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STUDY OF THE POPULATION OF IMMATURE NEURONS IN THE ADULT PIRIFORM CORTEX LAYER II

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“The growth of knowledge depends entirely upon disagreement.”
Karl Popper

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Abreviation List

AON	Anterior Olfactory Nucleus
BrdU	5-bromo-2'-deoxyuridine
CAM	Cell Adhesion Molecule
CAMKII	Ca+/calmodulin-dependent protein kinases II
CNS	Central Nervous System
CoA	Cortical Amygdala
DCX	Doublecortin
DIV	Days *in vitro*
DPN	Deep pyramidal neurons
GABA	γ-Aminobutyric acid
GAD67	Glutamic acid decarboxylase 67
GFAP	Glial Fibrillary acid protein
GFP	Green Fluorescent Protein
IL	Infralimbic cortex
IP	Intraperitoneal
LEnt	Lateral Entorhinal Cortex
LOT	Lateral Olfactory Tract
mPFC	Medial Prefrontal Cortex
NCAM	Neural Cell Adhesion Molecule
OB	Olfactory Bulb
PB	Phosphate Buffer
PBS	Phosphate buffered saline
PCX	Piriform Cortex
PSA-NCAM	Polysialylated form of the Neural Cell Adhesion Molecule
RMS	Rostral Migratory Stream
SL	Semilunar Cells
SLPTN	Semilunar-Pyramidal Transitional Neurons
SP	Superficial Pyramidal neurons
SVZ	Subventricular Zone
SYN	Synaptophysin
Tbr1	Transcriptional Factor *T-Brain 1*
TUC4	TOAD64/Ulip/CRMP-4

CONTENTS

CHAPTER 1

Introduction

INTRODUCTION

1. Structural plasticity

Work over the last 25 years has highlighted that the central nervous system (CNS) has a remarkable capacity to undergo significant modifications, as a consequence of alterations in the environment. This ability is called "neuronal plasticity" and, interestingly, it is not only present during early development, when precise patterns of connectivity are being established, but also during adulthood (Magalhães & Sandberg, 2005 for review). This plasticity is crucial for wide adaptive-responses, like memory and learning (Sutton & Schuman, 2006 for review) or recovery from brain damage or disease (Fox et al., 2008; Krägeloh-Mann, 2004; Sabael, 2008 for review), among others. Thus, these adaptive responses must involve a spectrum of neuronal modifications, which range from molecular to structural plasticity (see Xerri, 2008 for review). The term neuronal structural plasticity refers to modifications in the structure of neurons and glial cells, which include changes in the morphology and density of dendritic spines (reviewed in Alvarez & Sabatini, 2007), outgrowth/retraction of dendrites and axons (Geinisman et al., 2000) neuron-glia plasticity (Theodosis et al., 2008) and the maximal expression of plasticity: the production and incorporation of new neurons or adult neurogenesis.

2. "Canonical" and "non-canonical" neurogenic niches

There are two different niches, in which the production of new neurons in the healthy central nervous system of adult mammals continuously takes place: the subgranular zone of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Doetsch & Alvarez-Buylla, 1996; Doetsch et al., 1997; Eriksson et al., 1998; Gross, 2000; Kempermann & Gage, 1999; Kempermann et al. ,

2004; Lois & Alvarez-Buylla, 1994). In both regions, stem/progenitor cells proliferate and give rise to neuroblasts or immature neurons that differentiate, mature, and integrate functionally into pre-existing neuronal circuits. Therefore, these two areas are often referred to as the "canonical neurogenic niches".

Interestingly, the presence of immature and/or adult-born neurons has been reported as well outside the canonical neurogenic niches in mammals, including the cerebral cortex (Cai et al., 2009; Gómez-Climent et al., 2008; Varea et al., 2011; Zhang et al., 2009);the striatum (Ernst et al., 2014; Luzzati et al., 2007) the hypothalamus (Kokoeva, 2005; Pierce & Xu, 2011; Robins et al., 2013) and the spinal cord (Shechter et al., 2007). Consequently, these regions have been classified as putative "non-canonical neurogenic regions" (Bonfanti, 2006; Feliciano & Bordey, 2009; Feliciano et al., 2015; Luzzati et al., 2014; Peretto & Bonfanti, 2014).

3. Non-canonical neurogenic niches: the piriform cortex layer II

The piriform cortex (PCX) is one of these non-canonical neurogenic structures in the adult CNS, where immature neurons have been reported. It is a 3-layered structure and corresponds to one of the architecturally simplest and evolutionarily oldest cortices: the paleocortex or olfactory cortex. The layer II of the PCX contains densely packed principal neurons, which mainly comprise two morphologically distinctive cell types: superficial pyramidal (SP) and semilunar (SL) cells (Haberly, 1983; Neville & Haberly, 2003). Other excitatory neuronal types are the semilunar-pyramidal transitional neurons (SLPTN) located in layer II, the fusiform neurons, present in layer I, and the deep pyramidal neurons (DPN), residing in layer III. GABAergic interneurons are found in all layers and

modulate the activity of excitatory neurons (Gavrilovici et al., 2010; Löscher et al., 1998; Luna & Pettit, 2010; Luna & Schoppa, 2009; Suzuki & Bekkers, 2010).

The PCX has also a layer-specific distribution of efferent and afferent projections: while efferent pathways are related to SP, SL and SLPTN (located in layer II) and DPN (located in layer III), which are the projecting neurons of the PCX, the afferent target is basically the layer I, since SP, SL, SLPTN and DPN neurons extend their apical dendrites into this layer, being hence the place in which the PCX receives the afferent projections from other brain regions (Neville & Haberly, 2003).

3.1. Afferent projections to the PCX

Afferent pathways to the PCX are mainly correlated with the olfactory bulb (OB), since SL and SP cells receive afferent excitation from the lateral olfactory tract (LOT) axons (see **Figure 1**). The input to SL cells comes predominantly from the OB, while SP cells receive weaker afferent inputs from the OB and stronger intracortical excitatory input (Haberly & Feig, 1983; Hagiwara et al., 2012; Suzuki & Bekkers, 2010; Wiegand et al., 2011).

3.2. Efferent projections of the PCX

The PCX encodes, processes and finally transmits to a variety of olfactory and non-olfactory regions the information coming from the OB. These olfactory regions, such as the anterior olfactory nucleus (AON), the olfactory tubercle, cortical amygdala (CoA), and lateral entorhinal cortex (LEnt) (see **Figure 1**) (Ekstrand et al., 2001; Johnson et al., 2000; Kerr et al., 2007; Meyer et al., 2006). Interestingly, the PCX also has direct

connections with non-olfactory regions, such as the IL (Infralimbic cortex) (Diodato et al., 2016). Finally, the PCX sends consequent feedback projections to the OB (Haberly & Price, 1978; Shipley & Adamek, 1984) (see Figure 1).

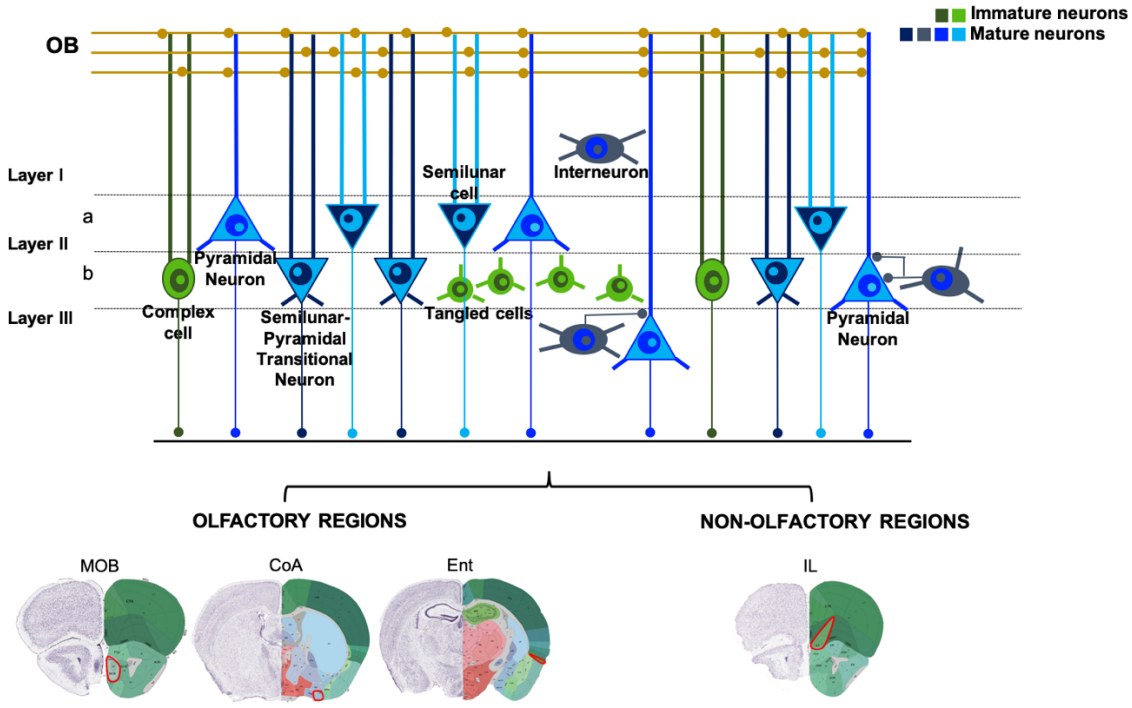

Figure 1. Diagram of the efferences and afferences of the PCX. Adapted from Bonfanti & Nacher 2012. The images of the mouse brain were exported from the Allen Brain Atlas. Projections from the Olfactory Bulb (OB) are represented in yellow. The OB projects to the PCX contacting a variety of principal neurons: semilunar-pyramidal transitional neurons (SLPTN), pyramidal neurons and semilunar cells (SL). Immature complex cells (Type II) also receive some scarce synapses from the OB. Olfactory and non-olfactory regions receive PCX projections. In particular, the olfactory regions are the OB, the Cortical Amygdala (CoA), and the Entorhinal cortex (Ent), and among the non-olfactory regions, the Infralimbic cortex (IL) is one of the most important.

SL, SP and DPN are the responsible of almost all these efferent projections. In fact, these neurons have different targets and specifically express different transcription factors, which allow their discrimination. The feedback projecting neurons to the OB are located predominantly in the anterior PCX and display an organized structure (SP and DPN located in close contact in the PCX, will project to similar regions in the OB);

these cells express the transcription factor Cux1. Neurons projecting to the mPFC, specifically to the IL region, express the transcription factor Ctip2. Interestingly, there are neurons which co-express Cux1 and Ctip2, which project to both structures, the OB and the IL. Finally, SL cells, located preferentially in layer IIa, project to the CoA and express the transcription factor Fezf2 (Diodato et al., 2016). There is still few information on the connectivity of SLPTN, but a report has described axonal projections of one of these cells in the amygdaloid nuclei, the agranular insular cortex, the olfactory tubercle and the dorsal endopiriform nucleus (Yang et al., 2004).

4. Immature neurons in the PCX layer II

To better understand neuronal differentiation in adult neurogenesis, Kemperman et al. (2004) classified the cell types in the hippocampal subgranular zone based on their phenotype and maturational stage (see **Figure 2**). This classification starts from the type-1 cells, which are nestin and GFAP expressing multipotential precursors. These precursors give rise to three different putative amplifying progenitor cells, which no longer express GFAP: type-2a (nestin expressing cells), type-2b (nestin and doublecortin [DCX] expressing cells) and type-3 (DCX expressing cells). These progenitors in turn give rise to immature neurons, which retain DCX expression, together with other immature neuronal markers, such as the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) or TUC4. Finally, these cells develop into mature NeuN expressing neurons.

Non proliferative cells expressing immature neuronal markers have been described in all the extension of the adult rodent olfactory system, including the layer II and layer III of the PCX, the endopiriform nucleus, and the main and accessory bulb (Nacher et al.,

2001, reviewed in Bonfanti & Nacher, 2012). It is known for some years that immature neurons in the olfactory bulb are derived from progenitors residing in the SVZ, which have migrated through the rostral migratory stream (RMS)(Lois & Alvarez-Buylla, 1994). However, the origin of the immature neurons in the PCX has been a matter of debate until recently.

Two cell subpopulations with different morphologies have been described in the layer II of the adult PCX based on the detection of markers characteristic for immature neurons (Gomez-Climent et al., 2008). This description was initially performed in rats, but similar cells have been found in the PCX of other mammals (Bonfanti & Ponti, 2008; Luzzati et al., 2009; Varea et al., 2011; Xiong et al., 2008).The first population is constituted by the so called tangled cells, which have a small soma diameter (~9 µm) and a few short intricate dendrites. These small cells appear frequently in closely apposed clusters and strongly express markers for immature neurons, like doublecortin (DCX) or the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) (Bonfanti et al., 1994; Brown et al., 2003; Couillard-Despres et al., 2005; Gomez-Climent et al., 2008; Luzzati et al., 2009; Nacher et al., 2002; Nacher et al., 2001; Seki & Arai, 1991) but they do not express markers of mature neurons (e.g. NeuN, CaMKII, GAD67), or glial cells (Gomez-Climent et al., 2008) (see **Figure 2**). Moreover, they do not receive synaptic contacts from other neurons and are surrounded by astroglial lamellae (Gomez-Climent et al., 2008). The second group of immature neurons that have been detected in the PCX layer II, the complex cells, have larger dendritic trees and in many cases resemble SLPTN, a typical excitatory neuronal type in the PCX layer II. However, cells with intermediate morphologies between tangled cells and SLPTN are also commonly

observed. The complex cells show a larger soma than the tangled cell population (~15 µm diameter) and their elaborated dendritic trees bear some spines and occasional synaptic contacts. These larger immature cells also co-express DCX and PSA-NCAM, but the expression level of these molecules is lower than that found in the tangled cells. Occasionally, low levels of NeuN expression have been detected in this cell population (Gomez-Climent et al., 2008) (see **Figure 2**).

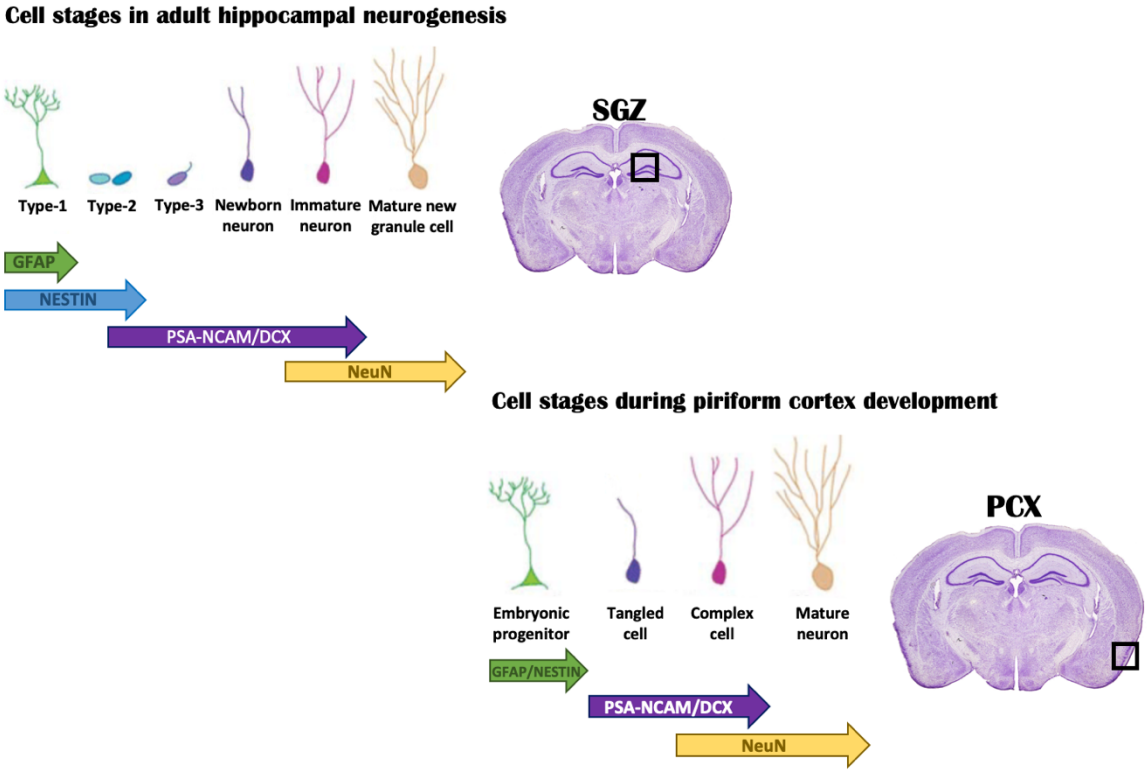

Figure 2. Graphics showing the different precursors and stages of cell differentiation during adult hippocampal neurogenesis and the development of immature neurons in the adult piriform cortex. The upper part of the panel represents Kempermann's classification of the different stages that can be observed during adult hippocampal neurogenesis. The lower part of the panel shows the different stages that can be found during the development of tangled cells in the adult piriform cortex. The diagrams also include the marker profile of cells at every stage.

Although it is tempting to consider tangled cells as type-3 cells according to Kemperman's classification, these are not truly type-3 cells, since, at least in rats (Gomez-Climent et al., 2008) and cats (Varea et al., 2011), they do not proliferate and,

consequently, they do not appear to be progenitor cells. Nevertheless, we cannot discard completely the presence of some progenitor cells in the PCX layer II. The isolation and culture of the cells expressing immature neuronal markers in the PCX layer II and their ability to form neurospheres would be an excellent approach to analyse the presence of progenitor cells in this cortical region.

4.1. Origin

There are different hypotheses regarding the origin of the immature neurons in the PCX layer II. On the one hand, it has been explored, using BrdU pulse-chase experiments, the possibility of an adult generation of these immature cells. Some studies have provided solid proof of the lack of generation or incorporation of recently generated neurons to the PCX layer II of adult rats (Nacher et al., 2001), rabbits (Luzzati et al., 2003) or cats (Varea et al., 2011). By contrast, some other reports in rats, mice and primates have described the generation of new neurons, although in very low numbers, in the paleocortex layer II during adulthood (Bernier et al., 2002; Pekcec et al., 2006; Shapiro et al., 2007). Bernier et al. (2002) and Pekcec et al. (2006) found cells double labelled with BrdU and markers for immature neurons in PCX layer II when animals were sacrificed 28 days after BrdU administration but not 12 weeks after. These results lead them to suggest that the vast majority of these newly generated neurons degenerate and do not become incorporated to the circuitry. Shapiro et al., (2007) also found BrdU/DCX double-labelled cells in this cortical region, but the precise layer location of these double-labelled cells was not described.

On the other hand, it has been suggested that in young mammals a population of cells generated in the adult SVZ can migrate via a stream to the PCX and integrate in this region as new neurons (Bernier et al., 2002; Shapiro et al., 2007, 2009). However, putative adult periventricular-derived cells as well as locally *de novo* generated neurons have not been detected by other studies (Gomez-Climent et al., 2008; Luzzati et al., 2009; Nacher et al., 2002; Varea et al., 2011) and might only occur at very low levels or might have transient existence (Pekcec et al., 2006; Shapiro et al., 2007, 2009). Other studies suggested that a local neural/glial antigen 2 (NG2) expressing cell population with low DCX expression (Guo et al., 2010; Rivers et al., 2008; Tamura et al., 2007) may be the origin of these immature neurons. Finally, Xiong et al., (2007) proposed that a population of progenitors residing in layer I may be the origin of the immature neurons observed in the PCX layer II. Nevertheless, the number of new cells coming from this cortical layer was extremely low.

Despite all these inconclusive studies seeking the origin of the immature neurons of the PCX in the adult brain, BrdU experiments with pregnant rats, have clearly demonstrated that the majority of the immature neurons in this region are generated during embryonic development, in E15.5 (Gomez-Climent et al., 2008). Thereby, these results give support to the idea that adult neurogenesis in the paleocortex of adult rodents does not exist, or must occur at very low levels, and that the vast majority of the immature neurons in the PCX layer II have been generated during embryonic stages.

4.2. Fate

The decrease in the number of PSA-NCAM/DCX expressing cells in layer II of the PCX throughout the course of aging has been broadly documented in different species, like rats (Abrous et al., 1997; Varea et al., 2009), cats (Cai et al., 2009) or guinea pigs (Xiong et al., 2008). Since no evidences of cell death have been observed in structures bearing the immature neuronal populations (see Bonfanti & Nacher, 2012 for review), the alternative hypothesis is that these PSA-NCAM/DCX expressing cells may be differentiating in the course of aging into mature neurons. Thereby, they would lack the expression of these immature markers, being in this way no longer detectable. As there are different intermediate stages between tangled cells and complex cells bearing the morphology of SLPTN, it seems that this second hypothesis is the most plausible (Bonfanti & Nacher, 2012). In fact, this later hypothesis has been supported by immunohistochemical (Gomez-Climent et al., 2008; Luzzati et al., 2009) and electrophysiological analyses (Klempin et al., 2011). Although there is a general consensus regarding their differentiation into mature neurons, the neurochemical nature of these mature neurons is still controversial: while some authors support the idea of a major interneuronal differentiation for these immature neurons in cortical layer II (Cai et al., 2009; Xiong et al., 2008; Zhang et al., 2009), others, including our laboratory, claim against it, suggesting an excitatory neuronal fate (Gomez-Climent et al., 2008; Luzzati et al., 2009). The evidence supporting the inhibitory hypothesis is based mainly on the analysis of DCX expressing cells. These studies have reported expression of parvalbumin, calbindin or somatostatin in large interneurons with DCX faint expression in guinea pigs (Xiong et al., 2008), cats (Cai et al., 2009) and non-human primates (Zhang et al., 2009), suggesting a putative interneuronal phenotype. By

contrast, several evidences are in favour of an excitatory fate for most of the immature neurons in PCX layer II. The morphology is one of these evidences, since the larger immature neurons residing in PCX layer II do not resemble interneurons, and the vast majority of them can be classified as SLPTN or SP, which are excitatory neurons commonly found in the PCX layer II (Suzuki & Bekkers, 2010). The neurochemical phenotype is the second and most important evidence. DCX/PSA-NCAM expressing cells in PCX layer II do not express markers for mature interneurons, such as GAD67, GABA, calbindin, parvalbumin, calretinin or somatostatin (Gomez-Climent et al., 2008). However, they also do not express markers for mature excitatory neurons, such as Ca(2+)/CaM dependent protein kinase II (CAMKII) (Gomez-Climent et al., 2008; Varea et al., 2011). The analysis of the expression of certain transcription factors has shed light on the fate of these immature neurons. Transcription factors specific for certain neuronal populations are expressed during neural development and maintain their expression during the lifespan. Consequently, as they are not restricted to a specific maturational stage, it makes them suitable for determining the fate of progenitors or immature neurons. Therefore, since most of the immature neurons in PCX layer II express Tbr1, a transcription factor specific for pallium-derived principal neurons (Luzzati et al., 2009) and they do not express Lhx6 or pan distalless (DLL), which are the transcription factors expressed by the cells coming from the subpallium (a region where most cortical interneurons originate) (Luzzati et al., 2009), it suggests that the vast majority of them may differentiate into excitatory neurons.

4.3. Distribution

Although in rats and mice this population of post-mitotic immature neurons reside almost exclusively in the layer II of the PCX (some tangled cells can be located in the perirhinal, insular and entorhinal cortices layer II of adult rats) (Bonfanti, 2006; Gomez-Climent et al., 2008; Nacher et al., 2002; Seki & Arai, 1991; Shapiro et al., 2007) the same population with a wider distribution has been found in various mammalian species with more complex cerebral cortices e.g. in guinea pigs, cats, rabbits, dogs, non-human-primates, and humans, including neocortical regions (Ni Dhuill et al., 1999; Bonfanti, 2006; Gomez-Climent et al., 2008; Xiong et al., 2008; Cai et al., 2009; Luzzati et al., 2009; Zhang et al., 2009; Varea et al., 2011; De Nevi et al., 2013; He et al., 2014; Patzke et al., 2014; Rossi et al., 2014; Yang et al., 2015).

4.4. Function

Despite extensive research, there is no definitive data yet on the function of the immature neurons in the adult cerebral cortex layer II. Interestingly, this cell population appears to be somehow related with the olfactory bulb, since after unilateral bulbectomy, the number of these immature cells is reduced, while the number of mature neurons increases (Gómez-Climent et al., 2011). However, the fact that in mammals with larger cerebral cortices immature neurons in layer II have a more widespread distribution, indicate that these cells may participate in circuits and related functions general to cerebral cortex and not only to olfactory processing. In fact, the "test-potentiated odor aversion learning", a learning paradigm dependent on olfactory function, does not affect the immature population of cells in the rat PCX (Gómez-Climent et al., 2011).

It is interesting to note that these immature neurons may have an important role in mediating the effects of stress and stress-related hormones. Chronic stress increases the number of PSA-NCAM/DCX expressing cells in the PCX layer II, while chronic corticosterone administration decreases it (Nacher et al., 2004). As these PSA-NCAM/DCX expressing neurons do not express glucocorticoid receptors (Gomez-Climent et al., 2008), it is possible that the effects of the stress were mediated by NMDA receptors, since these glutamate receptors are present in immature SLPTN (Gomez-Climent et al., 2008), and the number of PSA-NCAM expressing cells in PCX layer II increases after NMDA receptor antagonist treatment (Nacher et al., 2002).

Objectives

Research during the last twenty years has revealed the presence of an intriguing population of immature neurons in the PCX and other cortical regions of adult mammals. Although the prenatal origin of these cells appears to be widely accepted, we still have many questions on the fate of these cells, their distribution and phenotype in different mammalian species and their function on the adult brain. Therefore, the **main objective** of this thesis is to deepen into these questions by analysing in detail this immature neuronal population in mice. This would also allow us to use a transgenic approach to study the fate of these cells *in vivo* and to perform analyses *in vitro* to determine the possibility to isolate and culture these cells and to explore their differentiation and proliferative potential. Finally, we will also manipulate some of the molecules expressed by the immature neurons in the PCX layer II in order to promote their differentiation and study their fate.

From this main objective, derive the following **specific objectives** of the present thesis:

1. To characterize the population of immature neurons in the PCX layer II of adult mice, using immunohistochemical methods.
2. To evaluate the time of origin of the population of immature neurons in the PCX layer II of adult mice by birthdating experiments with halogenated nucleosides performed at different developmental stages and during adulthood.
3. To isolate and culture cells from the adult PCX layer II in order to know whether they are able to proliferate/differentiate under mitogenic conditions, and

whether there are progenitor cells with the capacity to form neurospheres in this cortical region.

4. To trace the fate of the immature neurons in the PCX layer II by using an inducible DCX-Cre mouse line as a fate-mapping tool.
5. To study the impact of the genetic and enzymatic depletion of PSA on the population of immature neurons in the PCX layer II of mice.

CHAPTER 2

Characterization and Isolation of Immature Neurons of the Adult Mouse Piriform Cortex

Characterization and Isolation of Immature Neurons of the Adult Mouse Piriform Cortex

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ABSTRACT: Physiological studies indicate that the piriform or primary olfactory cortex of adult mammals exhibits a high degree of synaptic plasticity. Interestingly, a subpopulation of cells in the layer II of the adult piriform cortex expresses neurodevelopmental markers, such as the polysialylated form of neural cell adhesion molecule (PSA-NCAM) or doublecortin (DCX). This study analyzes the nature, origin, and potential function of these poorly understood cells in mice. As previously described in rats, most of the PSA-NCAM expressing cells in layer II could be morphologically classified as tangled cells and only a small proportion of larger cells could be considered semilunar-pyramidal transitional neurons. Most were also immunoreactive for DCX, confirming their immature nature. In agreement with this, detection of PSA-NCAM combined with that of different cell lineage-specific antigens revealed that most PSA-NCAM positive cells did not co-express markers of glial cells or mature neurons. Their time of origin was evaluated by birthdating experiments with halogenated nucleosides performed at different developmental stages and in adulthood. We found that virtually all cells in this paleocortical region, including PSA-NCAM-positive cells, are born during fetal development. In addition, proliferation analyses in adult mice revealed that very few cells were cycling in layer II of the piriform cortex and that none of them was PSA-NCAM-positive. Moreover, we have established conditions to isolate and culture these immature neurons in the adult piriform cortex layer II. We find that although they can survive under certain conditions, they do not proliferate *in vitro* either. © 2015 Wiley Periodicals, Inc. Develop Neurobiol 76: 748–763, 2016

Keywords: **adult neurogenesis; PSA-NCAM; neuroblast; neuronal birthdating; proliferation**

INTRODUCTION

The piriform or olfactory cortex is one of the most plastic regions in the adult CNS. Different authors

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have linked this plasticity to the fact that it receives input from the olfactory bulb, via the entorhinal cortex, and sends its projections to the hippocampus, two regions in which continuous neuronal turnover takes place during adult life (Gheusi et al., 2009). In adult rodents, this trilaminar paleocortex includes a highly cellularized layer II, containing the densely packed cell bodies of mature projecting excitatory neurons as well as a population of immature neurons, characterized by the expression of neuroblast markers, such as the polysialylated form of neural cell adhesion molecule (PSA-NCAM) or doublecortin (DCX) that are transiently expressed during both developmental and adult neurogenesis (Nacher et al., 2001; Nacher et al., 2002; Luzzati et al., 2009). Moreover, recent studies have expanded these findings to descriptions of similar immature cells, with a wider distribution, in the cerebral cortex of cats, guinea pigs, rabbits, monkeys, and humans (Ni Dhuill et al., 1999; Xiong et al., 2008; Cai et al., 2009; Luzzati et al., 2009; Zhang et al., 2009; Varea et al., 2011). Although most of these cortical cells with an immature neuronal phenotype in adulthood appear to be generated during fetal development (Gomez-Climent et al., 2008), some evidences have indicated that a small fraction could be generated during adult life (Bernier et al., 2002; Pekcec et al., 2006; Shapiro et al., 2007; Shapiro et al., 2007; Bonfanti and Nacher, 2012). The fate of these immature neurons in the piriform cortex layer II is also a matter of debate. This cell population disappears progressively with aging and is almost undetectable in old animals, suggesting their differentiation into mature neurons and their integration into the cortical circuitry (Varea et al., 2009). Moreover, olfactory bulbectomy decreases the number of these immature neurons and increases that of mature neurons in the layer II of the adult piriform cortex. Since this procedure does not result in an increase in the number of inhibitory neurons in this layer, it seems that it promotes the differentiation of immature neurons into an excitatory phenotype (Gomez-Climent et al., 2011). In line with this, most of these immature neurons express Tbr1 (Luzzati et al., 2009; Varea et al., 2011), a transcription factor characteristically expressed by excitatory neurons of pallial origin.

Despite extensive research, there is still no definitive data on whether intrinsic neuronal progenitor cells that could generate new neurons *in vivo* or *in vitro* exist in the piriform cortex layer II. Some studies have suggested the presence of a pool of NG2-expressing progenitors in this region, which may differentiate into excitatory neurons (Rivers et al., 2008; Guo et al., 2010) but these findings are still controversial (Richardson et al., 2011). In this study, we describe the different cell populations found in the piriform cortex layer II of adult mice and analyze in detail whether immature neurons and progenitor cells are present in this region. The time of origin of the different cell types in the adult piriform cortex layer II is also evaluated using pulse-chase experiments, in which thymidine analogs are administered during embryonic development or in adulthood. We analyze the presence of proliferative activity and the putative integration of new neuronal or glial cells. We have also developed different *in vitro* strategies directed to explore whether the putative progenitor cells and/or the immature neurons present in this paleocortical region during adulthood can be isolated and expanded.

METHODS

Animals and Treatments

Experiments were performed using 1–3-month-old CD1 mice (Harlan Iberica) and CAG-EGFP transgenic mice (The Jackson Labs), in which enhanced green fluorescent protein (EGFP) is expressed in a constitutive manner by the CMV-IE enhancer/chicken β-actin/rabbit β-globin hybrid promoter (Hadjantonakis et al., 1998). All the mice were housed under standard conditions in the Servicio de Producción Animal at the University of Valencia and all the experiments were approved by the Committee on Bioethics of the same institution, following EU guidelines (directive 2010/63).

Pregnant CD-1 mice received 2 intraperitoneal injections of 5′-chloro-2′-deoxyuridine (CldU; Sigma-Aldrich; 50 mg/kg, in sterile saline solution), 24 h apart, at gestational days E13.5 and E14.5 or at E15.5 and E16.5 (0.5 is at noon of the day following the night of mating). One-month-old males born from these crosses were used for immunohistochemistry. To study the cells generated during adulthood, 3-month-old male mice received 4 intraperitoneal injections, one every 12 h, of 5′-bromo-2′-deoxyuridine (BrdU; Sigma-Aldrich; 50 mg/kg, in sterile saline solution) and were sacrificed 21 days after the last injection.

Histological Analysis

Mice were deeply anesthetized with 4% chloral hydrate in saline and perfused transcardially with 4% paraformaldehyde (PFA) in phosphate buffer 0.1 *M*, pH 7.4 (PB). Brains were removed and sectioned with a vibratome (Leica VT 1000E, Leica) and 40-μm-thick coronal sections were collected and kept in cold PB (4°C). Then tissue was processed "free-floating" for immunohistochemistry as previously described (Gomez-Climent et al., 2008).

For anti-PSA-NCAM staining, an antigen-retrieval step was performed, consisting in a pretreatment with 10 m*M*

Table 1 Primary and Secondary Antibodies

Primary antibodies							
Antigen	Host	Isotype	Dilution in vivo	Dilution in vitro	Incubation	Company	Reference
BrdU	Rat	IgG2a	1:200		O/N, T_R	Immunological Direct, Oxford Biotechnology	OBT0030CX
BrdU	Rat	IgG2a	1:800	1:1000	O/N, T_R	Abcam	ab6326
DCX	Rabbit	IgG	1:1000		O/N, T_R	Abcam	Ab18723
DCX	Goat	IgG		1:400	O/N, T_R	Santa Cruz	sc-8066
GAD67	Mouse	IgG2a	1:500		O/N, T_R	Millipore	MAB5406
GFAP	Rabbit	IgG	1:500	1:500	O/N, T_R	DAKO	Z0334
GFP	Chicken	IgY		1:2000	O/N, T_R	Abcam	ab13970
Ki67	Rabbit	IgG	1:100	1:400	O/N, T_R	Abcam	ab15580
MAP2	Mouse	IgG		1:2000	O/N, T_R	Sigma	
NeuN	Mouse	IgG1	1:500		O/N, T_R	Chemicon-Millipore	MAB377
Nestin (Rat 401)	Mouse	IgGs1		1:40	O/N, T_R	DSHB	
NG2	Rabbit	IgG	1:500	1:400	O/N, T_R	Millipore	ab5320
PSA-NCAM	Mouse	IgM	1:700	1:700	48 h, 4°C	Abcys	abC0019
RIP	Mouse	IgG1	1:1000		O/N, T_R	DSHB	
Tbr1	Rabbit	IgG	1:500		O/N, T_R	Abcam	ab31940

Secondary antibodies						
Abbrevation	Host	Label	Dilution	Incubation	Company	Reference
Anti-Chicken IgY	Donkey	DyLight™488	1:400	1 h, T_R	Jackson immunoResearch	703-485-155
Anti-Goat IgG	Donkey	Alexa Fluor® 555	1:400	1 h, T_R	Invitrogen	A27012
Anti-Mouse IgG	Donkey	Alexa Fluor® 647	1:400	1 h, T_R	Jackson immunoResearch	A31570
Anti-Mouse IgG	Donkey	Alexa Fluor® 555	1:400	1 h, T_R	Invitrogen	
Anti-Mouse IgM μ	Donkey	DyLight™549	1:400	1 h, T_R	Jackson immunoResearch	715-505-140
Anti-Mouse IgM μ	Donkey	DyLight™649	1:400	1 h, T_R	Jackson immunoResearch	715-495-020
Anti-Mouse IgG	Donkey	Alexa Fluor® 555	1:400	1 h, T_R	Invitrogen	A31570
Anti-Rabbit IgG	Donkey	Alexa Fluor® 488	1:400	1 h, T_R	Invitrogen	
Anti-Rabbit IgG	Donkey	Alexa Fluor® 555	1:400	1 h, T_R	Invitrogen	A21206
Anti-Rabbit IgG	Donkey	Alexa Fluor® 647	1:400	1 h, T_R	Invitrogen	A31573
Anti-Rat IgG	Donkey	Alexa Fluor® 488	1:400	1 h, T_R	Invitrogen	A21208
Anti-Rat IgG	Donkey	Cyanine Cy™5	1:400	1 h, T_R	Jackson immunoResearch	712-175-153

Abbreviations: t, tissue; c, culture sample; O/N, overnight; T_R, room temperature; LP, light-protected.

sodium citrate buffer (pH 6.0) for 1 min at 100°C. Then, sections were incubated with 5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories) in phosphate-buffered saline (PBS; 0.9% NaCl in PB) for 1 h to block nonspecific binding. Later, sections were incubated for 48 h at 4°C with mouse monoclonal IgM anti-PSA-NCAM in PBS containing 0.2% Triton-X-100 and 3% NDS (this solution was used to dilute primary and secondary antibodies). After washing, sections were incubated for 1 h with donkey anti-mouse IgM secondary antibody (Table 1).

The BrdU/CldU immunohistochemistry protocol was performed after that of PSA-NCAM. Some immunostainings required to be performed before the denaturation of DNA in order to preserve antibody antigenicity. In brief, the sections were incubated in 2 *M* HCl in 0.1 *M* PBS for 15 min at room temperature (HCl was previously prewarmed at 37°C). Then, the sections were processed for immunohistochemistry as described above, using either anti-BrdU or anti-CldU antibodies by themselves or in combination with another primary antibody. To stain microglial cells, sections were incubated with a biotinylated tomato lectin (*Lycopersicon esculentum*) (Sigma-Aldrich, 10 μg/mL; 2 h) and then with fluorescent Alexa Fluor® 488-conjugated avidin (Invitrogen, 1:200; 1 h). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, 1 mg/mL; 5 min). Primary and secondary antibodies were diluted in PBS with 3% NDS and 0.2%

Triton X-100 as indicated in Table 1. All sections were mounted using Dako Cytomation fluorescent mounting medium (Dako North America, Inc.).

Cell Culture

We cultured cells from the mouse piriform cortex and from the subventricular zone (SVZ) as previously described (Ferron et al., 2007). To isolate the piriform cortex layer II, the brain was cut longitudinally along the midline to separate both hemispheres and two transversal incisions at −0.5 and −2.5 mm from Bregma were done to get a 1–2-mm-thick slice that contained the piriform cortex. After removing the meninges, the layer II was dissected making 2 incisions, one to separate the piriform cortex and another one to take apart definitely the layer III of the piriform cortex. *Corpus callosum* and amygdala were discarded. For neurosphere assays, disaggregated cells from the piriform cortex and the SVZ cells were seeded in neurosphere growth medium containing epidermal growth factor (EGF, 20 ng/mL, Invitrogen) and basic fibroblast growth factor 2 (FGF2, 10 ng/mL, Sigma-Aldrich) in uncoated plates (Ferron et al., 2007). To study the piriform cortex cells in adhesive conditions, they were seeded on Matrigel (1:100 in control medium; BD)-coated coverslips and they were grown in different media: (1) growth medium, (2) neurosphere growth medium with 10% fetal bovine serum (FBS, Thermo Scientific), and (3) neurosphere growth medium without EGF or FGF2, but supplemented with 10% FBS. To co-culture cortical cells with SVZ cells, single cells were obtained from growing neurospheres and seeded onto Matrigel-coated coverslips placed in 48-well plates at 80,000 cells *per* well. SVZ cells were allowed to adhere for an hour and, immediately afterward, enzymatically disaggregated cells from the piriform cortex of CAG-EGFP-transgenic mice were seeded on top in neurosphere growth medium with 10% FBS. Some co-cultures were pulsed for 1 h with 2 μM BrdU at 2 div and fixed 5 after the treatment.

Immunocytochemistry

Cell cultures were fixed with 4% PFA in PBS for 10 min at room temperature and processed for immunofluorescence. For BrdU detection, cells were previously treated with 2 *M* HCl in 0.1 *M* PBS for 15 min at room temperature. All coverslips were washed twice in 0.1 *M* PBS, blocked with 0.1 *M* glycine with 5% NDS and 0.2% Triton X-100 in PBS for 1 h, and incubated with primary antibodies overnight (Table 1). After washing, appropriate fluorescently labeled secondary antibodies were added (Table 1) for 1 h at room temperature. DAPI was used to stain nuclei (1 mg/mL; 5 min) and Fluorsave (Calbiochem) was used as mounting medium.

Microscopy and Quantification

Images were captured using a confocal microscope (Leica TCS-SP or Olympus FluoView® FV10i - O). Z-series of optical sections (1 μm apart) were obtained using sequential scanning mode. These stacks were processed with FV10-ASW 2.1 viewer (Olympus) and the free Java image-processing program Fiji (Schindelin et al., 2012). The soma diameters of 50 tangled cells and 20 semilunar-pyramidal transitional neurons *per* animal were measured using Fiji in sections from three different animals.

All data are represented as the mean calculated between different animals/cultures and the variation between animals/cultures is depicted as the standard error of the mean (SEM). For each experiment, "*n*" indicates the number of independent mice or cultures used. Analyses of significant differences between means were performed using two-tailed Student's *t* tests. Statistical significance was set at: $*p < 0.05$, $**p < 0.01$.

RESULTS

Cell Populations in the Piriform Cortex Layer II of Adult Mice

We analyzed the proportions of the main cell populations present in layer II of the piriform cortex of young adult mice. For this purpose, sections from this cortical region were immunostained for different cell markers and the cell density of positive cells was analyzed using the nuclear staining DAPI as a reference. Most of the cells were positive for Tbr1, a marker of postmitotic glutamatergic projection neurons (Englund et al., 2005), and for NeuN, a marker of mature neurons (Mullen et al., 1992) [71.7 ± 3.7% for Tbr1 and 70.8 ± 1.4% for NeuN, $n = 3$; Fig. 1(A,B)]. In contrast, very few cells expressed the glutamate decarboxylase isoform 67 (GAD67) characteristic of GABAergic interneurons [2.6 ± 0.3%, $n = 3$; Fig. 1(C)]. Although in smaller proportions, cells expressing glial features were also present in the piriform cortex layer II. We found that 14.6 ± 2.9% of the cells expressed the astrocytic marker GFAP [$n = 3$; Fig. 1(D)], whereas 6.2 ± 0.5% expressed the proteoglycan NG2, a marker of polydendrocytes and oligodendrocyte progenitors [$n = 3$; Fig. 1(E)]. On the other hand, oligodendrocytes detected with antibodies to RIP or microglial cells stained with tomato lectin were rarely found (data not shown).

Interestingly, we also found a population of scattered cells, which expressed the neuroblast markers PSA-NCAM or DCX (Bonfanti and Theodosis, 1994; Francis et al., 1999; Gleeson et al., 1999). PSA-NCAM expressing cells constituted around 10% of the total cell population in layer II [12.5 ± 0.5%, $n = 3$; Fig. 1(F)]. Most of them were small (main diameter of the soma 6.21 ± 0.02 μm, $n = 3$ animals), frequently grouped in nest-like clusters of 2–5 cells,

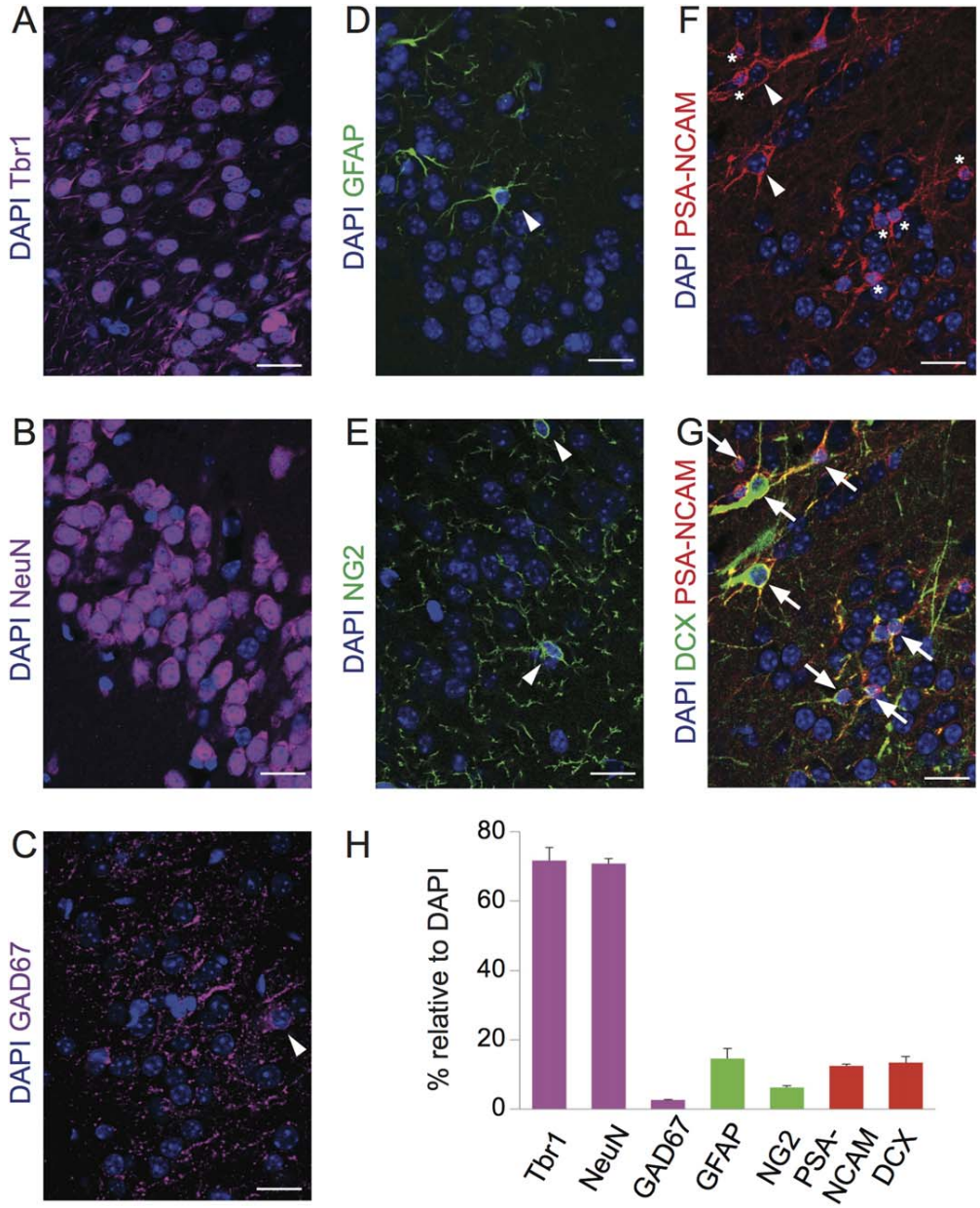


Figure 1 Characterization of the cell populations in the adult piriform cortex layer II. (A–G) Immunostaining for Tbr1 (purple), NeuN (purple), GAD67 (purple), GFAP (green), NG2 (green), PSA-NCAM (red), and DCX (green). DAPI was used as nuclear counterstaining (blue in all the panels). In F, white arrowheads indicate semilunar-pyramidal transitional neurons positive for PSA-NCAM and asterisks indicate tangled cells. In C–E, white arrowheads indicate immunoreactive somata for the indicated markers. In G, same area as F, white arrows indicate double positive cells for PSA-NCAM and DCX. (H) Graph showing the percentages of Tbr1, NeuN, GAD67, GFAP, NG2, PSA-NCAM, and DCX positive cells relative to DAPI. Data are shown as mean values ± SEM ($n = 3$). Images in A and E were taken from a unique confocal plane; B and C were 2D projections from two consecutive confocal planes located 1 μm apart, and D, F, and G were from three consecutive confocal planes. Scale bar: 20 μm.[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and could be classified as tangled cells (Gomez-Climent et al., 2008). A lower proportion of PSA-NCAM expressing cells were larger (main diameter of the soma 9.64 ± 0.22 μm, $n = 3$) and were morphologically similar to those previously described as semilunar-pyramidal transitional neurons (Haberly, 1983; Gomez-Climent et al., 2008). The morphology of PSA-NCAM expressing cells was indeed similar

to that previously observed in other species (Xiong et al., 2008; Cai et al., 2009; Luzzati et al., 2009; Zhang et al., 2009; Varea et al., 2011; Marti-Mengual et al., 2013). The proportion of DCX expressing cells in this cortical area was similar to those of PSA-NCAM-positive cells [$13.4 \pm 1.7\%$, $n = 3$; Fig. 1(G)], as it has been described in previous reports in different species. As also described in rats (Nacher et al., 2001; Gomez-Climent et al., 2008; Luzzati et al., 2009), a large fraction of PSA-NCAM expressing cells were also immunoreactive for DCX ($59.3 \pm 4.0\%$, $n = 3$), suggesting cell diversity or different stages of maturation within this group of cells with neuroblast features. All these data indicated the presence of a significant fraction of immature neurons in the mouse piriform cortex [see all quantifications in Fig. 1(H)].

Phenotype of PSA-NCAM Expressing Cells in Layer II of the Mouse Piriform Cortex

We next studied the nature of PSA-NCAM expressing cells in the mouse piriform cortex layer II by co-immunodetection of different cell lineage markers. In agreement with the lack of mature oligodendrocytes and microglial cells in layer II, we did not find any PSA-NCAM-positive cells that were also positive for RIP or tomato lectin (data not shown). Moreover, none of the PSA-NCAM expressing cells were positive for GFAP [Fig. 2(A)]. Despite the overall absence of glial markers in PSA-NCAM-positive cells, a small proportion of PSA-NCAM-expressing tangled cells were positive for NG2 [$5.9 \pm 0.5\%$, $n = 3$; Fig. 2(B,C)], as previously described in adult rats (Gomez-Climent et al., 2008). Regarding neuronal markers, most of the PSA-NCAM expressing cells in piriform cortex layer II also expressed Tbr1 [$77.7 \pm 2.3\%$, $n = 3$; Fig. 2(D)] and none of them expressed GAD67 [Fig. 2(E)]. These data indicated that most of these cells belong to the neuronal lineage and likely have a pallial origin and a putative excitatory phenotype.

Despite their apparent neuronal nature, most PSA-NCAM-positive cells did not express NeuN [Fig. 2(F)], further suggesting that they were immature. However, a small proportion of PSA-NCAM and DCX immunoreactive cells did display a homogeneous level of NeuN immunoreactivity in their nuclei [$6.1 \pm 0.8\%$, $n = 3$; Fig. 2(F,G)]. Although the staining for NeuN in some of the PSA-NCAM-positive cells was remarkably lower than that of mature neurons in the same layer, it indicated that some of the immature cells could be more differentiated toward a mature neuronal phenotype than others.

Interestingly, the percentage of NeuN/PSA-NCAM double positive cells within the PSA-NCAM population [$6.1 \pm 0.8\%$, $n = 3$; Fig. 2(F)], and the proportion of NeuN/PSA-NCAM/DCX triple positive cells among the PSA-NCAM/DCX double positive population [$5.9 \pm 0.5\%$, $n = 3$; Fig. 2(G)] were significantly lower than the proportion of NeuN/DCX expressing cells within the DCX positive population [$19.2 \pm 1.5\%$, $n = 3$; $p < 0.01$; Fig. 2(H)] suggesting that PSA-NCAM expression might be related to a more immature status. These data together with the additional observation that none of PSA-NCAM positive cells expressed nestin (data not shown) suggested that PSA-NCAM positive cells constitute a population of maturation-halted neurons.

Embryonic Origin of Cells in the Piriform Cortex Layer II

To determine the time of origin of the cells present in layer II of the piriform cortex, we injected the nucleoside CldU, an S-phase label, in pregnant mothers as one daily injection at two different embryonic intervals (E13.5–E14.5 and E15.5–E16.5) and studied the frequency of CldU-positive cells when born mice were 1 month old. We observed that CldU-positive cells were more frequent in the piriform cortex layer II when the thymidine analog was injected at E13.5–E14.5 than at E15.5–E16.5 [$29.0 \pm 2.1\%$ *vs* $4.6 \pm 0.5\%$, respectively, $n = 3$, $p < 0.01$, Fig. 3(A)].

In order to ascertain the identity of the cells born at the two developmental time periods studied, we combined the immunostaining for CldU with antibodies to neuronal, glial, and progenitor antigens. We found that the majority of the cells generated at E13.5–E14.5 were NeuN-positive mature neurons [$88.4 \pm$;4.3%, $n = 3$; Fig. 3(B,C)], and only a very small proportion expressed GAD67 [$2.6 \pm 0.8\%$, $n = 2$; Fig. 3(B,D)]. In animals injected at E15.5–E16.5, in which fewer cells appeared labeled by the nucleoside, we found that only $15.0 \pm 1\%$ ($n = 3$) of the CldU-positive cells were neurons and none were GAD67 positive ($n = 2$) [Fig. 3(B,E)].

In contrast to neurons, glial cells expressing GFAP were the prevailing cell type generated at E15.5–E16.5 [$64.4 \pm 2.1\%$ *vs* E13.5–E14.5 values of $1.1 \pm 0.7\%$, $n = 3$, $p < 0.01$, Fig. 3(B,F,G)]. These data were consistent with previous reports that described an early peak of neurogenesis followed by a late peak of astrogliogenesis during cortical embryonic development (Levers et al., 2001; Sarma et al., 2011). The proportion of NG2 expressing cells that

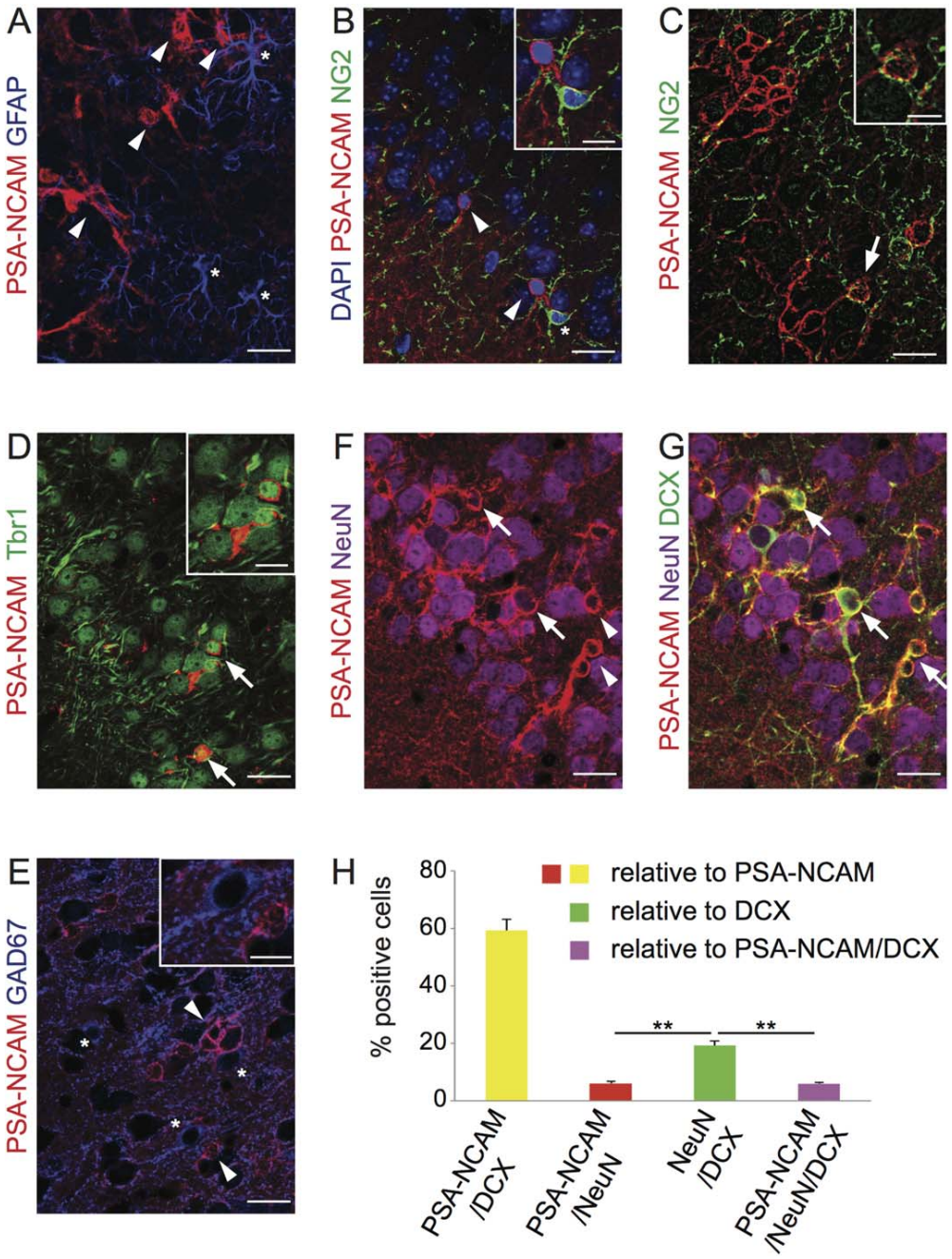


Figure 2 Characterization of PSA-NCAM expressing cells in the adult piriform cortex layer II. (A) Immunostaining for PSA-NCAM (red) and GFAP (blue). (B) Immunostaining for PSA-NCAM (red) and NG2 (green). DAPI (blue) was used as nuclear counterstaining. The inset enlarges the PSA-NCAM positive cell in close apposition to the NG2-positive cell. (C) Immunostaining for PSA-NCAM (red) and NG2 (green). The double positive cell is enlarged in the inset. (D) Immunostaining for PSA-NCAM (red) and Tbr1 (green). A double positive cell is shown in the inset. (E) Immunostaining for PSA-NCAM (red) and GAD67 (blue). Two cells are magnified in the inset. (F) Immunostaining for PSA-NCAM (red) and NeuN (purple). Few PSA-NCAM expressing cells present a faint NeuN stain. (G) Same area as F stained with the DCX antibody (green). In all the panels, white arrowheads indicate PSA-NCAM single positive cells and arrows indicate PSA-NCAM expressing cells positive for another marker. Asterisks indicate positive cells for a marker different than PSA-NCAM. (H) Bar diagram showing the proportions of PSA-NCAM colocalization with DCX or NeuN relative to PSA-NCAM positive cells, the proportion of DCX and NeuN double positive cells relative to DCX cells and the proportion of triple positive cells (PSA-NCAM/NeuN/DCX) relative to PSA-NCAM/DCX-positive cells. Data are shown as mean values ± SEM ($n = 3$, $^{**}p < 0.01$). C and D were taken from a unique confocal plane, E, F, and G were 2D projections from two consecutive confocal planes located 1 μm apart and A and B were three confocal planes. Scale bar: 20 μm and 10 μm in the inset. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

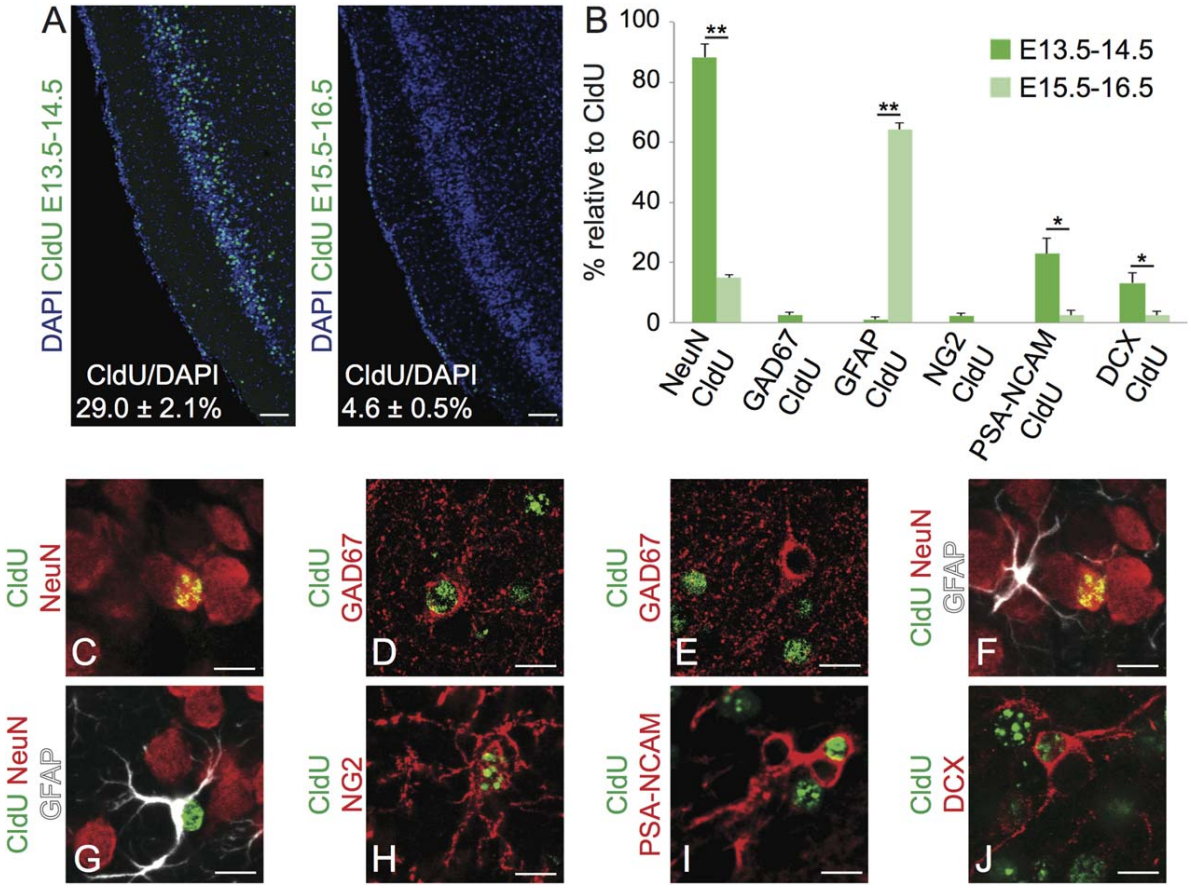


Figure 3 Cells expressing immature neuronal markers in the adult piriform cortex layer II are generated during embryonic development. (A) In the left panel, panoramic image of the piriform cortex stained with the CldU antibody (green) and with DAPI (blue). The thymidine analog CldU was injected at embryonic stages (E13.5–E14.5) and mice were sacrificed when they were 1 month old. Same image as A in the right panel, but in this case CldU was injected at E15.5–E16.5. (B) Percentages of CldU colocalization with NeuN, GAD67, GFAP, NG2, PSA-NCAM, and DCX relative to CldU cells in animals injected at E13.5–E14.5 or E15.5–E16.5. Data are shown as mean values ± SEM, $n = 2$–3, $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Immunostaining for CldU (green) and NeuN (red). (D) Immunostaining for CldU (green) and GAD67 (red). (E) Example of GAD67 (red)-positive cell negative for CldU (green). (F) Same confocal image as C, showing GFAP immunostaining (white). (G) Examples of NeuN (red)-positive cells negative for CldU (green) and a cell stained with CldU (green) and immunoreactive for GFAP (white). (H) Immunostaining for CldU (green) and NG2 (red). (I) Immunostaining for CldU (green) and PSA-NCAM (red). (J) Example of a double positive cell stained with CldU (green) and DCX antibodies (red). Images in A and D, E, I were taken from a unique confocal plane; J was a 2D projection from two consecutive confocal planes located 1 μm apart; H is from three consecutive confocal planes; C and F, G are from 5 and 7 consecutive confocal planes respectively. Scale bars: 200 μm in A and 10 μm in C–J. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

originated during E13.5–E14.5 was <5%, and was even lower at E15.5–E16.5 [Fig. 3(B,H)]. Oligodendrocytes expressing RIP were generated at late embryonic stages (0% for E13.5–E14.5 vs 1.3 ± 0.9% for E15.5–E16.5, $n = 3$) and microglial cells were not labeled at all, suggesting that these cells were mostly generated during later developmental stages (Chan et al., 2007).

Concerning the origin of the cells expressing immature neuronal markers in layer II, we observed that 23.0 ± 5.0% of the CldU-positive cells were also PSA-NCAM positive when the S-phase marker was injected at E13.5–E14.5, whereas only 2.6 ± 1.5% of all CldU-retaining cells expressed PSA-NCAM when the tracer was injected at E15.5–E16.5 [$n = 3$, $p < 0.05$, Fig. 3(B,I)]. Similar results were found when the proportion of CldU-positive cells co-expressing DCX was examined [13.3 ± 3.4% for E13.5–E14.5 injections *vs* 2.5 ± 1.4% for E15.5–E16.5, $n = 3$, $p < 0.05$, Fig. 3(B,J)].

We also quantified the proportion of PSA-NCAM expressing cells that were positive for CldU injected during the fetal period. We found that 64.6 ± 2.1% of all PSA-NCAM expressing cells contained the thymidine analog when injected at E13.5–E14.5, whereas only 1.2 ± 0.6% were double labeled when CldU was administered at E15.5–E16.5 ($n = 3$, $p < 0.01$). Taken together, these data indicated that the generation of these immature neurons in layer II of the piriform cortex follows a temporal pattern that is similar to that of the projecting mature neurons in the same layer (Bayer, 1986). In summary, CldU injections at E13.5–E14.5 mainly labeled neurons, whereas injections at E15.5–E16.5 preferentially labeled glial cells in layer II of the mouse piriform cortex and many of the cells expressing immature neuronal markers PSA-NCAM and DCX during adulthood in this layer were generated during embryonic development together with their neighbor mature neurons.

Proliferative Activity in the Piriform Cortex Layer II of Adult Mice

Our data indicated that many of the cells exhibiting markers of immature neurons in layer II of the piriform cortex could be labeled with a nucleoside during fetal development and retained the label until adulthood. This could indicate that these cells had exited the cell cycle and had become postmitotic, but their differentiation was partially halted. Alternatively, it could be a reflection of a slow cell cycling in a replication-competent cell expressing features of an immature neuron. Nonetheless, some of the cells were not labeled in our fetal regime of CldU injections and, therefore, we next decided to assess whether some of these cells could be generated during adulthood by performing BrdU injections into young adult mice. Mice received 4 BrdU injections (one every 12 h) and were sacrificed 21 days after. BrdU label-retention is commonly used to study adult neurogenesis and BrdU immunoreactive cells were readily found in the SVZ. In contrast, very few (3 to none *per* section) BrdU-labeled cells were observed in layer II of the piriform cortex. Some of the BrdU-positive cells were localized close to, but not within layer II. When we performed an immunostaining with both BrdU and PSA-NCAM antibodies, we found that none of the few BrdU-positive cells was also PSA-NCAM positive [Fig. 4(A)]. These data indicated that immature neurons in the adult piriform cortex layer II were not generated in the adult, at least under normal homeostatic conditions.

In order to investigate the proliferative activity in the piriform cortex layer II of adult mice, we performed an immunostaining to detect Ki67, a nuclear protein expressed in all phases of the cell cycle except the resting phase and a short period at the beginning of the phase G1 (Kee et al., 2002). While Ki67 expressing cells were frequently observed in the SVZ, very few Ki67-labeled nuclei could be found scattered in the piriform cortex layer II. As with BrdU, some of these nuclei were found in close apposition to layer II, but not within this layer. When we combined Ki67 and PSA-NCAM antibodies, we could not find any double labeled cell [Fig. 4(B)]. Thus, these data indicated that very few cells were cycling in the adult piriform cortex layer II. We also confirmed our previous results in rats, showing that the cells expressing immature neuronal markers in layer II were not actively dividing (Gomez-Climent et al., 2008).

Isolation and Culture of Immature Neurons From the Piriform Cortex Layer II

Cell culture can be used to isolate and enrich for specific cell populations that adequately survive/expand in the *in vitro* conditions *vs* those that do not. In an effort to isolate immature neurons and putative neuronal progenitors from the adult piriform cortex layer II, we first decided to analyze whether this cortical area would yield progenitors capable of extensive proliferation and self-renewal. Therefore, we independently dissected the SVZ (Ferron et al., 2007) and the layer II of the piriform cortex [Fig. 5(A)] from 1-month-old mice and seeded disaggregated cells in neurosphere growth medium containing the mitogens EGF and FGF2. When the cultures were monitored at 5 days *in vitro* (div), we observed spheres in all the SVZ cultures; however, no grown neurospheres or signs of incipient clone formation were observed in any of three independent experiments with cortical cultures during the same culturing period [Fig. 5(B)]. These data indicated that the layer II of the adult piriform cortex does not contain expandable self-renewing progenitor cells under conditions that promote the growth of multipotential SVZ neural stem cells.

SVZ cells can also be acutely dissociated and seeded onto Matrigel for the *in vitro* analysis of the *in vivo* cell composition. We decided to follow this approach with the piriform cortex layer II cells using three different conditions, which consisted in (1) neurosphere growth medium, (2) neurosphere growth medium with 10% FBS, and (3) neurosphere growth medium without the mitogens EGF or FGF2 but supplemented with 10% FBS. Twenty-four hours after plating, the number of piriform cortex cells appeared qualitatively higher in the condition 2 and we could

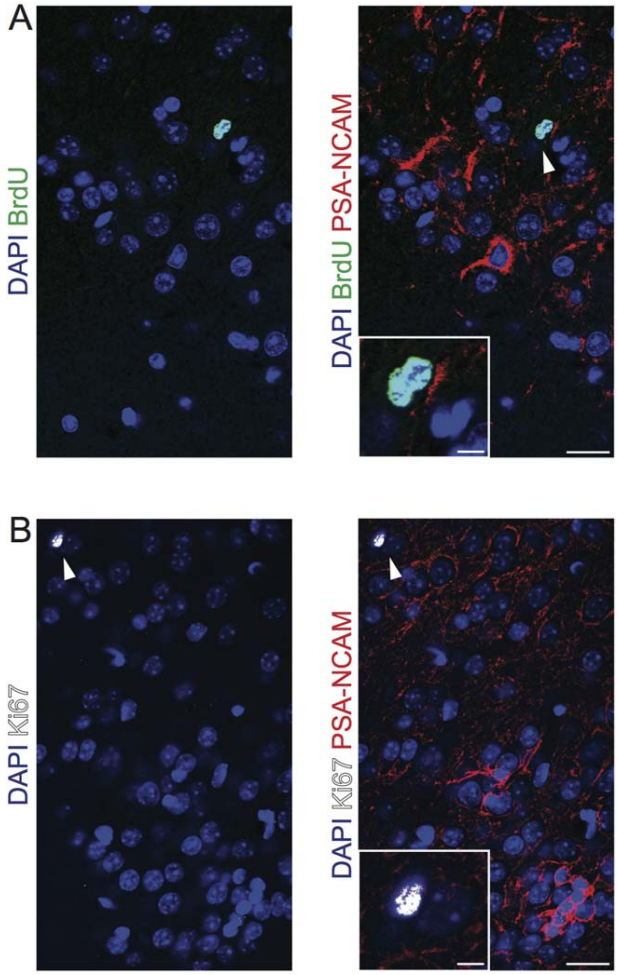


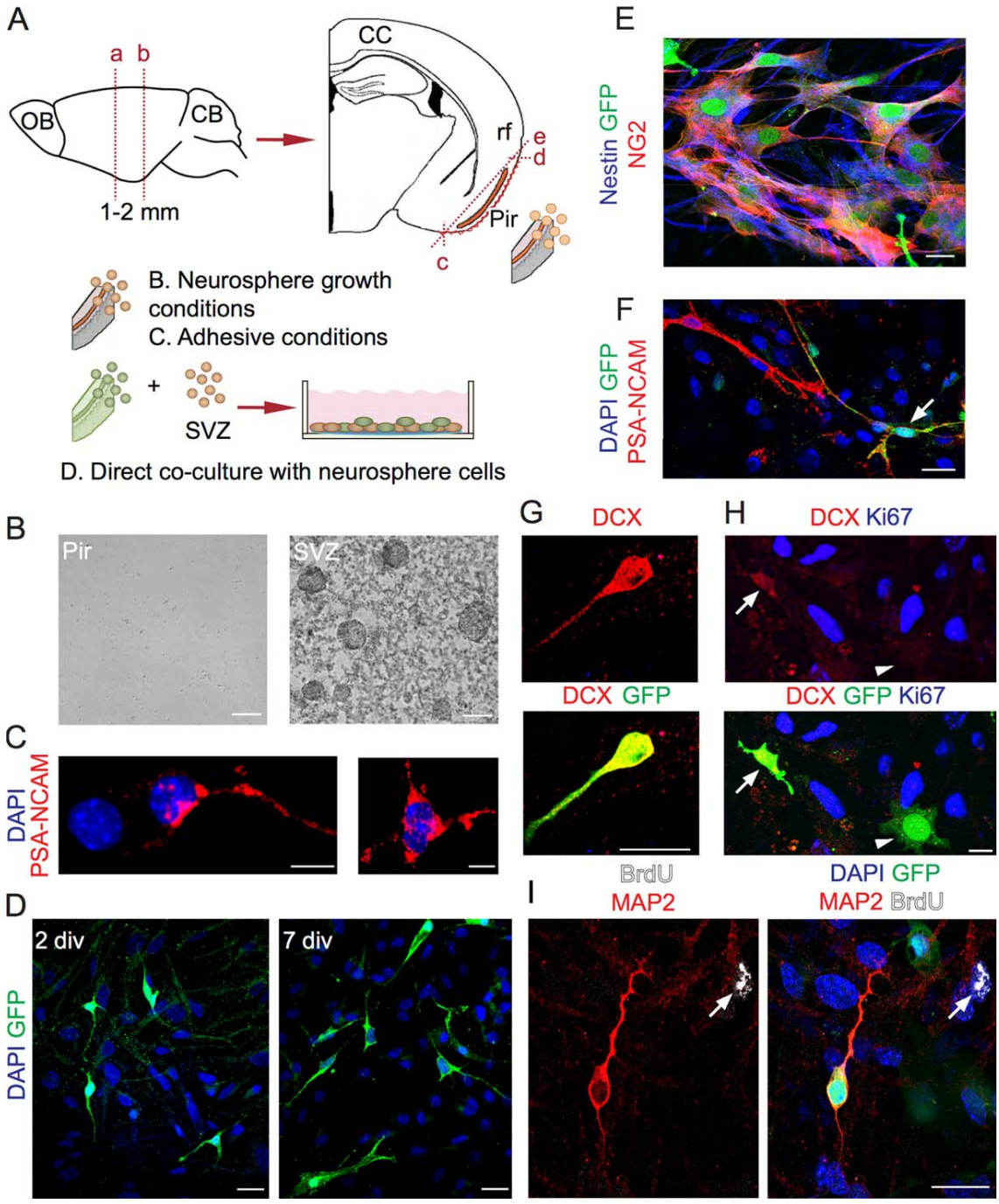
Figure 4 Cells expressing immature neuronal markers in the piriform cortex layer II do not proliferate and are not generated during adulthood. (A) Immunostaining for BrdU (green) and PSA-NCAM (red). Adult mice received four intraperitoneal injections of the thymidine analog BrdU and were sacrificed 21 days after the last injection. White arrowhead indicates a BrdU-positive cell negative for PSA-NCAM. The BrdU-positive cell is magnified in the inset. (B) Immunostaining for Ki67 (white) and PSA-NCAM (red). White arrowhead indicates a Ki67-positive cell that is also negative for PSA-NCAM. The inset enlarges the Ki67-positive cell. DAPI is used as nuclear counterstaining (in blue in A, B). All the images are 2D projections from 2 consecutive confocal planes located 1 μm apart. Scale bars: 20 μm and 5 μm in the inset. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

observe that a fraction of the cells were clearly PSA-NCAM positive [Fig. 5(C)]. Although the culture conditions appeared to promote the adhesion and initial survival of PSA-NCAM expressing cells from the adult piriform cortex layer II, most of them had disappeared by 5 div suggesting that the culture did not promote their long-term survival. In contrast, many cells from the SVZ survived for at least 5 div in the three culture conditions.

In an attempt to promote longer survival of piriform cortex immature neurons in the cultures, we next decided to test whether a cell substrate would increase maintenance of these cells. To do so, we designed a procedure consisting in the co-culture of piriform cortex dissociates on a feeder layer of SVZ cells [Fig. 5(A)]. Briefly, SVZ cells dissociated from growing neurospheres were seeded onto Matrigel-coated coverslips. SVZ cells were allowed to adhere for an hour and, immediately afterward, enzymatically disaggregated cells from the piriform cortex of CAG-EGFP-transgenic mice were seeded on top and cultured in neurosphere growth medium with 10% FBS [Fig. 5(A)]. The co-cultures were analyzed at 2 and 7 div and cortical cells were identified by the expression of the fluorescent reporter [Fig. 5(D)]. Under our culture conditions, SVZ cells proliferated

extensively forming a confluent feeder layer by 7 div, although the plating density needed to be precisely adjusted to avoid inconvenient overgrowth of the feeder cells during the culture period. The feeder SVZ cells together with the medium supplemented with mitogens and FBS supported the survival of the cortical cells for at least 7 div [Fig. 5(D)]. In contrast, the use of neurosphere medium without FBS promoted a dramatic increase in the proliferation of SVZ cells that led to the formation of aggregates in the feeder layer that interfered with the maintenance of the cortical cells (data not shown).

To study the phenotypic identity of the cortical cells in co-culture with neurosphere-derived cells, the



cultures were fixed at 2 and 7 div and stained with antibodies to GFP, alone, or in combination with antibodies for different cell-specific antigens. None of the GFP-positive cells in the cultures was GFAP-positive, although GFAP-positive cells were evident and numerous among GFP-negative SVZ cells (data not shown). Around 6% of the GFP-expressing cells were nestin positive ($6.13 \pm 1.73\%$ at 2 div, 6.65 ± 1.37 at 7 div, $n = 3$ independent cultures). A large proportion of these GFP/nestin-double positive cells was flat and polygonal and hardly ever positive for PSA-NCAM or MAP2. NG2 expressing cells were also present in the cultures and their frequency increased with time ($6.13 \pm 0.91\%$ at 2 div to $13.18 \pm 1.58\%$ at 7 div, $n = 3$; $p < 0.05$). Among the GFP-positive cells, we also observed a fraction of nestin/NG2-double positive cells, which increased more than fivefold during the differentiation period ($0.91 \pm 0.53\%$ at 2 div to $5.22 \pm 1.52\%$ at 7 div, $n = 3$; $p < 0.05$). Interestingly, at the end of the 7 div, most nestin-positive cells among the GFP expressing cells were also positive for NG2 ($76 \pm 11\%$, $n = 3$) [Fig. 5(E)].

Another fraction of GFP-positive cells displayed a neuron-like morphology, characterized by a more round cell body and 2–3 thin processes with varicosities, expressed detectable levels of PSA-NCAM [Fig. 5(F)] and never stained for nestin. PSA-NCAM-positive neuron-like cells were never seen forming small clusters and their number did not appear to increase with time *in vitro*. Since it is possible that PSA may be partially ablated from NCAM during the process of cell dissociation, in order to avoid an underestimation of the density of piriform cortex-derived immature neurons present in the co-cultures, we also detected them with DCX immunocytochemistry. DCX-positive cells constituted 5–10% of all GFP-positive cells in each culture and also survived during the whole culturing period ($11.03 \pm 0.06\%$ at 2 div and $6.59 \pm 0.87\%$ at 7 div, $n = 3$; $p < 0.05$). In order to know whether some of these piriform cortex-derived cells were proliferative, we studied them at 2 div by Ki67 immunohistochemistry. In cultures established from three independent animals, we were able to score around 60 DCX-positive cells among 500 piriform cortex-derived GFP-positive cells in each culture and none of them had detectable levels of Ki67 [Fig. 5(G)].

MAP2-positive neurons were very low in number, but their frequency increased during the culturing period [from $0.27 \pm 0.07\%$ at 2 div to $0.95 \pm 0.18\%$ at 7 div, $n = 3$, $p < 0.05$; Fig. 5(H)]. To investigate whether the new MAP2-positive neurons could have been generated from proliferating progenitor populations, we pulsed our 2 div cultures with 2 μ*M* BrdU and analyzed BrdU incorporation at 7 div [Fig. 5(H)]. None of the MAP2-positive neurons was immunepositive for the nucleoside, suggesting that our culture conditions had promoted their differentiation from nonproliferating cells.

Our data together indicated that we could isolate from the piriform cortex and maintain *in vitro*, at least for a short period of time, a nonproliferative PSA-NCAM/DCX-positive immature neuronal cell population. In addition, the data suggested that these cells might be able to differentiate into mature neurons.

DISCUSSION

In accordance with previous reports in other species, our data in mice indicate that layer II of the adult

Figure 5 Isolation and culture of piriform cells. (A) Schemes depicting the dissection, dissociation, and culture of piriform cortex layer II cells as explained in the text. (a–e) Dashed lines indicate surgical cuts, red line indicates meninges removal. OB, olfactory bulb; CB, cerebellum; CC, *corpus callosum*; Pir, piriform cortex; rf, rhinal fissure. Dissociated piriform cortex cells were seeded onto plastic to grow as neurospheres or onto Matrigel-coated coverslips to grow in adhesion conditions. In other experiments, piriform cells obtained from CAG-EGFP-transgenic mice were co-cultured with SVZ cells onto Matrigel-coated coverslips. (B) Phase contrast images taken of piriform cortex cells grown in neurospheres conditions (left panel) and SVZ neurospheres after 5 div (right panel). (C) Immunostaining for PSA-NCAM (red) of piriform cortex cells grown for 1 div in adhesion conditions. D–I: Different immunostainings performed in co-cultures of EGFP expressing piriform cells and SVZ cells. (D) Immunostaining for GFP (green) in co-cultures of 2 or 7 div. (E) Example of non-neuronal cells triple positive for GFP (green), nestin (blue), and NG2 (red). (F) Immunostaining for GFP (green) and PSA-NCAM (red) showing a neuron-like cell from the piriform cortex that expresses PSA-NCAM (arrow). (G) Neuron-like cell expressing DCX (red) and GFP (green). (H) Immunostaining for Ki67 (blue), DCX (red), and GFP (green). Note the presence of a DCX expressing neuron-like cell (arrow) derived from the piriform cortex. Another GFP-labeled cell lacking DCX expression can be observed (arrowhead). Several Ki67 expressing nuclei can be observed in the microphotograph, but none of them corresponds to a GFP-positive cell. (I) Example of a neuron-like cell double positive for GFP (green) and MAP2 (red) and negative for BrdU (gray, arrow). In some panels, as indicated, DAPI was used to stain nuclei. Scale bars: 100 μm in B, 10 μm in C and H, and 20 μm in D–G & I. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

piriform cortex contains, in addition to a predominant population of excitatory neurons and a smaller proportion of GABAergic inhibitory interneurons (Sarma et al., 2011), a population of PSA-NCAM/DCX-positive immature neurons. Despite their immature nature, these cells did not exhibit any markers of proliferative activity and were never labeled by birthdating experiments performed in adulthood. In contrast, PSA-NCAM-positive cells in layer II of the piriform cortex appear to be generated during fetal development. In agreement with a previous report in the same strain of mice (Sarma et al., 2011), we have demonstrated that the proportion of cells generated at the earlier embryonic stage studied (E13.5 and E14.5) was higher than of those generated at later stages (E15.5–E16.5). In addition, we have found that most of the cells in layer II generated at the earlier stage preferentially differentiate into mature and immature neurons (i.e., DCX- or PSA-NCAM-positive cells), while most of the cells generated later become glia. These data are consistent with an early developmental peak of neurogenesis and a late peak of gliogenesis observed in both paleo- and neocortex (Levers et al., 2001; Sarma et al., 2011). The percentage of immature neurons observed in this study is similar to that found previously in rats, although most of the immature neurons were generated at E15.5 in the rat (Gomez-Climent et al., 2008), in agreement with the differences in gestational period between these two species.

Our experiments indicate that very few cells in the adult murine piriform cortex layer II are either cycling or generated during adulthood and that none of them expressed PSA-NCAM, as it has been described in rats (Gomez-Climent et al., 2008). These data are also in agreement with work (Klempin et al., 2011), showing that BrdU-positive cells in the piriform cortex of adult mice 1, 3, or 15 days after BrdU injection were scarce and expressed NG2 or the glial marker S100β but not DCX (Klempin et al., 2011). Neurogenesis in the adult rodent piriform cortex under physiological conditions seems to be marginal (Pekcec et al., 2006; Shapiro et al., 2007; Chen et al., 2009) and it has been related to the migration of newly generated neurons from the SVZ (Shapiro et al., 2007). However, an intrinsic origin for some of the immature neurons in the adult cerebral cortex layer II should not be discarded. Recently generated interneurons in the adult rat neocortex may arise from intrinsic NG2 expressing progenitors (Dayer et al., 2005). Some of the NG2 expressing cells in the adult cerebral cortex are actively dividing and have been found to generate oligodendrocytes and also cells with a neuronal phenotype in the adult hippocampus (Belachew et al., 2003). We have observed that a very small subpopulation of tangled cells in the adult piriform cortex layer II co-expresses NG2 and PSA-NCAM, both in mice (present results) and in rats (Gomez-Climent et al., 2008), and the presence of DCX/NG2 double-positive cells has also been described in this layer (Tamura et al., 2007). Two recent reports have also demonstrated, using tamoxifen inducible transgenic mice with reporters under the control of the NG2 promoter, that a, most likely local, population of NG2 positive precursors is able to differentiate into projection neurons in the adult murine piriform cortex (Rivers et al., 2008; Guo et al., 2010). However, a similar study has failed to observe reporter-expressing neurons in the piriform cortex after tamoxifen injection (Richardson et al., 2011; Huang et al., 2014).

In this work, we have used *in vitro* conditions to isolate the population of immature neurons and to assess their activity under mitogenic stimulation. We have observed that this region does not appear to contain progenitors with the ability to form neurospheres. In addition, we could not maintain isolated cells from the piriform cortex layer II *in vitro* for a long period. Some reports have described the culture of adult cells from "classical" neurogenic zones, such as the SVZ (Reynolds and Weiss, 1992; Ferron et al., 2007) or the hippocampus (Babu et al., 2011). Neurons have been also cultivated from adult septum or striatum (Palmer et al., 1995), where neurogenesis has been described after BDNF infusion (Pencea et al., 2001). Cultivating cells from non-neurogenic regions seem more challenging and, as reported previously with adult rat or mouse cortex, it may imply the use of a density gradient to separate neurons from debris (Brewer and Torricelli, 2007). In fact, to maintain them *in vitro*, we used a feeder layer made by SVZ cells. Our culturing conditions could isolate from the piriform cortex a population of PSA-NCAM/DCX-positive immature neurons, which do not proliferate *in vitro* and that, most probably, differentiates into MAP2-positive neurons. Our BrdU analysis supports this hypothesis, showing that these neurons have not been generated from proliferating progenitors in the culture. We observed that our co-cultures also contained NG2-positive cells, as described *in vivo*, and that their number increased markedly *in vitro*. It is possible that some of these NG2-positive cells were indeed progenitors that could have proliferated after some time *in vitro*, generating new NG2 expressing cells. In fact, at 7 div, there is a dramatic increase in the proportion of nestin cells that express NG2.

Although we have tried removing completely the meninges from the tissue, we could not exclude the possibility that some of the cells found in our culture could be originated from the differentiation of stem cells present in some remnants of these mesodermic structures (Decimo et al., 2012), from oligodendrocyte precursors (Ben-Hur et al., 1998) or from microglia (Yokoyama et al., 2004; Takamori et al., 2009). Future experiments using this approach would be needed to follow the fate of immature neurons *in vitro*. One possibility to completely exclude the presence of contaminating cells could be purifying the immature neuronal cell population using immunopanning (Ben-Hur et al., 1998; Schmandt et al., 2005), magnetic cell sorting (MACS), or fluorescent activated cell sorting (FACS) (Pennartz et al., 2004; Seidenfaden et al., 2006; Azari et al., 2011)

Due to the singularity on these immature neurons in the adult piriform cortex, it will be interesting to clarify which is their potential function. Some evidences suggest that they could probably differentiate into mature neurons, constituting a reservoir of cells that progressively integrates in the existing circuitry of this cortical region. In this direction, it has been described that the number of PSA-NCAM/DCX expressing cells is reduced with aging in different species (Xiong et al., 2008; Cai et al., 2009; Varea et al., 2009; Zhang et al., 2009), suggesting that these cells can probably differentiate into mature neurons. This hypothesis is plausible considering that some DCX-expressing cells in the piriform cortex exhibit electrophysiological properties of mature neurons (Klempin et al., 2011) and that a minor percentage of PSA-NCAM- and DCX-expressing cells in layer II also express NeuN (Gomez-Climent et al., 2008; present results). To elucidate the different developmental phases that these immature neurons undergo during their differentiation and their timing, it will be interesting to trace them directly *in vivo* using a Cre-lox recombination system in transgenic mice.

Our present results support the hypothesis that immature neurons in the piriform cortex layer II differentiate into excitatory neurons. In consonance with previous findings in mice, rats, rabbits, and guinea pigs (Gomez-Climent et al., 2008; Luzzati et al., 2009), we have found that these cells express Tbr1, a transcription factor specific for pallium-derived principal neurons and do not express markers of interneurons. This is in contrast with some studies suggesting a major interneuronal fate for immature neurons in layer II (Xiong et al., 2008; Cai et al., 2009; Zhang et al., 2009; Bonfanti and Nacher, 2012).

The population of immature neurons in the piriform cortex layer II may be maintained as a potential reservoir of cells available for plasticity. These cells, particularly the tangled subtype, are almost completely isolated from the circuitry by the presence of PSA-NCAM in their membranes. The progressive loss of PSA may favor the formation of new synapses on these neurons once they have started their final development, probably becoming semilunar-pyramidal transitional neurons, in which scattered excitatory axospinous synapses have been found (Gomez-Climent et al., 2008). It is likely that these new synapses come from the olfactory bulb, but this still remains to be proven. In fact, the immature neurons in layer II seem to be linked to olfactory function. After unilateral olfactory bulbectomy, the number of PSA-NCAM- and DCX-expressing cells is significantly reduced in the ipsilateral piriform cortex layer II of adult rats (Gomez-Climent et al., 2011). Moreover, this reduction is paralleled by an increase in the number of NeuN-expressing cells, suggesting that some of the cells that stop expressing immature neuronal markers may differentiate into mature neurons (Gomez-Climent et al., 2011). Similar results have been found after naris-occlusion in adult guinea pigs: a reduction in the number and dendritic processes of DCX expressing cells (He et al., 2014). However, the possibility that these synaptic inputs were associational should not be disregarded, especially in the posterior piriform cortex (Bekkers and Suzuki, 2013). In fact, in contrast with bulbectomy and olfactory deprivation, at least a type of olfactory learning does not seem to have an impact on the immature neuronal population in the piriform cortex (Gomez-Climent et al., 2008).

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REFERENCES

Azari H, Osborne GW, Yasuda T, Golmohammadi MG, Rahman M, Deleyrolle LP, Esfandiari E, et al. 2011. Purification of immature neuronal cells from neural stem cell progeny. PLoS One 6:e20941.

Babu H, Claasen JH, Kannan S, Runker AE, Palmer T, Kempermann G. 2011. A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. Front Neurosci 5:89.

Bayer SA. 1986. Neurogenesis in the rat primary olfactory cortex. Int J Dev Neurosci 4:251–271.

Bekkers JM, Suzuki N. 2013. Neurons and circuits for odor processing in the piriform cortex. Trends Neurosci 36: 429–438.

Belachew S, Chittajallu R, Aguirre AA, Yuan X, Kirby M, Anderson S, Gallo V. 2003. Postnatal NG2 proteoglycan-expressing progenitor cells are intrinsically multipotent and generate functional neurons. J Cell Biol 161:169–186.

Ben-Hur T, Rogister B, Murray K, Rougon G, Dubois-Dalcq M. 1998. Growth and fate of PSA-NCAM+ precursors of the postnatal brain. J Neurosci 18:5777–5788.

Bernier PJ, Bedard A, Vinet J, Levesque M, Parent A. 2002. Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci USA 99:11464–11469.

Bonfanti L, Nacher J. 2012. New scenarios for neuronal structural plasticity in non-neurogenic brain parenchyma: The case of cortical layer II immature neurons. Prog Neurobiol 98:1–15.

Bonfanti L, Theodosis DT. 1994. Expression of polysialylated neural cell adhesion molecule by proliferating cells in the subependymal layer of the adult rat, in its rostral extension and in the olfactory bulb. Neuroscience 62: 291–305.

Brewer GJ, Torricelli JR. 2007. Isolation and culture of adult neurons and neurospheres. Nat Protoc 2:1490–1498.

Cai Y, Xiong K, Chu Y, Luo DW, Luo XG, Yuan XY, Struble RG, et al. 2009. Doublecortin expression in adult cat and primate cerebral cortex relates to immature neurons that develop into GABAergic subgroups. Exp Neurol 216:342–356.

Chan WY, Kohsaka S, Rezaie P. 2007. The origin and cell lineage of microglia: New concepts. Brain Res Rev 53: 344–354.

Chen J, Kwon CH, Lin L, Li Y, Parada LF. 2009. Inducible site-specific recombination in neural stem/progenitor cells. Genesis 47:122–131.

Dayer AG, Cleaver KM, Abouantoun T, Cameron HA. 2005. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol 168:415–427.

Decimo I, Fumagalli G, Berton V, Krampera M, Bifari F. 2012. Meninges: From protective membrane to stem cell niche. Am J Stem Cells 1:92–105.

Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, et al. 2005. Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247–251.

Ferron SR, Andreu-Agullo C, Mira H, Sanchez P, Marques-Torrejon MA, Farinas I. 2007. A combined ex/in vivo assay to detect effects of exogenously added factors in neural stem cells. Nat Protoc 2:849–859.

Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, et al. 1999. Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. Neuron 23:247–256.

Gheusi G, Ortega-Perez I, Murray K, Lledo PM. 2009. A niche for adult neurogenesis in social behavior. Behav Brain Res 200:315–322.

Gleeson JG, Lin PT, Flanagan LA, Walsh CA. 1999. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. Neuron 23:257–271.

Gomez-Climent MA, Castillo-Gomez E, Varea E, Guirado R, Blasco-Ibanez JM, Crespo C, Martinez-Guijarro FJ, et al. 2008. A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. Cereb Cortex 18:2229–2240.

Gomez-Climent MA, Hernandez-Gonzalez S, Shionoya K, Belles M, Alonso-Llosa G, Datiche F, Nacher J. 2011. Olfactory bulbectomy, but not odor conditioned aversion, induces the differentiation of immature neurons in the adult rat piriform cortex. Neuroscience 181:18–27.

Guo F, Maeda Y, Ma J, Xu J, Horiuchi M, Miers L, Vaccarino F, et al. 2010. Pyramidal neurons are generated from oligodendroglial progenitor cells in adult piriform cortex. J Neurosci 30:12036–12049.

Haberly LB. 1983. Structure of the piriform cortex of the opossum. I. Description of neuron types with Golgi methods. J Comp Neurol 213:163–187.

Hadjantonakis AK, Gertsenstein M, Ikawa M, Okabe M, Nagy A. 1998. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. Mech Dev 76:79–90.

He X, Zhang XM, Wu J, Fu J, Mou L, Lu DH, Cai Y, et al. 2014. Olfactory experience modulates immature neuron development in postnatal and adult guinea pig piriform cortex. Neuroscience 259:101–112.

Huang W, Zhao N, Bai X, Karram K, Trotter J, Goebbels S, Scheller A, et al. 2014. Novel NG2-CreERT2 knock-in mice demonstrate heterogeneous differentiation potential of NG2 glia during development. Glia 62:896–913.

Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. 2002. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods 115:97–105.

Klempin F, Kronenberg G, Cheung G, Kettenmann H, Kempermann G. 2011. Properties of doublecortin-(DCX)-expressing cells in the piriform cortex compared to the neurogenic dentate gyrus of adult mice. PLoS One 6:e25760

Levers TE, Edgar JM, Price DJ. 2001. The fates of cells generated at the end of neurogenesis in developing mouse cortex. J Neurobiol 48:265–277.

Luzzati F, Bonfanti L, Fasolo A, Peretto P. 2009. DCX and PSA-NCAM expression identifies a population of neurons preferentially distributed in associative areas of different pallial derivatives and vertebrate species. Cereb Cortex 19:1028–1041.

Marti-Mengual U, Varea E, Crespo C, Blasco-Ibanez JM, Nacher J. 2013. Cells expressing markers of immature neurons in the amygdala of adult humans. Eur J Neurosci 37:10–22.

Mullen RJ, Buck CR, Smith AM. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. Development 116: 201–211.

Nacher J, Alonso-Llosa G, Rosell D, McEwen B. 2002. PSA-NCAM expression in the piriform cortex of the

adult rat. Modulation by NMDA receptor antagonist administration. Brain Res 927:111–121.

Nacher J, Crespo C, McEwen BS. 2001. Doublecortin expression in the adult rat telencephalon. Eur J Neurosci 14:629–644.

Ni Dhuill CM, Fox GB, Pittock SJ, O'Connell AW, Murphy KJ, Regan CM. 1999. Polysialylated neural cell adhesion molecule expression in the dentate gyrus of the human hippocampal formation from infancy to old age. J Neurosci Res 55:99–106.

Palmer TD, Ray J, Gage FH. 1995. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci 6: 474–486.

Pekcec A, Loscher W, Potschka H. 2006. Neurogenesis in the adult rat piriform cortex. Neuroreport 17:571–574.

Pencea V, Bingaman KD, Wiegand SJ, Luskin MB. 2001. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. J Neurosci 21:6706–6717.

Pennartz S, Belvindrah R, Tomiuk S, Zimmer C, Hofmann K, Conradt M, Bosio A, et al. 2004. Purification of neuronal precursors from the adult mouse brain: Comprehensive gene expression analysis provides new insights into the control of cell migration, differentiation, and homeostasis. Mol Cell Neurosci 25:692–706.

Reynolds BA, Weiss S. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255:1707–1710.

Richardson WD, Young KM, Tripathi RB, McKenzie I. 2011. NG2-glia as multipotent neural stem cells: Fact or fantasy? Neuron 70:661–673.

Rivers LE, Young KM, Rizzi M, Jamen F, Psachoulia K, Wade A, Kessaris N, et al. 2008. PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. Nat Neurosci 11:1392–1401.

Sarma AA, Richard MB, Greer CA. 2011. Developmental dynamics of piriform cortex. Cereb Cortex 21:1231–1245.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, et al. 2012. Fiji: An open-source platform for biological-image analysis. Nat Methods 9:676–682.

Schmandt T, Meents E, Gossrau G, Gornik V, Okabe S, Brustle O. 2005. High-purity lineage selection of embryonic stem cell-derived neurons. Stem Cells Dev 14:55–64.

Seidenfaden R, Desoeuvre A, Bosio A, Virard I, Cremer H. 2006. Glial conversion of SVZ-derived committed neuronal precursors after ectopic grafting into the adult brain. Mol Cell Neurosci 32:187–198.

Shapiro LA, Ng KL, Kinyamu R, Whitaker-Azmitia P, Geisert EE, Blurton-Jones M, Zhou QY, et al. 2007. Origin, migration and fate of newly generated neurons in the adult rodent piriform cortex. Brain Struct Funct 212:133–148.

Shapiro LA, Ng KL, Zhou QY, Ribak CE. 2007. Olfactory enrichment enhances the survival of newly born cortical neurons in adult mice. Neuroreport 18:981–985.

Takamori Y, Mori T, Wakabayashi T, Nagasaka Y, Matsuzaki T, Yamada H. 2009. Nestin-positive microglia in adult rat cerebral cortex. Brain Res 1270:10–18.

Tamura Y, Kataoka Y, Cui Y, Takamori Y, Watanabe Y, Yamada H. 2007. Multi-directional differentiation of doublecortin- and NG2-immunopositive progenitor cells in the adult rat neocortex in vivo. Eur J Neurosci 25: 3489–3498.

Varea E, Belles M, Vidueira S, Blasco-Ibanez JM, Crespo C, Pastor AM, Nacher J. 2011. PSA-NCAM is expressed in immature, but not recently generated, neurons in the adult cat cerebral cortex layer II. Front Neurosci 5:17.

Varea E, Castillo-Gomez E, Gomez-Climent MA, Guirado R, Blasco-Ibanez JM, Crespo C, Martinez-Guijarro FJ, et al. 2009. Differential evolution of PSA-NCAM expression during aging of the rat telencephalon. Neurobiol Aging 30:808–818.

Xiong K, Luo DW, Patrylo PR, Luo XG, Struble RG, Clough RW, Yan XX. 2008. Doublecortin-expressing cells are present in layer II across the adult guinea pig cerebral cortex: Partial colocalization with mature interneuron markers. Exp Neurol 211:271–282.

Yokoyama A, Yang L, Itoh S, Mori K, Tanaka J. 2004. Microglia, a potential source of neurons, astrocytes, and oligodendrocytes. Glia 45:96–104.

Zhang XM, Cai Y, Chu Y, Chen EY, Feng JC, Luo XG, Xiong K, et al. 2009. Doublecortin-expressing cells persist in the associative cerebral cortex and amygdala in aged nonhuman primates. Front Neuroanat 3:17.

CHAPTER 3

Cellular Plasticity in the Adult Murine Piriform Cortex: Continuous Maturation of Dormant Precursors Into Excitatory Neurons

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ORIGINAL ARTICLE

Cellular Plasticity in the Adult Murine Piriform Cortex: Continuous Maturation of Dormant Precursors Into Excitatory Neurons

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Abstract

Neurogenesis in the healthy adult murine brain is based on proliferation and integration of stem/progenitor cells and is thought to be restricted to 2 neurogenic niches: the subventricular zone and the dentate gyrus. Intriguingly, cells expressing the immature neuronal marker doublecortin (DCX) and the polysialylated-neural cell adhesion molecule reside in layer II of the piriform cortex. Apparently, these cells progressively disappear along the course of ageing, while their fate and function remain unclear. Using DCX-CreER^{T2}/Flox-EGFP transgenic mice, we demonstrate that these immature neurons located in the murine piriform cortex do not vanish in the course of aging, but progressively resume their maturation into glutamatergic (TBR1^{+}, CaMKII^{+}) neurons. We provide evidence for a putative functional integration of these newly differentiated neurons as indicated by the increase in perisomatic puncta expressing synaptic markers, the development of complex apical dendrites decorated with numerous spines and the appearance of an axonal initial segment. Since immature neurons found in layer II of the piriform cortex are generated prenatally and devoid of proliferative capacity in the postnatal cortex, the gradual maturation and integration of these cells outside of the canonical neurogenic niches implies that they represent a valuable, but nonrenewable reservoir for cortical plasticity.

Key words: doublecortin, integration, maturation, neuroplasticity, piriform cortex, PSA-NCAM

Introduction

Inducible cortical neurogenesis outside the canonical neurogenic niches of the adult rodent brain can be detected in lesions, such as stroke, and mostly depends on the migration of neuroblasts from the neurogenic regions towards the lesion site (Arvidsson et al. 2002; Jin et al. 2003). However, some studies suggested the presence of immature and/or adult-born neurons outside of the canonical neurogenic regions even under physiological conditions (Dayer et al. 2005; Takemura 2005; Gould 2007; Luzzati et al. 2009; Shapiro et al. 2009). The piriform cortex is one of these structures where immature neurons have been reported, although no local proliferative neuroblasts have been detected under physiological conditions (Gómez-Climent et al. 2008; Luzzati et al. 2009; Klempin et al. 2011; Yang et al. 2015; Rubio et al. 2016). Whether these brain regions in which immature neurons have been detected support the events that define adult neurogenesis, that is, proliferation, differentiation, maturation, and network-integration, is currently under debate (Bonfanti and Peretto 2011; Bonfanti and Nácher 2012; Feliciano and Bordey 2013; Ernst et al. 2014; Wang et al. 2014; Feliciano et al. 2015).

In adult mice and rats, immature neurons are mainly restricted to layer II of the paleocortex. However, a more extensive distribution of immature neurons residing in this cortical layer, including the neocortex, has been reported in mammals with larger cerebral cortices, for example, in guinea pigs, rabbits, cats, nonhuman-primates, and humans, (Ní Dhúill et al. 1999; Bonfanti 2006; Gómez-Climent et al. 2008; Luzzati et al. 2009; Zhang, Cai, et al. 2009; Varea et al. 2011; De Nevi et al. 2013; He et al. 2014; Patzke et al. 2014; Rubio et al. 2016), reviewed in (König et al. 2016).

The majority of the immature neurons detected in layer II of the piriform cortex of mice, rats, guinea pigs, cats, and most likely in all mammals, appears to have been generated prenatally (Gómez-Climent et al. 2008; Varea et al. 2011; Bonfanti and Nácher 2012; Yang et al. 2015; König et al. 2016; Rubio et al. 2016). Nevertheless, some studies have suggested that immature neurons of the piriform cortex can also originate from progenitors generated in the SVZ of young mammals that migrate towards the piriform cortex and integrate locally (Bernier et al. 2002; Pekcec et al. 2006; Shapiro et al. 2007, 2009). Other investigations reported that a local cell population expressing neural/glial antigen 2 (NG2) with low level of doublecortin (DCX) (Tamura et al. 2007; Rivers et al. 2008; Guo et al. 2010) or progenitors residing in layer I (Xiong et al. 2010) are the origin of the immature neurons detected in the piriform cortex layer II. However, these findings could not be reproduced by other studies (Nácher et al. 2002; Gómez-Climent et al. 2008; Luzzati et al. 2009; Varea et al. 2011), which suggests that, at best, these sources of immature neurons might only contribute at a very low level or have a transient existence (Pekcec et al. 2006; Shapiro et al. 2007; 2009).

Intriguingly, 2 subpopulations of immature neurons with distinct cell morphologies have been detected in layer II of the adult piriform cortex (Gómez-Climent et al. 2008; Rubio et al. 2016). The first population represents so-called "tangled cells", which are endowed with a small diameter (~9 μm) and a few short intricate dendrites. These small cells strongly express DCX, the polysialylated neural cell adhesion molecule (PSA-NCAM) and the neuron-specific class III beta-tubulin (TuJ-1), which are classical markers found in immature neurons (Seki and Arai 1991; Bonfanti et al. 1992; Nàcher et al. 2001; Nácher et al. 2002; Brown et al. 2003; Couillard-Despres et al. 2005; Gómez-Climent et al. 2008; Luzzati et al. 2009). In contrast, tangled cells do not express markers of stem cells (nestin), mature neurons (e.g., NeuN, CaMKII, GAD67), oligodendrocytes (RIP), or astrocytes (GFAP) (Gómez-Climent et al. 2008; Rubio et al. 2016). Tangled cells appear to be inactive according to the lack of expression of functionality markers like c-Fos or the activity-regulated cytoskeleton-associated protein (Arc) (Gómez-Climent et al. 2008; Carceller et al. 2016) and due to the ensheathment of their soma by astrocytic end feet (Gómez-Climent et al. 2008).

The second group of immature neuronal cells residing in layer II of the piriform cortex displays a larger soma than tangled cells, that is, a diameter of ~15 μm, and a rather elaborated dendritic tree decorated with some dendritic spines. These larger immature neurons express DCX and also PSA-NCAM, albeit at a lower intensity than observed in tangled cells. Furthermore, the occasional low expression of NeuN could be detected as well (Gómez-Climent et al. 2008; Rubio et al. 2016). These larger cells have the typical morphology of semilunar–pyramidal transitional neurons, a common population of excitatory neurons found in the piriform cortex layer II. These cells will be thereafter referred to as "complex cells."

Complex cells with morphology between those of tangled cells and semilunar–pyramidal transitional neurons are found in layer II as well. Since tangled cells appear more immature than semilunar–pyramidal transitional neurons, it has been hypothesized that the former progressively differentiate into semilunar–pyramidal neurons. Considering that both subpopulations of immature neurons express the transcription factor Tbr1, which is exclusively expressed by pallial-derived excitatory neurons (Gómez-Climent et al. 2008; Luzzati et al. 2009; Varea et al. 2011; Rubio et al. 2016), we hypothesized that tangled cells could generate immature complex cells, which turn into glutamatergic pyramidal neurons upon maturation.

In rats (Gómez-Climent et al. 2008; Varea et al. 2009), dogs (De Nevi et al. 2013), guinea pigs (Xiong et al. 2008), and nonhuman primates (Cai et al. 2009; Zhang, Cai, et al. 2009), the immature cortical neurons gradually vanished with age. Two hypotheses have been formulated to explain the disappearance of the immature neurons in the cortical layer II (Abrous et al. 1997; Varea et al. 2009; Gómez-Climent et al. 2010). On the one hand, the immature neurons may simply die due to their lack of functional integration. However, no evidence of augmented cell death has been observed in structures bearing the immature neuron populations (Bonfanti and Nácher 2012). On the other hand, the immature neurons in layer II may remain in a dormant or standby stage until they resume their delayed final maturation step and become functionally integrated neurons. The latter has been supported by recent immunohistochemical (Gómez-Climent et al. 2008; Luzzati et al. 2009; Rubio et al. 2016) and electrophysiological analyses (Klempin et al. 2011) of cells expressing immature neuronal markers in adult cortical layer II.

Until now, the lack of an adequate fate-mapping tool precluded the demonstration that immature neurons residing in the adult piriform cortex can differentiate and incorporate as principal neurons. To this end, we made use of an inducible

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transgenic mouse model in which DCX-expressing cells can be permanently labeled following tamoxifen administration and monitored with a green fluorescent protein (GFP) reporter (short DCX-CreER^{T2}/Flox-EGFP mouse) (Zhang et al. 2010). With this approach, we have been able to show that immature neurons residing in layer II of the mouse adult piriform cortex remain alive in the course of ageing, developing the morphological requirements for synaptic input and action potential output (i.e., synapses and axonal initial segments). Taken together, our observations suggest the gradual integration of the immature neurons of the piriform cortex into the surrounding network as local principal neurons.

Material and Methods

Animals

Transgenic DCX-CreER^{T2}/Flox-EGFP mice were used to label and follow the fate of neuronal precursor cells (Zhang et al. 2010). All experiments were performed in accordance with the guidelines of the "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes" and were approved by the national animal care authorities.

Induction of the GFP Reporter Gene in DCX-Expressing Cells

Mice were divided into 3 experimental groups and tamoxifen (100 mg/kg of bodyweight dissolved in corn oil, Sigma-Aldrich) was administered orally by gavage once daily for 5 consecutive days (days 1–5) (Fig. 1A). To study the fate and distribution of GFP$^+$ cells over time, mice of group 1 received tamoxifen at the age of 3 months and were sacrificed on day 8 following the first tamoxifen administration (3m-t, $n = 7$). Mice from group 2 received tamoxifen at 9 months of age and were sacrificed on day 8 after the first administration (9m-t, $n = 5$). Finally, mice of group 3 received tamoxifen at 3 months of age and were sacrificed 6 months later at the age of 9 months (3m-t→9, $n = 8$).

To scrutinize whether tangled cells in the piriform cortex proliferate in the adult brain or remain postmitotic, mice ($n = 5$) were injected intraperitoneally with BrdU (50 mg/kg bodyweight) at the age of 3 months once daily for 5 consecutive days. Simultaneously, mice also received standard oral application of tamoxifen (100 mg/kg bodyweight). Mice were sacrificed on day 8 following the first BrdU administration and the brains were further processed for immunohistochemistry. In a second group, pregnant mice received BrdU (50 mg/kg bodyweight) to label the brain of developing fetuses at embryonic age E14 and E15 (estimated by plug-check of the mother). The progeny ($n = 5$) of these pregnant mice received the standard oral application of tamoxifen (100 mg/kg bodyweight) as for the 3m-t group and sacrificed on day 8. Moreover, possible leakage of the system resulting in the activation of the EGFP reporter expression in the absence of tamoxifen was addressed in 2-year-old DCX-CreER^{T2}/Flox-EGFP naive mice ($n = 2$) which where compared with 2-year-old transgenic mice treated with tamoxifen (100 mg/kg bodyweight daily for 5 consecutive days) at the age of 3 months ($n = 2$).

Immunohistochemistry and Image Analysis

For immunohistochemistry, mice were transcardially perfused with 0.9% NaCl for 5 min followed by 0.1 M phosphate buffered 4% paraformaldehyde pH 7.4 for 10 min. Brains were dissected and postfixed in the same paraformaldehyde solution overnight at 4 °C and then transferred in 0.1 M phosphate buffered 30% sucrose solution pH 7.4 at 4 °C for at least 48 h. Brains were cut in 40 µm sagittal sections using a sliding microtome (Leica) on dry ice and sections were stored at −20 °C until further processing in cryoprotectant (25% v/v glycerol, 0.05 M sodium phosphate buffer pH 7.4, 25% v/v ethylene glycol).

Following antigen-retrieval (citrate buffer pH 6.0 [Sigma-Aldrich], 10 min at 100 °C), fluorescent immunohistological analyses were performed as previously described (Couillard-Despres et al. 2005; Rubio et al. 2016). Antibodies: rat anti-BrdU (Bio-Rad AbD Serotec) 1:500; mouse anti-CaMKII (Abcam) 1:500; goat anti-ChAT (Novus Biologicals) 1:100; rabbit anti-DCX (Cell Signaling Technology) 1:300; mouse anti-GAD67 (Millipore) 1:500; guinea pig anti-GFAP (Progen) 1:500; chicken anti-GFP (Invitrogen) 1:500; guinea pig anti-NeuN (Millipore) 1:500; rabbit anti-NG2 (Millipore) 1:200; mouse anti-PSA-NCAM (Millipore) 1:1000; rabbit anti- βIV-spectrin (selfmade) (Schlüter et al. 2017) 1:500; goat anti-Sox2 (Santa Cruz Biotechnology) 1:1000; mouse anti-synaptophysin (Sigma Aldrich) 1:500; rabbit anti-Tbr1 (Abcam) 1:500; rabbit anti-VGAT (Synaptic Systems) 1:500. Fluorescence images were acquired using a LSM 710 confocal microscope and ZEN 2011 Black Software (Carl Zeiss) and a TSC SPE confocal microscope (Leica). Z-stacks were acquired over the whole thickness of the section and co-localization was confirmed by the analysis of successive optical slices. For image-analysis, ImageJ Software 1.46r (National Institutes of Health) and FIJI based on ImageJ 1.50a (Schindelin et al. 2012) were used.

The analysis of the marker profile and fate of GFP$^+$ cells involved 50 GFP$^+$ cells in layer II of the piriform cortex per mouse and staining and were analyzed for double- and triple-labeling with cell type-specific antibodies.

The leakiness of the system in the absence of tamoxifen was evaluated based on the density of GFP-expressing cells in 2-year-old naive transgenic mice, compared with the density observed in mice induced at 3 months of age and perfused at 2 years. The density of GFP-expressing cells in the layer II of the piriform cortex was assessed in 10 randomly chosen fields of view per mice acquired with a ×20 objective (Z-stack step size = 1 µm).

Cell morphometric analysis was performed using confocal microscopy with a ×63 oil immersion objective and z-series of optical sections (0.2 µm step size). The soma diameter of GFP-expressing cells was measured in 10 randomly chosen cells of each type from 5 mice per group. The confocal stacks were then processed using FIJI to render 3D reconstructions (Fig. 2A–C).

Spine density on the apical dendrite of GFP-expressing neurons and the density of immunoreactive puncta in their perisomatic region were analyzed as described previously (Guirado et al. 2014). For the analysis of the spine density, a ×63 oil immersion objective with an additional ×3.5 digital zoom and a 0.8 µm Z-step size was used. Five randomly chosen apical dendrites of GFP-positive neurons within the piriform cortex layer II of 5 mice per group were analyzed ($n = 25$). Selected dendrites from GFP-positive neurons had to meet the following criteria: measure at least 150 µm from the soma and not be intersected by another dendrite along their trajectory. Apical dendrite thickness was also measured at a distance of 120 µm from the cell soma.

For the analyses of perisomatic puncta containing synaptophysin (SYN) and vesicular GABA transporter (VGAT) apposed

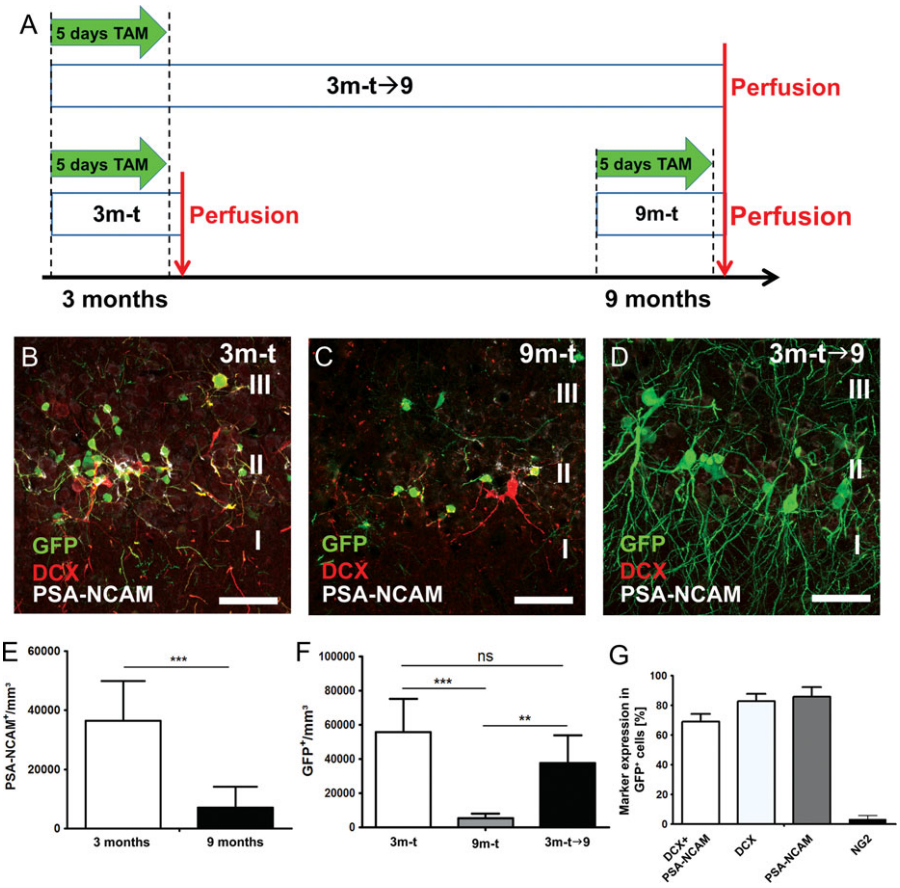

Figure 1. Overview of the experimental groups and detection of immature neurons in the piriform cortex. (*A*) DCX-CreER^{T2}/Flox-EGFP transgenic mice were split into 3 experimental groups and administered daily with tamoxifen for 5 consecutive days. First and second group received tamoxifen with 3 months (3m-t) or 9 months (9m-t) of age and were sacrificed on day 8 following administration. The third group (3m-t→9) received tamoxifen with 3 months of age and was perfused with 9 months of age. Numerous DCX / PSA-NCAM-expressing cells (red) could be detected in layer II of the piriform cortex in 3-month-old mice (*B*) and to lesser extent at 9 months of age (*C* and *D*). The majority of cells expressing GFP (green) coexpressed PSA-NCAM (white) or DCX (red) in mice sacrificed shortly after tamoxifen administration (*B* and *C*). Expression of immature markers in GFP$^+$ was seldom detected in the 3m-t→9 group (*D*). Density of cells expressing PSA-NCAM (*E*) and GFP (*F*) in the layer II of the piriform cortex. (*G*) Coexpression of markers for immature neurons and NG2 in GFP$^+$ cells of the 3m-t group., Scale bars = 50 μm.

to GFP$^+$ neurons, micrographs were acquired using a ×63 oil objective. From each animal, 5 GFP$^+$ expressing cells from piriform cortex layer II were randomly selected. Z-series of optical sections covering all 3D extensions of the somata were acquired using sequential scanning mode (Z-step size, 0.5 μm). Stacks were processed with Fiji software. The profile of every soma was delimited manually and puncta placed within a range of 0.5 μm from the edge of this profile were analyzed. SYN and VGAT containing puncta were defined as having an area larger than 0.15 μm^2 and smaller than 2.5 μm^2. Puncta linear density values were determined and expressed as number of puncta per micron of soma perimeter.

Micrographs of the axonal initial segment were acquired using a ×63 oil objective (Z-series step size 1 μm). Analysis of NeuN and βIV-spectrin coexpression was performed in at least 100 GFP-expressing cells per transgenic mouse.

GraphPad Prism 5 (GraphPad Software Inc.) with 2-tailed one-way ANOVA and Bonferroni post hoc test or a 2-tailed unpaired *t*-test was used for statistical analyses. The Mann Whitney test was used to analyze the proportion of complex cells carrying an axon initial segment. Graphs show mean values with standard deviation as error bars. Significance: $P > 0.05$ ns, $P < 0.05$*, $P < 0.01$**, $P < 0.001$***.

Results

DCX-CreER^{T2}/Flox-EGFP transgenic mice were divided into 3 experimental groups and either received tamoxifen at 3 or 9 months of age and were perfused one week later (3m-t and 9m-t), or received tamoxifen at 3 months of age and were perfused 6 months later at the age of 9 months (3m-t→9) (see Material and Methods and Fig. 1A). We confirmed that the density of immature neurons expressing PSA-NCAM in the piriform cortex layer II of these mice (Fig. 1B–D, Supplementary Fig. S1) decreased in the course of aging as previously reported (Varea et al. 2009). Indeed, the density of PSA-NCAM$^+$ cells decreased significantly from 36 443 ± 13 468 cells/mm^3 in the 3-month-old transgenic mice to 7049 ± 7036 cells/mm^3 in the 9-month-old mice (two-tailed *t*-test, $P = 0.0002$***) (Fig. 1E). The density of GFP$^+$ cells in layer II of the piriform cortex was thereafter determined in the 3 experimental groups (Fig. 1B–D, Supplementary Fig. S1). Similar to the PSA-NCAM$^+$ cell population, the density of GFP$^+$ cells decreased significantly in 9-month-old mice when compared with 3-month-old (3m-t: 55 829 ± 19 444 cells/mm^3 vs. 9m-t: 5405 ± 2516 cells/mm^3, one-way ANOVA, $P = 0.0002$***) (Fig. 1F).

The overwhelming majority of GFP$^+$ cells expressed markers of immature neurons when animals were sacrificed 1 week

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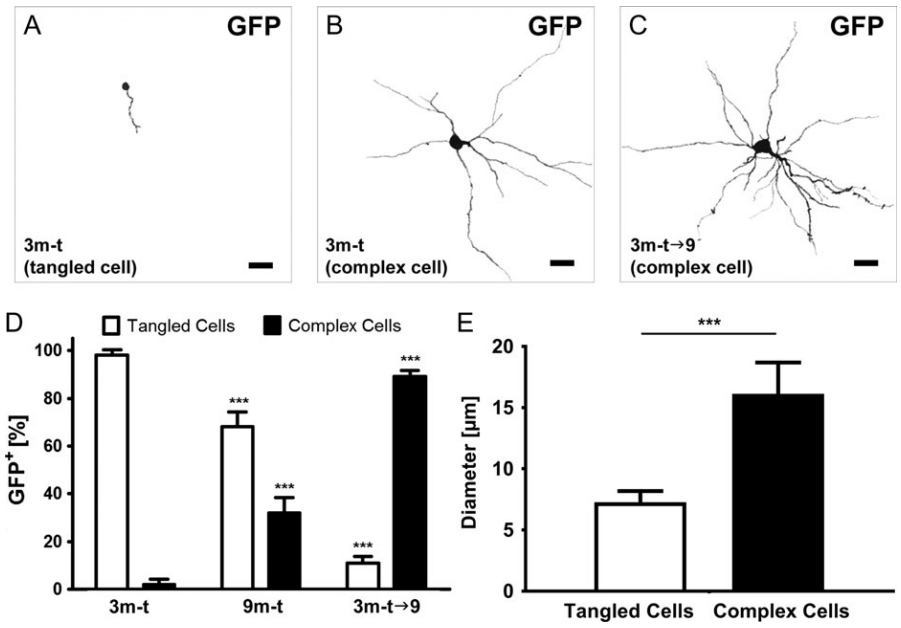

Figure 2. Maturation of immature neurons in the layer II of the piriform cortex. According to a 3D reconstruction, the majority of GFP$^+$ cells in group 3m-t exhibited a tangled cell morphology (*A*), whereas a smaller population could be classified as semilunar–pyramidal transitional neuron (*B*). When cells were given 6 months to mature (group 3m-t→9), cells morphologically increased in complexity (*C*). Scale bars for *A*–*C*: 15 µm. (*D*) In group 3m-t, the majority of GFP$^+$ cells could be morphologically classified as tangled cells and only a small fraction showed a more complex morphology. The tangled cell population decreased significantly in group 9m-t and 3m-t→9, while cells gained a more complex morphology. (*E*) Mean diameter of the soma of tangled cells in the 3m-t group in comparison to cells with a complex morphology of the 3m-t→9.

after tamoxifen administration, both at 3 months (PSA-NCAM$^+$ 85.7 ± 2.7%, DCX$^+$ 82.7 ± 2.0%, DCX$^+$/PSA-NCAM$^+$ 69.0 ± 2.1%) and at 9 months (PSA-NCAM$^+$ 63.1 ± 8.9%, DCX$^+$ 77.8 ± 3.3%, DCX$^+$/PSA-NCAM$^+$ 58.1 ± 8.6%) (Fig. 1*B*,*C*,*G*). These results indicated that shortly after tamoxifen induction at 3 months or 9 months of age, most GFP$^+$ cells in the piriform cortex layer II of our transgenic mice were immature neurons. BrdU was also injected in 2 different groups of transgenic mice, either at embryonic age E14–15 or at the age of 3 months, to label proliferating cells. Tamoxifen induction took place at 3 months of age in both groups. Overlap between BrdU and GFP-expression could be detected in the piriform cortex layer II only in mice that were labeled with BrdU at E14–15, but not in mice that received BrdU at the age of 3 months, thus confirming the postmitotic nature of the tangled cells in the adult brain (Supplementary Fig. S2). In contrast, numerous GFP-expressing cells had integrated BrdU in the dentate gyrus, a proliferative neurogenic niche of the adult mouse brain (Supplementary Fig. S2).

The number of GFP$^+$ cells coexpressing markers of immature neurons decreased dramatically when the animals were sacrificed 6 months after tamoxifen administration (3m-t→9) (PSA-NCAM$^+$ 4.7 ± 1.7%, $P < 0.0001^{***}$; DCX$^+$ 6.0 ± 1.4%, $P < 0.0001^{***}$; DCX$^+$/PSA-NCAM$^+$ 3.25 ± 1%, $P < 0.0001^{***}$) (Fig. 1*D*, Supplementary Fig. S1). In contrast, a nonsignificant reduction of the density of GFP-expressing cells was detected between the mice that were treated with tamoxifen at 3 months of age and sacrificed after one week and the mice that were sacrificed 6 months later (3m-t: 55 829 ± 19 444 cells/mm^3 vs. 3m-t→9:37 562 ± 16 173 cells/mm^3) (Fig. 1*F*). While it cannot be excluded that some tangled cells died along ageing, the large number of surviving cells expressing GFP in old mice and the prominent decline in expression of immature markers indicate that most of the GFP$^+$ cells, which were expressing DCX and/or PSA-NCAM in the piriform cortex at 3 months of age, had survived for 6 months and by then were no longer immature.

The distribution of GFP signal within expressing cells allows for a detailed morphological analysis (Fig. 2*A*–*C*). Mice of the 3m-t group exhibited a high proportion of small GFP$^+$ cells in layer II of the piriform cortex (98.0 ± 2.3% of GFP$^+$ cells; mean soma diameter 7.3 ± 0.9 µm), displaying a very low number of dendrites, and were therefore classified as tangled cells (Fig. 2*A*,*D*,*E*). Very few larger and morphologically complex cells could be detected (2.0 ± 2.3% of GFP$^+$ cells; mean soma diameter 14.6 ± 2.1 µm), which were classified as semilunar–pyramidal transitional neurons or intermediate cell types (Fig. 2*B*). By contrast, in the 9m-t group, the proportion of tangled cells significantly decreased to 68.0 ± 6.3% of GFP$^+$ cells ($P < 0.0001^{***}$), whereas the percentage of GFP$^+$ complex cells increased to 32.0 ± 6.3% of all GFP$^+$ cells ($P < 0.0001^{***}$) (Fig. 2*D*). Six months following tamoxifen administration (3m-t→9 group), GFP-expressing cells were densely intermingled and most of them exhibited a complex morphology (tangled cells 11.0 ± 2.8% vs. complex cells 89.0 ± 2.8%) (Fig. 2*D*). In comparison to the tangled cells of the 3m-t group, complex cells of the 3m-t→9 group had remarkably larger cell soma ($P < 0.001$) with average diameter equal to 16.1 ± 2.5 µm (Fig. 2*E*, Supplementary Table S1). These data indicate a progressive development from small tangled cells to complex cells resembling semilunar–pyramidal transitional neurons over time.

To further monitor this progressive maturation, we measured and compared the thickness of the principal apical dendrite of GFP$^+$ cells at a distance of 120 µm from the soma (Fig. 3*A*–*C*). Since tangled cells only have few stunted nonspinous dendrites, we compared the few complex GFP$^+$ cells in the 3m-t group with the complex GFP$^+$ cells found in the 3m-t→9 group. The apical dendrite thickness increased significantly in the 3m-t→9 group and reached 0.98 ± 0.30 µm as compared

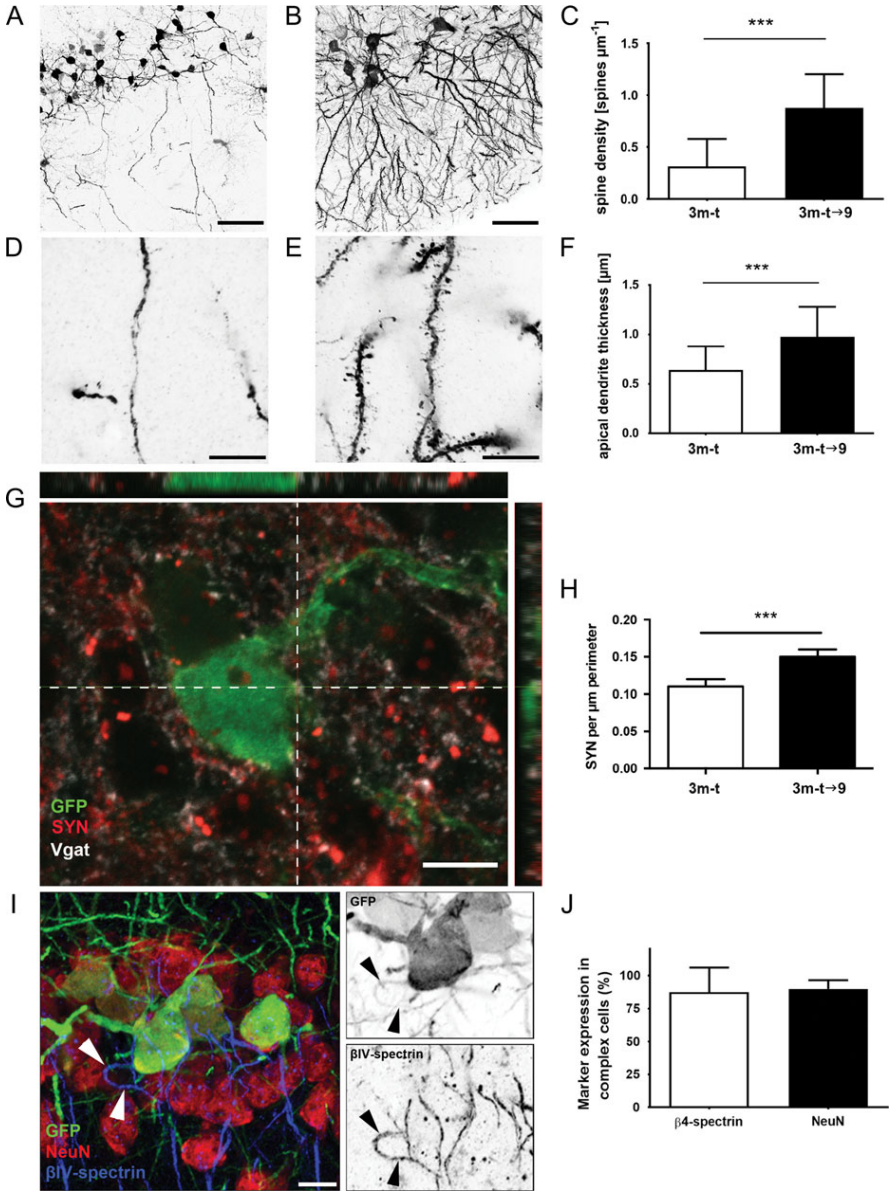

Figure 3. Morphological maturation of synapses, dendrites and axons over time. (*A–C*) Maturation of dendrites from GFP$^+$ cells projecting in the layer I. (*A*) Few thin and sparse dendrites were detected in the GFP$^+$ cells from the 3m-t group. (*B*) dense distribution of broader dendrites could be detected in the 3m-t→9 group. (*C*) The apical dendrite thickness increased significantly from group 3m-t to group 3m-t→9. Dendrites of cells in 3m-t mice were almost devoid of synaptic spine structures (*D*), while numerous spines were decorating the dendrites of group 3m-t→9 mice (*E*). (*F*) Comparison of dendritic spine densities between the 3m-t and 3m-t→9 groups suggesting functional integration of complex cells. Scale bars = 50 µm (*A*, *B*) and 10 µm (*D*, *E*). To measure the synaptic innervation of GFP$^+$ cells in layer II of the piriform cortex, a linear density of synaptophysin (SYN) containing puncta apposed to the cell soma of GFP$^+$ cells was calculated. (*G*) GFP$^+$ complex cell (green) surrounded by SYN (red) and VGAT (white) immunoreactive puncta. Scale bar = 10 µm. (*H*) The linear density of SYN significantly increased between GFP$^+$ cells found in group 3m-t compared with GFP$^+$ cells measured in group 3m-t→9. (*I*, *J*) In 2-year-old transgenic mice that received tamoxifen at 3 months of age, the large majority of GFP$^+$ complex cells were endowed with a βIV-spectrin-positive AIS and expressed the mature neuronal marker NeuN. Scale bar = 20 µm.

with 0.64 ± 0.24 µm in the 3m-t group (two-tailed *t*-test, $P < 0.0001^{***}$). This observation regarding apical dendrite thickness further indicates a maturation from the GFP$^+$ tangled cell morphology into GFP$^+$ semilunar–pyramidal transitional neurons with increasing complexity over the follow-up period of 6 months.

In order to functionally integrate into the existing network, immature neurons must be able to receive inputs via their dendritic tree and further propagate the information in form of action potentials. Therefore, we analyzed the density of spines decorating a 40 µm dendritic segment starting from a distance of 100 µm from the cell body (Fig. 3*D–F*). The spine density detected on GFP-labeled dendrites increased markedly and significantly during the 6 months separating the 3m-t group (0.31 ± 0.27 spines/µm, $n = 25$) from the 3m-t→9 group (0.88 ± 0.32 spines/µm, $n = 25$; 2-tailed *t*-test, $P < 0.0001^{***}$) Structural integration of immature neurons into the surrounding neuronal network was further examined using the linear density of synaptophysin (SYN) containing puncta, a marker of active synapses, apposed to the soma of GFP$^+$ cells. This parameter has been described as a surrogate marker of synaptic integration (Calhoun et al. 1996). The density of SYN$^+$-puncta on GFP$^+$

cells increased significantly over time (3m-t: 0.11 ± 0.01 puncta/μm vs. 3m-t→9:0.15 ± 0.01 puncta/μm; one-way ANOVA, P = 0.012*), demonstrating that neurons of the surrounding network increased their connectivity with the maturing GFP$^+$ neurons (Fig. 3G,H). Similar results were observed when analyzing the density of VGAT immunoreactive puncta (3m-t: 0.1 ± 0.01 puncta/μm vs. 3m-t→9:0.14 ± 0.01 puncta/μm; one-way ANOVA, $P < 0.0001$***).

Similarly, we investigated the appearance of an axonal initial segment (AIS), a specialized axonal microdomain usually localized at the emergence of the axon (Leterrier 2016). It fulfills 2 main functions: 1) it is the initiation site of action potentials, with a 40-fold higher density of voltage-gated sodium channels compared with the soma (Kole and Stuart 2012) and 2) it has been shown to be a major player for the regulation of cellular excitability (Wefelmeyer et al. 2016). A main molecular component of the AIS is the master scaffolding protein AnkyrinG (AnkG), which in turn recruits other proteins enriched at the AIS (Jones and Svitkina 2016). Voltage-gated sodium channels, voltage-gated potassium channels, as well as scaffolding proteins such as βIV-spectrin and neurofascins cluster at the AIS (Yoshimura and Rasband 2014). Although the presence of an AIS based on the detection of βIV-spectrin could not be detected in tangled cells, the vast majority of immature neurons with a complex morphology displayed a clear AIS. In transgenic mice treated with tamoxifen at 3 months of age and analyzed at 2 years, AIS could be detected in 87 ± 18% of GFP-expressing cells (Fig. 3I,J). At this time point, 90% ± 6% of the GFP$^+$ complex cells expressed NeuN, thereby substantiating further the advanced structural and functional maturation of the labeled cells.

The possibility that induction of GFP expression occurs stochastically in the absence of tamoxifen was investigated. Such a leakiness of the system could lead to the accumulation of GFP-expressing mature neurons over time. We therefore quantified the number of GFP$^+$ neurons in layer II of the piriform cortex of 2-year-old transgenic DCX-CreER^{T2}/Flox-EGFP mice, which either received tamoxifen at the age of 3 months or remained untreated. Without the application of tamoxifen, we detected 1310 ± 1388 GFP$^+$ cells/mm^3 in the piriform cortex of the naive 2-year-old transgenic mice, whereas 44 590 ± 11 309 GFP$^+$ cells/mm^3 could still be detected in the 2-year-old transgenic mice induced with tamoxifen at the age of 3 months, corresponding to a leakiness of less than 3% over 2 years (Supplementary Fig. S3). Hence, we concluded that the leakiness of our transgenic DCX-CreER^{T2}/Flox-EGFP model did not interfere with the fate analysis of the tangled cells.

We subsequently analyzed the expression of a collection of mature neuronal and glial markers in GFP$^+$ cells in layer II of the piriform cortex of our 3 experimental groups, with a particular attention to the fate of GFP$^+$ cells 6 months after tamoxifen administration (3m-t→9). Three types of excitatory neurons were examined, namely dopaminergic neurons (expressing tyrosine hydroxylase, TH), cholinergic neurons (expressing choline acetyltransferase, ChAT) and glutamatergic principal neurons (expressing the transcription factor T-Box brain 1 (Tbr1) or Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)). Tbr1 was coexpressed in virtually all GFP$^+$ cells (3m-t: 100%; 9m-t: 91.5 ± 3.4%; 3m-t→9:100%) (Fig. 4A–C,L, Supplementary Fig. S4). The proportion of GFP$^+$CaMKII$^+$ coexpressing cells increased significantly between the 3m-t group (2.0 ± 2.3% of GFP$^+$ cells) and 9m-t group (12 ± 2.3% of GFP$^+$ cells; one-way ANOVA, $P <$ 0.05*) and this increase was even higher in GFP$^+$ cells that were given 6 months to mature (3m-t→9: 80.0 ± 7.7%; one-way ANOVA, $P < 0.0001$***) (Fig. 4D–F,M, Supplementary Fig. S4). No GFP$^+$ cells were found to coexpress TH (data not shown) or ChAT (Fig. 4G). A few cells (6 out of 1000 GFP$^+$ cells) were found to express GAD67, a marker for GABAergic neurons. These GFP$^+$GAD67$^+$ cells were found exclusively in the 9m-t group, while this scarce cell population was not detected in the other experimental groups (Fig. 4H, Supplementary Fig. S5). The detection of glial fibrillary acidic protein (GFAP) was used to identify astrocytes, in addition to the transcription factor SRY-box 2 (Sox2), which is expressed in astrocytes as well as in neural stem cells. No GFP$^+$ cell was found to coexpress GFAP and/or Sox2 in any of the 3 experimental groups (Fig. 4I,J). This indicates that the vast majority of former immature GFP$^+$ neurons became mature glutamatergic neurons within the 6 months after induction of the GFP reporter gene.

A small population of GFP$^+$ cells in our study presented a morphology resembling that of polydendrocytes and expressed NG2 (Fig. 4K, Supplementary Fig. S4). Previous studies have also reported a subpopulation of PSA-NCAM/DCX$^+$ cells expressing NG2 in layer II of the piriform cortex of both mice and rats (Gómez-Climent et al. 2008; Rubio et al. 2016). Therefore, we further analyzed the NG2$^+$ population by measuring its density in layer II of the piriform cortex in all 3 experimental groups. Our comparison did not reveal any statistical difference in the NG2$^+$ cell densities of 3 and 9-month-old mice (3m-t: 14 622 ± 3432 NG2$^+$ cells/mm^3; 9m-t: 15 510 ± 2845 NG2$^+$ cells/mm^3; 2-tailed t-test, P = 0.6677) (Fig. 4O). However, the GFP$^+$NG2$^+$ cells density was slightly decreased following a survival of 6 months post tamoxifen administration, that is, 3m-t versus 3m-t→9 (3m-t: 7387 ± 844; 9m-t: 4861 ± 521; 2-tailed t-test P = 0.0142*), thus, suggesting a slow turnover of this cell population (Fig. 4N).

Discussion

In this study, we detected and mapped the fate of immature neurons in the murine piriform cortex layer II, by the use of the transgenic DCX-CreER^{T2}/Flox-EGFP mouse. We demonstrate that immature neurons in the piriform cortex do not die in the course of aging, but structurally integrate as newly matured neurons, as hypothesized by previous studies (Gómez-Climent et al. 2008; Varea et al. 2011; Yang et al. 2015; Rubio et al. 2016). This conclusion was supported by the pattern of marker expression together with the morphological features of GFP$^+$ cells after tamoxifen induction, including the sprouting of synapses and axons. Further evidence against the elimination of immature neurons in the piriform cortex via cell death comes from the lack of elevated TUNEL activity (Xiong et al. 2008) or the absence of pyknotic nuclei (Gómez-Climent et al. 2010). GFP$^+$ cells in layer II of the piriform cortex were found to be postmitotic. Hence, application of BrdU during adulthood did not label any neurons of the piriform cortex. However, in line with previous studies (Gómez-Climent et al. 2008; Luzzati et al. 2009; Zhang, Cai, et al. 2009; Rubio et al. 2016), we could detect abundant incorporation of BrdU at E14–15, which remained detectable in the immature neurons of the piriform cortex at 3 months of age. Under physiological conditions, newly generated cells in the rodent adult piriform cortex thus appear to be marginal and might originate from cells migrating out of the SVZ (Shapiro et al. 2007).

Virtually all GFP$^+$ immature neurons in the piriform cortex expressed the Tbr1 transcription factor, suggesting a determined glutamatergic phenotype and fate. This observation substantiated previous reports indicating that immature neurons in the piriform cortex express Tbr1 (Gómez-Climent et al. 2008;

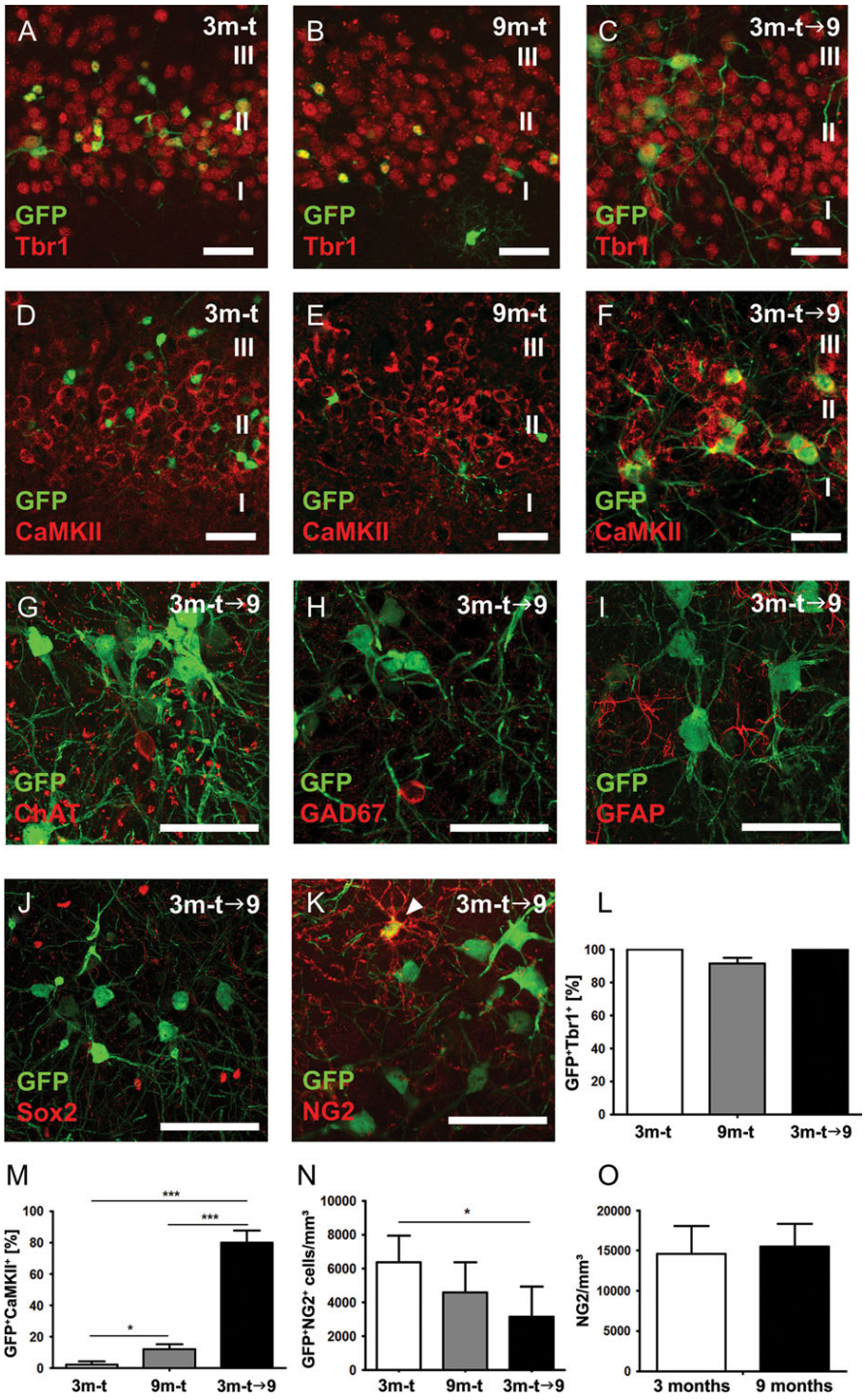

Figure 4. Fate of GFP$^+$ cells. (*A–C*) Virtually all GFP-expressing cells in layer II of the piriform cortex coexpressed Tbr1, a transcription factor expressed in glutamatergic neurons. (*D–F*) Expression of CaMKII, a marker of mature glutamatergic neurons could be detected in GFP$^+$ cells of all 3 experimental groups at various levels. (*G*) GFP$^+$ cells did not coexpress the cholinergic neuronal marker ChAT. (*H*) GFP$^+$ cells only rarely expressed the GABAergic marker GAD67 (see also Supplementary Fig 5). (*I–J*) No coexpression with astrocyte and stem cell markers GFAP and Sox2 was detected. (*K*) A small subpopulation of GFP-expressing cells with polydendrocytic morphology expressed NG2. (*M*) The coexpression of CaMKII in GFP$^+$ cells increased significantly between the 3m-t group and 9m-t group and increased to even higher extent in GFP$^+$ cells that were given 6 months to mature. (*N*) The density of GFP$^+$NG2$^+$ cells did not significantly differ between groups 3m-t and 9m-t, however, if GFP-labeled cells were analyzed 6 months post tamoxifen administration (3m-t→9), their density was significantly decreased compared with 3m-t. (*O*) The density of NG2-expressing cells in layer II of the piriform cortex remained stable comparing 3- and 9-month-old mice. Scale bars = 50 μm.

Luzzati et al. 2009; Rubio et al. 2016), while they were very rarely found to express the LIM/homeobox protein Lhx6 or distal-less DLL, arguing therefore for a pallial origin, rather than subpallial structures in which most cortical interneurons originate (Luzzati et al. 2009). In contrast to Tbr1, CaMKII, a Ca^{2+}/calmodulin-dependent protein kinase, is expressed only in

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mature principal neurons. We observed that coexpression of GFP with CaMKII, which was marginal shortly after application of tamoxifen in DCX-CreER^{T2}/Flox-EGFP, irrespective of the age of mice (3m-t and 9m-t groups), impressively increased to approximately 80% following a maturation period of 6 months. This marker profile suggests the progressive maturation of tangled cells into mature glutamatergic neurons.

To explore the integration of these "newly matured" glutamatergic neurons within the neuronal network, we analyzed morphological correlates of functionality, namely, dendritic spines and axon initial segments (AIS). Upon 6 months of maturation, the apical dendrites of GFP$^+$ complex cells in layer I displayed an increase in their thickness and in the linear density of spines, suggesting an increase in synaptic input.

Similarly, we detected the appearance of an AIS upon maturation. Since AIS are necessary and sufficient structures to define any cell as a neuron able to generate action potentials, an effective strategy for tracing the transition between tangled cells and neurons is to evaluate the maturation of an AIS according to the presence of AIS-specific scaffolding proteins (Gutzmann et al. 2014; Yoshimura and Rasband 2014; Schlüter et al. 2017). Although tangled cells were devoid of AIS, based on the detection of βIV-spectrin, this key feature of neuronal identity was present in the vast majority of GFP-expressing cells that underwent 6 months of maturation. Taken together, we observed that the GFP-expressing cells, which corresponded to tangled cells shortly after tamoxifen application, adopted a morphology consistent with the functional semilunar–pyramidal transitional neurons or pyramidal neurons upon 6 months of maturation.

Moreover, the surrounding neuronal network increasingly contacted the GFP-expressing immature neurons upon maturation. The analysis of SYN$^+$ and VGAT$^+$ perisomatic puncta, which correspond to presynaptic structures that could be used as a surrogate marker of synaptic integration (Calhoun et al. 1996), has revealed that, regardless of the age of the mice analyzed, the densities of these puncta on tangled cells were very low. In contrast, in the 3m-t→9 group, the GFP$^+$ neurons which acquired a complex morphology at this time point had significantly higher density of these SYN$^+$ and VGAT$^+$ presynaptic structures, arguing for an increased synaptic connectivity with the surrounding network. These observations are in accordance with a study performed in the piriform cortex by Klempin and colleagues (2011), who reported that cells with a low DCX expression and a complex morphology, reminiscent of immature semilunar–pyramidal transitional neurons, exhibited an electrophysiological profile consistent with neurons undergoing maturation (Klempin et al. 2011).

Studies investigating various cortical structures in different mammalian species suggested that immature cortical neurons located in layer II can mature into GABAergic interneurons (Xiong et al. 2008; Cai et al. 2009; Zhang, Zhang, et al. 2009; König et al. 2016). This possibility that some GFP$^+$ neurons belong to an interneuronal population was examined using the marker GAD67 expressed in inhibitory neurons (Young and Sun 2009). Only a few GFP$^+$/GAD67$^+$ cells could be detected in the 9m-t group. Due to the very low abundance of this cell population, we can neither exclude that they are the result of a labeling artifact, nor that they remained undetected in the 2 other experimental groups. On the other hand, we could clearly exclude that GFP$^+$ cells in the piriform cortex undergo cholinergic or dopaminergic maturation.

Intriguingly, we found low frequency of coexpression of the proteoglycan NG2 in GFP$^+$ cells. Previous studies in rats and mice have also indicated that a small subpopulation of NG2-expressing cells in the piriform cortex layer II coexpressed markers usually found in immature neurons (Gómez-Climent et al. 2008; Rubio et al. 2016). NG2$^+$ cells have been generally considered oligodendrocyte precursors, but many of them remain in the adult CNS as polydendrocytes, a specific glial cell type displaying a distinct morphology capable of lineage plasticity (Nishiyama et al. 2009; Huang et al. 2014). However, in contrast to the PSA-NCAM$^+$ and DCX$^+$ cell populations progressively disappearing, the density of NG2$^+$ cells in layer II remained stable during aging. The very low number of GFP$^+$ cells expressing NG2 and the absence of significant cell proliferation, rules out this cell population as potential source for the numerous glutamatergic neurons observed in the 3m-t→9. Similarly, the absence of coexpression of GFP, shortly after tamoxifen application or following 6 months of maturation, with GFAP or Sox2 ruled out the association with an astrocytic or neural stem cell phenotype.

The mechanisms enabling postmitotic tangled cells to survive in a dormant state during adulthood still have to be deciphered. Similarly, the nature of the signals triggering completion of their neuronal maturation processes and the functional relevance of such immature cells in the piriform cortex remain largely unknown. Although the maturation of the immature neurons in the piriform cortex does not seem to play a crucial role in olfactory memory, an activity-dependent control of maturation has been suggested by the observation that the number of PSA-NCAM/DCX-expressing cells dramatically decreased following bulbectomy, whereas the expression of mature markers increased (Gómez-Climent et al. 2011). The continuous maturation of glutamatergic neurons under physiological conditions offers a precious opportunity for neuronal plasticity in piriform cortex layer II. Analyses of the classical neurogenic niches demonstrated that the integration of immature neurons not only adds new synapses in the neuronal circuitry, but also showed that the activity of the surrounding neuronal network is profoundly influenced by immature neurons due to their distinct electrophysiological properties (Couillard-Despres et al. 2006; Ge et al. 2007).

In summary, we demonstrated that in layer II of the murine adult piriform cortex, a population of postmitotic immature neurons is continuously providing newly matured glutamatergic principal neurons, which structurally integrate in the surrounding neuronal network according to the increase in synaptic contacts and the appearance of an AIS. These findings suggest that new functional neurons could mature and integrate within in the adult central nervous system outside of the classical neurogenic niches. It is tempting to speculate that these cortical immature neurons, which are more widely distributed in mammals with gyrencephalic brains, such as humans (reviewed in König et al. 2016), progressively integrate into the neuronal network throughout adulthood and thereby provide an unsuspected capacity of network plasticity. Such matter will be of crucial relevance for the neurobiology of human aging, because the maturation and integration of postmitotic immature neurons will also result in a progressive depletion of this resource. In light of the reported scarce proliferative neurogenesis in the adult human brain (Sanai et al. 2011; Wang et al. 2011; Bergmann et al. 2012; Obernier et al. 2018), the existence of latent nonproliferative neuronal precursor populations in several brain areas might constitute an unsuspected resource for the plasticity of neuronal networks in higher mammals (König et al. 2016).

Supplementary Material

Supplementary material is available at *Cerebral Cortex* online.

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Notes

Conflict of Interest: None declared.

References

Abrous DN, Montaron MF, Petry KG, Rougon G, Darnaudéry M, Le Moal M, Mayo W. 1997. Decrease in highly polysialylated neuronal cell adhesion molecules and in spatial learning during ageing are not correlated. Brain Res. 744:285–292.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. 2002. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med. 8:963–970.

Bergmann O, Liebl J, Bernard S, Alkass K, Yeung MSY, Steier P, Kutschera W, Johnson L, Landén M, Druid H, et al. 2012. The age of olfactory bulb neurons in humans. Neuron. 74: 634–639.

Bernier PJ, Bedard A, Vinet J, Levesque M, Parent A. 2002. Newly generated neurons in the amygdala and adjoining cortex of adult primates. Proc Natl Acad Sci USA. 99:11464–11469.

Bonfanti L. 2006. PSA-NCAM in mammalian structural plasticity and neurogenesis. Prog Neurobiol. 80:129–164.

Bonfanti L, Nácher J. 2012. New scenarios for neuronal structural plasticity in non-neurogenic brain parenchyma: the case of cortical layer II immature neurons. Prog Neurobiol. 98:1–15.

Bonfanti L, Olive S, Poulain DA, Theodosis DT. 1992. Mapping of the distribution of polysialylated neural cell adhesion molecule throughout the central nervous system of the adult rat: an immunohistochemical study. Neuroscience. 49:419–436.

Bonfanti L, Peretto P. 2011. Adult neurogenesis in mammals—a theme with many variations. Eur J Neurosci. 34:930–950.

Brown JP, Couillard-Despres S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG. 2003. Transient expression of doublecortin during adult neurogenesis. J Comp Neurol. 467:1–10.

Cai Y, Xiong K, Chu Y, Luo D-W, Luo X-G, Yuan X-Y, Struble RG, Clough RW, Spencer DD, Williamson A, et al. 2009. Doublecortin expression in adult cat and primate cerebral cortex relates to immature neurons that develop into GABAergic subgroups. Exp Neurol. 216:342–356.

Calhoun ME, Jucker M, Martin LJ, Thinakaran G, Price DL, Mouton PR. 1996. Comparative evaluation of synaptophysin-based methods for quantification of synapses. J Neurocytol. 25:821–828.

Carceller H, Rovira-Esteban L, Nácher J, Castrén E, Guirado R. 2016. Neurochemical phenotype of reelin immunoreactive cells in the piriform cortex layer II. Front Cell Neurosci. 10:65.

Couillard-Despres S, Winner B, Karl C, Lindemann G, Schmid P, Aigner R, Laemke J, Bogdahn U, Winkler J, Bischofberger J, et al. 2006. Targeted transgene expression in neuronal precursors: watching young neurons in the old brain. Eur J Neurosci. 24:1535–1545.

Couillard-Despres S, Winner B, Schaubeck S, Aigner R, Vroemen M, Weidner N, Bogdahn U, Winkler J, Kuhn H-G, Aigner L. 2005. Doublecortin expression levels in adult brain reflect neurogenesis. Eur J Neurosci. 21:1–14.

Dayer AG, Cleaver KM, Abouantoun T, Cameron HA. 2005. New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. J Cell Biol. 168: 415–427.

De Nevi E, Marco-Salazar P, Fondevila D, Blasco E, Pérez L, Pumarola M. 2013. Immunohistochemical study of doublecortin and nucleostemin in canine brain. Eur J Histochem. 57:e9.

Ernst A, Alkass K, Bernard S, Salehpour M, Perl S, Tisdale J, Possnert G, Druid H, Frisén J. 2014. Neurogenesis in the striatum of the adult human brain. Cell. 156:1072–1083.

Feliciano DM, Bordey A. 2013. Newborn cortical neurons: only for neonates? Trends Neurosci. 36:51–61.

Feliciano DM, Bordey A, Bonfanti L. 2015. Noncanonical sites of adult neurogenesis in the mammalian brain. Cold Spring Harbor Perspect Biol. 7:a018846.

Ge S, Yang C-H, Hsu K-S, Ming G-L, Song H. 2007. A critical period for enhanced synaptic plasticity in newly generated neurons of the adult brain. Neuron. 54:559–566.

Gould E. 2007. How widespread is adult neurogenesis in mammals? Nat Rev Neurosci. 8:481–488.

Guirado R, Perez-Rando M, Sanchez-Matarredona D, Castillo-Gómez E, Liberia T, Rovira-Esteban L, Varea E, Crespo C, Blasco-Ibáñez JM, Nácher J. 2014. The dendritic spines of interneurons are dynamic structures influenced by PSA-NCAM expression. Cereb Cortex. 24:3014–3024.

Guo F, Maeda Y, Ma J, Xu J, Horiuchi M, Miers L, Vaccarino F, Pleasure D. 2010. Pyramidal neurons are generated from oligodendroglial progenitor cells in adult piriform cortex. J Neurosci. 30:12036–12049.

Gutzmann A, Ergül N, Grossmann R, Schultz C, Wahle P, Engelhardt M. 2014. A period of structural plasticity at the axon initial segment in developing visual cortex. Front Neuroanat. 8:11.

Gómez-Climent MA, Castillo-Gómez E, Varea E, Guirado R, Blasco-Ibáñez JM, Crespo C, Martínez-Guijarro FJ, Nàcher J. 2008. A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. Cereb Cortex. 18:2229–2240.

Gómez-Climent MA, Guirado R, Varea E, Nàcher J. 2010. "Arrested development." Immature, but not recently generated, neurons in the adult brain. Arch Ital Biol. 148:159–172.

Gómez-Climent MA, Hernández-González S, Shionoya K, Belles M, Alonso-Llosa G, Datiche F, Nàcher J. 2011. Olfactory bulbectomy, but not odor conditioned aversion, induces the differentiation of immature neurons in the adult rat piriform cortex. Neuroscience. 181:18–27.

He X, Zhang X-M, Wu J, Fu J, Mou L, Lu D-H, Cai Y, Luo X-G, Pan A, Yan X-X. 2014. Olfactory experience modulates immature neuron development in postnatal and adult guinea pig piriform cortex. Neuroscience. 259:101–112.

Huang W, Zhao N, Bai X, Karram K, Trotter J, Goebbels S, Scheller A, Kirchhoff F. 2014. Novel NG2-CreERT2 knock-in mice demonstrate heterogeneous differentiation potential of NG2 glia during development. Glia. 62:896–913.

Jin K, Sun Y, Xie L, Peel A, Mao XO, Batteur S, Greenberg DA. 2003. Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. Mol Cell Neurosci. 24: 171–189.

Jones SL, Svitkina TM. 2016. Axon initial segment cytoskeleton: architecture, development, and role in neuron polarity. Neural Plast. 2016:1–19.

Klempin F, Kronenberg G, Cheung G, Kettenmann H, Kempermann G. 2011. Properties of doublecortin-(DCX)-expressing cells in the piriform cortex compared to the neurogenic dentate gyrus of adult mice. PLoS One. 6:e25760.

Kole MHP, Stuart GJ. 2012. Signal processing in the axon initial segment. Neuron. 73:235–247.

König R, Benedetti B, Rotheneichner P, O' Sullivan A, Kreutzer C, Belles M, Nácher J, Weiger TM, Aigner L, Couillard-Despres S. 2016. Distribution and fate of DCX/PSA-NCAM expressing cells in the adult mammalian cortex: a local reservoir for adult cortical neuroplasticity? Front Biol. 11: 193–213.

Leterrier C. 2016. The axon initial segment, 50 years later: a nexus for neuronal organization and function. Curr Top Membr. 77:185–233.

Luzzati F, Bonfanti L, Fasolo A, Peretto P. 2009. DCX and PSA-NCAM expression identifies a population of neurons preferentially distributed in associative areas of different pallial derivatives and vertebrate species. Cereb Cortex. 19: 1028–1041.

Nishiyama A, Komitova M, Suzuki R, Zhu X. 2009. Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. Nat Rev Neurosci. 10:9–22.

Nàcher J, Crespo C, McEwen BS. 2001. Doublecortin expression in the adult rat telencephalon. Eur J Neurosci. 14:629–644.

Nácher J, Alonso-Llosa G, Rosell D, McEwen B. 2002. PSA-NCAM expression in the piriform cortex of the adult rat. Modulation by NMDA receptor antagonist administration. Brain Res. 927:111–121.

Ní Dhúill CM, Fox GB, Pittock SJ, O'Connell AW, Murphy KJ, Regan CM. 1999. Polysialylated neural cell adhesion molecule expression in the dentate gyrus of the human hippocampal formation from infancy to old age. J Neurosci Res. 55:99–106.

Obernier K, Cebrián-Silla A, Thomson M, Parraguez JI, Anderson R, Guinto C, Rodriguez JR, Garcia-Verdugo JM, Alvarez-Buylla A. 2018. Adult neurogenesis is sustained by symmetric self-renewal and differentiation. Stem Cell. 22:221–234.e228.

Patzke N, LeRoy A, Ngubane NW, Bennett NC, Medger K, Gravett N, Kaswera-Kyamakya C, Gilissen E, Chawana R, Manger PR. 2014. The distribution of doublecortin-immunopositive cells in the brains of four afrotherian mammals: the Hottentot golden mole (Amblysomus hottentotus), the rock hyrax (Procavia capensis), the eastern rock sengi (Elephantulus myurus) and the four-toed sengi (Petrodromus tetradactylus). Brain Behav Evol. 84:227–241.

Pekcec A, Löscher W, Potschka H. 2006. Neurogenesims in the adult rat piriform cortex. NeuroReport. 17:571–574.

Rivers LE, Young KM, Rizzi M, Jamen F, Psachoulia K, Wade A, Kessaris N, Richardson WD. 2008. PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. Nat Neurosci. 11:1392–1401.

Rubio A, Belles M, Belenguer G, Vidueira S, Fariñas I, Nàcher J. 2016. Characterization and isolation of immature neurons of the adult mouse piriform cortex. Dev Neurobiol. 76:748–763.

Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai H-H, Wong M, Gupta N, Berger MS, Huang E, Garcia-Verdugo JM, et al. 2011. Corridors of migrating neurons in the human brain and their decline during infancy. Nature. 478:382–386.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al. 2012. Fiji – an Open-Source platform for biological image analysis. Nat Methods. 9:676–682.

Schlüter A, Del Turco D, Deller T, Gutzmann A, Schultz C, Engelhardt M. 2017. Structural plasticity of synaptopodin in the axon initial segment during visual cortex development. Cereb Cortex. 27:4662–4675.

Seki T, Arai Y. 1991. Expression of highly polysialylated NCAM in the neocortex and piriform cortex of the developing and the adult rat. Anat Embryol. 184:395–401.

Shapiro LA, Ng KL, Kinyamu R, Whitaker-Azmitia P, Geisert EE, Blurton-Jones M, Zhou Q-Y, Ribak CE. 2007. Origin, migration and fate of newly generated neurons in the adult rodent piriform cortex. Brain Struct Funct. 212:133–148.

Shapiro LA, Ng K, Zhou Q-Y, Ribak CE. 2009. Subventricular zone-derived, newly generated neurons populate several olfactory and limbic forebrain regions. Epilepsy Behav. 14 (Suppl 1):74–80.

Takemura NU. 2005. Evidence for neurogenesis within the white matter beneath the temporal neocortex of the adult rat brain. Neuroscience. 134:121–132.

Tamura Y, Kataoka Y, Cui Y, Takamori Y, Watanabe Y, Yamada H. 2007. Multi-directional differentiation of doublecortin- and NG2-immunopositive progenitor cells in the adult rat neocortex in vivo. Eur J Neurosci. 25:3489–3498.

Varea E, Belles M, Vidueira S, Blasco-Ibáñez JM, Crespo C, Pastor AM, Nácher J. 2011. PSA-NCAM is expressed in immature, but not recently generated, neurons in the adult cat cerebral cortex layer II. Front Neurosci. 5:17.

Varea E, Castillo-Gómez E, Gómez-Climent MA, Guirado R, Blasco-Ibáñez JM, Crespo C, Martínez-Guijarro FJ, Nácher J. 2009. Differential evolution of PSA-NCAM expression during aging of the rat telencephalon. Neurobiol Aging. 30:808–818.

Wang C, Liu F, Liu Y-Y, Zhao C-H, You Y, Wang L, Zhang J, Wei B, Ma T, Zhang Q, et al. 2011. Identification and characterization of neuroblasts in the subventricular zone and rostral migratory stream of the adult human brain. Cell Res. 21:1534–1550.

Wang C, You Y, Qi D, Zhou X, Wang L, Wei S, Zhang Z, Huang W, Liu Z, Liu F, et al. 2014. Human and monkey striatal interneurons are derived from the medial ganglionic eminence but not from the adult subventricular zone. J Neuroscii. 34:10906–10923.

Wefelmeyer W, Puhl CJ, Burrone J. 2016. Homeostatic plasticity of subcellular neuronal structures: from inputs to outputs. Trends Neurosci. 39:656–667.

Xiong K, Cai Y, Zhang X-M, Huang J-F, Liu Z-Y, Fu G-M, Feng J-C, Clough RW, Patrylo PR, Luo X-G, et al. 2010. Layer I as a putative neurogenic niche in young adult guinea pig cerebrum. Mol Cell Neurosci. 45:180–191.

Xiong K, Luo D-W, Patrylo PR, Luo X-G, Struble RG, Clough RW, Yan X-X. 2008. Doublecortin-expressing cells are present in layer II across the adult guinea pig cerebral cortex: partial colocalization with mature interneuron markers. Exp Neurol. 211:271–282.

Yang Y, Xie M-X, Li J-M, Hu X, Patrylo PR, Luo X-G, Cai Y, Li Z, Yan X-X. 2015. Prenatal genesis of layer II doublecortin expressing neurons in neonatal and young adult guinea pig cerebral cortex. Front Neuroanat. 9:109.

Yoshimura T, Rasband MN. 2014. Axon initial segments: diverse and dynamic neuronal compartments. Curr Opin Neurobiol. 27:96–102.

Young A, Sun Q-Q. 2009. GABAergic inhibitory interneurons in the posterior piriform cortex of the GAD67-GFP mouse. Cereb Cortex. 19:3011–3029.

Zhang X-M, Cai Y, Chu Y, Chen E-Y, Feng J-C, Luo X-G, Xiong K, Struble RG, Clough RW, Patrylo PR, et al. 2009. Doublecortin-expressing cells persist in the associative cerebral cortex and amygdala in aged nonhuman primates. Front Neuroanat. 3:17.

Zhang L-M, Zhang Y-Z, Liu Y-Q, Gong Z-H, Zhao Y-M, Li Y-F. 2009. CTN-986, a compound extracted from cottonseeds, increases cell proliferation in hippocampus in vivo and in cultured neural progenitor cells in vitro. Eur J Pharmacol. 607:110–113.

Zhang J, Giesert F, Kloos K, Weisenhorn DMV, Aigner L, Wurst W, Couillard-Despres S. 2010. A powerful transgenic tool for fate mapping and functional analysis of newly generated neurons. BMC Neurosci. 11:158.

CHAPTER 4

The enzymatic removal of PSA from the NCAM promotes neuronal differentiation in the piriform cortex of adult mice

The enzymatic removal of PSA from NCAM promotes neuronal differentiation in the piriform cortex of adult mice

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ABSTRACT: The layer II of the adult rodent piriform cortex contains a population of immature neurons that have been generated during embryonic stages. Two different cell types can be detected among these immature neurons: i) tangled cells, which are small cells with short neurites and ii) complex cells, which are more developed and resemble semilunar-pyramidal transitional neurons, a typical excitatory neuron in this cortical region. During the life span tangled cells progressively differentiate into complex cells, which in turn mature into semilunar-pyramidal transitional neurons. Both tangled and complex cells express immature neuronal markers such as doublecortin or the polysialylated form of the neural cell adhesion molecule (PSA-NCAM), the later confering them anti-adhesive properties and limiting the membrane space available for the establishment of synaptic contacts. There are two different enzymes which catalyze the addition of PSA to NCAM: the polysialyltransferases St8SiaII and St8SiaIV. PSA-NCAM expression is downregulated dramatically during the final differentiation of the piriform cortex immature neurons. In order to understand the role that PSA-NCAM may play in the development of the immature neurons in the adult piriform cortex layer II we have depleted PSA from NCAM in this region using the enzyme EndoN. We found that after EndoN injection the number of immature neurons expressing doublecortin decreased in layer II. Moreover, the percentage of tangled cells diminished, while that of complex cells was increased. The depletion of PSA-NCAM from the piriform cortex also increased the density of nuclei expressing NeuN, a mature neuronal marker, in layer II. This increase was paralleled by increases in the expression of the synaptic marker synaptophysin and MAP2, a marker of mature dendrites, in layer I. Together, these results indicate a pro-maturation effect of PSA depletion. We have also tested the effect of the absence of PSA through genetic deletions of both polysialyltransferases or NCAM. The deletion of both polysialyltransferases decreases dramatically the number of immature doublecortin expressing neurons in the adult piriform cortex. By contrast, the deletion of NCAM, increases this number, suggesting the presence of compensatory effects.

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Keywords: piriform cortex, PSA-NCAM, EndoN, polysialyltransferases, plasticity

INTRODUCTION

In the healthy adult mammalian brain, the presence of newly generated immature neurons is thought to be restricted to the two classical neurogenic niches: the subventricular zone/RMS/olfactory bulb and the subgranular zone of the dentate gyrus. However, a population of post-mitotic immature neurons can be also observed in the layer II of the piriform or olfactory cortex (PCX) of adult rodents and with a wider distribution in the cerebral cortex of gyrencephalic mammals (Gomez-Climent et al. 2010; Bonfanti & Nacher 2012). The PCX is a three-layered region, in which layer II contains the densely packed cell bodies of principal neurons. In this layer, particularly in its lower half, there is a population of cells expressing doublecortin (DCX), among other immature neuronal markers (Nacher et al. 2001; Nacher et al. 2002; Luzzati et al. 2009). Experiments in rats (Gomez-Climent et al. 2008), mice (Rubio et al. 2016) and guinea pigs (Yang et al. 2015) have demonstrated that most, if not all, the immature neurons in the adult cerebral cortex layer II have been generated during embryonic development.

Two populations of immature neurons, with different maturational stages, can be observed in the adult PCX layer II: small cells with very short and intricate processes (tangled cells/Type I) and larger cells (complex cells/Type II), which have a more developed morphology, similar to one of the typical excitatory neurons in this layer (semilunar-pyramidal transitional neurons, SLPTN). Interestingly, these immature neurons diminish their number[i] progressively as the life span progresses (Gomez-Climent et al. 2008; Xiong et al. 2008; Cai et al. 2009; Varea et al. 2009; Zhang et al. 2009). As previous studies

have not found evidence of an increased TUNEL activity in the cortical layer II in aged guinea pigs (Xiong et al. 2008) or elevated numbers of pyknotic nuclei during aging in rats (Gomez-Climent and Nacher, unpublished results), they allow us to suggest that tangled cells do not die, but instead they mature into complex cells, and subsequently into SLPTN. In fact, a recent study has demonstrated, using DCX-Cre inducible transgenic animals, that during the course of the life span the immature neurons in the PCX do not die and that they differentiate and incorporate to the circuitry as typical excitatory neurons (Rotheneichner et al. 2018).

The immature neurons in the cerebral cortex layer II also express the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) (Seki et al., 1991). The addition of PSA to NCAM confers antiadhesive properties to this molecule and, consequently, PSA-NCAM is widely expressed during neuronal development, participating in different phenomena such as migration or neurite extension (Rutishauser et al., 2008). In fact, PSA-NCAM is highly expressed in immature neurons, both during development and in the adult brain. In the immature neurons present in the adult brain, including those in the PCX layer II, PSA is added to NCAM by the polysialyltransferase St8SiaII (Nacher et al. 2010). It has been hypothesized that PSA-NCAM, through its anti-adhesive properties, isolates the immature neurons from the neuropil, probably preventing the formation of synaptic contacts onto these cells. The presence of PSA-NCAM may also promote the progressive growth of their dendritic arbors and axons once they have started their differentiation (Gomez-Climent et al. 2008; Bonfanti & Nacher 2012).

The PSA moiety can be successfully depleted from NCAM using an specific phage enzyme, the endoneuraminidase N (EndoN) (Vimr et al. 1984). This depletion *in vivo* results in different structural modifications in both excitatory and inhibitory neurons: When injected in the adult hippocampus it increases the dendritic arborization of CA3 pyramidal neurons (McCall et al. 2013) and alters the spine density of a subpopulation of interneurons (Guirado et al. 2014), whereas when injected in the prefrontal cortex, it increases the density of perisomatic inhibitory puncta on pyramidal neurons (Castillo-Gomez et al. 2016).

The objective of the present work is to study the impact of the enzymatic depletion of PSA from the population of immature neurons in the PCX layer II and to investigate whether this depletion may accelerate the differentiation of these cells. For this purpose, we have injected intracerebrally Endo-N and have studied this population of immature neurons by using DCX immunohistochemistry. In order to evaluate putative neurotoxic effects, we have evaluated cell death studying the presence of pyknotic nuclei. Additionally, we have explored whether PSA depletion may induce the differentiation of the immature neurons in the PCX layer II by analyzing the expression of the mature neuronal marker NeuN (Mullen et al. 1992). The expression of synaptophysin (Syn) and MAP2, in layer I have also been analyzed in order to evaluate the presence of new synapses and apical dendrites belonging to recently matured neurons. In addition, we have studied this population of immature neurons in the PCX of adult transgenic mice lacking PSA expression due to genetic deletions of NCAM (Cremer et al. 1994), the major carrier of PSA in the adult cerebral cortex, or of the 2 polysialyltransferases that attach PSA to NCAM (Hildebrandt et al. 2009).

MATERIALS AND METHODS

Animals and administration of EndoNeuraminidase-N

All animals were maintained under controlled conditions of temperature and humidity, with food and water *ad libitum* and normal light/dark cycle (lights on: 8:00–20:00). All animal experimentation was performed in accordance with the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and was approved by the Committee on Bioethics of the Universitat de València. Every effort was made to minimize the number of animals used and their suffering.

Twelve young–adult male (3 months-old) FVB/NJ mice (Jackson laboratories, Bar Harbor, Maine, USA) were used in this study. Mice were deeply anaesthetized (5 mg/g xylazine and 0.5mL/kg ketamine i.p.) and placed in a stereotaxic instrument (David Kopf Instruments). Using a 10 μL Flexitip syringe (World Precision Instruments), the animals were injected intracranially in the ectorhinal cortex (Bregma -2 mm, lateral 4 mm, deep -1.5 mm; Paxinos and Franklin 2001). The needle was left in position for 1 min and then 1 µL of the solution was injected, over a five minutes period in the right hemisphere. Seven animals were injected with EndoN (0.7 U/μL in glycerol; AbCys, Paris, France). When the injection was completed, the needle was left in place for 2 extra minutes to reduce reflux of the solution into the track of the injection needle and then withdrawn. In order to exclude effects due to the stereotaxic injection by itself, an additional group of five animals received a vehicle injection.

Histological processing

After recovery from the surgery, animals were returned to their original cages, where they remained for fifteen days. At this point, animals were perfused transcardially under deep pentobarbital anaesthesia (0.01mL/g in 0.9% NaCl solution, Sigma Aldrich), first with saline and then with 4% paraformaldehyde in sodium phosphate buffer 0.1 M, pH 7.4 (PB). Brains were extracted and sectioned with a

vibratome (Leica VT 1000E, Leica) and 50-µm-thick coronal sections (6 subseries) were collected and kept in cold PB (4°C).

Immunohistochemistry for conventional light microscopy

In order to check whether PSA in the PCX had been depleted by EndoN, one of the subseries of sections was used for "free-floating" immunohistochemistry using the avidin-biotin-peroxidase (ABC) method. In brief, for anti-PSA-NCAM staining, an antigen retrieval step was performed, consisting in a pretreatment with sodium citrate buffer (10 mM, pH 6.0) for 1 min at 100°C. After cooling down the sections to room temperature, they were incubated for 10 minutes with 3% H_2O_2 in 0.1 M phosphate-buffered saline (PBS) to block the endogenous peroxidase. Then, non-specific binding was blocked with 5% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories) in PBS with 0.2% Triton X-100 (Sigma-Aldrich) for 1 h. Sections were incubated for 48h at 4°C with mouse IgM anti-PSA-NCAM (1:700, Millipore). After rinsing with PBS, slides were incubated for 1 hour with biotinylated donkey anti-mouse IgM antibody (1:200; Jackson Immunoresearch) followed by an avidin-biotin-peroxidase complex (ABC, Vector Laboratories) for 30 min. Finally, sections were incubated with 3,3´-diaminobenzidine tetrahydrochloride (DAB, 0.5%, Sigma, St Louis, MO) and 0.0033% H_2O_2 for 4 min for color development.

The quantifications of the different parameters analysed in this study were performed in an "area of interest", considered as the region in which PSA was not present in the PCX after EndoN treatment. Since the extension of this region was very similar in all the animals analysed and its limits were always between Bregma -1.46 to Bregma -2.18 (anterio-posterior axis) and from 3 to 5'5 (dorso-ventral axis) of the ipsilateral hemisphere (figure 1), all the analysis have been performed between these two rostrocaudal levels.

Immunohistochemistry for fluorescence microscopy

For the immunofluorescence procedures, sections were processed for single or double-labelling with cell type-specific primary antibodies as follows: slices were first incubated "free-floating" in 10% normal donkey serum (NDS; AbCys) for 1 hour. Then, they were incubated overnight at room temperature with i) rabbit IgG anti-NG2 antibody (1:200, Millipore), ii) with a cocktail containing rabbit IgG anti-DCX (1:1000, Abcam) and Mouse IgG1 anti-NeuN (1:500, Millipore) antibodies or iii) with a cocktail of Guinea Pig anti-synaptophysin (Syn) (1:1000, Synaptic Systems) and mouse IgG anti-MAP2 (1:1000, sigma) antibodies. After rinsing with PBS, sections were incubated for 1 hour at room temperature with Goat anti-Mouse IgG1 A647, Goat anti-Rabbit A555 or with a Donkey anti-mouse IgG A555 and Goat anti-Guinea pig A635 (1:400, Invitrogen) secondary antibodies**.** Finally, sections were mounted on slides and covered using Fluorescence Mounting Medium with DAPI (Dako Omnis). All serum, primary and secondary antibodies were diluted in PBS with 0.2% Triton-X 100 (Sigma). All the sections were processed simultaneously in order to minimize any difference from immunohistochemical staining itself. To avoid any bias, all slides were coded prior to the analysis and the codes were not broken until the experiment was finished.

Sections processed for immunohistochemistry were observed under a confocal microscope (Leica TCS-SPE) using a 63x objective or with a conventional fluorescence microscope (Olympus BX41). Stacks of images with Z-step size of 0,5µm were taken without any digital zoom.

Quantification

To measure the impact of the depletion of PSA from NCAM on immature neurons, using DCX immunohistochemistry, we quantified the density of DCX expressing cells inside the total volume of the region of interest, using confocal Z-stacks, comparing between hemispheres. Moreover, as there are two different subpopulations of DCX expressing cells (tangled and complex cells), which can be distinguished by their morphological characteristics, we measured as well the percentage of each of these subpopulations.

In order to detect EndoN-induced changes in the density of mature neurons in layer II, we measured the density of NeuN expressing nuclei in 96-128 randomly located squares (single confocal planes, 50 x 50 µm) in all the extension of the area of interest. Four slices of the region of interest were analysed in each animal (24-32 squares analysed per section). The mature neuronal marker NeuN cannot be detected in the nuclei of tangled cells and it is only faintly expressed in some complex cells (Gomez-Climent et al. 2008). We also investigated the putative differentiation of immature neurons induced by EndoN treatment by measuring in layer I the expression of MAP2, a protein strongly expressed in mature dendrites but absent from the dendrites tangled cells and only faintly expressed in those of the complex cells (Gomez-Climent et al. 2008). Tangled cells, which comprise the majority of immature neurons in the PCX layer II, do not have apical dendrites extending to layer I. We quantified dendritic processes in the deeper portion of layer I (30 micrometers from the limit between layer II and I) by measuring the fluorescence intensity of MAP2 immunostaining in 96-128 randomly located squares (single confocal planes, 50 x 50 µm) inside the area of interest, as described above for NeuN analysis. We also explored putative increases in synapse density due to the development of new postsynaptic elements on the EndoN-induced recently matured neurons. For this, the density of active synapses

was estimated using synaptophysin immunohistochemistry and the same methodology employed for MAP2 analysis.

A NG2 fluorescent immunostaining, combined with DAPI, was used to quantify the number of polydendrocytes (or NG2+ cells), and the presence of cell death (pyknotic nuclei), respectively. To do so, we quantified the density of NG2+ cells inside the total volume of the region of interest in the PCX layer II, and compared it with the density of NG2+ cells in the same area of the contralateral hemisphere. The same procedure was used to analyse the density of pyknotic nuclei.

Analysis of NCAM$^{-/-}$ and St8SiaII$^{-/-}$ St8SiaIV$^{-/-}$ knockout mice

We have also studied the consequences of the elimination of PSA by using genetic approaches. We studied this in two knockout mice strains, one lacking NCAM, the main carrier of PSA in the CNS, and one lacking the two polysialyltransferases that add this complex sugar to NCAM. We used 5 NCAM$^{-/-}$ mice and 5 wild-type littermates (Cremer et al. 1994); 3 St8SiaII$^{-/-}$ St8SiaIV$^{-/-}$ mice and 3 wild-type littermates (Eckhardt et al. 2000; Angata et al. 2004). When mice were 3-4 months old, they were deeply anesthetized, perfused transcardially and their brains were processed for histology as described above. Conventional immunohistochemistry for bright field microscopy was performed to detect DCX expressing cells in these transgenic mice as described above for PSA-NCAM, but using rabbit IgG anti-DCX (1:1000, Abcam) as primary antibody and biotinylated donkey anti-rabbit IgG as secondary antibody (1:200; Jackson Immunoresearch). The number of DCX immunoreactive cells was estimated using a modified version of the fractionator method (West et al. 1993; Nacher et al. 2002).

Statistics

All data were analyzed using two-tailed paired t-tests (except the sham vs control analysis, which was unpaired t-test) comparing the hemisphere injected with EndoN and the contralateral one. GraphPad Prism 5 software (GraphPad Software Inc.) was used for statistical analyses. Significance: P>0.05 ns, 0.05<p<0,1 #, P<0.05 *, P<0.01 **, P<0.001 ***.

RESULTS

Enzymatic depletion of PSA-NCAM

In all animals injected with EndoN, we observed that the expression of PSA-NCAM in the portion of the PCX located between coordinates Bregma -1.46 and -2.18 of the ipsilateral hemisphere was extremely low in comparison with the contralateral hemisphere, which showed the habitual intensity and distribution of immunolabeling (Nacher et al. 2002) (figure 1). Consequently, all the microscopic analyses were performed between these two rostrocaudal levels.

The injection of EndoN markedly diminished the density of DCX expressing cells in the PCX layer II of the ipsilateral hemisphere when compared with the contralateral one (p<0,01, figures 2A, D & E). No differences in the density of DCX expressing cells were detected when comparing the hemispheres of animals injected with vehicle (P>0.05) (Figure 3).

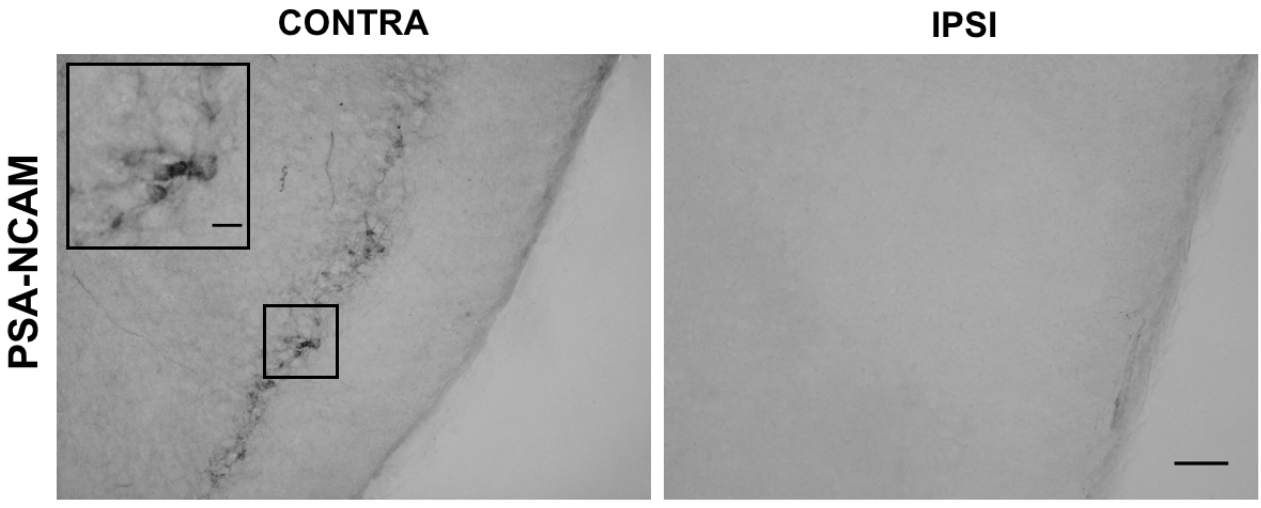

Figure 1. EndoN injection depletes PSA from the PCX. Images of PSA-NCAM immunostained sections showing the posterior PCX of the contralateral (control, left) and ipsilateral (injected, right) hemispheres. Inset shows an enlarged view of the squared area. Scale bar = 40µm. Scale bar of the inset = 8 µm.

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In order to better understand the effects of EndoN, we subdivided the analysis of the density of DCX expressing neurons by cell type. The DCX expressing immature neurons in the PCX layer II can be classified in 2

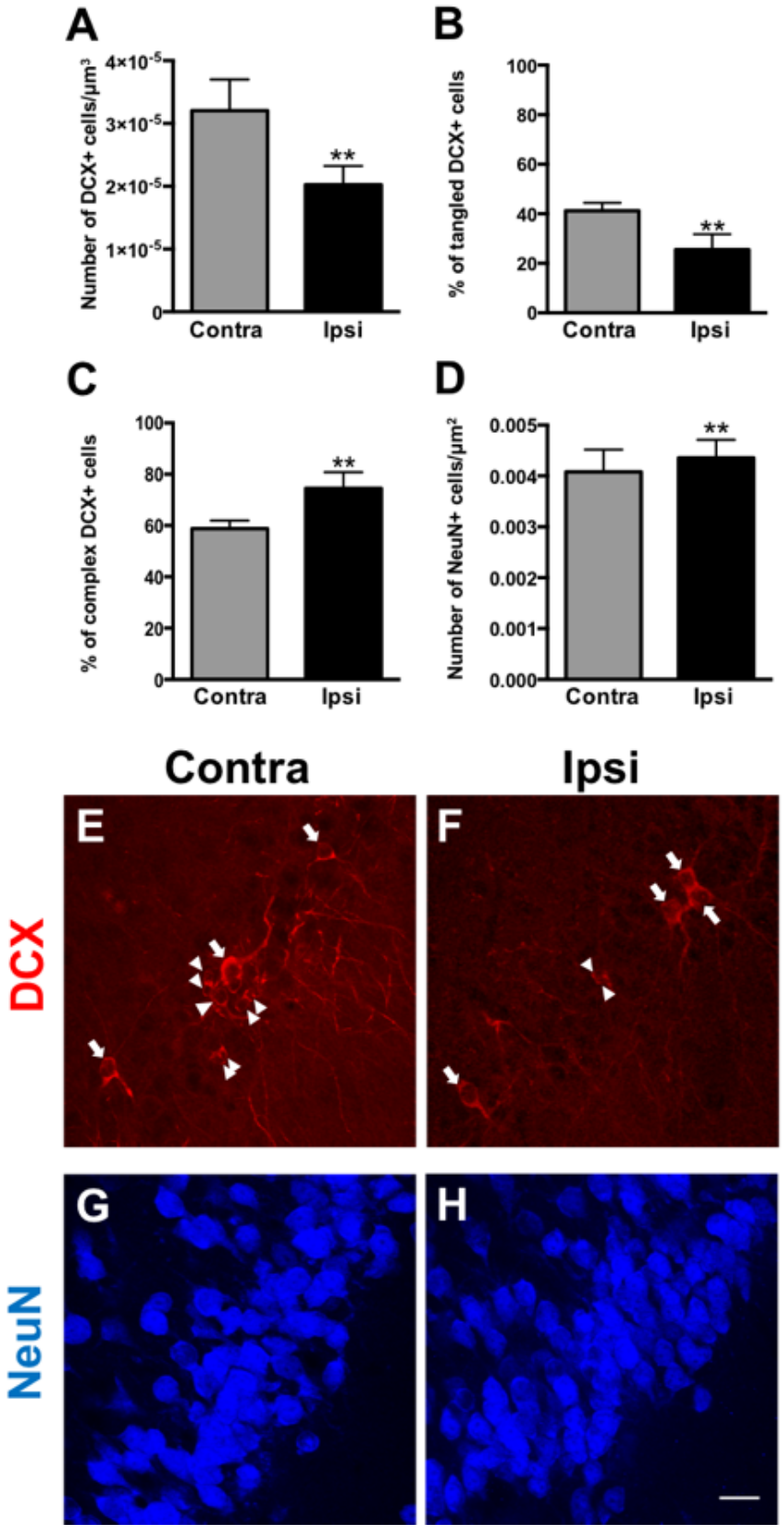

Figure 2. EndoN injection decreases the density of DCX expressing cells while it increases the density of NeuN expressing cells in the PCX layer II. **A**: The density of DCX expressing cells is significantly lower in the ipsilateral hemisphere, after the injection of EndoN ($p<0,01$). **B & C:** This decrease does not affect equally the different subpopulations of DCX expressing cells: the percentage of tangled cells is significantly lower ($p<0,01$) (B), and the percentage of complex cells significantly higher ($p<0,01$) (C) in the injected hemisphere. **E & F:** Microphotographs of DCX expressing cells in the PCX layer II of the contralateral (E), and ipsilateral (F) hemisphere. Complex cells are indicated with arrows and tangled cells are tagged with arrowheads. **D:** The density of NeuN expressing nuclei is significantly higher in the ipsilateral hemisphere ($p<0,01$). **G & H**: Microphotographs of NeuN immunoreactive nuclei in the contralateral (G) and ipsilateral (H) hemispheres. Microphotographs in this figure are Z stack 2D projections of 6 confocal planes located 0.5 µm apart (z step size). Scale bar = 25µm.

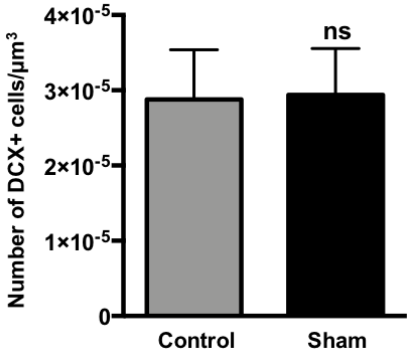

Figure 3. No differences in the density of DCX expressing cells can be found between Control and Sham group (P>0.05).

subcategories following morphological criteria (Bonfanti & Nacher 2012): i) tangled or type I cells, which are very small cells with very short intricate processes and ii) complex or type II cells, larger cells with more complex dendritic arborization, which resemble in many cases semilunar-pyramidal transitional neurons (SLPTN), a common excitatory cell type in this layer (Haberly, 1983). The density of tangled cells decreased dramatically in the ipsilateral hemisphere (p<0,01, figures 2B, D & E), while the density of complex cells was increased (p<0,01 figures 2C, D & E), suggesting an EndoN-induced differentiation of the tangled cells into complex cells.

In order to know whether this decrease on the density of DCX expressing immature neurons was the consequence of an increase in cell differentiation promoted by PSA depletion, we analysed the expression of NeuN, a marker of fully developed neurons (Mullen et al. 1992), which is only expressed faintly in some complex DCX+ cells. The analysis of NeuN immunofluorescence revealed that in the PCX layer II of the injected hemisphere, the density of NeuN expressing nuclei increased significantly (p< 0,05, figures 2F, G & H).

To study whether new synapses had been established on the apical dendrites of these recently differentiated neurons, we studied the intensity of synaptophysin immunofluorescence in layer I. A significant increase of this parameter was observed in the ipsilateral hemisphere (p< 0,001, figures 4A, B & C). In order to know whether

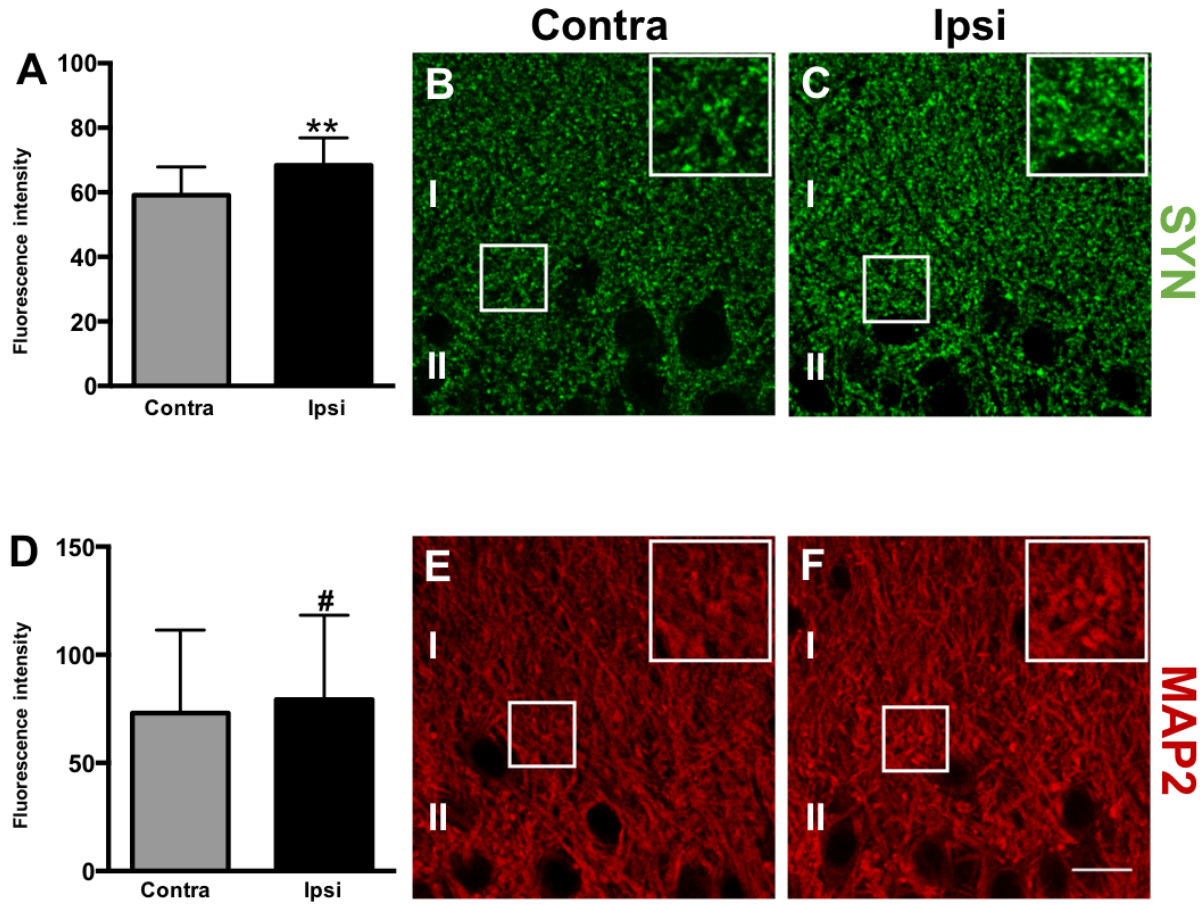

Figure 4. PSA depletion increases the intensity of fluorescence of MAP2 and SYN in layer I of the PCX. **A:** The intensity of synaptophysin (Syn) immunofluorescence increases in the ipsilateral hemisphere after the injection of the enzyme EndoN (p<0,01). **B & C**: Microphotographs of the PCX layer I immunostained with anti-SYN antibody. Insets show enlarged views of small squared regions in the contralateral (B) and the ipsilateral (C) hemispheres. These areas represent examples of regions of interest used for the analysis. **D:** The intensity of MAP2 immunofluorescence increases in the ipsilateral hemisphere (0.05<p<0,1). **E & F**: Microphotographs of the PCX layer I immunostained with anti-MAP2 antibody. Insets show enlarged views of small squared regions in the contralateral (E) and the ipsilateral (F) hemispheres. These areas represent examples of regions of interest used for the analysis. Microphotographs in this panel are Z stack 2D projections of 3 confocal planes with 0.5 µm z step size. Scale bar 20µm.

new dendritic branches from the putative recently differentiated neurons had reached layer I, we analysed the intensity of fluorescence of MAP2. The results also showed a significant increase when comparing the injected hemisphere with the contralateral one ($p< 0,05$, figure 4D, E & F).

No differences in the density of pyknotic nuclei were observed between the ipsilateral and contralateral hemispheres, indicating that the injection of EndoN did not produce cell death in the PCX layer II.

Since a small subpopulation of the DCX expressing cells in the PCX layer II also expresses NG2 (Gomez-Climent et al. 2008; Rubio et al. 2016), we also compared the density of these glial cells between hemispheres. No differences were found in the density of this cell type.

Genetic depletion of PSA

We have also studied the population of DCX expressing immature neurons in the PCX layer II in different genetic models lacking PSA-NCAM expression. Mice devoid of both polysialyltransferases, the 2 enzymes that incorporate PSA to NCAM ($St8SiaII^{-/-}$ $St8SiaIV^{-/-}$) (Hildebrandt et al. 2009), showed a remarkable reduction in the number of DCX expressing cells in the PCX layer II when compared with their wildtype littermates ($p<0,001$) (figure 5A). By contrast, NCAM knockout animals (Cremer et al. 1994), showed an increase in the number of DCX expressing cells in the PCX layer II when compared with their wildtype littermates ($p<0,01$, figure 5B).

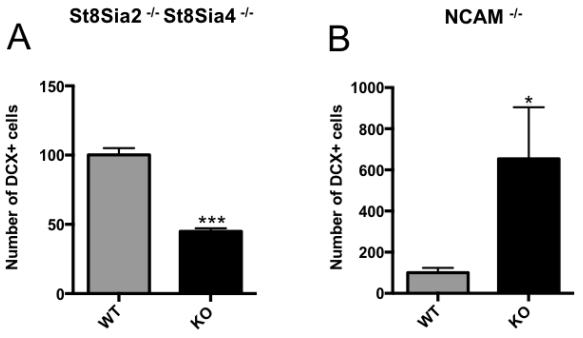

Figure 5. Impact of the genetic depletion of PSA on the immature neurons of the PCX layer II. **A**: Knockout animals for both polysialyltransferases ($St8Sia2^{-/-}$ $St8Sia4^{-/-}$) have a dramatic decrease in the number of DCX expressing cells ($p<0,001$). **B**: NCAM knockout mice have a significantly higher number of DCX expressing cells ($p<0,05$).

DISCUSSION

Two different populations of immature neurons have been described in the layer II of the adult PCX: tangled cells and complex cells (Gomez-Climent et al. 2008; Rubio et al. 2016). Tangled cells (also known as type I cells) are small and round shaped cells usually grouped in intricate clusters, which highly express markers for immature neurons, like DCX or PSA-NCAM, but lack expression of markers of mature neurons (e.g. NeuN, CaMKII, GAD67), or glial cells (Gomez-Climent et al. 2008). The complex cells (or type II cells) are larger than the tangled cells and most of them have the morphology of semilunar-pyramidal transitional neurons (SLPTN). The complex cells appear to be in a more mature stage than tangled cells, since they have larger somata, elaborated dendritic trees and lower levels of expression of DCX and PSA-NCAM. Interestingly, a low level of NeuN expression has been detected in this cell population (Gomez-Climent et al. 2008; Rubio et al. 2016). DCX/PSA-NCAM expressing cells with intermediate morphologies between tangled and complex cells are frequently observed in the PCX layer II, suggesting a transition between these two cell types. Our results showing an increased proportion of complex cells in the PCX layer II, and a decreased proportion of tangled cells after PSA depletion support this hypothesis. We can speculate that after EndoN injection there are maturating events that accelerate the final stages of development of tangled cells, inducing their transformation into complex cells. The increased NeuN expression in layer II after PSA depletion can reflect this transformation of tangled cells into complex cells, since the later exhibit low but detectable levels of this nuclear protein (Gomez-Climent et al. 2008; Rubio et al. 2016). This increase can also reflect the final maturation of some complex cells into semilunar-pyramidal transitional neurons, since we have also observed a decrease in the total number of DCX-expressing immature neurons after EndoN treatment.

One of the most important postranslational modifications on the NCAM is the addition of long chains of a complex sugar, the polysialic acid (PSA). PSA chains are highly hydrated and negatively charged, meaning that after the addition of PSA to the NCAM, there are steric impediments for homotypic and heterotypic interactions (Rutishauser et al. 2008). Due to its anti-adhesive properties, PSA increases the range and magnitude of intermembrane repulsion, by increasing the nonspecific repulsive force between cells (Yang et al. 1992). Thus, where PSA is present there are no regions of membrane available for adhesion, and consequently for the establishment of synaptic contacts (Johnson et al. 2005, reviewed in Rutishauser 2008) Thereby, the removal of PSA from the NCAM molecule with EndoN may release more free membrane space, available hence for synaptic contacts (Castillo-Gomez et al. 2011) .

Given the anti-adhesive proprieties of PSA-NCAM, its role has been studied in different neurodevelopmental processes involving immature neurons. The classical studies were focused on progenitor cells, mainly in the neurogenic regions. Many of these studies used EndoN to ablate PSA and explore the role of PSA-NCAM on the development of these cells. In most of the cases, as in the present study, EndoN promoted the differentiation of

precursor cells. In the rostral migratory stream, large numbers of PSA-NCAM-positive neuroblasts migrate towards the olfactory bulb, where they become interneurons. This migration can be specifically blocked by *in vivo* administration of EndoN in the SVZ of young animals (2-to 3-months old), (Ono et al. 1994), which results in an important inhibition of the migration of neuronal precursors from the SVZ to the OB. This inhibition is caused, at least partially, by the premature differentiation of neuronal precursors in the SVZ after PSA removal by EndoN administration, as evidenced by the promotion of neurite outgrowth and tyrosine hydroxylase synthesis (Petridis et al. 2004). In the subgranular zone of the dentate gyrus, where PSA-NCAM expression is found in immature granule neurons, the administration of EndoN also increases neural differentiation (Burgess et al. 2008). Similar effects are observed *in vitro*: the removal of PSA from adult-derived neural progenitors also enhances neuronal differentiation (Burgess et al. 2008). Outside the canonical niches, results are pointing out as well to an enhancement of neuronal differentiation/maturation after EndoN treatment: PSA removal from primary cultures of septal neurons significantly increases neurite outgrowth (Burgess et al. 2008).

Interestingly, this pro-plastic effects of EndoN treatment are not restricted to progenitors and immature neurons, since the addition of EndoN to hippocampal organotypic cultures, promotes an increase in the apparition rate of spines in mature interneurons (Guirado et al. 2014).

Thus, *in vitro* and *in vivo* findings are supporting the idea that PSA removal induces the maturation of neuronal precursors and immature neurons. The hypothesis that EndoN treatment induces the differentiation of immature neurons in the adult PCX is also supported by our analysis of MAP-2 and synaptophysin expression. The Microtubule-associated protein 2 (MAP-2) is a dynamic molecule, involved in the growth, differentiation, and plasticity of neurons (reviewed in Johnson and Jope 1992). In the adult brain, MAP-2 is mainly concentrated in mature dendrites and it is virtually absent from axons (Bernhardt & Matus 1984; De Camilli et al. 1984; Kosik & Finch 1987). MAP-2 seems to be rather important for dendritic function, since in areas where there is a loss of this protein, dendrites collapse and in the regions where its expression increases there is dendritic sprouting (Caceres et al. 1992). Tangled cells lack MAP2 expression, but faint MAP2 labelling can be found in some complex cells with SLPTN morphology (Gomez-Climent et al. 2008). In the present study we have used MAP2 immunohistochemistry to explore whether EndoN expression induces an increase in the number of mature dendrites in the PCX layer I, the stratum where typical excitatory neurons in layer II extend their dendrites to. Additionally, we have used immunohistochemistry for synaptophysin as an index of active synapses (Thiele et al. 2000) in the PCX layer I. Presumably, if immature neurons in the PCX layer II are differentiating after EndoN administration, they will receive new synaptic contacts on their dendritic trees. Our results support this hypothesis, since after PSA removal the density of synaptophysin immunoreactive puncta and that of MAP-2 expressing dendritic segments increased in layer I.

Previous results from our laboratory, both in rats and mice, have shown that a subpopulation of PSA-NCAM/DCX expressing cells in the PCX layer II also express NG2, a marker of polydendrocytes or NG2+ cells (Gomez-Climent et al. 2008; Rubio et al. 2016). Some authors have suggested the possibility that NG2+ cells can give rise to neurons in the healthy adult PCX (Rivers et al. 2008; Guo et al. 2010). Moreover, Honsa et al. (2012), have identified cells of polydendrocyte origin that displayed the immunohistochemical and electrophysiological properties of neuronal precursor cells after focal cerebral ischemia. Therefore, we have studied the number of NG2 expressing cells after EndoN treatment. As our results failed to show differences in the number of NG2 expressing cells when comparing both hemispheres (EndoN injected and control), it seems rather unlikely that the NG2+ population might be involved in the reduction of the density of DCX expressing cells induced by EndoN.

In order to discard nonspecific toxicity induced by the EndoN treatment, we have measured the density of pyknotic nuclei. In accordance with our results, previous studies have also failed to find alterations in the viability/survival of cells after EndoN treatment: Muller et al. (1996) did not find alterations in the viability of organotypic hippocampal cultures; similarly, Burgess et al. (2007) found no differences in the survival of primary septal neurons plated on laminin. *In vivo* findings appear to follow the same direction: Burgess et al. (2008) also failed to find differences after assessing the effect intra-hippocampal EndoN administration on the survival and proliferation of adult rat dentate gyrus progenitors.

The NCAM protein core is submitted to several post-translational modifications, such the addition of PSA (Hildebrandt et al. 1998; Bork et al. 2007; Hildebrandt et al. 2007). There are two different enzymes that catalyse the synthesis of PSA: St8SiaII and St8SiaIV. St8SiaII is highly expressed during prenatal development, while St8SiaIV is more abundant in the adult brain (Kojima et al. 1996; Nakayama & Fukuda 1996; Hildebrandt et al. 2007). As previously reported by Nacher et al. (2010), both polysialyltransferases seem to play a role on the development and maintenance of the immature neuronal population in the PCX In St8Sia II$^{-/-}$ St8SiaIV $^{+/+}$ knockout mice, the number of PSA-NCAM expressing cells is markedly reduced in the PCX layer II, but the genetic depletion of this polysialyltransferase has no impact on the number of DCX positive cells, suggesting that this enzyme is not critical for the initial development and migration of these cells. By contrast, in St8Sia II$^{+/+}$St8SiaIV $^{-/-}$ knockout mice the number of immature neurons, both expressing

DCX and PSA-NCAM is much higher than in wildtype mice, suggesting that St8SiaIV plays an important role on the differentiation of these cells. Surprisingly, the effects that we observe in the double knockouts are very different from the single knockouts: there is a dramatic reduction in the number of DCX expressing cells in the PCX. A possible explanation for this discrepancy may be that some compensatory effects may be occurring in the single knockouts, which are helping the population of immature neurons to develop. In the absence of St8SiaII, the main polysialyltransferase during development, St8SiaIV may be operating to allow the migration and incorporation of many DCX positive cells to the PCX layer II. In the absence of the two polysialyltransferases this compensation cannot occur, probably producing a disruption in the migratory pathway during development, which might explain the dramatic decrease in the DCX expressing cells in the double knockout. Since there is no PSA-NCAM in the double knockouts, it is possible that the progenitors were not able to migrate from their site of origin to the PCX layer II, and, as a consequence, the number of immature neurons in the PCX decreases. Future experiments should determine whether these alterations in the migration and final positioning also affect the mature excitatory neurons located in the layer II. Finally, it is possible as well that the absence of PSA may accelerate the development in such a way that it is not possible to see immature cells in the layer II of the PCX, since almost all of them would be already mature at 3 months, when we perform our analyses of immature neuronal markers. Studies on the density of mature neurons in the layer II of these transgenic animals or at earlier postnatal stages may shed light on this matter.

Although the RMS is one of the most affected areas in NCAM knockout mice, alterations as a consequence of the genetic removal of the NCAM protein can also be observed in other regions bearing populations of immature neurons during the adulthood, such as the hippocampus (Tomasiewicz et al. 1993). In fact, NCAM $^{-/-}$ deficient mice have reduced adult neurogenesis in the dentate gyrus (Aonurm-Helm et al. 2008) . Although the proliferation of the dentate gyrus neural precursors was unaffected in this NCAM deficient mice, the survival of these progenitors was reduced. Moreover, additional studies with cultures employing neuronal progenitor cells obtained from rodent hippocampus, have demonstrated that when soluble NCAM is added, it promotes a reduction in cell proliferation and induces the differentiation of the progenitors into the neuronal lineage (Amoureux et al. 2000). Similar results were found when soluble NCAM was added to hippocampal cultures prepared from NCAM knockout mice, which allows to suggest that NCAM promotes differentiation (Amoureux et al. 2000). In the PCX layer II, we have described an increase in the number of the DCX expressing cells in the NCAM knockout mice, which suggests that the survival of the progenitors is not affected by the lack of the NCAM, while their differentiation may be impaired.

Despite extensive research, we still do not know which are the mechanisms that underlie the final stages of development of the immature neurons in the PCX layer II. Recent data from Dr. Couillard-Despres laboratory and our own (Rotheneichner et al. 2018), has shown how these cells mature into excitatory neurons, and do not die over the lifespan. Some candidate molecules like reelin (Carceller et al. 2016) or BDNF (Vutskits et al. 2001) have been proposed as mediators of this final differentiation, but it is still necessary to deepen further in the knowledge of their effects on this population of immature neurons to completely understand the process. As previously described by Rubio et al. (2015), it is possible to isolate and cultivate PCX layer II cells. Thus, it would be interesting to study the effect of the addition of BDNF to PCX cultures, since it has been shown that the addition of BDNF to embryonic progenitor and adult mice striatum cultures, can induce the differentiation of neuronal precursors (Ahmed et al. 1995). In the same way, it would be interesting to test *in vitro* whether the addition of EndoN to PCX cultures also induces neuronal differentiation. Similarly, intracranial injections of BDNF in the PCX or the induction of its overexpression using viral vectors may allow us to explore whether the differentiation of SVZ precursors *in vitro* is reproducible *in vivo* in the PCX layer II.

BIBLIOGRAPHY

Ahmed S, Reynolds B a, Weiss S. 1995. BDNF enhances the differentiation but not the survival of CNS stem cell-derived neuronal precursors. J. Neurosci. 15:5765–5778.

Amoureux MC, Cunningham B a, Edelman GM, Crossin KL. 2000. N-CAM binding inhibits the proliferation of hippocampal progenitor cells and promotes their differentiation to a neuronal phenotype. J. Neurosci. 20:3631–3640.

Angata K, Chan D, Thibault J, Fukuda M. 2004. Molecular dissection of the ST8Sia IV polysialyltransferase: Distinct domains are required for neural cell adhesion molecule recognition and polysialylation. J. Biol. Chem. 279:25883–25890.

Aonurm-Helm A, Jurgenson M, Zharkovsky T, Sonn K, Berezin V, Bock E, Zharkovsky A. 2008. Depression-like behaviour in neural cell adhesion molecule (NCAM)-deficient mice and its reversal by an NCAM-derived peptide, FGL. Eur. J. Neurosci. 28:1618–1628.

Bernhardt R, Matus A. 1984. Light and electron microscopic studies of the distribution of microtubule-associated protein 2 in rat brain: a difference between

dendritic and axonal cytoskeletons. J. Comp. Neurol. 226:203–21.

Bonfanti, Luca, & Nacher J. 2012. New scenarios for neuronal structural plasticity in non-neurogenic brain parenchyma: the case of cortical layer II immature neurons. Prog Neurobiol 98:1–15.

Bork K, Gagiannis D, Weidemann W, Kontou M, Reutter W. 2007. Experimental approaches to interfere with the polysialylation of the neural cell adhesion molecule in vitro and in vivo §. J. Neurochem. 103:65–71.

Burgess A, Wainwright SR, Shihabuddin LS, Rutishauser U, Seki T, Aubert I. 2008. Polysialic acid regulates the clustering, migration, and neuronal differentiation of progenitor cells in the adult hippocampus. Dev. Neurobiol. 68:1580–1590.

Burgess A, Weng Y, Rebecca A, Cui X, Caines G, Aubert I. 2007. Polysialic acid limits septal neurite outgrowth on laminin. Brain Res 44:52–58.

Caceres A, Mautino J, Kosik K. 1992. Suppression of MAP2 in cultured cerebellar macroneurons inhibits minor neurite formation. Neuron 9:607–18.

Cai Y, Xiong K, Chu Y, Luo DW, Luo XG, Yuan XY, Struble RG, Clough RW, Spencer DD, Williamson A, et al. 2009. Doublecortin expression in adult cat and primate cerebral cortex relates to immature neurons that develop into GABAergic subgroups. Exp Neurol 216:342–356.

De Camilli P, Miller PE, Navone F, Theurkauf WE, Vallee RB. 1984. Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. Neuroscience 11:819–846.

Carceller H, Rovira-esteban L, Nacher J, Castrén E, Guirado R et al. 2016. Neurochemical Phenotype of Reelin Immunoreactive Cells in the Piriform Cortex Layer II. Front cell Neurosci. 10:1–11.

Castillo-Gomez E, Varea E, Blasco-Ibañez JM, Crespo C, Nacher J. 2016. Effects of chronic dopamine D2R agonist treatment and polysialic acid depletion on dendritic spine density and excitatory neurotransmission in the mPFC of adult rats. Neural Plast. 2016:12.

Cremer H, Lange R, Christoph A, Plomann M, Vopper G, Roes J, Kraemer P, Scheff S, Barthels D, Rajewsky K, et al. 1994. © 19 9 4 Nature Publishing Group. Lett. to Nat. 367:455–459.

Eckhardt M, Bukalo O, Chazal G, Wang L, Goridis C, Schachner M, Gerardy-Schahn R, Cremer H, Dityatev a. 2000. Mice deficient in the polysialyltransferase ST8SiaIV/PST-1 allow discrimination of the roles of neural cell adhesion molecule protein and polysialic acid in neural development and synaptic plasticity. J. Neurosci. 20:5234–5244.

Gomez-Climent MA, Castillo-Gomez E, Varea E, Guirado R, Blasco-Ibanez JM, Crespo C, Martinez-Guijarro FJ, Nacher J. 2008. A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. Cereb Cortex 18:2229–2240.

Gomez-Climent MA, Guirado R, Varea E, Nàcher J. 2010. " Arrested development ". Immature , but not recently generated , neurons in the adult brain. Arch Ital Biol 148:159–172.

Guirado R, Perez-Rando M, Sanchez-matarredona D, Castillo-gómez E, Liberia T, Rovira-esteban L, Varea E, Crespo C, Blasco-ibáñez JM, Nacher J. 2014. The dendritic spines of interneurons are dynamic structures influenced by PSA-NCAM expression. Cereb. Cortex 24:3014–3024.

Guo F, Maeda Y, Ma J, Xu J, Horiuchi M, Miers L, Vaccarino F, Pleasure D. 2010. Pyramidal neurons are generated from oligodendroglial progenitor cells in adult piriform cortex. J. Neurosci. 30:12036–12049.

Hildebrandt H, Becker C, Mürau M, Gerardy-schahn R. 1998. Heterogeneous Expression of the Polysialyltransferases ST8Sia II and ST8Sia IV During Postnatal Rat Brain Development.

Hildebrandt H, Mu M, Weinhold B, Gerardy-schahn R. 2007. Dissecting polysialic acid and NCAM functions in brain development. J. Neurochem. 103:56–64.

Hildebrandt H, Mühlenhoff M, Oltmann-Norden I, Röckle I, Burkhardt H, Weinhold B, Gerardy-Schahn R. 2009. Imbalance of neural cell adhesion molecule and polysialyltransferase alleles causes defective brain connectivity. Brain 132:2831–2838.

Honsa P, Pivonkova H, Dzamba D, Filipova M, Anderova M. 2012. Polydendrocytes Display Large Lineage Plasticity following Focal Cerebral Ischemia. 7.

Johnson CP, Fujimoto I, Rutishauser U, Leckband DE. 2005. Direct evidence that neural cell adhesion molecule (NCAM) polysialylation increases intermembrane

repulsion and abrogates adhesion. J Biol Chem 280:137–145.

Johnson GVW, Jope RS. 1992. The Role of Microtubule-Associated Protein 2 (MAP-2) in Neuronal Growth , Plasticity , and Degeneration. 512.

Kojima N, Kono M, Yoshida Y, Tachida Y, Nakafuku M, Tsuji S, Ii S. 1996. Biosynthesis and Expression of Polysialic Acid on the Neural Cell Adhesion Molecule Is Predominantly Directed by ST8Sia II / STX during in Vitro Neuronal Differentiation . 271:22058–22062.

Kolkova K, Novitskaya V, Pedersen N, Berezin V, Bock E. 2000. Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. J. Neurosci. 20:2238–2246.

König R, Benedetti B, Rotheneichner P, O' Sullivan A, Kreutzer C, Belles M, Nacher J, Weiger T, Aigner L, Couillard-Despres S. 2016. Distribution and fate of DCX / PSA-NCAM expressing cells in the adult mammalian cortex : A local reservoir for adult cortical neuroplasticity ? Front. Biol. (Beijing). 11:193–213.

Kosik KS, Finch E a. 1987. MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites: an immunocytochemical study of cultured rat cerebrum. J. Neurosci. 7:3142–3153.

Luzzati F, Bonfanti L, Fasolo A, Peretto P. 2009. DCX and PSA-NCAM expression identifies a population of neurons preferentially distributed in associative areas of different pallial derivatives and vertebrate species. Cereb Cortex 19:1028–1041.

Maness PF, Schachner M. 2007. Neural recognition molecules of the immunoglobulin superfamily : signaling transducers of axon guidance and neuronal migration. Nat. Neurosci. 10:19–26.

McCall T, Weil ZM, Nacher J, Bloss EB, Marouf A El, Rutishauser U, McEwen BS. 2013. NIH Public Access. Exp. Neurobiol. 6:2166–2171.

Mullen RJ, Buck CR, Smith AM. 1992. NeuN, a neuronal specific nuclear protein in vertebrates. Development 116:201–211.

Muller D, Mendez P, DeRoo M, Klauser P, Steen S, Poglia L. 2010. Role of NCAM in Spine dynamics and synaptogenesis. Adv. Exp. Med. Biol. 663:245–256.

Muller D, Wang C, Skibo G, Toni N, Cremer H, Calaora V, Rougon G, Kiss JZ. 1996. PSA – NCAM Is Required for Activity-Induced Synaptic Plasticity. Cell 17:413–422.

Nacher J, Alonso-Llosa G, Rosell D, McEwen B. 2002. PSA-NCAM expression in the piriform cortex of the adult rat . Modulation by NMDA receptor antagonist administration. Brain Res 927:111–121.

Nacher J, Crespo C, McEwen B. 2001. Doublecortin expression in the adult rat telencephalon. Eur J Neurosci 14:629–644.

Nacher J, Guirado R, Varea E, Alonso-Llosa G, Röckle I, Hildebrandt H. 2010. Divergent impact of the polysialyltransferases ST8SiaII and ST8SiaIV on polysialic acid expression in immature neurons and interneurons of the adult cerebral cortex. Neuroscience 167:825–837.

Nakayama J, Fukuda M. 1996. A Human Polysialyltransferase Directs in Vitro Synthesis of Polysialic Acid *. J. Biol. Chem. 271:1829–1833.

Ono K, Tomasiewicz H, Magnuson T, Rutishauser U. 1994. N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. Neuron 13:595–609.

Petridis AK, Maarouf A El, Rutishauser U. 2004. Polysialic Acid Regulates Cell Contact-Dependent Neuronal Differentiation of Progenitor Cells from the Subventricular Zone. :675–684.

Rivers LE, Young KM, Rizzi M, Jamen F, Psachoulia K, Wade A, Kessaris N, Richardson WD. 2008. PDGFRA/NG2 glia generate myelinating oligodendrocytes and piriform projection neurons in adult mice. Nat Neurosci 11:1392–1401.

Rotheneichner P, Belles M, Benedetti B, König R, Dannehl D, Kreutzer C, Zaunmair P, Engelhardt M, Aigner L, Nacher J, et al. 2018. Cellular Plasticity in the Adult Murine Piriform Cortex: Continuous Maturation of Dormant Precursors Into Excitatory Neurons. Cereb. Cortex:1–12.

Rubio A, Belles M, Belenguer G, Vidueira S, Fariñas I, Nacher J, Farinas I, Nacher J. 2016. Characterization and Isolation of Immature Neurons of the Adult Mouse Piriform Cortex. Dev Neurobiol 76:748–763.

Rutishauser U. 2008. Polysialic acid in the plasticity of the developing and adult vertebrate nervous system. Nat Rev Neurosci 9:26–35.

Seki T, Arai Y. 1991. Expression of highly polysialylated

NCAM in the neocortex and piriform cortex of the developing and the adult rat. Anat. Embryol. (Berl). 184:395–401.

Shapiro LA, Ng KL, Kinyamu R, Whitaker-Azmitia P, Geisert EE, Blurton-Jones M, Zhou QY, Ribak CE. 2007. Origin, migration and fate of newly generated neurons in the adult rodent piriform cortex. Brain Struct Funct 212:133–148.

Thiele C, Hannah M, Fahrenholz F, Huttner HB. 2000. Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. Nat. Cell Biol. 2:42–9.

Tomasiewicz H, Ono K, Yee D, Thompson C, Goridis C, Rutishauser U, Magnuson T. 1993. Genetic deletion of a neural cell adhesion molecule variant (N- CAM-180) produces distinct defects in the central nervous system. Neuron 11:1163–1174.

Varea E, Castillo-Gomez E, Gomez-Climent MA, Guirado R, Blasco-Ibanez JM, Crespo C, Martinez-Guijarro FJ, Nacher J. 2009. Differential evolution of PSA-NCAM expression during aging of the rat telencephalon. Neurobiol Aging 30:808–818.

Vimr ER, McCoy RD, Vollger HF, Wilkison NC, Troy FA. 1984. Use of prokaryotic-derived probes to identify poly(sialic acid) in neonatal neuronal membranes. Proc. Natl. Acad. Sci. 81:1971–1975.

Vutskits L, Djebbara-Hannas Z, Zhang H, Paccaud JP, Durbec P, Rougon G, Muller D, Kiss JZ. 2001. PSA-NCAM modulates BDNF-dependent survival and differentiation of cortical neurons. Eur. J. Neurosci. 13:1391–1402.

West MJ. 1993. New Stereological Methods for Counting Neurons. Neurobiol. Aging 14:275–285.

Xiong K, Luo DW, Patrylo PR, Luo XG, Struble RG, Clough RW, Yan XX. 2008. Doublecortin-expressing cells are present in layer II across the adult guinea pig cerebral cortex: partial colocalization with mature interneuron markers. Exp Neurol 211:271–282.

Yang P, Yin X, Rutishauser U. 1992. Intercellular Space Is Affected by the Polysialic Acid Content of NCAM. J. cel Biol. 116:1487–1496.

Yang Y, Xie MX, Li JM, Hu X, Patrylo PR, Luo XG, Cai Y, Li Z, Yan XX. 2015. Prenatal genesis of layer II doublecortin expressing neurons in neonatal and young adult guinea pig cerebral cortex. Front Neuroanat 9:11.

Zhang XM, Cai Y, Chu Y, Chen EY, Feng JC, Luo XG, Xiong K, Struble RG, Clough RW, Patrylo PR, et al. 2009. Doublecortin-expressing cells persist in the associative cerebral cortex and amygdala in aged nonhuman primates. Front Neuroanat 3:17.

CHAPTER 5

Discussion

DISCUSSION

In the present thesis, using *in vivo* and *in vitro* conditions, we have studied the origin and fate of the immature neurons residing in the layer II of the adult piriform cortex (PCX). Additionally, we have studied the impact of the genetic or enzymatic removal of polysialic acid (PSA) from this population of cells.

Immature neurons in PCX layer II are generated during embryonic development

In accordance with previous reports in other species, our data in mice indicate that the layer II of the adult PCX contains, in addition to a predominant population of excitatory neurons and a smaller proportion of GABAergic inhibitory interneurons (Sarma et al., 2011), a population of PSA-NCAM/DCX-positive immature neurons. As previously described in rats (Gomez-Climent et al., 2008) and guinea pigs (Yang et al., 2015), we have demonstrated that most, if not all, of these immature neurons in the adult cerebral cortex layer II of mice, have been generated during embryonic development. Interestingly, in agreement with a previous report (Sarma et al., 2011), we have found that the percentage of cells generated at the earlier embryonic stage studied (E13.5 and E14.5) was higher than that of those cells that were generated at later stages (E15.5–E16.5). Additionally, their fate was also different depending on the date of birth: while most of the cells in layer II that were generated at the earlier stage preferentially differentiate into neurons, those cells generated at later stages become glia. These data are consistent with an early developmental peak of neurogenesis and a late peak of gliogenesis observed in both paleo- and neocortex (Sarma et al., 2011).

Isolation, culture and proliferative activity of immature neurons in the adult PCX

Using *in vitro* conditions to isolate the population of immature neurons in the adult PCX layer II, we have assessed their proliferative activity under mitogenic stimulation. In this regard, we have observed that this region does not seem to contain progenitors with the ability to form neurospheres. In addition, we were able to maintain *in vitro* a small subpopulation of isolated cells from the PCX layer II for at least seven days. While many reports have described the culture of adult cells from "classical" neurogenic zones of the adult CNS: SVZ (Ferrón et al., 2007; Reynolds & Weiss, 2011), hippocampus (Babu et al., 2011) or other regions where neurogenesis has been described after BDNF infusion, like adult septum or striatum (Palmer et al., 1995; Pencea et al., 2001), cultivating cells from non-neurogenic regions is more challenging and, as reported previously with adult rat or mouse cortex, it implies the use of a density gradient to separate neurons from debris (Brewer & Torricelli, 2007). In fact, to maintain our cells *in vitro*, we used a feeder layer composed by SVZ cells. By using our culturing conditions, we were able to isolate from the PCX a population of PSA-NCAM/ DCX-positive immature neurons, which does not proliferate *in vitro* and, most likely, differentiates into microtubule-associated protein 2 (MAP-2)-positive neurons. Our 5'BrdU analysis supports this hypothesis, showing that these neurons have not been generated from proliferating progenitors in the culture.

In vivo, in adult animals, the immature neurons in the PCX did not express any marker of proliferative activity and were never labelled by birth-dating experiments performed in adulthood. In line with previous studies (Gómez-Climent et al., 2008; Luzzati et al., 2009; Zhang et al., 2009), we could not detect the incorporation of BrdU within the tangled cells, neither shortly after the injection of the tracer nor after 3 weeks

of survival. Therefore, under physiological conditions, newly generated neurons in the rodent PCX seem to be marginal, if existent, and might be originated from cells migrating out of the SVZ in early life (Shapiro et al., 2007).

PSA-NCAM/DCX expressing immature neurons in the adult PCX differentiate into excitatory neurons

Using inducible DCX-CreER^{T2}/Flox-EGFP transgenic mouse line, we have been able to trace the fate of immature neurons of the PCX layer II, and convincingly demonstrate that these cells do not die in the course of aging, but integrate as newly excitatory neurons, as hypothesized by previous studies (Gómez-Climent et al., 2008; Varea et al., 2011). The marker profile together with the morphological features of GFP+ cells after tamoxifen induction, suggests a continuous functional integration of newly matured neurons in the adult PCX. Thus, the observation that virtually all GFP$^+$ immature neurons expressed the Tbr1 transcription factor, and very rarely found to express the LIM/homeobox protein Lhx6 or distal-less DLL, allows us to suggest a pallial origin, rather than from subpallial structures, in which most cortical interneurons originate (Luzzati et al., 2009). In contrast to Tbr1, CaMKII, a Ca2$^+$/calmodulin-dependent protein kinase, is expressed only in mature principal neurons. We observed that co-expression of GFP with CaMKII, which was marginal shortly after application of tamoxifen in DCX-CreER^{T2}/Flox-EGFP, impressively increased to approximately 80% following a maturation period of 6 months, regardless of the age of mice (3m-t and 9m-t groups).

Mature excitatory neurons residing in layer II are not an homogenic group. In fact, depending on their target they express different transcriptional factors and can be therefore divided into different subpopulations: the ones that project to the OB express

Cux1; when the IL is the main target, they express Ctip2, while they express Fezf2 when project to the CoA (Diodato et al., 2016). Thus, considering this pattern of expression, it would be interesting to perform further studies using inducible DCX-CreER^{T2}/Flox-EGFP transgenic mouse line, but analysing each subpopulation of cells separately.

Removal of PSA promotes the differentiation of immature neurons in the adult PCX

Previous studies in the neurogenic niches (Burgess et al., 2008; Petridis et al., 2004), but also outside of them (Burgess et al., 2007; Guirado et al., 2013), have demonstrated that the removal of PSA from the NCAM molecule induces neuronal maturation. In accordance with these reports, we have found that after treatment with EndoN, an enzyme that specifically depletes PSA from NCAM, the number of immature neurons in the PCX layer II decreases, while the number of those expressing mature neuronal markers increases.

The hypothesis that EndoN treatment induces the differentiation of immature neurons in the adult PCX is also supported by our analysis of MAP-2 and synaptophysin (Syn) expression. MAP-2 is a dynamic molecule, involved in the growth, differentiation, and plasticity of neurons (reviewed in Johnson, 1992). In the adult CNS, this protein is expressed principally in mature dendrites and it is mainly absent from axons (Bernhardt & Matus, 1984; De Camilli et al., 1984; Kosik & Finch, 1987). Interestingly, at least in PCX, there seems to be a relationship between the neuronal maturation stage and the MAP 2 expression: tangled cells never express this protein, while complex cells faintly express it (Gomez-Climent et al., 2008), and the dendrites of mature neurons are intensely labelled by this marker. Thus, as we have found an increase in the density of MAP2 expressing dendritic segments in layer I, in which layer II neurons receive their main

synaptic input, we speculate these new MAP2 positive dendrites belong to recently matured neurons. Additionally, we have quantified the expression of Syn using immunohistochemistry and confocal microscopy, as an index of active synapses (Thiele et al., 2000) in the PCX layer I. Presumably, if immature neurons in the PCX layer II are differentiating after EndoN administration, they will receive new synaptic contacts in their dendritic trees located in layer I. Our results confirm this hypothesis, since after PSA removal there was an increase in the density of Syn immunoreactive puncta.

The genetic depletion of NCAM and PSA induces alterations in the immature neuronal population of the adult PCX

There are two different enzymes that catalyse the synthesis of PSA: the polysialyltransferases ST8SiaII and ST8SiaIV. ST8SiaII is strongly expressed during prenatal development, while ST8SiaIV is more abundant in the adult brain (Kojima et al., 1996; Nakayama & Fukuda, 1996; Hildebrandt et al., 2007). As previously reported by Nacher et al., (2010), both polysialyltransferases seem to play a role on the development and maintenance of the immature neuronal population in the adult PCX. In ST8Sia $II^{-/-}$ ST8SiaIV $^{+/+}$ knockout mice, the number of PSA-NCAM expressing cells is markedly reduced in the PCX layer II, but the genetic depletion of this polysialyltransferase has no impact on the DCX positive population, suggesting that this enzyme is not critical for the initial development and migration of these cells. These results also indicate that St8SiaII is the main polysialyltransferase in immature PCX neurons during adulthood. By contrast, in ST8Sia $II^{+/+}$ST8SiaIV $^{-/-}$ knockout mice, the number of immature neurons, both expressing DCX and PSA-NCAM, is higher than in wildtype mice, suggesting that St8SiaIV plays an important role on the differentiation of these cells. Surprisingly, the effects

that we observe in the present thesis in the double knockout mice (ST8Sia II$^{-/-}$ ST8SiaIV $^{-/-}$) are very different from the single knockouts: there is a dramatic reduction in the number of DCX expressing cells in the PCX. A possible explanation to this discrepancy could be that some compensating effects may be occurring in the single knockouts, which are helping the population of immature neurons to develop. In the absence of ST8SiaII, the main polysialyltransferase during development, ST8SiaIV may be operating to allow the migration and incorporation of many DCX positive cells to the PCX layer II. In the absence of the two polysialyltransferases this compensation cannot occur. On the other hand, a disruption in the migratory pathway during development, could explain the dramatic decrease in the DCX expressing cells in the double knockout. Thus, as there is no PSA, it is possible that the progenitors are not able to migrate from their original site to the PCX, and, as a consequence, the number of immature neurons in the PCX decreases markedly. Finally, it is also possible that the lack of PSA might accelerate the development of these cells in such a way that it would not be possible to see immature neurons in the layer II of the PCX, since almost all of them might have already lost DCX expression.

In NCAM mutant mice, there is no chance for adding PSA to the NCAM molecule. Thus, this phenotype should be comparable to those found in the double knockouts (ST8Sia II$^{-/-}$ST8SiaIV $^{-/-}$). In this way, we should expect a decrease in the number of DCX expressing cells. However, the lack of NCAM produces an increase in the number of these immature neurons in the PCX layer II. Thus, these results are indicating that some other molecules may be playing similar roles to that of NCAM during the generation, migration and differentiation of the population of immature neurons in the adult PCX.

Which are the mechanisms that underlie the development of the immature neurons in the adult PCX layer II?

The mechanisms enabling post-mitotic tangled cells to survive in a dormant stage during adulthood still have to be deciphered. Similarly, the nature of the signals triggering the completion of their neuronal maturation processes and the functional relevance of such immature cells in the PCX are still largely unknown. Although the maturation of the immature neurons in the PCX does not seem to play a crucial role in olfactory memory, an olfactory activity-dependent control of maturation has been suggested by the observation that the number of PSA-NCAM/DCX-expressing cells dramatically decreased following bulbectomy, whereas the expression of mature markers increased (Gómez-Climent et al., 2011).

Interestingly, these immature neurons may have an important role in mediating the effects of stress and stress-related hormones: chronic stress increases the number of PSA-NCAM/DCX expressing cells in the PCX layer II, while chronic corticosterone administration decreases it (Nacher et al., 2004). As these PSA-NCAM/DCX expressing neurons do not express glucocorticoid receptors (Gómez-Climent et al., 2008), it is possible that the effects of stress were mediated by NMDA receptors, since these glutamate receptors are present in immature SLPTN (Gómez-Climent et al., 2008), and the number of PSA-NCAM expressing cells in PCX layer II increases after NMDA receptor antagonist treatment (Nacher et al., 2002).

In addition to glutamate (acting through NMDA receptors), other neurotransmitters may play an important role in the regulation of this population of immature neurons. Noradrenaline (NE), which is also involved in the stress response, seems to regulate synaptic and structural plasticity on PCX layer II, since the depletion

of this monoamine by a noradrenergic neurotoxin (DSP-4) significantly increases the number of PSA-NCAM/DCX immunopositive cells in the adult PCX of rats (Vadodaria et al., 2017).

In summary, we have demonstrated that in the layer II of the adult murine PCX, a population of post-mitotic immature neurons composed by the so-called tangled cells, and to a lesser extent by complex cells with SLPTN morphology, is continuously providing newly matured glutamatergic principal neurons, which appear to integrate in the surrounding neuronal network. This observation demonstrates that it is possible to integrate new functional neurons in the adult central nervous system outside the classical neurogenic niches. We propose that these cortical immature neurons, which are more widely distributed in mammals with gyrencephalic brains, such as cats or humans (see Bonfanti & Nacher, 2012 and König et al., 2016 for review) progressively integrate into the neuronal network throughout adulthood and thereby provide an unsuspected capacity of cellular plasticity.

CHAPTER 6

Conclusions

CONCLUSIONS

1. The layer II of the PCX of adult mice contains, in addition to a predominant population of excitatory neurons and a smaller proportion of GABAergic inhibitory interneurons, a population of PSA-NCAM/DCX-positive immature neurons.
2. The vast majority of the immature neurons in the layer II of the PCX of adult mice, have been generated during embryonic development.
3. Immature neurons in the layer II of the PCX of adult mice do not express markers of proliferative activity and are not labelled by short or long term 5'BrdU birth-dating experiments performed in adulthood.
4. Cells from the layer II of the PCX of adult mice can be cultured *in vitro* using a feeder layer made by SVZ cells.
5. It is possible, at least with our *in vitro* conditions, to maintain isolated cells from the adult PCX layer II of mice, for at least 7 days.
6. Some of the cells isolated from the adult PCX layer II of mice are able to differentiate into MAP-2 immunoreactive mature neurons.
7. The layer II of the PCX of adult mice does not contain progenitors with the ability to form neurospheres.
8. The PSA-NCAM/DCX-positive immature neurons in the layer II of the adult PCX of mice, do not proliferate *in vitro*.
9. The study of the inducible DCX-CreER^{T2}/Flox-EGFP transgenic mouse line demonstrates that immature neurons in the adult PCX layer II do not die in the course of aging.

10. The study of the inducible DCX-CreER^{T2}/Flox-EGFP transgenic mouse line demonstrates that tangled cells integrate in the surrounding neuronal network as mature neurons, according to the increase in the density of synaptic contacts and the appearance of the AIS.
11. The pattern of expression of the DCX-CreER^{T2}/Flox-EGFP transgenic mouse line confirms that the vast majority of immature neurons in the adult PCX layer II integrate to the circuitry as newly matured glutamatergic principal neurons, since almost all of them express TBR1 and CaMKII.
12. The enzymatic removal of PSA from NCAM in the PCX layer II of adult mice decreases the density of DCX-expressing immature neurons and increases the density of NeuN expressing nuclei.
13. The enzymatic removal of PSA from NCAM in the PCX layer II of adult mice, leads to an increased expression of Syn and MAP-2 in the layer I.
14. After the enzymatic removal of PSA from NCAM, the percentage of tangled cells residing in layer II of the adult PCX of mice decreases, while the percentage of complex cells increases.
15. Adult ST8SiaII$^{-/-}$ST8SiaIV$^{-/-}$ knockout mice display a dramatic reduction in the number of DCX expressing cells in the PCX layer II when compared with their wildtype littermates.
16. NCAM knockout mice show an increase in the number of DCX expressing cells in the adult PCX layer II when compared with their wildtype littermates.

CHAPTER 7

Bibliography

BIBLIOGRPHY

Abrous, D. N., Montaron, M. F., Petry, K. G., Rougon, G., Darnaudery, M., Le Moal, M., & Mayo, W. (1997). Decrease in Highly Polysialylated Neuronal Cell Adhesion Molecules and in Spatial Learning During Aging Are Not Correlated. *Brain Res.*, *744*, 285–292. https://doi.org/10.1016/S0006-8993(96)01115-8

Alvarez, V. A., & Sabatini, B. L. (2007). Anatomical and Physiological Plasticity of Dendritic Spines. *Annual Review of Neuroscience*, *30*(1), 79–97. https://doi.org/10.1146/annurev.neuro.30.051606.094222

Babu, H., Claasen, J. H., Kannan, S., Rünker, A. E., Palmer, T., & Kempermann, G. (2011). A protocol for isolation and enriched monolayer cultivation of neural precursor cells from mouse dentate gyrus. *Frontiers in Neuroscience*, *5*(JUL), 1–10. https://doi.org/10.3389/fnins.2011.00089

Bernhardt, R., & Matus, A. (1984). Light and electron microscopic studies of the distribution of microtubule-associated protein 2 in rat brain: a difference between dendritic and axonal cytoskeletons. *The Journal of Comparative Neurology*, *226*(2), 203–221. https://doi.org/10.1002/cne.902260205

Bernier, P. J., Bedard, A., Vinet, J., Levesque, M., & Parent, A. (2002). Newly generated neurons in the amygdala and adjoining cortex of adult primates. *PNAS*, *99*(17), 11464–11469.

Bonfanti, L. (2006). PSA-NCAM in mammalian structural plasticity and neurogenesis. *Prog Neurobiol*, *80*(3), 129–164. https://doi.org/10.1016/j.pneurobio.2006.08.003

Bonfanti, L., & Nacher, J. (2012). New scenarios for neuronal structural plasticity in non-neurogenic brain parenchyma: The case of cortical layer II immature neurons. *Progress in Neurobiology*, *98*(1), 1–15.

https://doi.org/10.1016/j.pneurobio.2012.05.002

Bonfanti, L., Olive, S., Poulain, D. A., & Theodosis, D. T. (1992). Mapping of the distribution of polysialylated neural cell adhesion melecule throughout the central nervous system of the adult rat: an immunohistochemical study. *Neuroscience*, *49*(2), 419–436.

Bonfanti, L., & Ponti, G. (2008). Adult mammalian neurogenesis and the New Zealand white rabbit. *Veterinary Journal*, *175*(3), 310–331. https://doi.org/10.1016/j.tvjl.2007.01.023

Brewer, G. J., & Torricelli, J. R. (2007). Isolation and culture of adult neurons and neurospheres. *Nature Protocols*, *2*(6), 1490–1498. https://doi.org/10.1038/nprot.2007.207

Brown, J. P., Couillard-Després, S., Cooper-Kuhn, C. M., Winkler, J., Aigner, L., & Kuhn, H. G. (2003). Transient Expression of Doublecortin during Adult Neurogenesis. *Comp Neurol*, *467*(1), 1–10. https://doi.org/10.1002/cne.10874

Burgess, A., Wainwright, S. R., Shihabuddin, L. S., Rutishauser, U., Seki, T., & Aubert, I. (2008). Polysialic acid regulates the clustering, migration, and neuronal differentiation of progenitor cells in the adult hippocampus. *Developmental Neurobiology*, *68*(14), 1580–1590. https://doi.org/10.1002/dneu.20681

Burgess, A., Weng, Y. Q., Ypsilanti, A. R., Cui, X., Caines, G., & Aubert, I. (2007). Polysialic acid limits septal neurite outgrowth on laminin. *Brain Research*, *1144*(1), 52–58. https://doi.org/10.1016/j.brainres.2007.01.072

Cai, Y., Xiong, K., Chu, Y., Luo, D., Luo, X., Yuan, X., … Jeffrey, H. (2009). Doublecortin expression in adult cat and primate cerebral cortex relates to immature neurons that develop into GABAergic subgroups. *Experimental Neurobiology*, *216*(2), 342–

356. https://doi.org/10.1016/j.expneurol.2008.12.008.Doublecortin

Couillard-Despres, S., Winner, B., Schaubeck, S., Aigner, R., Vroemen, M., Weidner, N., … Aigner, L. (2005). Doublecortin expression levels in adult brain reflect neurogenesis. *Eur J Neurosci*, *21*(1), 1–14. https://doi.org/10.1111/j.1460-9568.2004.03813.x

De Camilli, P., Miller, P. E., Navone, F., Theurkauf, W. E., & Vallee, R. B. (1984). Distribution of microtubule-associated protein 2 in the nervous system of the rat studied by immunofluorescence. *Neuroscience*, *11*(4), 819–846. https://doi.org/10.1016/0306-4522(84)90194-5

De Magalhães, J. P., & Sandberg, A. (2005). Cognitive aging as an extension of brain development: A model linking learning, brain plasticity, and neurodegeneration. *Mechanisms of Ageing and Development*, *126*(10), 1026–1033. https://doi.org/10.1016/j.mad.2005.04.004

De Nevi, E., Marco-Salazar, P., Fondevila, D., Blasco, E., Perez, L., Pumarola, M., … Cerdanyola, B. (2013). Immunohistochemical study of doublecortin and nucleostemin in canine brain. *Eur J Histochem*, *57*(1), e9. https://doi.org/10.4081/ejh.2013.e9

Diodato, A., Ruinart De Brimont, M., Yim, Y. S., Derian, N., Perrin, S., Pouch, J., … Fleischmann, A. (2016). Molecular signatures of neural connectivity in the olfactory cortex. *Nature Communications*, *7*, 1–10. https://doi.org/10.1038/ncomms12238

Doetsch, F., & Alvarez-Buylla, A. (1996). Network of tangential pathways for neuronal migration in adult mammalian brain. *Proceedings of the National Academy of Sciences*, *93*(25), 14895–14900. https://doi.org/10.1073/pnas.93.25.14895

Doetsch, F., García-Verdugo, A., & Alvarez-Buylla, J. (1997). Cellular Composition and Three-Dimensional Organization of the Subventricular Germinal Zone in the Adult Mammalian Brain. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *17(13)*(13), 5046–5061. https://doi.org/10.1002/cne.902890106

Ekstrand, J. J., Domroese, M. E., Feig, S. L., Illig, K. R., & Haberly, L. B. (2001). Immunocytochemical analysis of basket cells in rat piriform cortex. *Journal of Comparative Neurology*, *434*(3), 308–328. https://doi.org/10.1002/cne.1179

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., & Gage, F. H. (1998). Neurogenesis in the adult human hippocampus. *Nat Med*, *4*(11), 1313–1317. https://doi.org/10.1038/3305

Ernst, A., Alkass, K., Bernard, S., Salehpour, M., Perl, S., Tisdale, J., … Frisén, J. (2014). Neurogenesis in the striatum of the adult human brain. *Cell*, *156*(5), 1072–1083. https://doi.org/10.1016/j.cell.2014.01.044

Feliciano, D., & Bordey, A. (2009). NIH Public Access, *19*(1), 389–399. https://doi.org/10.1016/j.asieco.2008.09.006.EAST

Feliciano, D., Bordey, A., & Bonfanti, L. (2015). Noncanonical Sites of Adult Neurogenesis in the Mammalian Brain Noncanonical Sites of Adult Neurogenesis in the Mammalian Brain, (October), 1–14. https://doi.org/10.1101/cshperspect.a018846

Ferrón, S. R., Andreu-Agulló, C., Mira, H., Sánchez, P., Ángeles Marqués-Torrejón, M., & Fariñas, I. (2007). A combined ex/in vivo assay to detect effects of exogenously added factors in neural stem cells. *Nature Protocols*, *2*(4), 849–859. https://doi.org/10.1038/nprot.2007.104

Fox, R. J., McColl, R. W., Lee, J.-C., Frohman, T., Sakaie, K., & Frohman, E. (2008). A

Preliminary Validation Study of Diffusion Tensor Imaging as a Measure of Functional Brain Injury. *Archives of Neurology*, *65*(9), 1179–1184. https://doi.org/10.1001/archneur.65.9.1179

Gavrilovici, C., D'Alfonso, S., & Poulter, M. O. (2010). Diverse interneuron populations have highly specific interconnectivity in the rat piriform cortex. *Journal of Comparative Neurology*, *518*(9), 1570–1588. https://doi.org/10.1002/cne.22291

Geinisman, Y., Disterhoft, J. F., Gundersen, H. J. G., Mcechron, M. D., Persina, I. S., Power, J. M., … West, M. J. (2000). Remodeling of hippocampal synapses after hippocampus-dependent associative learning. *Journal of Comparative Neurology*, *417*(1), 49–59. https://doi.org/10.1002/(SICI)1096-9861(20000131)417:1<49::AID-CNE4>3.0.CO;2-3

Gómez-Climent, Á., Hernández-González, S., Shionoya, K., Belles, M., Alonso-Llosa, G., Datiche, F., & Nacher, J. (2011). Olfactory bulbectomy, but not odor conditioned aversion, induces the differentiation of immature neurons in the adult rat piriform cortex. *Neuroscience*, *181*, 18–27. https://doi.org/10.1016/j.neuroscience.2011.03.004

Gomez-Climent, M. A., Castillo-Gomez, E., Varea, E., Guirado, R., Blasco-Ibanez, J. M., Crespo, C., … Nacher, J. (2008). A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. *Cerebral Cortex*, *18*(10), 2229–2240. https://doi.org/10.1093/cercor/bhm255

Gómez-Climent, M. Á., Castillo-Gómez, E., Varea, E., Guirado, R., Blasco-Ibáñez, J. M., Crespo, C., … Nácher, J. (2008). A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal phenotype into adulthood. *Cerebral Cortex*, *18*(10), 2229–2240. https://doi.org/10.1093/cercor/bhm255

Gross, C. (2000). Neurogenesis in the adult brain: death of a dogma. *Nature Reviews Neuroscience*, *1*, 67–73.

Guirado, R., Perez-Rando, M., Sanchez-Matarredona, D., Castillo-Gómez, E., Liberia, T., Rovira-Esteban, L., … Nacher, J. (2013). The dendritic spines of interneurons are dynamic structures influenced by PSA-NCAM expression. *Cerebral Cortex*, *24*(11), 3014–3024. https://doi.org/10.1093/cercor/bht156

Guo, F., Maeda, Y., Ma, J., Xu, J., Horiuchi, M., Miers, L., … Pleasure, D. (2010). Pyramidal neurons are generated from oligodendroglial progenitor cells in adult piriform cortex. *The Journal of Neuroscience*, *30*(36), 12036–12049. https://doi.org/10.1523/JNEUROSCI.1360-10.2010.Pyramidal

Haberly, L. B. (1983). Structure of the piriform cortex of the opossum. I. Description of neuron types with golgi methods. *Journal of Comparative Neurology*, *213*(2), 163–187. https://doi.org/10.1002/cne.902130205

Haberly, L. B., & Feig, S. L. (1983). Structure of the Pirifom Cortex of the Opossum . 11 . Fine Structure of Cell Bodies and Neuropil, *88*.

Haberly, L. B., & Price, J. L. (1978). Association and commissural fiber systems of the olfactory cortex of the rat. *The Journal of Comparative Neurology*, *178*(4), 711–740. https://doi.org/10.1002/cne.901780408

Hagiwara, A., Pal, S. K., Sato, T. F., Wienisch, M., & Murthy, V. N. (2012). Optophysiological analysis of associational circuits in the olfactory cortex. *Frontiers in Neural Circuits*, *6*(April). https://doi.org/10.3389/fncir.2012.00018

He, X., Zhang, X. M., Wu, J., Fu, J., Mou, L., Lu, D. H., … Yan, X. X. (2014). Olfactory experience modulates immature neuron development in postnatal and adult guinea pig piriform cortex. *Neuroscience*, *259*, 101–112.

https://doi.org/10.1016/j.neuroscience.2013.11.056

Hildebrandt, H., Mühlenhoff, M., Weinhold, B., & Gerardy-Schahn, R. (2007). Dissecting polysialic acid and NCAM functions in brain development. *Journal of Neurochemistry*, *103 Suppl 1*, 56–64. https://doi.org/10.1111/j.1471-4159.2007.04716.x

Johnson, D. M. G., Illig, K. R., Behan, M., & Haberly, L. B. (2000). New Features of Connectivity in Piriform Cortex Visualized by Intracellular Injection of Pyramidal Cells Suggest that "Primary" Olfactory Cortex Functions Like "Association" Cortex in Other Sensory Systems. *Journal of Neurosciene*, *20*(18), 6974–6982. https://doi.org/20/18/6974 [pii]

Johnson GV, J. R. (1992). The role of microtubule-associated protein 2 (MAP-2) in neuronal growth, pasticity, and degeneration. *J Neurosci Res*, *33*, 505–512.

Kempermann, G., & Gage, F. H. (1999). Experience-dependent regulation of adult hippocampal neurogenesis : effects of long-term stimualation and stimulus withdrawal. *Hippocampus*, *9*(3), 321–332. https://doi.org/10.1002/(SICI)1098-1063(1999)9:3<321::AID-HIPO11>3.0.CO;2-C

Kempermann, G., Jessberger, S., Steiner, B., & Kronenberg, G. (2004). Milestones of neuronal development in the adult hippocampus. *Trends in Neurosciences*, *27*(8), 447–452. https://doi.org/10.1016/j.tins.2004.05.013

Kerr, K., Agster, K., Furtak, S., & Burwell, R. (2007). From Grid Cells to Place Cells: A Mathematical Model. *Hippocampus*, *17*(9), 801–812. https://doi.org/10.1002/hipo

Klempin, F., Kronenberg, G., Cheung, G., Kettenmann, H., & Kempermann, G. (2011). Properties of doublecortin-(DCX)-expressing cells in the piriform cortex compared

to the neurogenic dentate gyrus of adult mice. *PLoS One*, *6*(10), e25760. https://doi.org/10.1371/journal.pone.0025760

Kojima, N., Kono, M., Yoshida, Y., Tachida, Y., Nakafuku, M., & Tsuji, S. (1996). Biosynthesis and expression of polysialic acid on the neural cell adhesion molecule is predominantly directed by ST8Sia II/STX during in vitro neuronal differentiation. *Journal of Biological Chemistry*, *271*(36), 22058–22062. https://doi.org/10.1074/jbc.271.36.22058

Kokoeva, M. V. (2005). Neurogenesis in the Hypothalamus of Adult Mice: Potential Role in Energy Balance. *Science*, *310*(5748), 679–683. https://doi.org/10.1126/science.1115360

König, R., Benedetti, B., Rotheneichner, P., O' Sullivan, A., Kreutzer, C., Belles, M., … Couillard-Després, S. (2016). Distribution and fate of DCX/PSA-NCAM expressing cells in the adult mammalian cortex: A local reservoir for adult cortical neuroplasticity? *Frontiers in Biology*, *11*(3), 193–213. https://doi.org/10.1007/s11515-016-1403-5

Kosik, K. S., & Finch, E. a. (1987). MAP2 and tau segregate into dendritic and axonal domains after the elaboration of morphologically distinct neurites: an immunocytochemical study of cultured rat cerebrum. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *7*(10), 3142–3153. https://doi.org/1987/10/01 00:01

Krägeloh-Mann, I. (2004). Imaging of early brain injury and cortical plasticity. *Experimental Neurology*, *190*(SUPPL. 1), 84–90. https://doi.org/10.1016/j.expneurol.2004.05.037

Lois, C., & Alvarez, A. (1994). Long-Distance Neuronal Migration in the Adult

Mammalian Brain. *Science, New Series*, *264*(5162), 1145–1148. Retrieved from http://www.jstor.org/stable/2885135

Löscher, W., Lehmann, H., & Ebert, U. (1998). Differences in the distribution of GABA- and GAD-immunoreactive neurons in the anterior and posterior piriform cortex of rats. *Brain Research*, *800*(1), 21–31. https://doi.org/10.1016/S0006-8993(98)00488-0

Luna, V. M., & Pettit, D. L. (2010). Cortex, *13*(5), 533–535. https://doi.org/10.1038/nn.2524.Asymmetric

Luna, V. M., & Schoppa, N. E. (2009). Cortex. *Biophysics*, *28*(35), 8851–8859. https://doi.org/10.1523/JNEUROSCI.2385-08.2008.GABAergic

Luzzati, F., Bonfanti, L., Fasolo, A., & Peretto, P. (2009). DCX and PSA-NCAM expression identifies a population of neurons preferentially distributed in associative areas of different pallial derivatives and vertebrate species. *Cereb Cortex*, *19*(5), 1028–1041. https://doi.org/10.1093/cercor/bhn145

Luzzati, F., Bonfanti, L., Fasolo, A., & Peretto, P. (2009). DCX and PSA-NCAM Expression identifies a population of neurons preferentially distributed in associative areas of different pallial derivatives and vertebrate species. *Cerebral Cortex*, *19*(5), 1028–1041. https://doi.org/10.1093/cercor/bhn145

Luzzati, F., De Marchis, S., Fasolo, A., & Peretto, P. (2007). Adult neurogenesis and local neuronal progenitors in the striatum. *Neurodegenerative Diseases*, *4*(4), 322–327. https://doi.org/10.1159/000101889

Luzzati, F., Nato, G., Oboti, L., Vigna, E., Rolando, C., Armentano, M., … Peretto, P. (2014). Quiescent neuronal progenitors are activated in the juvenile guinea pig lateral striatum and give rise to transient neurons. *Development*, *141*(21), 4065–

4075. https://doi.org/10.1242/dev.107987

Luzzati, F., Peretto, P., Aimar, P., Ponti, G., Fasolo, a, & Bonfanti, L. (2003). Glia-independent chains of neuroblasts through the subcortical parenchyma of the adult rabbit brain. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(22), 13036–13041. https://doi.org/10.1073/pnas.1735482100

Meyer, E. A., Illig, K. R., & Brunjes, P. C. (2006). Differences in chemo- and cytoarchitectural features within pars principalis of the rat anterior olfactory nucleus suggest functional specialization. *Journal of Comparative Neurology*, *498*(6), 786–795. https://doi.org/10.1002/cne.21077

Nacher, J., Alonso-Llosa, G., Rosell, D., & McEwen, B. (2002). PSA-NCAM expression in the piriform cortex of the adult rat. Modulation by NMDA receptor antagonist administration. *Brain Research*, *927*(2), 111–121. https://doi.org/10.1016/S0006-8993(01)03241-3

Nacher, J., Alonso-Llosa, G., Rosell, D., & McEwen, B. (2002). PSA-NCAM expression in the piriform cortex of the adult rat . Modulation by NMDA receptor antagonist administration. *Brain Res*, *927*(2), 111–121.

Nacher, J., Crespo, C., & McEwen, B. . (2001). Doublecortin expression in the adult rat telencephalon. *Eur J Neurosci*, *14*(4), 629–644.

Nacher, J., Crespo, C., & Mcewen, B. S. (2001). Doublecortin expression in the adult rat telencephalon - Nacher - 2001 - European Journal of Neurosc.pdf, *14*, 629–644.

Nacher, J., Guirado, R., Varea, E., Alonso-Llosa, G., Röckle, I., & Hildebrandt, H. (2010). Divergent impact of the polysialyltransferases ST8SiaII and ST8SiaIV on polysialic acid expression in immature neurons and interneurons of the adult cerebral

cortex. *Neuroscience*, *167*(3), 825–837. https://doi.org/10.1016/j.neuroscience.2010.02.067

Nacher, J., Pham, K., Gil-Fernandez, V., & Mcewen, B. S. (2004). Chronic restraint stress and chronic corticosterone treatment modulate differentially the expression of molecules related to structural plasticity in the adult rat piriform cortex. *Neuroscience*, *126*(2), 503–509. https://doi.org/10.1016/j.neuroscience.2004.03.038

Nakayama, J., & Fukuda, M. (1996). A human polysialyltransferase directs in vitro synthesis of polysialic acid. *Journal of Biological Chemistry*, *271*(4), 1829–1832. https://doi.org/10.1074/jbc.271.4.1829

Neville, K. R., & Haberly, L. B. (2003). Beta and Gamma Oscillations in the Olfactory System of the Urethane-Anesthetized Rat. *Journal of Neurophysiology*, *90*(6), 3921–3930. https://doi.org/10.1152/jn.00475.2003

Ni Dhuill, C. M., Fox, G. B., Pittock, S. J., O'Connell, A. W., Murphy, K. J., & Regan, C. M. (1999). Polysialylated Neural Cell Adhesion Molecule Expression in the Dentate Gyrus of the Human Hippocampal Formation From Infancy to Old Age. *J Neurosci Res*, *55*(1), 99–106. https://doi.org/10.1002/(SICI)1097-4547(19990101)55:1<99::AID-JNR11>3.0.CO;2-S [pii]

Palmer, T. D., Ray, J., & Gage, F. H. (1995). FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. *Molecular and Cellular Neurosciences*. https://doi.org/10.1006/mcne.1995.1035

Patzke, N., LeRoy, A., Ngubane, N. W., Bennett, N. C., Medger, K., Gravett, N., … Manger, P. R. (2014). The distribution of doublecortin-immunopositive cells in the brains of four afrotherian mammals: the Hottentot golden mole (Amblysomus

hottentotus), the rock hyrax (Procavia capensis), the eastern rock sengi (Elephantulus myurus) and the four-toed sengi (. *Brain Behav Evol*, *84*(3), 227–241. https://doi.org/10.1159/000367934

Pekcec, A., Löscher, W., & Potschka, H. (2006). Neurogenesis in the adult rat piriform cortex. *Neuroreport*, *17*(6), 571–574.

Pencea, V., Bingaman, K. D., Wiegand, S. J., & Luskin, M. B. (2001). Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience*, *21*(17), 6706–6717. https://doi.org/21/17/6706 [pii]

Peretto, P., & Bonfanti, L. (2014). Major unsolved points in adult neurogenesis: Doors open on a translational future? *Frontiers in Neuroscience*, *8*(8 JUN), 1–6. https://doi.org/10.3389/fnins.2014.00154

Petridis, A. K., El Maarouf, A., & Rutishauser, U. (2004). Polysialic acid regulates cell contact-dependent neuronal differentiation of progenitor cells from the subventricular zone. *Developmental Dynamics*, *230*(4), 675–684. https://doi.org/10.1002/dvdy.20094

Pierce, A. a, & Xu, A. W. (2011). Compensatory Mechanism To Regulate Energy Balance, *30*(2), 723–730. https://doi.org/10.1523/JNEUROSCI.2479-09.2010.De

Reynolds, B. A., & Weiss, S. (2011). Nervous System Generation of Neurons and Astrocytes from Isolated Cells of the Adult Mammalian Central Nervous System. *Science*, *255*(5052), 1707–1710.

Rivers, L. E., Young, K. M., Rizzi, M., Jamen, F., Psachoulia, K., Wade, A., … Richardson, W. D. (2008). PDGFRA/NG2 glia generate myelinating oligodendrocytes and

piriform projection neurons in adult mice. *Nat Neurosci*, *11*(12), 1392–1401. https://doi.org/10.1038/nn.2220

Robins, S. C., Trudel, E., Rotondi, O., Liu, X., Djogo, T., Kryzskaya, D., … Kokoeva, M. V. (2013). Evidence for NG2-glia derived, adult-born functional neurons in the hypothalamus. *PLoS ONE*, *8*(10). https://doi.org/10.1371/journal.pone.0078236

Rossi, S. L., Mahairaki, V., Zhou, L., Song, Y., & Koliatsos, V. E. (2014). Remodeling of the piriform cortex after lesion in adult rodents. *Neuroreport*, *25*(13), 1006–1012. https://doi.org/10.1097/WNR.0000000000000203

Rubio, A., Belles, M., Belenguer, G., Vidueira, S., Fariñas, I., Nacher, J., … Nacher, J. (2016). Characterization and isolation of immature neurons of the adult mouse piriform cortex. *Dev Neurobiol*, *76*(7), 748–763. https://doi.org/10.1002/dneu.22357

Sabael, B. A. (2008). Plasticity and restoration of vision after visual system damage: an update. *Restor Neurol Neurosci*, *26*(4), 243–247.

Sarma, A. A., Richard, M. B., & Greer, C. A. (2011). Developmental dynamics of piriform cortex. *Cerebral Cortex*, *21*(6), 1231–1245. https://doi.org/10.1093/cercor/bhq199

Seki, T. & A., & Arai, Y. (1991). Expression of highly polysialylated NCAM in the neocortex and piriform cortex of the developing and the adult rat. *Anat Embryol*, *184*(4), 395–401.

Shapiro, L. A., Ng, K. L., Kinyamu, R., Whitaker-Azmitia, P., Geisert, E. E., Blurton-Jones, M., … Ribak, C. E. (2007). Origin, migration and fate of newly generated neurons in the adult rodent piriform cortex. *Brain Struct Funct*, *212*(2), 133–148. https://doi.org/10.1007/s00429-007-0151-3

Shapiro, L. A., Ng, K. L., Zhou, Q. Y., & Ribak, C. E. (2007). Olfactory enrichment enhances the survival of newly born cortical neurons in adult mice. *Neuroreport*, *18*(10), 981–985. https://doi.org/10.1097/WNR.0b013e3281532bc1

Shapiro, L. A., Ng, K., Zhou, Q. Y., Ribak, C. E., & Ribak, C. E. S. L. A. N. K. Z. Q. Y., Shapiro, L. A., … Ribak, C. E. (2009). Subventricular zone-derived, newly generated neurons populate several olfactory and limbic forebrain regions. *Epilepsy Behav*, *14 Suppl 1*(Suppl 1), 74–80. https://doi.org/10.1016/j.yebeh.2008.09.011

Shechter, R., Ziv, Y., & Schwartz, M. (2007). New GABAergic Interneurons Supported by Myelin-Specific T Cells Are Formed in Intact Adult Spinal Cord. *Stem Cells*, *25*(9), 2277–2282. https://doi.org/10.1634/stemcells.2006-0705

Shipley, M. T., & Adamek, G. D. (1984). the Connections of the Mouse Olfactory-Bulb - a Study Using Orthograde and Retrograde Transport of Wheat-Germ-Agglutinin Conjugated To Horseradish-Peroxidase. *Brain Research Bulletin*, *12*(6), 669–688. https://doi.org/10.1016/0361-9230(84)90148-5

Sutton, M. A., & Schuman, E. M. (2006). Dendritic Protein Synthesis, Synaptic Plasticity, and Memory. *Cell*, *127*(1), 49–58. https://doi.org/10.1016/j.cell.2006.09.014

Suzuki, N., & Bekkers, J. M. (2010). Distinctive classes of GABAergic interneurons provide layer-specific phasic inhibition in the anterior piriform cortex. *Cerebral Cortex*, *20*(12), 2971–2984. https://doi.org/10.1093/cercor/bhq046

Tamura, Y., Kataoka, Y., Cui, Y., Takamori, Y., Watanabe, Y., Yamada, Y., & Yamada, H. (2007). Multi-directional differentiation of doublecortin- and NG2-immunopositive progenitor cells in the adult rat neocortex in vivo. *Eur J Neurosci*, *25*(12), 3489–3498. https://doi.org/10.1111/j.1460-9568.2007.05617.x

Theodosis DT, Poulain DA, O. S. (2008). Activity-Dependent Structural and Functional

Plasticity of Astrocyte-Neuron Interactions.pdf. *Physiological Reviews*, 983–1008. https://doi.org/10.1152/physrev.00036.2007.

Thiele, C., Hannah, M., Fahrenholz, F., & Huttner, W. (2000). Cholesterol binds to synaptophysin and is required for biogenesis of synaptic vesicles. *Nat Cell Biol*, *1*(2), 42–49.

Vadodaria, K. C., Yanpallewar, S. U., Vadhvani, M., Toshniwal, D., Liles, L. C., Rommelfanger, K. S., … Vaidya, V. A. (2017). Noradrenergic regulation of plasticity marker expression in the adult rodent piriform cortex. *Neuroscience Letters*, *644*, 76–82. https://doi.org/10.1016/j.neulet.2017.02.060

Varea, E., Belles, M., Vidueira, S., Blasco-Ibanez, J. M., Crespo, C., Pastor, A. M., & Nacher, J. (2011). PSA-NCAM is Expressed in Immature, but not Recently Generated, Neurons in the Adult Cat Cerebral Cortex Layer II. *Front Neurosci*, *5*(17), 9. https://doi.org/10.3389/fnins.2011.00017

Varea, E., Belles, M., Vidueira, S., Blasco-Ibáñz, J. M., Crespo, C., Pastor, Á. M., & Nacher, J. (2011). PSA-NCAM is expressed in immature, but not recently generated, neurons in the adult cat cerebral cortex layer II. *Frontiers in Neuroscience*, *5*(FEB), 1–9. https://doi.org/10.3389/fnins.2011.00017

Varea, E., Castillo-Gómez, E., Gómez-Climent, M. Á., Guirado, R., Blasco-Ibáñez, J. M., Crespo, C., … Nácher, J. (2009). Differential evolution of PSA-NCAM expression during aging of the rat telencephalon. *Neurobiology of Aging*, *30*(5), 808–818. https://doi.org/10.1016/j.neurobiolaging.2007.08.016

Wiegand, H. F., Beed, P., Bendels, M. H. K., Leibold, C., Schmitz, D., & Johenning, F. W. (2011). Complementary Sensory and Associative Microcircuitry in Primary Olfactory Cortex. *Journal of Neuroscience*, *31*(34), 12149–12158.

https://doi.org/10.1523/JNEUROSCI.0285-11.2011

Xerri, C. (2008). Imprinting of idyosyncratic experience in cortical sensory maps: Neural substrates of representational remodeling and correlative perceptual changes. *Behavioural Brain Research*, *192*(1), 26–41. https://doi.org/10.1016/j.bbr.2008.02.038

Xiong, J., Wang, Y., Zhu, Z., Liu, J., Wang, Y., Zhang, C., … Feng, Y. (2007). NG2 proteoglycan increases mesangial cell proliferation and extracellular matrix production. *Biochemical and Biophysical Research Communications*, *361*(4), 960–967. https://doi.org/10.1016/j.bbrc.2007.07.113

Xiong, K., Luo, D. W., Patrylo, P. R., Luo, X. G., Struble, R. G., Clough, R. W., & Yan, X. X. (2008). Doublecortin-expressing cells are present in layer II across the adult guinea pig cerebral cortex: partial colocalization with mature interneuron markers. *Exp Neurol*, *211*(1), 271–282. https://doi.org/10.1016/j.expneurol.2008.02.003

Yang, Y., Xie, M. X., Li, J. M., Hu, X., Patrylo, P. R., Luo, X. G., … Yan, X. X. (2015). Prenatal genesis of layer II doublecortin expressing neurons in neonatal and young adult guinea pig cerebral cortex. *Front Neuroanat*, *9*(109), 11. https://doi.org/10.3389/fnana.2015.00109

Zhang, X. M., Cai, Y., Chu, Y., Chen, E. Y., Feng, J. C., Luo, X. G., … Yan, X. X. (2009). Doublecortin-expressing cells persist in the associative cerebral cortex and amygdala in aged nonhuman primates. *Front Neuroanat*, *3*, 17. https://doi.org/10.3389/neuro.05.017.2009