction of BrdU+ cells (magenta) stained for the Pax6 (green). Reconstructed orthogonal projections are presented as viewed in the *x–z* (bottom) and *y–z* (right) planes. (**D**) The histograms show the percentage of cells expressing Pax6 born either 21 (BrdU 21d►Occl) or 60 days (BrdU 60d►Occl) before or 21 days after (Occl►BrdU) sensory deprivation Arrows point to double labeled cells, whereas arrowheads indicate cells stained only with BrdU. Scale bars: 50 µm for **A, B** and 10 µm for **C**.

CHAPTER V

5. General Discussion:

Olfaction is a sense present in most animals, and provides essential information for their survival. The rodent olfactory system comprises the peripherally situated olfactory epithelium housing the olfactory receptor neurons that transmit sensory information into the olfactory bulb where the incoming receptor neuron axon synapses with the primary dendrites of output neurons. Local interneurons granule and periglomerular cells modulate information processing in the OB. Even though this well organized multi-layered sensory modality is studied in great details, there are still many questions left to be answered. My thesis work on the rodent olfactory system allowed me to ask several questions as to how the sensory inputs impact the bulb or what are the mechanisms governing the production, migration and acquisition of interneurons in the adult olfactory system and how are they different from those derived in the developing olfactory bulb? Finally, are the adult generated granule interneurons represents a uniform group of cells or are composed from functionally distinct subpopulations?

5.1 Sensory activity and the interneurons of the OB:

Sensory input has been shown to play crucial roles in the OB and affect both GC and PG populations. Studies have shown that sensory deprivation reduces the number of GCs in the OB and influences the spine density and the complexity of dendritic arborisation (Saghatelyan et al., 2005; Brunjes, 1994; Corotto et al., 1994; Friske and Brunjes, 2001; Yamaguchi and Mori, 2005; Mandairon et al., 2006). We studied the effect of sensory activity on the periglomerular interneurons that are a molecularly varied population of cells expressing a wide variety of proteins (Kosaka and Kosaka, 2007).

Many studies have reported that in response to odor deprivation, amongst the various subpopulations of periglomerular interneruons, the TH expressing

dopaminergic neurons are the ones most affected (Baker et al., 1984,1993; Stone et al., 1991; Saino-Saito et al., 2004; Mandairon et al., 2006; Vallejo et al., 2000). Our results and those of other studies show that this loss of TH is due to the downregulation of the enzyme and not due to the death of the cells themselves. Contrary to the downregulation of TH, the expression of Pax6 the transcription factor responsible for the fate determination of dopaminergic cells is up regulated following naris closure. These data are in agreement with earlier reports (Cave and Baker, 2009). This increase was seen to be transient with levels of Pax6 returning to normal after 42 days. Thus our results along with earlier work reiterate the importance of sensory activity in the normal maintenance of the OB and the fate maintenance of specific subpopulations of interneurons. But how do these interneurons get to the OB?

5.2 Tenascin-R affects adult but not perinatal neurogenesis:

Neurogenesis; the production of neurons, happens continuously in the sub ventricular region of the forebrain (Luskin, 1993; Lois and Alvarez-Buylla, 1994). This region is one of only two regions in the adult brain that preserves its ability to give rise to new neurons destined for the olfactory bulb (Ming and Song, 2005, 2011; Lledo et al., 2006). The progenitors produced in the sub ventricular zone then migrate via the rostral migratory steam to reach the bulb. The region of production and path taken by interneuron progenitors to reach the bulb is remodeled from an embryonic organism till it reached adulthood (Law et al., 1999; Peretto et al., 2005; Puche and Shipley, 2001; Emsley et al., 2012; Bozoyan et al., 2012). Thus the production, migration, differentiation, integration and survival of these progenitors are orchestrated by a huge number of intrinsic and extrinsic factors (Lledo et al., 2006). However, to date, it is not clear if any of these factors could be involved exclusively with adult neurogenesis without having any role in the earlier developmental neurogenesis.

Here I presented a study where we have shown that the extracellular matrix glycoprotein Tenascin-R (TNR) is expressed comparative late in the olfactory bulb.

Since earlier work showed impaired neurogenesis in adult mice deficient for this molecule (Saghatelyan et al., 2004), TNR became a viable candidate as a molecular cue for its participation in adult neurogenesis exclusively. Through our work we have shown that a lack of TNR significantly reduced the number of interneurons integrated in the adult bulb by impairing radial migration of these interneurons, and these deficits were not seen in young/perinatal mice. This is in agreement with previous studies showing that TNR is a detachment signal for tangentially migrating interneurons and a guidance cue to those migrating radially in adult olfactory bulb. Thus, we describe the first molecular cue shown to be specifically implicated in adult neurogenesis, opening up the possibilities to investigate for other cues that are exclusive for adult or perinatal neurogenesis. Knowledge that could allow for manipulating endogenous progenitor cells for repair of brain injury and disease.

Using these mice we were also able to show that adult generated interneurons are crucial to the proper network activity of the bulb. Any impairment in neurogenesis during adulthood severely affects the mouse's ability in short-term odor memory tasks, which is also in agreement with earlier published work (Breton-Provencher et al., 2009).

5.3 CaMKIIα expressing GCs form a distinct subpopulation of adult generated GCs:

Knowing that adult generated interneurons are crucial to the proper functioning of the olfactory bulb, we next wanted to know if all adult generated granule interneurons are the same. It is well documented that adult generated periglomerular interneurons are a heterogeneous population (Kosaka and Kosaka, 2007; Batista-Brito et al., 2008), however the possible molecular differences in granule cells have not been characterised yet. The granule cell layer in itself is not exclusively inhabited by granule cells, other interneuron types have been documented in the GCL (Schneider and Macrides, 1978). Amongst granule cells only one subpopulation of cells expressing CR has been documented to be

produced during adulthood (Batista-Brito et al., 2008). Intriguingly, earlier studies have shown that the granule cells express the calcium/calmodulin kinase (CaMKIIα) (Zou et al., 2002). However, it was not known if these interneurons could be produced as a subpopulation amongst other adult generated interneurons. By using the DNA synthesis marker BrdU coupled with immunohistochemistry for CaMKIIα, we have shown that 50% of all newly generated interneurons also express CaMKIIα. By making electrophysiological recordings from adult generated CaMKIIα expressing cells and those that do not express this kinase, we have shown that cells positive for CaMKIIα experience lesser inhibition when compared to the population negative for CaMKIIα. OBs of mice expressing tomato-red under the CaMKIIα promoter when labelled for the immediate early gene cfos, showed that 90% of cfos positive cells were also CaMKIIα positive. Together with our electrophysiological data it is clear that CaMKIIα positive cells are a distinct subpopulation of adult generated granule interneurons. This finding now opens up the possibility to selectively activate or inactivate this subpopulation of adult generated GCs using pharmacogenetics. By using designer receptors exclusively activated by designer drugs (DREADDs) expressed under the CaMKIIα promoter and its synthetic ligand clozapine-N-oxide (CNO) we can activate or inactivate adult generated GCs that express CaMKIIα. We stereotaxically injected adeno associated virus (AAV) under the CaMKIIα promoter expressing either GqDREADDs that leads and neuronal firing or GiDREADDs that leads to neuronal silencing following binding with CNO. Preliminary results show that these vectors express the transgene in adult generated OB interneuron progenitors (**Fig. 5.1**).Thus these tools will allow us to selectively activate or silence CamKIIa expressing sub-populations of adult-born GCs in the adult OB and assess their role in odor behavior.

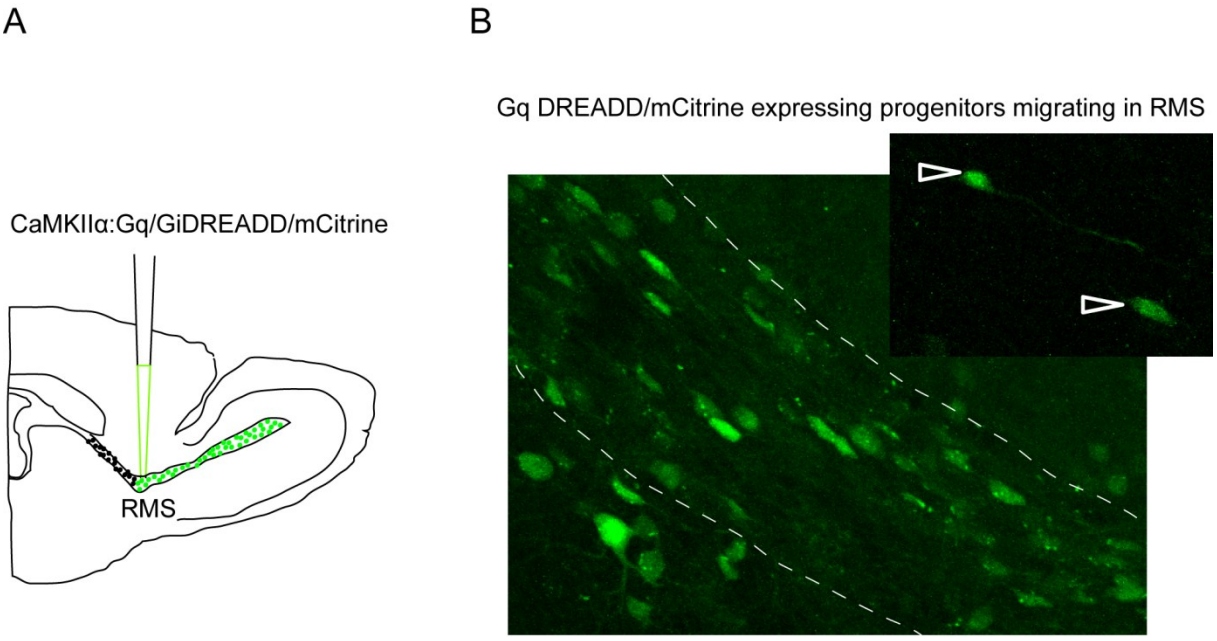

Figure 5.1: GqDREADD/mCitrine is expressed in adult generated interneurons

A Stereotaxic injection of viral vectors expressing Gq/GiDREADD along with mCitrine was made in the RMS of adult mice. **B** Two weeks after stereotaxic injection of AAV viral vectors expressing GqDREADD, interneuron progenitors expressing mCitrine were seen migrating in the RMS towards OB; inlay shows cells with migratory morphology.

5.4. Future perspectives and prospects:

From the first study that showed the presence of adult neurogenesis to present day, the knowledge about neurogenesis has been added upon by many studies over the years. Our knowledge of the neurogenic niches, factors, cells involved in neurogenesis during physiological and disease conditions has led to many important hypothesis and studies to better understand diseases. However, before meaningful therapy options are designed a more deep and fundamental understanding of the stem cells and factors that control them must be achieved.

In order to better understand stem cells of neural origin we need to answer fundamental questions like are SVZ and SGZ derived stem cells similar? Can adult stem cells be treated like those from embryos? There have already been reports demonstrating the feasibility of promoting healing and functional recovery by transplanting stem/progenitor cells or mobilizing the stem/progenitor cells from within the animal's body for repair. Skeletal muscle stem cells when transplanted into dystrophin-deficient (mdx) mice resulted in 94% more myofibers, restored dystrophin expression along with improved muscle histology and better contractile functions (Cerletti et al., 2008). Likewise cells can be recruited from within the organism itself to achieve repair. Cardiac stem cells (CSCs) resident within the heart were isolated and expanded ex vivo and re introduced into the injured heart of SCIDs mice resulting in engraftment, migration, myocardial regeneration and improved heart function (Barile et al., 2007). From these studies it can be said that both stem cells introduction from external sources or recruitment of those present in all adult tissues can be both effective in achieving some order of repair. Thus it maybe possible that a combining two approaches of transplanting exogenous cells and recruiting endogenous can improve the functional recovery. Is such therapy possible to be replicated in CNS healing? Can neurodegenerative diseases, psychiatric disorders, aging and depression all of which have been shown to be implicated with neurogenesis in some form be curable? There have been many reports of transdifferentiation that report either successful of unsuccessful trials to make neurons from other cell types. Several types of cells like skin-derived precursors (SKPs), bone marrow cells, muscle progenitor cells (MCPs) and peripheral blood cells for example have been reported to have failed to produce functional neurons even if they produced neuron like cells (Castro et al., 2002; Fernandes et al., 2006; Sarig et al., 2010; Liu et al., 2011). However, other studies have shown that functional neurons can be made from reprogramming specialized cells like fibroblasts and hepatocytes and embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) using factors like Oct4, Sox2, Klf4, c-Myc, neurogenin2 (Ngn2) and NeuroD1. These neurons were electrophysiologically

functional and capable of making synapses (Kim et al., 2011; Marro et al., 2011; Liu et al., 2013). A new study has unveiled a new protocol to transdifferentiate human myeloid lineage CD34+ bone marrow stem cells into neurons using antibodies that act as agonists for the granulocyte colony stimulating factor (GCSF) (Xie et al., 2013).

As discussed earlier it could be beneficial to not only transplant cells into a diseased brain, but to also recruit the brains native stem/ progenitors to the site of injury and bring about repair and healing. Studies have shown that the brain in itself is capable of responding to insults such as ischemic stroke, traumatic brain injury or even in cases of degenerative diseases. Neural precursors from the SGZ and more efficiently from the SVZ leave their normal migratory route via the RMS and migrate into the region of the infarct. The rerouted cells migrate in this new direction in response to chemokines and cytokines released by the inflammatory response at the site of injury along with growth and neurotropic factors. Several growth factors like brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), fibroblast growth factor (FGF), glial cell derived neurotrophic factor (GDNF), insulin-like growth factor (IGF), pituitary adenylate cyclase-activating polypeptide (PACAP) and vascular endothelial factor (VEGF) along with morphogens like bone morphogenic protein (BMP), Wnt and sonic hedgehog (shh) may be important for the internal repair (Christie and Turnley, 2013). These factors are good candidates to target proliferation, survival, migration and differentiation and possibly even survival of these new cells in the region where they ultimately need to undertake repairs. Thus it becomes clearer that better understanding of adult generated cells and the factors that control their proliferation, migration and survival is crucial. It is also essential to understand the permissive and disruptive factors present in the adult brain environment. A balanced knowledge of all these aspects and trails will allow for better therapy for debilitating neural diseases that are still incurable.

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