University of Turin

DEPARTMENT OF VETERINARY SCIENCES

Thesis submitted for the degree of "Doctor Philosophiae" in
'Veterinary Sciences for Animal Health and Food Safety'

XXXV CYCLE

"Immature" neurons in mammals: phylogenetic variation in brain regions and ages

CANDIDATE: Marco Ghibaudi

SCIENTIFIC SUPERVISOR: Prof. Luca Bonfanti

PhD COORDINATOR: Prof. Maria Teresa Capucchio

ACADEMIC YEARS: 2020/2021/2022

SCIENTIFIC DISCIPLINARY SECTION: VET/01

Summary

Most biomedical research, including the neurosciences, is largely carried out using laboratory rodents as animal models, yet differences are emerging with respect to humans. This fact can lead to an incorrect interpretation of preclinical data, and consequent problems in translation. In the last few years, remarkable interspecies differences have emerged in brain plasticity: the ability of the nervous tissue to modify its structure, allowing to refine cellular contacts and to "sculpt" neuronal circuits based on external/internal stimuli (both in physiological and pathological states). For this reason, studies in comparative neuroplasticity are needed, possibly performed in a "comparable" way.

Despite being substantially stable, the adult brain displays plasticity in different forms. A striking example is adult neurogenesis: the addition of new neurons from active division of neural stem cells, hosted in restricted "neurogenic niches". Yet, neurogenic processes are heterogeneous in mammals, as to their types (canonical and non-canonical), and remarkable interspecies variation. In this complexity, a new form of plasticity is represented by populations of prenatally generated, "immature" or "dormant" neurons (INs): non-dividing cells that continue to express molecules of immaturity through life (some of which shared with the newly born neurons, such as the cytoskeletal protein doublecortin; DCX). The INs can "awake" at different time points by completing their maturation and functionally integrating into the preexisting circuitries (neurogenesis without division). Though restricted to paleocortex in rodents, the INs extend within the entire neocortex in other mammals, being particularly abundant in large-brained, gyrencephalic species. DCX+ cells are known to exist in subcortical regions, such as claustrum and amygdala, yet their nature (newly generated or dormant), as well as their interspecies variation, remain uncertain.

Here we pursued the mapping of the "immature" neuronal cell populations moving from the cortex to the subcortical regions of mammals (from mouse to chimpanzee). To obtain "comparable" results despite the high number of variables encountered (brain availability, brain size, tissue fixation, antibody specificity, real interspecies difference), we firstly set the best conditions for detecting our antigens of interest in widely different species. The study, carried out for DCX and Ki67-antigen in brain regions hosting INs (neocortex; paleocortex) and newly born neurons (hippocampus; subventricular zone), revealed that antibody species-specificity and real interspecies differences can be true limits, rather than different types of fixation.

On these bases, the occurrence, topographical distribution, and amount of DCX+ cells were investigated in the amygdala of 7 mammalian species at different ages. The analysis was extended to cell division (Ki67 antigen), in search for possible marker coexpression. While the DCX+ cells were more abundant in the amygdala of non-rodent species (especially in primates), the dividing cells showed an inverse pattern. Coexpression of the two antigens was never found, and the two cell populations displayed very different topographical distribution in all the species investigated. These results strongly indicate that the amygdalar DCX+ cells are INs with high interspecies variation, being particularly important in complex brains of long-living species. Interestingly, their amount remained substantially unchanged between young and aged individuals, suggesting that they may persist as a reservoir of undifferentiated neurons in the adult/aging amygdala. This raised the question whether INs of the cortex behave similarly through the animal lifespan. Hence, the variation in number and features of cINs was investigated in the mouse paleocortex at different ages (from 1 to 15 months). We found a marked decrease in cINs during juvenile stages, reminiscent of that observed for hippocampal neurogenesis, and only a small amount of cINs persisted up to advanced ages. Results obtained in the mouse cortex were very different from those observed in the amygdala (and cortex) of gyrencephalic species: while reaching a slow exhaustion in short-living species, the INs form a long-lasting reservoir of "plastic" cells in long-living mammals.

Overall, a new landscape emerges, likely sculpted by evolution: INs represent the prevalent choice in gyrencephalic, long-living species, not only being more abundant but diluted in time to grant a reservoir of plastic neurons in strategic brain regions.

Contents

REFERENCES

Acknowledgements

§§§

Figures and Tables

This work includes **41** Figures (**251** original micrographs, **34** original drawings/graphics, **10** images imported/modified from different sources) and **19** original Tables. A list of the Figures is reported below:

Boxes

Abbreviations

CNS	Central Nervous System
NSCs	Neural Stem Cells
SVZ	Subventricular Zone
SGZ	Subgranular Zone
INs	Immature Neurons
cINs	Cortical Immature Neurons
DCX	Doublecortin
PSA-NCAM	Polysialylated form of the Neural Cell Adhesion Molecule
BrdU	5-bromo-2′-deoxyuridine
NeuN	Neuronal Nuclear Antigen
NG2	Neuron Glia Antigen
GFAP	Glial Fibrillary Acidic Protein
PFA	Paraformaldehyde
PMI	Post Mortem Interval
OPCs	Oligodendrocyte Progenitor Cells

CHAPTER 1

Introduction

In the early 1900s, the adult brain was considered an immutable and stable system, in which no changes can occur (Cajal, 1928). A century later, it was discovered that this assumption was not completely true, since the brain tissue can change, even in its mature form (Altman and Das, 1965; Lois and Alvarez-Buylla, 1994). Yet, the discoveries on brain structural plasticity opened many questions and some controversies, revealing a highly complex landscape (Bonfanti and Nacher, 2012; Bonfanti and Seki, 2021).

1.1 Brain plasticity: the ability to change

Plasticity is defined as "the ability to make adaptive changes related to the structure and the functions of a system" (Davidson and McEwen, 2012; Bonfanti and Nacher, 2012; Bonfanti and Charvet, 2021; Leuner and Gould, 2010). This feature allows to adapt to environmental changes as well as to modifications in the system itself (changes in the homeostasis, tissue damages, pathologies). In the central nervous system (CNS), the adaptations involve the refinement of interneuronal connections (Bonfanti and Peretto, 2011; Martino et al., 2011; Ming and Song, 2005; Imayoshi et al., 2008; Kempermann et al., 2004; Lim and Alvarez-Buylla, 2016) or the genesis of new cells (such as neurons, astrocytes or oligodendrocytes, the main cell types in the brain), and are strongly affected by factors like the individual behavior or the environment in which the animal lives (Vivar and Praag, 2017; Kempermann, 2019; Kempermann et al., 2022)[1].

Different types of plasticity do exist, and they can be divided in "functional" and "structural". The former occurs at the molecular level, involving changes at synapses such as presynaptic transmitter release, postsynaptic receptor trafficking, signal transduction pathways, gene activation and protein synthesis, which can affect the synaptic transmission. These functional changes are usually involved in learning and memory. Structural plasticity instead involves modifications in the shape and/or number of cells, including formation/elimination of synapses (Holtmaat and Svoboda 2009; Chen and Nedivi 2010) and the genesis of new neurons (adult neurogenesis; Lois and Alvarez-Buylla, 1994). During embryogenesis, structural changes occur in high amount and rate, allowing

[1] It is worth noting that several studies carried out on natural populations of wild-living rodents did not find any environmental effects exerted by physical activity (Amrein et al., 2004; Hauser et al., 2009; Klaus and Amrein, 2012), nor these effects seem to be present in animals with imposed running (e.g., sheep; Swanson et al., 2017), which is not a natural behaviour for these animals. These results strongly suggest that an increase of neurogenesis after physical activity might be detectable only in inbreed animal models, which are born in the lab and kept in artificial conditions through their life.

the correct shaping of the CNS (massive changes involving cell proliferation, cell migration, axonal/dendritic growth, synapse formation/elimination). From the postnatal period, a general stabilization of the system takes place, with a downregulation of these processes (with regional differences; Bonfanti and Charvet, 2021; Kast and Levitt, 2019) that fix tissue architecture and neural connections, thus building up a prevalently non-renewable tissue (Götz et al., 2016; Bonfanti and Peretto, 2011; Bonfanti, 2006).

1.1.1 Brain structural plasticity: theme and variations

Different forms of structural plasticity can be found in the adult CNS (Figure 1.1). One at the synaptic level (synaptic plasticity) involving changes in contacts among pre-existing neurons such as formation/elimination of synapses, axonal sprouting/pruning, changes in dendritic spines and modification of cell shape (Holtmaat and Svoboda, 2009), thus being considered a form of *non-neurogenic* plasticity (not changing the number of neurons). A further level of plasticity can be present at the cellular level (whole cell plasticity, in which the overall shape of the cell is affected; e.g., neuro-glial plasticity; Theodosis et al., 2008). While synaptic changes are widespread in the adult brain, the neurogenic processes are highly restricted to "neurogenic sites" which contain neural stem cell niches. This kind of plasticity can be referred to as stem cell-driven neurogenesis, depending on the occurrence and activity of neural stem/progenitor cells (NSCs; Reynolds and Weiss 1992; Gritti et al., 1996; Doetsch et al., 1999). Since the discovery of the NSCs (at the beginning of the Nineties), two main stem cell niches were studied: the subventricular zone of the lateral ventricles (SVZ; Lim and Alvarez-Buylla, 2016), and the subgranular zone of the hippocampal dentate gyrus (SGZ; Gonçalves et al., 2016). Nevertheless, over the years the landscape of adult neurogenesis has gained complexity (see below and Box 1).

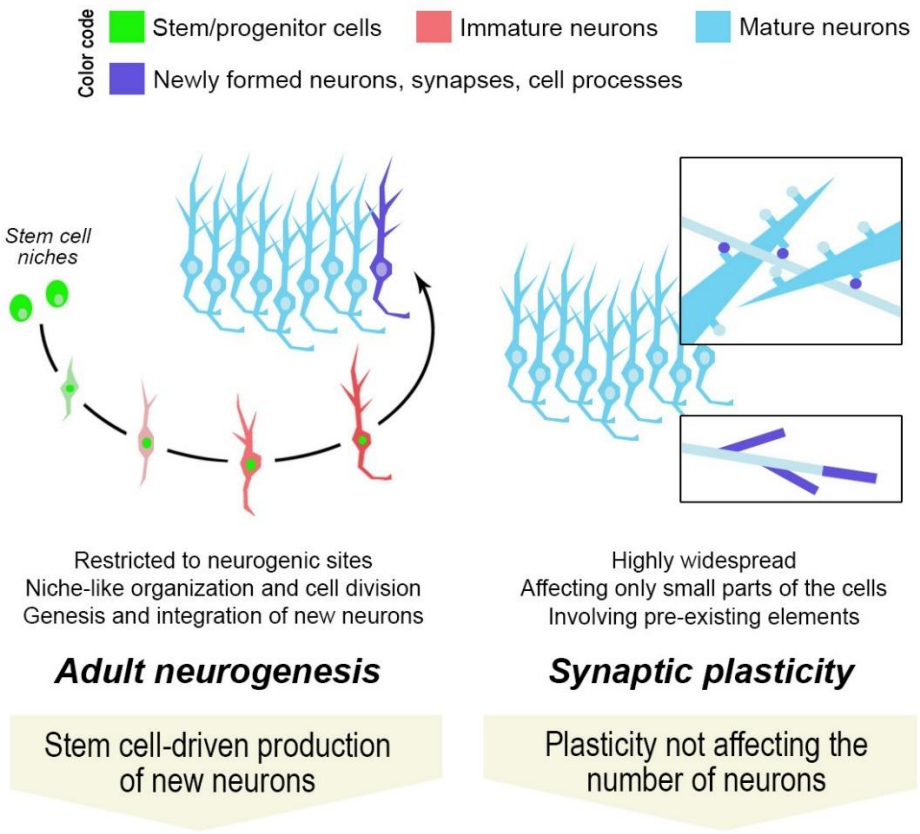


Figure 1.1 - Different ways for achieving structural plasticity in the adult mammalian brain. Stem cell-driven genesis of new neurons (adult neurogenesis) and synaptic/axonal plasticity represent two extremes of plastic events which can modify the neural circuits (modified from La Rosa and Bonfanti, 2021).

1.1.2 Neurogenic plasticity (adult neurogenesis)

Two main neurogenic sites are present in the adult brain: the SVZ and the SGZ. Both regions contain neural stem cells (NSCs), which originate from radial glia, then remaining within the mature brain tissue in the form of astrocytes forming "niches", namely remnants of the embryonic germinal layers (Doetsch et al., 1999; Noctor et al., 2001; reviewed in Alvarez-Buylla et al., 2001; Bonfanti and Peretto, 2007). The neural stem cell niches are composed by different types of cells: endothelial cells, astrocytes, ependymal cells, microglia, extracellular matrix and neuronal progeny. Herein, the proliferating NSCs give birth to neuronal precursors (intermediate progenitors) which are highly proliferative and produce neuroblasts that then migrate towards their final target: the granule and periglomerular layers of the olfactory bulb for SVZ, and the granule cell layer of the dentate gyrus for SGZ (Lois and Alvarez-Buylla, 1994; Kempermann et al., 2015).

Once reached their final target location (after selective death of around one half of the cells), the neuronal precursors can mature in different types of neurons that functionally integrate in the neuronal network (Obernier et al., 2014; Figure 1.2).

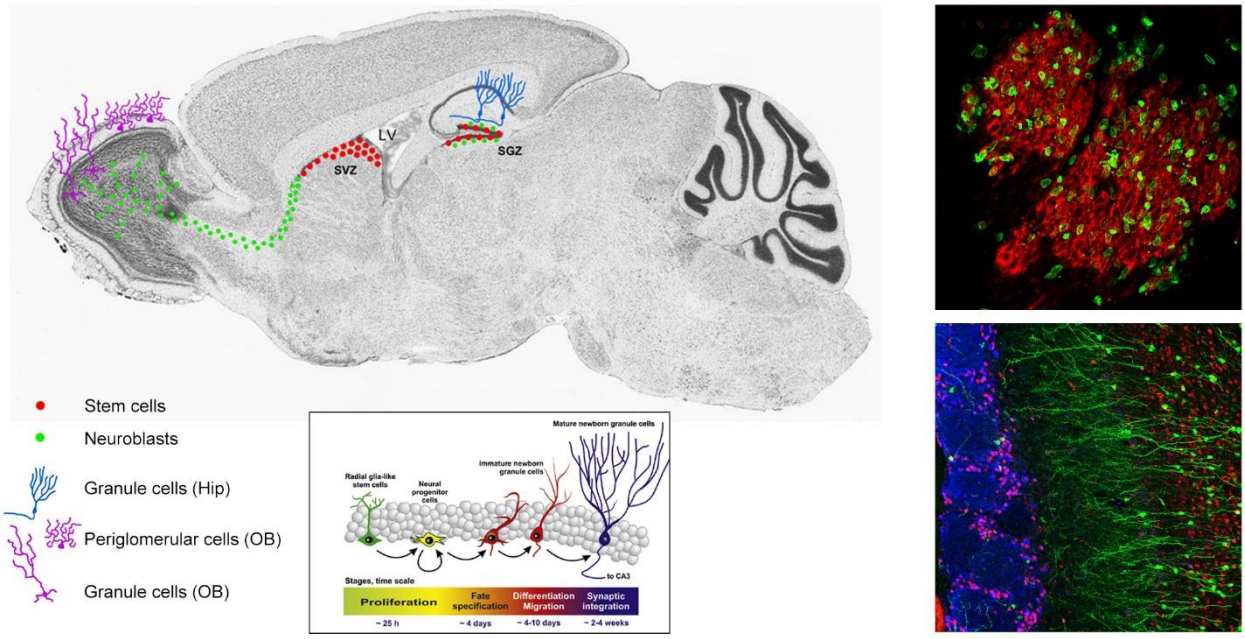


Figure 1.2 - Adult neurogenesis in rodents. Two main neurogenic sites (lateral ventricle wall - SVZ - and hippocampal dentate gyrus - SGZ) contain neural stem cells and provide a progeny of neuronal precursors for hippocampal granule cell layer and olfactory bulb. Images: schematic drawing on the bottom, Lucassen et al., 2010; photos on the right, Adult Neurogenesis group, Neuroscience Institute Cavalieri Ottolenghi.

More recently, a third neurogenic site has been described in the hypothalamus, wherein a population of tanycytes can act as stem cells (Pencea et al., 2001; Xu et al., 2005; see Box 1). In addition, other neurogenic processes have been described outside of the two/three main niches, representing the so-called "parenchymal" or "non-canonical neurogenesis" (reviewed in Bonfanti and Peretto, 2011; Feliciano et al., 2015; see Box 1).

The discovery of adult neurogenesis in the Nineties started a "gold rush" in the scientific community, in search for possible brain repair solutions for different kind of damage and neurological disorders (neurodegenerative diseases such as Alzheimer's disease). In the last 30 years, a huge number of publications were produced on this topic (now exceeding 13.000 articles in PubMed), mainly focusing on the mouse brain as a model. Yet, until now no promising therapies

to repair the CNS has been discovered, and most clinical trials approved for testing stem cell treatments (so-called regenerative medicine) failed to reach the end (see Namiot et al., 2022, and Chapter 5 for discussion).

There are several reasons (and interpretations) that might explain the general failure in exploiting neurogenic plasticity for therapeutic purposes (Bonfanti, 2016). First, the fact that adult neurogenesis is a mostly juvenile, protracted developmental process serving for refining specific neural circuits during brain growth/maturation, to allow the animal to adapt on the basis of experience/environmental changes (see Box 1). Second, but related with the previous point, adult neurogenesis can be highly heterogeneous in terms of location, age, physiological and pathological states (Bonfanti and Peretto, 2011), a variation that can increase when different animal species, living in different socio-ecological niches, are considered (Barker et al., 2011; Feliciano et al., 2015). Finally, a third important element of heterogeneity is the general reduction in neurogenesis/plasticity rate observed at different ages across the animal lifespan, an aspect that is present in all animal species, yet with substantial interspecies differences.

Before addressing the above-mentioned points (in Box 1), it is important to introduce a new form of plasticity that has been unveiled during recent years, as a new element of heterogeneity to the existing variation in types of neurogenesis: the so called "immature" or "dormant" neurons.[2]

1.2 "Immature" neurons: a new form of structural plasticity and neurogenesis?

While the "gold rush" raged in research laboratories, another cell population started to be studied in the brain structural plasticity field: the non-newly generated "immature" neurons (INs). Being firstly described in the layer II of rodent paleocortex (Bonfanti et al., 1992; Gómez-Climent et al., 2008) subsequent studies showed a high level of heterogeneity in terms of species and brain regions in which they occur. For instance, in rabbit, guinea pig (Luzzati et al., 2009; Xiong et al., 2008), cat (Cai et al., 2009; Varea et al., 2011), some bat species (Chawana et al., 2016) and some non-human primates (Cai et al., 2009; Zhang et al., 2009; Bloch et al., 2011; Fasemore et al., 2018), INs were found in layer II and upper II of the piriform, perirhinal and entorhinal cortices

[2] At present, being the topic of immature neurons a substantial novelty, many different names have been employed by different authors to define them (immature neurons, stand by neurons, dormant neurons, non-newly generated immature neurons). The researched involved in their discovery agree that it is time to update and uniform this terminology, and some of them are working at this task. Here, it can be useful to remind that "immature" is too vague and general, since a phase of immaturity is shared with the newly born neurons of adult neurogenesis; on the other hand, "dormant" is reminiscent of "quiescent", and quiescence is a property of stem cells, that in the case of the immature neurons are not involved.

and also in neocortical areas (cortical "immature" neurons; cINs), especially associative regions (Luzzati et al., 2009; Zhang et al., 2009). Despite these differences, two main subpopulations can be distinguished in all species based on their soma size and dendritic morphology: small cells with short and highly irregular trajectories and large cells with one or two long dendrites expanding into layer I and a pyramidal-semilunar morphology (Figure 1.3).

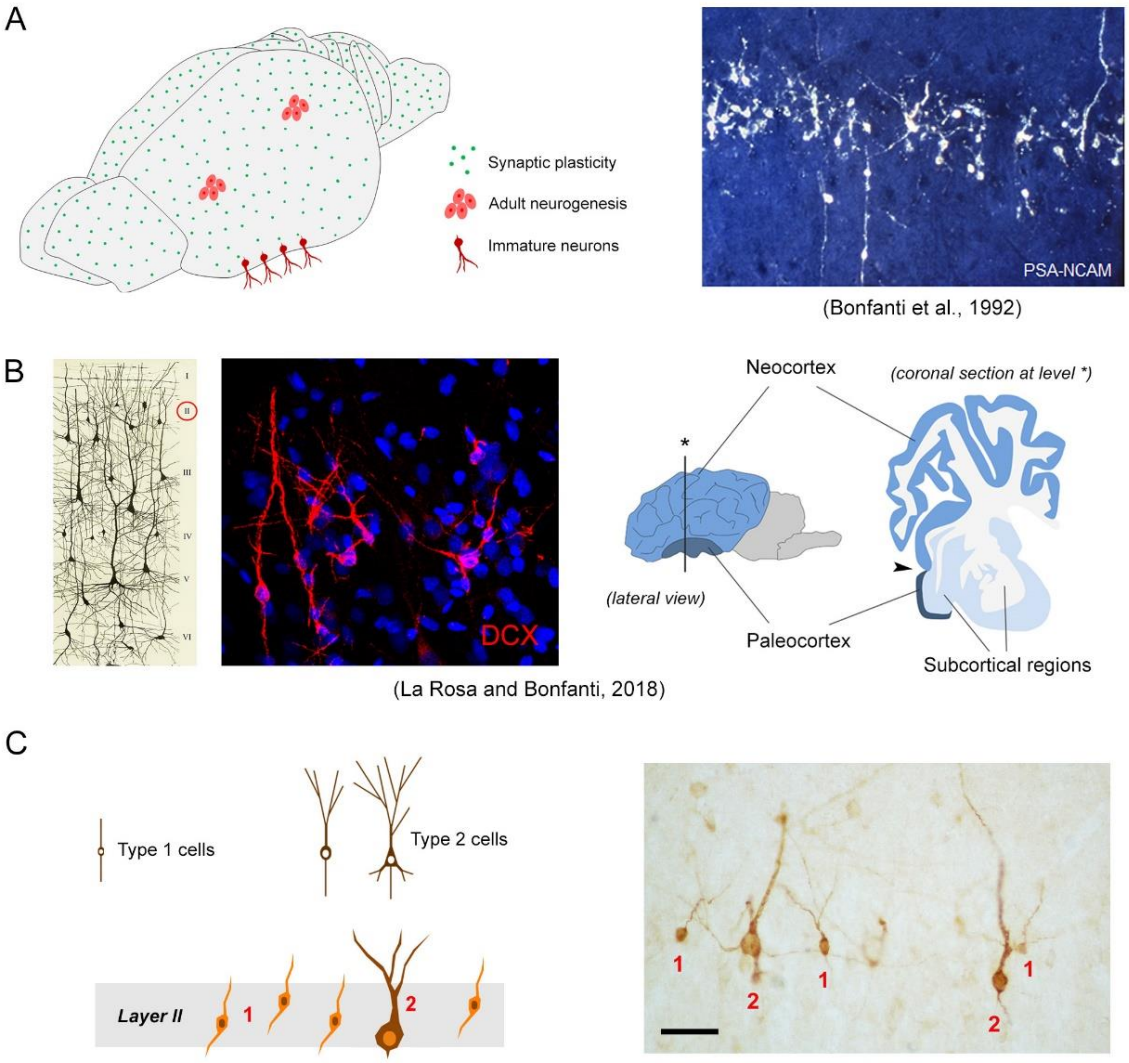


Figure 1.3 – Immature neurons: a third type of brain structural plasticity, in addition to synaptic plasticity and canonical adult neurogenesis (A, left); A, right, a population of neurons expressing PSA-NCAM ("embryonic" form of N-CAM) was identified in the nineties within the paleocortex of laboratory rodents. B, These neurons are located in the layer II of the piriform and entorhinal cortices (paleocortex), and express other molecules of immaturity (e.g., doublecortin, DCX). C, Two main types of cortical DCX+ neurons can be identified on the basis of their morphology and cell soma size: type 1 cells (small, bipolar) and type 2 cells (large and ramified); scale bar, 25 µm.

Initially, this type of cells was wrongly considered as a form of neurogenic plasticity, since they shared common immaturity markers used in the adult neurogenesis field (e.g. the cytoskeletal protein doublecortin, DCX, and the polysialylated form of the neural cell adhesion molecule, PSA-NCAM; Gómez-Climent et al., 2008; Bonfanti et al., 1992). In fact, great differences emerged in terms of origin and features of this cell population: newly generated neurons of adult neurogenesis are born in the adult brain, showing an active cell proliferation and giving birth to newly generated elements which can migrate to their final target and become fully-mature neurons. In contrast, studies involving BrdU injections followed by different survival times showed that the DCX+ cells of the cortical layer II are generated during embryogenesis and not in adulthood (Nacher et al., 2002; Gomez-Climent et al., 2008; Luzzati et al., 2009; Piumatti et al., 2018) and no evidence of cell migration has been proven. Hence, these neurons would be originated during embryonic development and then they would persist in a prolonged immature state in the adult CNS (Gomez-Climent et al., 2008; Figure 1.4).

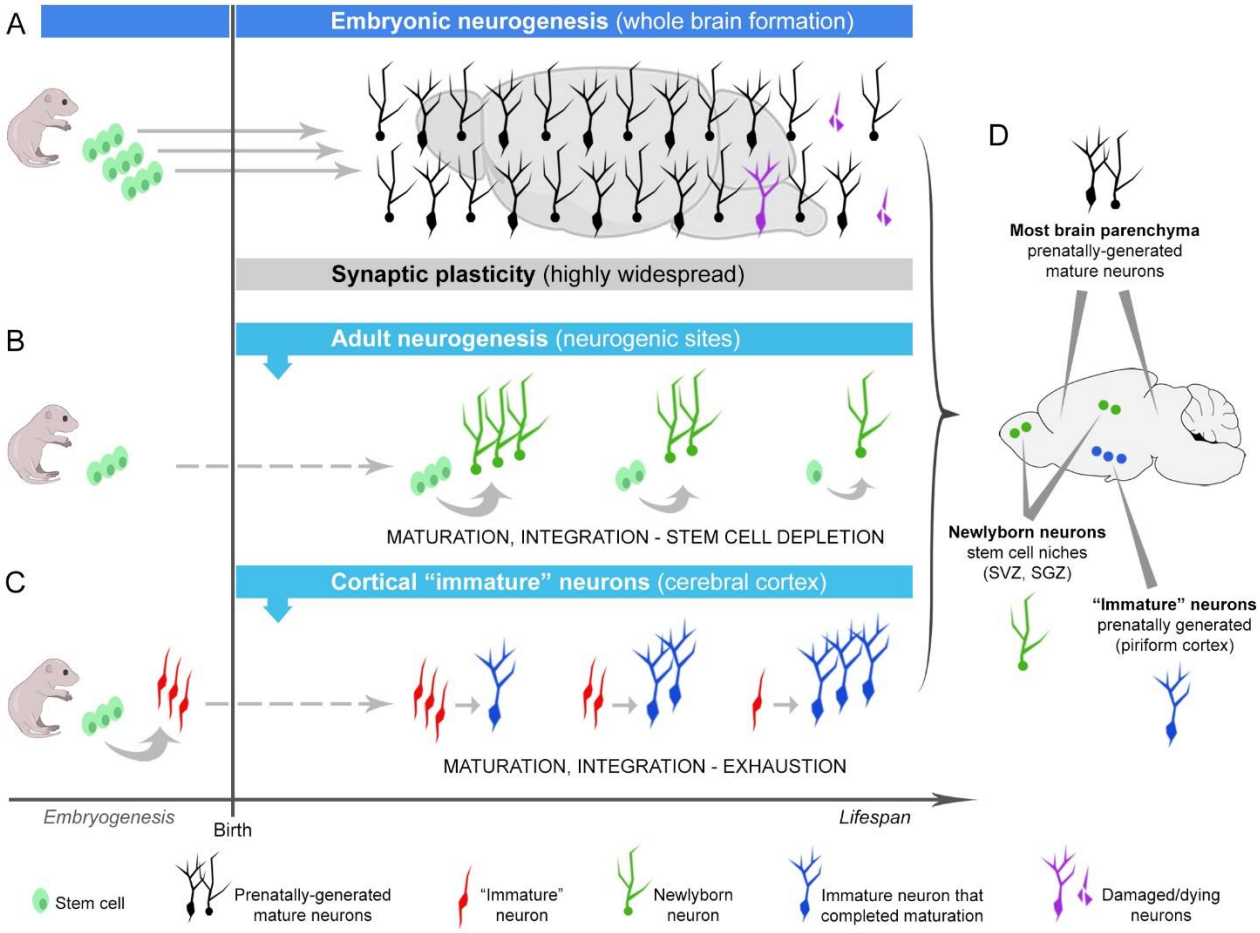
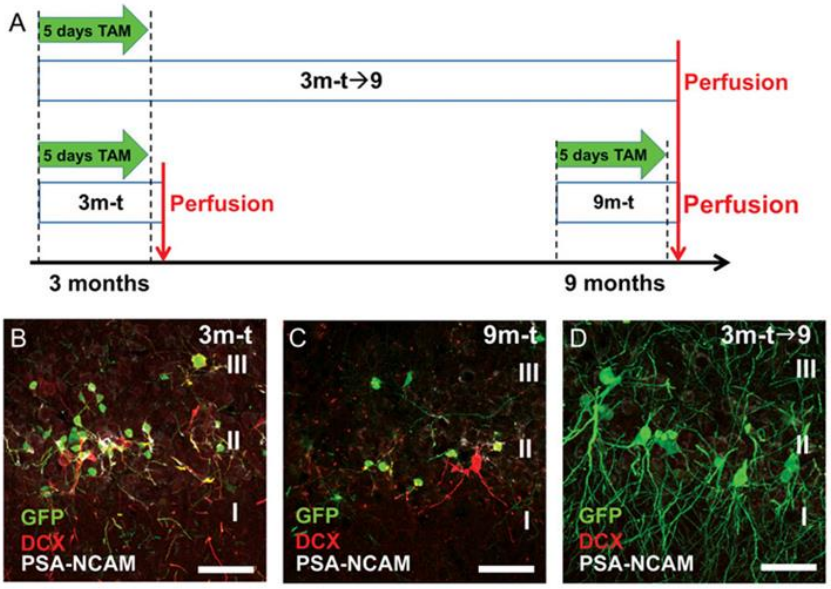


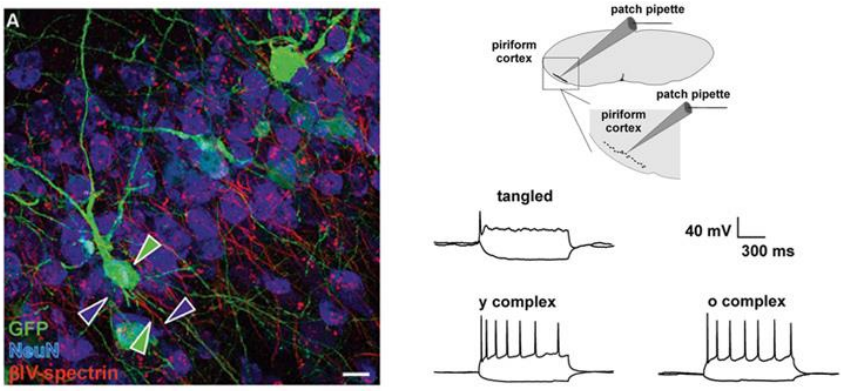
Figure 1.4 - Neurogenesis as a process to build up the brain (dark blue), and to provide new neurons during adulthood at specific locations (light blue). A, the vast majority of brain neurons are produced during embryogenesis, then reaching maturation during postnatal assembly and stabilization of the neural circuits (black). The life of these neurons spans the entire life of the animal, some of them undergoing damage/death because of aging or neurological diseases (purple). It is assumed that all these neurons can undergo synaptic plasticity (grey). B, neurogenic processes can last during adulthood in restricted neurogenic sites hosting stem cell niches. Full integration of functional mature neurons has been well documented in two brain sites: the olfactory bulb (from cells generated in the forebrain subventricular zone, SVZ) and the hippocampus (from cells generated in the subgranular zone of the dentate gyrus, SGZ). These processes undergo remarkable reduction through ages, due to stem cell depletion. C, neuronal integration of new elements in the circuits can also occur in the layer II of the cerebral cortex (piriform cortex in mice) through "awakening" and maturation of prenatally generated, "immature" neurons that had been blocked in an immature state since embryogenesis (see Piumatti et al., 2018). This "neurogenesis without division" can occur in the absence of active stem cells, undergoing exhaustion only after maturation of the entire reserve. D, at least three types of mature neurons are present in the adult brain on the basis of their origin: most of them were generated during embryogenesis and reach maturity in early postnatal periods (black), others are generated from stem cells in the neurogenic sites (green), and others come from delayed maturation of "immature" neurons (blue). Modified from Bonfanti et al., 2023.

Regarding their fate, it appears that the number of layer II cINs varies across animal ages (Xiong et al., 2008; Cai et al., 2009; Piumatti et al., 2018; Rotheneichner et al., 2018; Ai et al., 2021; La Rosa et al., 2020a; Li et al., 2023), with cIN becoming undetectable as aging progresses, indeed the number of PSA-NCAM+ cells in the rat paleocortex layer II dramatically declines during aging (Abrous et al., 1997; Murphy et al., 2001; Varea et al., 2009). Recent data obtained by Rotheneichner et al., using DCX-Cre-ERT2/Flox-EGFP transgenic mice suggest that cINs do not die with aging progression but mature as glutamatergic neurons. The maturation of these cells is supported by the increasing in density of dendritic spine and the appearance of the axon initial segments (Rotheneichner et al., 2018) and their functional integration in the cortical circuitry is demonstrated by the generation of electric activity (Benedetti et al., 2019; Figure 1.5). Even if these studies provide new information regarding their fate, no data are available in other "non-rodent" species and so if the same is happening in larger animals is far from clear. Anyway, they could constitute a pool of "reserve neurons" which might differentiate under physiological or pathological circumstances to be recruited in cortical circuits after completing maturation (Rotheneichner et al., 2018; Benedetti et al., 2020; reviewed in La Rosa et al., 2019; Benedetti & Couillard-Despres, 2022; Figure 1.6). Even if detailed reports about their age-related changes exist, it is not known if their reduction follow a pattern similar to that observed in adult neurogenesis (Ben Abdallah et al., 2010) or whether they might be considered as a reservoir of

young, undifferentiated neurons in the adult/aging brain (König et al., 2016; La Rosa et al., 2019; Benedetti & Couillard-Despres, 2022).



Rotheneichner et al.
2018, *Cerebral Cortex*



Benedetti et al.
2020, *Cerebral Cortex*

Figure 1.5 – Immature neurons progressively mature and functionally integrate in rodent paleocortex. Using a DCX-Cre-ERT2/Flox-EGFP transgenic mice, in which the green fluorescent protein (GFP) is permanently expressed in DCX^+ cells and in their progeny following tamoxifen administration, the research group of S. Couillard-Despres (Salzburg, Austria) demonstrated that most cINs do mature throughout life into glutamatergic neurons (top) and can be integrated into the pre-existing piriform cortex network (bottom).

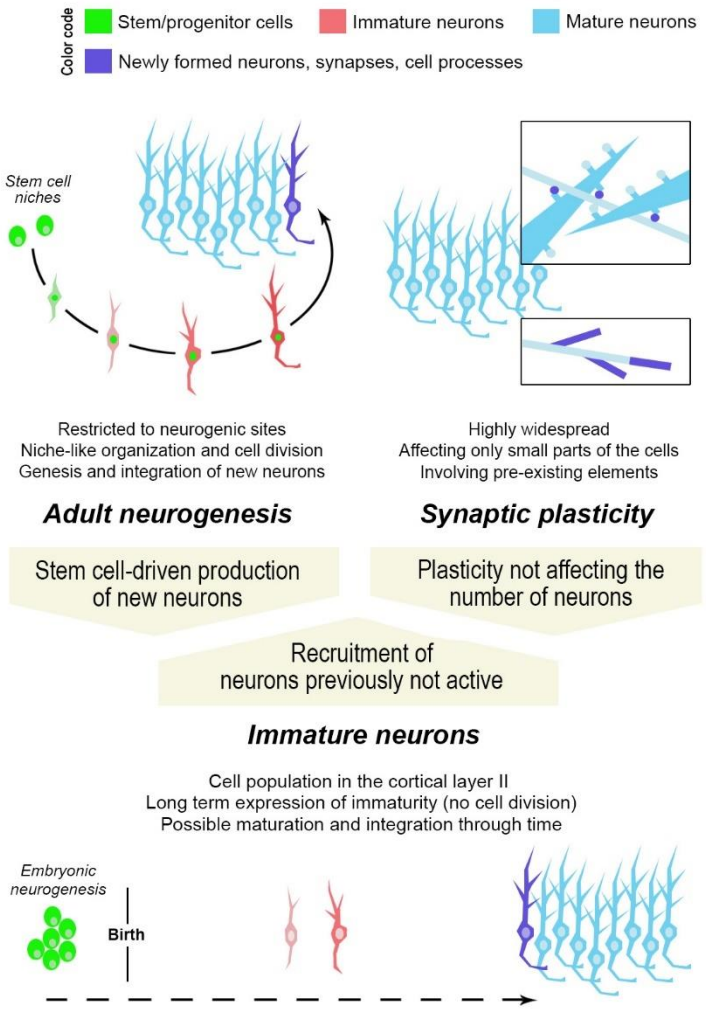


Figure 1.6 – Immature neurons can represent a third type of structural plastic processes as a non-canonical type of neurogenesis (neurogenesis without division). Indeed, the ultimate outcome of neurogenic processes consists of the addition of new neurons (here represented in dark blue), independently from the mechanisms used to produce them: stem cell/progenitor division or delayed maturation of immature neuronal precursors. Modified from La Rosa and Bonfanti, 2021.

BOX 1 – Canonical and non-canonical adult neurogenesis: the heterogeneity of brain structural plasticity

What is increasingly emerging in the research field of brain structural plasticity is the remarkable heterogeneity of this process across the animal world (Bonfanti, 2011), even among mammals, and especially concerning protracted events of neurogenesis (see for example Barker et al., 2011; Patzke et al., 2015; Alunni and Bally-Cuif, 2016; Paredes et al., 2016b). Our laboratory contributed to develop this hypothesis through previous PhD projects (Roberta Parolisi, in 2017, by demonstrating the absence of adult neurogenesis in dolphins, and Chiara La Rosa, in 2020, by showing the remarkable heterogeneity of cortical immature neurons in widely different mammals). Overall, this complex matter has sometimes generated confusion, mainly linked to ambiguity and consequent misinterpretation of some neural markers (reviewed in Lipp and Bonfanti, 2016; La Rosa et al., 2020b), but also to the simple fact that heterogeneity in plasticity is organized on different levels:

i) existence of different types of plasticity [TYPE VARIATION]
(see above the distinction between synaptic plasticity ad neurogenesis)

ii) heterogeneity of neurogenic processes (canonical neurogenesis, non-canonical neurogenesis, neurogenesis without division) [TYPE VARIATION WITHIN NEUROGENESIS]

iii) heterogeneity linked to animal age progression [TEMPORAL VARIATION]

iv) heterogeneity linked to interspecies variation [PHYLOGENETIC VARIATION]

The latter aspect is important and underestimated, since most researchers almost exclusively use (and refer to) laboratory rodents (reviewed and discussed in Bolker, 2012,2017; Brenowitz and Zakon, 2015; Faykoo-Martinez et al., 2017; La Rosa and Bonfanti, 2018; Cozzi et al., 2020). Examples of remarkable heterogeneity in plasticity

among mammals mostly came from research work carried during the last decade, and involving widely different mammalian species (Luzzati et al., 2006; Ponti et al., 2008; Sanai et al., 2011; Amrein, 2015; Patzke et al., 2015; Parolisi et al., 2017; Piumatti et al., 2018; Sorrells et al., 2018,2019; La Rosa et al., 2020a). This part is further developed below, in Section 1.4.

The present Box will deal with points ii-iv, concerning neurogenic processes. Our lab addressed this matter in previous review articles (Bonfanti and Peretto, 2011; Feliciano et al., 2015; Parolisi et al., 2018), yet the concept has evolved with time, by expanding more recently in the emerging story of “immature” neurons (Bonfanti and Nacher, 2012; Bonfanti and Charvet, 2021; Bonfanti and Seki, 2021).

Canonical and non-canonical adult neurogenesis: an evolving concept

In brief, the word “adult neurogenesis” can be used (and has been used across the years) with different (more restrictive or less restrictive) meanings, depending on the authors:

a) the genesis of new neurons in the adult brain (less restrictive)

b) the genesis of new neurons in the adult brain from well characterized stem cell niches

c) the genesis of new neurons in the adult brain from well characterized stem cell niches and a well demonstrated outcome consisting of the structural and functional integration of the new neurons in the pre-existing neural circuits (more restrictive).

In our vision, (a) and (b) consist of *non-canonical* neurogenesis, while only (c) falls into the *canonical* neurogenic process.

At present, the only neurogenic sites that fulfil the third point are the SVZ and SGZ, though some authors tend to include the hypothalamus in this group. Indeed, neurogenesis has been proven to exist in the postnatal and adult hypothalamus (Pencea et al., 2001; Kokoeva et al., 2005), in both ventricular and parenchymal regions (Yoo and Blackshaw 2018). The process has been shown to occur in different mammalian species including mice (Kokoeva et al., 2005) rats (Pencea et al., 2001; Xu et al., 2005; Perez-Martin et al., 2010) and sheep (Migaud et al., 2011; Batailler et al., 2013), involving a wide range of hypothalamic nuclei, although with different distribution patterns (Batailler et al., 2013). The specific site in which the hypothalamic progenitor cells are born is not known, but it is proposed that they could originate in the hypothalamus itself (Migaud et al., 2010). As

regards their fate, the expression of hypothalamus-specific neuropeptides (Kokoeva et al., 2005) and the development of a specific phenotype involved in neuroendocrine function (Batailler et al., 2013) suggest that these cells (or at least a fraction of them) might become mature. However, at present there is no definitive evidence that these cells are able to structurally and functionally integrate into the hypothalamic circuitry (Yoo and Blackshaw 2018). Though progresses have been made in the description and characterization of the hypothalamic neurogenic niche, several aspects (including its functional and evolutionary role) still need to be clarified; for this reason we would consider this process in the above-mentioned group (b) of non-canonical neurogenesis.

Overall, both canonical and non-canonical neurogenic processes might be considered as an exception to the prevalent stability (in terms of cell renewal) of the adult mammalian brain. Both canonical stem cell niches and the various example of cell genesis within the parenchyma consist of small events in the context of the whole brain, and progressively decrease in their rate as the animal age progresses (see Section below in this Box). In addition, beside the occurrence of non-canonical neurogenesis, the greater outcome of the "cell genesis" (cell division) detectable in the adult brain parenchyma is mostly represented by glial cells of the oligodendrocytic lineage (OPCs; Horner et al., 2000; Nishiyama et al., 2009; Boda et al., 2010; Bonfanti, 2013), whose cell population also appears to be far more stable in time (Semënov, 2021).

The recent identification of a population of "immature" or "dormant" neurons in the cerebral cortex (cINs; see Section 1.2 and Figures 1.3-1.6) introduced another category of non-canonical (parenchymal) neurogenesis (Bonfanti and Nacher, 2012; Feliciano et al., 2015; König et al., 2016). This "neurogenesis without division" does not require the existence of stem cells and stem cell niches (hence does not belong to points (b) and (c)), yet, in the mouse piriform cortex it has proven to yield neurons capable of full maturation and functional integration into the cortical circuits (Benedetti et al., 2020), thus fulfilling both point (a) and the second part of point (c). Although these distinctions might appear too theoretical and "semantic", they underline the remarkable (increasing) heterogeneity of brain plasticity in terms of neurogenic events (La Rosa et al., 2020b; Benedetti and Couillard-Despres, 2022).

Finally, other aspects contributing to adult neurogenesis heterogeneity and affecting both canonical and non-canonical forms are the *age-related decrease in the rate of*

neurogenesis through the animal life (existing in each species), and the *different decrease linked to interspecies variation*. This point has often created confusion, because the two variables are overlapping, and because different animal groups have different lifespan, thus requiring proper translating times (see Bonfanti and Charvet, 2021 for general review).

Adult Neurogenesis as a developmental process characterized by age-related decrease

It is well known that some forms of plasticity, especially the striking structural changes involving persistent neurogenesis, progressively decline as the animal age progresses (Ben Abdallah et al., 2010; Sanai et al., 2011; Snyder, 2019; Bonfanti and Charvet, 2021; Duque et al., 2022; Bond et al., 2022). In stem cell-driven adult neurogenesis, one of the recognized causes for age-related reduction is stem cell depletion consisting of a mix of real, progressive exhaustion of the stem cell pool (reduction of the stem cell number; Encinas et al., 2011) and/or entry in stem cell quiescence (Urbán et al., 2019). The final result is a progressive exhaustion of the active stem cell reservoir, with a progressive drop of the capacity to produce new neurons. Such age-related reduction has been carefully described both in the SVZ and SGZ (Luo et al., 2006; Ben Abdallah et al., 2010; Semënov, 2021) leading to deep knowledge of the cellular and molecular mechanisms regulating the neural stem cell biology (Aimone et al., 2014; Lim and Alvarez-Buylla, 2016; Encinas et al., 2011; Urbán et al., 2019).

In both canonical neurogenic sites, with some differences in the slope of reduction between SVZ and SGZ, a substantial genesis of new neurons is a juvenile event, even in mice (Ben Abdallah et al., 2010; Semënov, 2021). Concerning the evolutionary meaning of such a temporal pattern, the explanation seems to reside in the need for the CNS of young individuals to exploit the first part of life to refine or “sculpt” its capability (and thus the animal capability) to properly interact with the environment (Kempermann, 2019). In other words, plasticity in young pups will allow to grasp a vision of the world in which the animal will live by adapting some brain circuits to its features while these circuits are still highly plastic (Cushman et al., 2021). After this period, the brain (and the animal) needs substantial stability, what can explain the end of adult neurogenesis (or its decrease to very low levels). In this context, it is worth mentioning that adult neurogenesis, even in mice with their active stem cell niches, does not represent a “true stem cell system” as in

other tissues that undergo continuous cell renewal (e.g., skin, bone, blood), since in the olfactory bulb and hippocampus there is no continuous (and repeated, complete) cell renewal, but only addition of new elements, with progressive exhaustion of this capacity (Semënov, 2019). This fact can easily explain both the brain neurogenic potential as a "juvenile" feature, and its scarce possibility to repair after damage. In other words, adult neurogenesis is a process restricted in space and time, with physiological roles in brain circuit refinement during youth rather than in "regenerative events".

A further element of complexity consists of the fact that age-related reduction in neurogenic capacity is influenced by lifespan extension and its impact on the timing of neurodevelopmental events across species (Finlay and Darlington, 1995; Snyder, 2019; Charvet and Finlay, 2018; Bonfanti and Charvet, 2021; Bond et al., 2022). In fact, although the age-related reduction can be observed across the animal world, substantial differences do exist among species. Recent research carried out in comparative neuroplasticity has started to describe these differences in mammals.

Adult neurogenesis appears to be reduced from small-brained to large-brained mammals

Great differences exist between non-mammalian vertebrates (e.g., fish, whose brain has high capacity for renewal and repair) and mammals, since the former host neural stem cell niches at widespread locations, allowing substantial neuronal cell renewal throughout life and brain regions (Ganz and Brand, 2016; Kyritsis et al., 2012), while the latter are endowed with a far more static brain, both in terms of cell renewal and repair (Bonfanti, 2011; La Rosa and Bonfanti, 2018). Apart from differences between animal classes, recent research has revealed remarkable differences in adult neurogenesis even among mammals. Studies that went further humans and laboratory rodents as models (the latter being the most studied in the field of adult neurogenesis) highlighted a general heterogeneity in the occurrence of this process in the animal world. Especially in mammals, remarkable differences do exist in terms of occurrence and rate of neurogenesis. A wide comparative study carried out in different mammalian species did not find any DCX positivity in the hippocampus of cetaceans (Patzke et al., 2015). Accordingly, our laboratory has demonstrated that in dolphins, mammals devoid of olfaction, but descendant of ancestors provided with olfaction, there is a disappearance of neurogenesis in both neonatal and adult individuals (Parolisi et al., 2017). In parallel, several papers have demonstrated that forebrain and hippocampal neurogenesis seems

to disappear in human since infancy or young ages (Sanai et al., 2011; Cipriani et al., 2018; Sorrells et al., 2018), while in laboratory rodents a neurogenesis activity is present throughout life (Ben Abdallah et al., 2010). As regards other mammalian species, few studies investigated the rate of adult neurogenesis: in bats, low or absent level of hippocampal adult neurogenesis has been described comparing twelve different bat species (Amrein et al., 2007) while works involving non-human primates showed a lower rate of hippocampal neurogenesis with respect to rodents accompanied by an age-related decline (Leuner et al., 2007; Marlatt et al., 2011; Bunk et al., 2011; Kornack and Rakic 1999; Wang et al., 2022). The timing of hippocampal neurogenesis is therefore consistent with the broader comparative pattern of neurodevelopment, where humans and nonhuman primates are born with a mature nervous system (at least in terms of cell production) compared to that in rodents (Snyder, 2019). If we consider birth as T0, neurogenesis appears conspicuously low in primates and humans compared to rodents. The pattern of early neurogenesis is therefore likely to impact neurogenesis later in life, potentially contributing to species differences (e.g., accelerated dentate gyrus development in humans may lead to lower rates of neurogenesis in childhood and adulthood if dentate gyrus precursor cells undergo a finite number of divisions; Snyder, 2019). Collectively, these data highlight the need to study older animals that can better model adult humans (and animal species endowed with longer lifespan than in rodents).

In conclusion, an increasing number of studies is revealing the existence of high, unexpected levels of heterogeneity for neurogenic plasticity, linked to multifaceted aspects: different types of neurogenic events with different dependency from stem cell occurrence, interspecies variation concerning location, rate, amount, and temporal patterns, thus making necessary a systematic comparative approach to this topic.

1.3 Cortical immature neurons are heterogenous in mammals

The topic of cortical “immature” neurons, along with the concept of "neurogenesis without division," is relatively new and still not fully explored (Bonfanti and Seki, 2021; Benedetti and Couillard-Despres, 2022). Many questions remain unanswered, such as the molecular and cellular mechanisms that allow these neurons to halt their maturation before birth and subsequently "awaken" during adulthood. Additionally, their prevalence throughout the brain is not yet precisely

known (Ghibaudi and Bonfanti, 2022), and it remains unclear whether they can be activated in response to injury, inflammation, or neurological disorders (excluding recent reports on subcortical, putative immature cell populations; Chareyron et al., 2021). Some insight has been gained about the phylogenetic variation of cINs through systematic investigation of the cortex of different mammalian species widely varying in brain size, gyrencephaly and socioecological features, providing an unexpected twist in our understanding of comparative neuroplasticity (Piumatti et al., 2018; La Rosa et al., 2020a). Previous reports indicated that in laboratory rodents cINs are highly restricted to the piriform and entorhinal regions of the paleocortex (Seki and Arai, 1991; Bonfanti et al., 1992; Nacher et al., 2001), though DCX^+ neurons were also observed in the neocortex of some mammals, including guinea pigs, rabbits, and cats (Cai et al., 2009; Zhang et al., 2009; Varea et al., 2011; Luzzati et al., 2009; Xiong et al., 2008). Hence differences exist in the anatomical distribution of cINs among mammals, suggesting a more widespread presence in large-brained gyrencephalic species (Palazzo et al., 2018). Since most comparative studies on this subject were carried out on single animal species, by different laboratories, and using different methods of tissue processing and cell counting, we decided to perform a comparative study across mammals by addressing the occurrence, distribution, and amount of cINs in the whole cortical mantle (La Rosa et al., 2020a). In that study, 84 brains were processed by using the same method to identify and count the layer II DCX^+ cINs to obtain a linear density (number of cINs/mm of cortical layer II). The analysis revealed an extension of the presence of cINs from paleocortex in rodents to the entire neocortical mantle in gyrencephalic mammals (Figure 1.7), with remarkable variation in cell density (one order of magnitude when comparing the group of small-brained species with large-brained ones; Palazzo et al., 2018; La Rosa et al., 2020a; Figure 1.7). The presence of DCX^+ cINs has been confirmed in the cerebral cortex of humans (Coviello et al., 2022; Li et al., 2023; see also Chapter 2 and Ghibaudi et al., 2023a). Though comparable quantitative data in humans are not yet available, it has been shown that these neurons cover layer II of the entire cortical mantle, being preserved at adult and old ages (Li et al., 2023). Thus, it appears that the cINs could grant a reservoir of young cells for the neocortex of large-brained species. For the highly complex cerebral cortices of these mammals, to rely on pre-existing neurons that can be added functionally throughout life might be an evolutionarily advantageous, energetically inexpensive solution for overcoming the lack of stem cells and progenitor cells (La Rosa and Bonfanti, 2021).

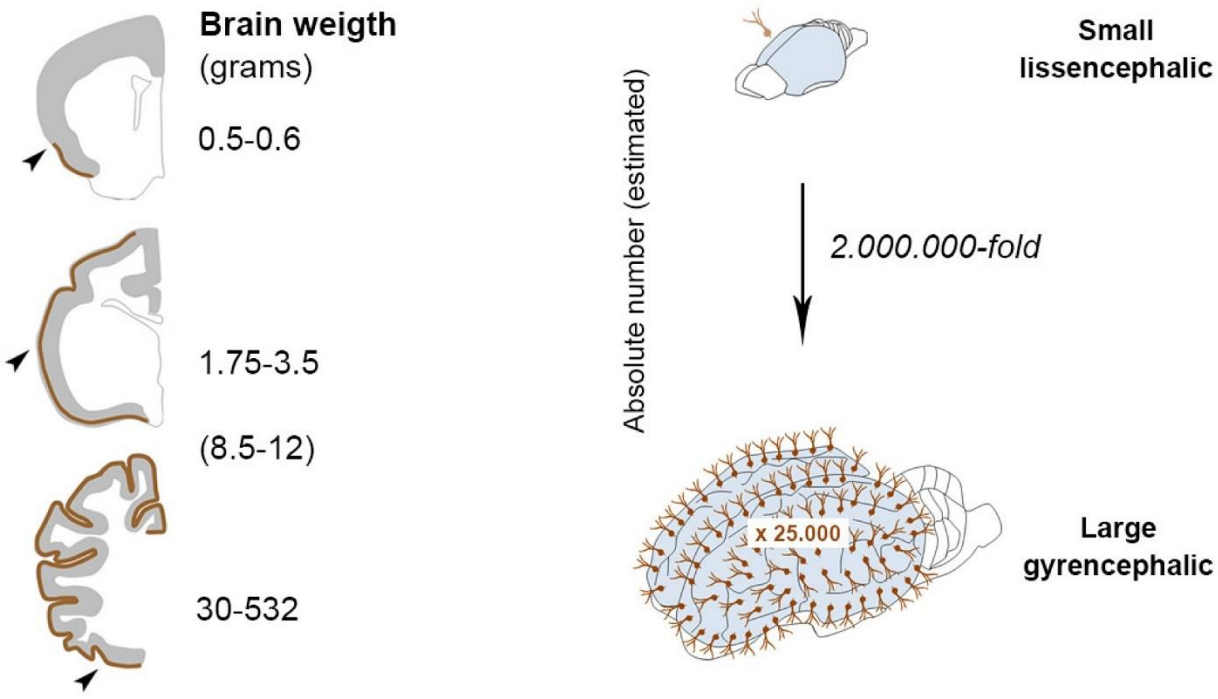


Figure 1.7 – "Immature" neurons are more abundant and widespread in large-brained species. Left, the occurrence (anatomical distribution) of cortical immature neurons (cINs; brown line) remarkably increases with increasing brain size and gyrencephaly (brains are not represented in scale; brain size is indicated by brain weight). Arrowheads: limit between paleocortex and neocortex. Right, higher density of cINs in the cortical layer II, in addition to higher extension of the neocortex in highly gyrencephalic species, lead to huge difference between mouse and primates (chimpanzee). Modified from La Rosa et al., 2020a.

1.3.1 Are parenchymal DCX+ cells (as INs) confined to the cortical mantle?

In addition to canonical and non-canonical neurogenic sites, as well as the cortical layer II, DCX+ cells can also be present in other regions of the brain. DCX+ cells have been described in subcortical regions such as claustrum and amygdala in several mammals, including humans (Piumatti et al., 2018; Li et al., 2023; Bernier et al., 2002; Fudge et al., 2012; Jhaveri et al., 2018; Lavenex et al., 2021; Marlatt et al., 2011; Marti-Mengual et al., 2013; Sorrells et al., 2019; Zhang et al., 2009). Although we currently lack systematic studies on the distribution and amount of "immature" neurons in these brain areas, several reports indicate that they might undergo interspecies variation (Chareyron et al., 2021; Ghibaudi and Bonfanti, 2022; Page et al., 2022). For example, in a detailed study conducted on the human amygdala from embryogenesis to adulthood, many DCX+, PSA-NCAM+ cells were found in the basolateral nucleus, in association with Ki67 antigen+ nuclei (but mostly no coexpression) whose number and distribution varied across different ages (Sorrells et al., 2019). The same research group recently studied the DCX+

cells of the amygdala in mice (only a few cells are detectable in rodents; see Chapter 4). By using transgenic animals, this study confirms that immature neurons in the amygdala are generated prenatally (Alderman et al., 2022), thus extending the concept of INs from the cortex to subcortical regions. The data obtained in humans and mice indicate that subcortical immature neurons may also display interspecies variation, nevertheless systematic comparative analyses are still lacking. Very few is known regarding the real nature/origin of the subcortical DCX+ cells, sometimes interpreted as newly generated in the past, when DCX was considered a proxy for neurogenesis (La Rosa et al., 2020b; Bonfanti and Seki, 2021). The literature concerning this topic, with some functional interpretations and hypotheses, is reported in Table 1.1**.**

Some DCX+ cell populations of unknown function have also been described in the postnatal brain white matter of different mammalian species (Luzzati et al. 2003; Ponti et al. 2006b, Piumatti et al., 2018), including primates and humans (Fung et al. 2011; Paredes et al. 2016a)[3].

Due to the importance of white matter, amygdala and claustrum for proper brain connectivity and functioning (see Box 2), linked to survival from emotions to conscience, a deeper knowledge of their "young" neurons is needed.

[3] In mouse and humans, the current explanation for these cells is that they are young neurons generated postnatally and migrating to the cortex (Inta et al. 2008; Le Magueresse et al. 2011, 2012; Riccio et al. 2012; Paredes et al. 2016a).

Table 1.1 Detection of young, undifferentiated neurons in subcortical brain regions of mammals: interspecies differences and heterogeneous interpretation in literature (from Ghibaudi and Bonfanti, 2022)

Brain region	Animal species	Age	Proposed nature		References
			PHYSIOLOGICAL CONDITION	*EXPERIMENTAL CONDITION*	
AMYGDALA	Mouse (*Mus musculus*)	All ages	No immature cells detectable		(mostly unpublished negative results and personal observations)
		7-12 weeks		Newly generated (YFP transgenic mice, neurosphere assay) (BrdU and DCX)	Jhaveri et al. 2018
	Rabbit (*Orictolagus cuniculus*)	3-6 months	Chains of immature neurons (DCX, PSA-NCAM), with a few newly generated elements (BrdU 40 mg/kg)		Luzzati et al. 2003
	Sheep (*Ovis aries*)	1 week 4 months 2 years	Pre-natally generated (BrdU 20 mg/kg, injected during pregnancy and in adulthood; confocal microscopy)		Piumatti et al. 2018
	Marmoset (*Callithrix jacchus*)	4 years	Newly generated (PSA-NCAM expression) (BrdU 200 mg/kg, light microscopy)		Marlatt et al. 2011
	Squirrel monkey (*Saimiri sciureus*) Cynomolgus monkeys (*Macaca fascicularis*)	3-6 years 6-12 years	Newly generated (PSA-NCAM expression and other markers) (BrdU 50 mg/kg twice a day for 3 days)		Bernier et al. 2002
	Macaque (*Macaca mulatta*)	1 day-9,5 years		Immature neurons - Cell migration suggested (Bcl-2 expression) [*hippocampal lesion*]	Chareyron et al. 2021
		12 years 21 years 31 years	Immature neurons (DCX and PSA-NCAM expression)		Zhang et al. 2009
	Macaque (*Macaca fascicularis, Macaca nemestrina*)	2,5 years	Immature neurons (DCX and PSA-NCAM expression)		Fudge et al. 2012
	Human (*Homo sapiens*)		Immature neurons (expression of PSA-NCAM but not Ki67)		Marti-Mengual et al. 2013
	Human (*Homo sapiens*)	from embryo to adult	Immature neurons until adolescence, then possibly undergoing maturation		Sorrells et al. 2019
	Mouse, Rat, Human	7 weeks (M) 2-4 months (R) 18-65 years (H)	Relation with behaviour (DCX expression: protein isolation;RNAseq, qRT-PCR)		Maheu et al. 2021
CLAUSTRUM	Sheep (*Ovis aries*)	1 week 4 months 2 years	Pre-natally generated (BrdU 20 mg/kg, injected during pregnancy and in adulthood; confocal microscopy)		Piumatti et al. 2018
WHITE MATTER	Rabbit (*Orictolagus cuniculus*)	3-6 months	Chains of PSA-NCAM+ cells		Luzzati et al. 2003
	Macaque and Human (*Macaca mulatta Homo sapiens*)	2 weeks-12 years (M) 6 weeks-49 years (H)	Migrating cells (DCX expression, DCX mRNA) Decreased during infancy	Decreased in schizofrenic patients *[schizofrenia]*	Fung et al. 2011
	Sheep (*Ovis aries*)	1 week 4 months 2 years	Clusters of immature neurons (non-newly generated in sheep; not coexpressing Ki67 in dolphin)		La Rosa et al. 2018
	Dolphin (*Tursiops truncatus Stenella coeruloalba*)	1-9 days to adult			

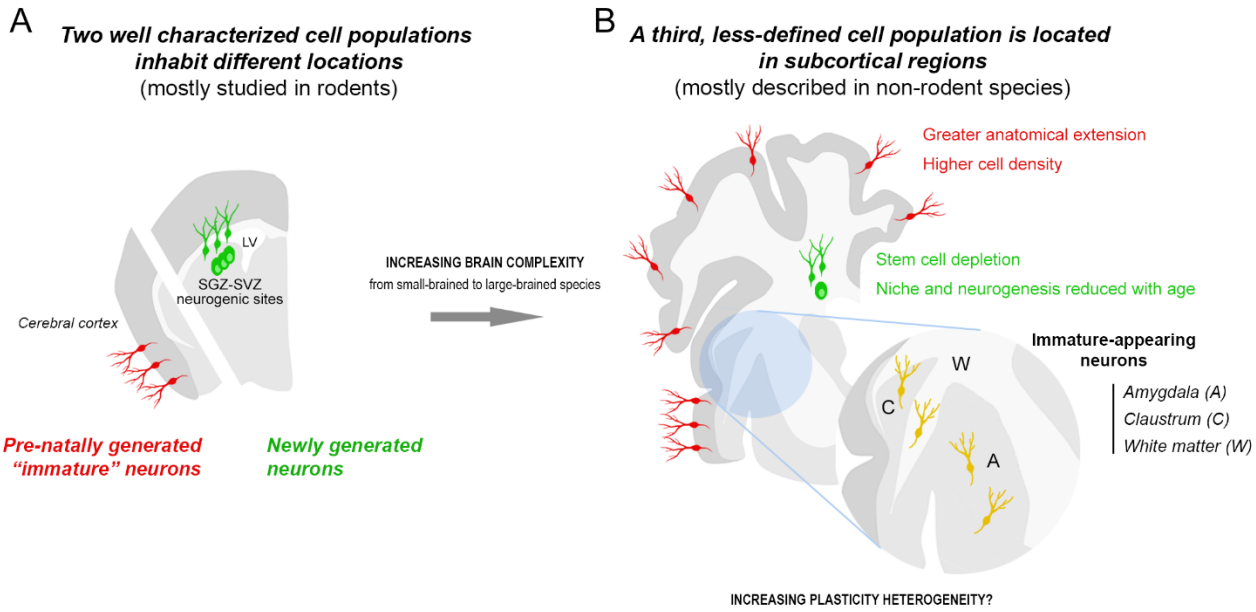


Figure 1.8 – Heterogeneity of "young", undifferentiated neurons existing in the adult mammalian brain, on the basis of the current knowledge. A, the two populations of young neurons corresponding to newly generated and non-newly generated "immature" neurons inhabit different brain regions: the forebrain/hippocampal neurogenic sites and the cerebral cortex layer II. Their anatomical distribution and amount appear to differ among mammals (A,B), with a prevalence of stem cell-driven neurogenesis in small-brained species and a greater abundance of "dormant" neurons in large-brained ones, extending to neocortex (La Rosa et al., 2020a). A third, less studied population (yellow cells), also expressing DCX, has been described in subcortical regions of different mammals (see Table 1.1). Here they are indicated as immature-appearing neurons since it is not clear whether they are composed of either "dormant" neurons, newly born neurons, or both (modified from Ghibaudi and Bonfanti, 2022).

BOX 2 – Claustrum and Amygdala: hubs of the cerebral cortex

Claustrum and amygdala can be seen as sorting centers of the brain. They receive inputs from the cerebral cortex, elaborate a response that immediately send out to produce an output. Here follows a summary highlighting their anatomical location, cellular components and their role inside the brain.

The claustrum is a sheet of neurons lying between the cortex and the striatum. It is composed of two different cell types (Type I and Type II neurons; Braak and Braak, 1982; Crick & Koch, 2005) of both glutamatergic and GABAergic nature. Additionally, three interneuron types are present (parvalbumin-, calbindin-, and calretinin-positive interneurons; Bruguier et al., 2020).
The claustrum may be present only in mammals and possibly absent in all monotremes (Jackson et al., 2020), but the existence of a claustrum homolog in non-mammalian forms is still under debate (discussed in Puelles et al., 2014). This, together with the fact that the claustrum volume increases in concert with the neocortex expansion (Kowianski et al., 1999) lead to the hypothesis that the claustrum has a role in higher-order cognitive processes. Studies of its function has revealed that it works as a central hub for the cortical circuitry, communicating most densely with medial regions of frontal cortex (e.g., cingulate cortex) and ventrolateral areas of the temporal lobe (e.g.,entorhinal cortex). Moreover, it receives inputs from brain regions processing limbic information such as the amygdala, hippocampus, and thalamus. These findings suggest that the claustrum may be involved in functions such as attention, memory, anxiety and vigilance, then directing such information to the cortex to modify perception and actions (Jackson et al., 2020).

The Amygdala is an almond-shaped structure located in the temporal lobe of the brain. It is composed of several nuclei and subdivisions, each one identifiable thanks to the different cell types present (the basolateral group, which includes the basal nucleus and the lateral nucleus; the centromedial group, that comprises the medial nucleus and the central nucleus and other amygdaloid nuclei which are the intercalated cell masses, the amygdalohippocampal area and the paralaminar nucleus; De Olmos et al., 2004;

Decampo & Fudge 2012). Different cell types are present according to each nucleus composing the amygdala: in the basolateral nucleus, 70% of the cell population is represented by the Class I glutamatergic neurons, which have a pyramidal-like somata. The remaining 30% is represented by the Class II GABAergic neurons which are local circuit interneurons. The centromedial group shows fusiform or ovoid spiny neurons with GABAergic projections (Sah et al., 2003). A comparative analysis of the basolateral group from small to large-brained mammals (guinea pig, rabbit, fox and pig) has demonstrated that its neuronal structure remains substantially stable and unchanged among the different species (Równiak et al., 2003), suggesting a possible conservation of this structure among mammals. Its function span from the genesis of emotions, fear, learning and memory consolidation (Sah et al., 2003). Anterograde and retrograde tracers injections demonstrated its strong connection with several brain regions, spanning from the cerebral cortex, the thalamus, the hypothalamus and the brainstem (Sah et al., 2003).

1.4 Comparative analysis: a nonconformist choice

Most neuroscience research is performed on laboratory rodents. Although the prevalence of this model has been repeatedly challenged (Manger et al., 2008; Bolker, 2012; Keifer and Summers, 2016), it remains the gold standard in translational research for most laboratories and it is not hard to understand why: its large availability and easy/fast manipulation certainly make it easier the life of a researcher. In addition, the undisputable advantage of generating a wide range of transgenic animals, makes it the model of choice to study molecular mechanisms and biological pathways. Nevertheless, while phylogenetically close to primates, laboratory rodents might not be the answer for all biological processes, and focusing only on mice and rats can hamper the correct translation to humans. About 5000 species, including 26 orders, form the class Mammalia. Until now, the exact number of species is not yet clear, and doubts still exist concerning how some orders and families are related to others (Burgin et al., 2018). New exciting information is coming from phylogenesis based on molecular evidence and from fossils. For example, the traditional subdivision of Chiroptera into megabats and microbats may not accurately reflect evolutionary history (Teeling et al., 2002). The Animal Diversity Web, a database we used for information about animal natural history, distribution, and classification, generally follows the arrangement used by Wilson and Reeder (2005).

As a matter of fact, remarkable differences do exist in mammals concerning complex biological processes such as structural plasticity (see Sections above), thus reducing the value of results obtained in rodents for translational purposes (see further discussion of this point in Chapter 5). Nevertheless, the use of larger mammals as animal models is still viewed with suspicion in the scientific field (a few publications have been dedicated to the brain of the domestic bovine (207), sheep (100), horse (4), or pig (414), out of 700,000 reports in the neurosciences during the last 20 years; Cozzi et al., 2020), a fact that is explainable with the large number of variables, limits, ethical and technical problems encountered when studying such models (summarized in Figure 1.9).

First of all, the number of samples obtainable for a study cannot be as large as the one used with laboratory rodents, given less tissue availability, longer times for processing larger brains (a simple example coming from our lab is the fact that the whole brain processing for a mouse could take not more than 2/3 weeks, while for a larger brain it can take months) and the high costs. This leads to a number of samples that may be close to the minimum requested for statistical analysis.

Then, there are some techniques that can be exclusively performed in small-brained species, both for technical and ethical reasons: preserving the marker detection integrity is a fundamental aspect in neuroscience, especially in brain structural plasticity, and this requires techniques which aim at preserving the tissue in the best condition possible. In the process of brain fixation, an easy way to reach this goal is intracardiac perfusion: it consists of the direct injection of a fixative (usually paraformaldehyde) in the animal blood circulation, thus reducing the time from the death of the animal and the fixation of the brain (postmortem interval, PMI) virtually to zero, preserving the tissue and the molecules of interest in the best condition possible. This is a process which cannot be performed on some larger animals (both for ethical and technical limits); for this reason, other techniques are requested, such as the extraction of the brain from the skull and its immersion in the fixative (usually formalin), which requires more time to penetrate the tissue thickness, thus increasing the PMI and possibly affecting the tissue and antigen integrity.

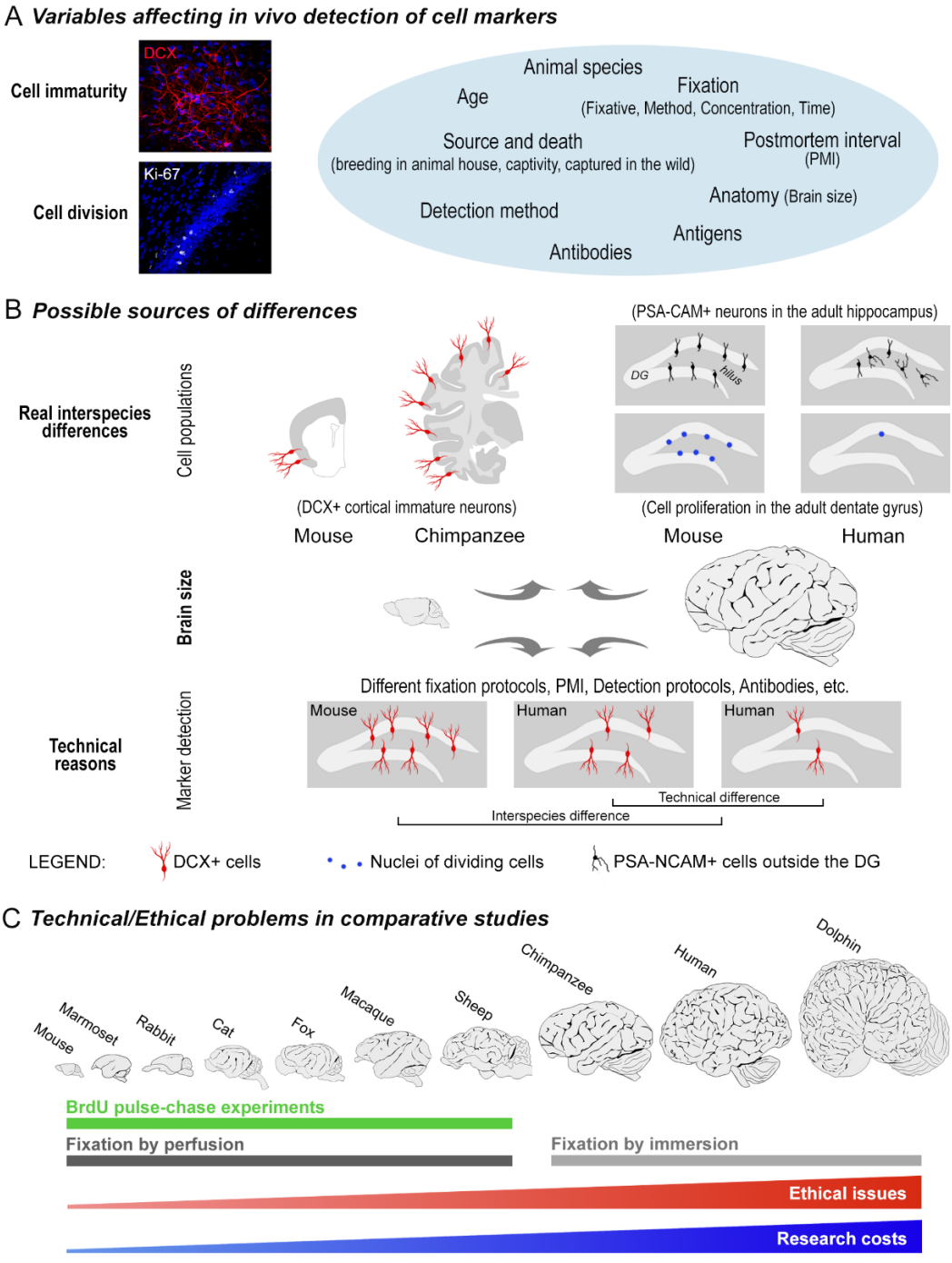


Figure 1.9 – The complex landscape of cell marker detection in comparative neuroplasticity. (A) The two most used markers for in vivo detection of neuronal immaturity (doublecortin, DCX) and cell division (Ki-67 antigen), and the main variables affecting their detection in the brain tissue; Image magnification: 40x. (B) The possible sources of variation can depend on several reasons, including differences linked to the animal species (neuroanatomy, evolutionary choices in the types of neuroplasticity, and intrinsic differences in the expression of cells/markers), and/or technical reasons linked to the procedures of brain sampling and fixation, potentially varying in relation to brain size. (C) Difficulties encountered in cell marker detection increase with increasing brain size and complexity of cognitive abilities (from mice to humans) on the basis of technical and ethical issues.

Another example is the long-term tracing of newly born elements (BrdU label-retaining cells), which cannot be successfully used in humans or in mammalian species that are protected by international law (e.g., human primates and cetaceans). Alternatively, local cell division can be detected by immunocytochemistry in post-mortem tissues (e.g., Ki67 antigen; Kee et al., 2002), limitedly to cells dividing at the exact time of animal death. Additionally, some discrepancies emerging after comparing the results with those obtained in laboratory rodents can be linked to real interspecific differences of the biological processes themselves (Lipp and Bonfanti, 2016; La Rosa et al., 2020b; Sorrells et al., 2018; Moreno-Jimènez et al., 2019; Parolisi et al., 2017; Seki 2020) or to antibody specificity in the different mammals. Hence, the overall issue of analysing large, gyrencephalic brains is more complex than simply fixation, which overlap with natural interspecies variation and/or different specificity of antibodies across phylogeny, the latter two aspects being often neglected.

BOX 3 – Main cellular markers for cell proliferation and neuronal maturity/immaturity used in this thesis

---

Cell proliferation and neuronal maturation are biological processes which allow the formation of the CNS during embryonic development but can also be retained in certain regions/cell populations of the postnatal/adult brain (in the latter case leading to “whole cell” structural plasticity). Some molecular/cellular steps and temporal stages of such processes can be identified/followed by using a series of “molecular markers” which are expressed transiently and can be visualized by immunocytochemistry (Figure 1.10).

Doublecortin (DCX). A microtubule-associated protein expressed by migrating neuroblasts in both developing and adult mammals (Francis et al., 1999; Gleeson et al., 1999; Brown et al., 2003). It plays a crucial role for microtubule stabilization (Gleeson et al., 1999), nuclear translocation during neuronal migration (Koizumi et al., 2006), and growth cone dynamics (Burgess and Reiner, 2000). In human, it is essential for normal brain development and mutations cause X-linked lissencephaly, with the characteristic defect in cortical layering (Gleeson et al., 1998; Sapir et al., 2000). This marker covers several cell populations being in a low state of maturation and spanning through various morphologies (Klempin et al., 2011). This protein starts to be expressed in dividing

neuronal precursor cells, where it colabels with the marker of cell proliferation Ki-67. Then it is downregulated to undetectable levels when neurons enter in the differentiation stage (Brown et al., 2003). These characteristics led sometimes to the misunderstanding that DCX could be a specific marker for neurogenesis, although in the CNS it is not restricted to newly generated cells. Indeed, DCX expression has been described in some mammalian species in a wide number of cells in the cortical layer II and they are generated during embryonic development (Verwer et al., 2007; Xiong et al., 2009; Luzzati et al., 2008; Gomez-Climent et al., 2008; Bloch et al., 2011).

PSA-NCAM (polysialylated form of N-CAM). An anti-adhesive form of the Neural Cell Adhesion Molecule (N-CAM). Polysialic acid (PSA) is a large carbohydrate added post-translationally to the extracellular domain of the transmembrane protein N-CAM, a member of the immunoglobulin superfamily of adhesion molecules. This process leads to a reduction in the interaction between cell expressing PSA-NCAM and the surrounding neuropil element. PSA-NCAM is widely distributed in the developing nervous system where it promotes dynamic cell interaction, like those responsible for axonal growth, terminal sprouting and target innervation. In adult nervous system, its expression is restricted to regions in which there are different form of plasticity (Bonfanti and Theodosis, 2009).

Ki-67 antigen. It is a nuclear protein expressed in dividing cells for the entire duration of their mitotic process. In interphase, it can be exclusively detected in the nucleus, while in mitosis the protein is relocated to the surface of the chromosomes. Considering the fact that it is expressed in all active phases of the cell cycle (G1, S, G2 and mitosis) and it is absent in resting cells (G0), it is an excellent marker to identify growing cells (Scholzen and Gerdes, 2000; Sobecki et al., 2016). Hence, it is not expressed by INs and it could be useful as negative control.

NeuN (Neuronal Nuclear Protein). In most species analysed until now in literature, a small fraction of INs expresses also *NeuN*, a protein localized in nuclei and perinuclear cytoplasm of most of the mature neurons in the central nervous system. There are few conflicting data about this marker: it is believed that NeuN emerges during early embryogenesis in postmitotic neuroblasts and remains in differentiating and terminally differentiated neurons throughout the whole subsequent ontogeny and its functions are still not fully clear (Gusel'nikova and Korzhevskiy, 2015).

NG2 chondroitin sulfate proteoglycan (Neuron Glia antigen 2). Also known as chondroitin sulphate proteoglycan 4 (CSPG4), it is a surface type I transmembrane core proteoglycan expressed in the developing and adult CNS by several cell types, including glial cells such as polydendrocytes or oligodendrocyte precursors (Ampofo et al., 2017). It is suggested that its function is to regulate both cell proliferation, migration and survival by interacting with extracellular and intracellular ligands (Stallcup 2002).

SOX10 (SRY-Box Transcription Factor 10). Expressed throughout oligodendrocyte development, is an important component of the transcriptional regulatory network in myelin-forming CNS glia (Küspert et al., 2011). Specifically, it is crucial in oligodendrocytes differentiation by promoting myelin gene expression and is necessary for the survival of myelinating oligodendrocytes (Takada et al., 2010)

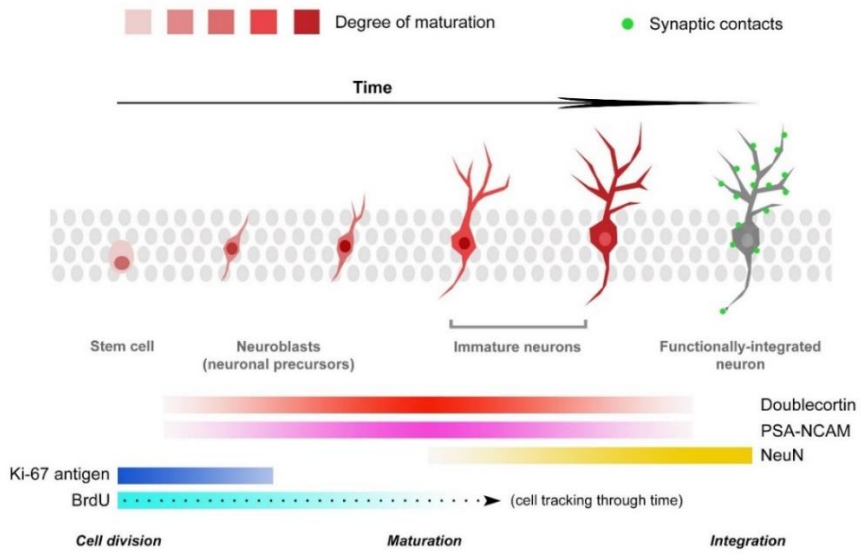


Figure 1.10 – An example of different temporal expression of some molecular markers commonly used for studying both newly generated and INs. Some of them are more specific of restricted phases (e.g., cell proliferation or differentiation) whereas others (e.g., the markers of immaturity and plasticity DCX and PSA-NCAM) can be expressed for longer periods of time and are usually lost as maturation starts.

1.5 Aims of this PhD project

The present work is a pursuing of a previous PhD project aimed at mapping and characterizing the "immature" neuronal cell populations in the brain of different mammals, based on the emerging evidence that brain neurogenic plasticity is more heterogeneous than previously thought, likely having undergone to evolutionary choices in different animal groups.

Starting from these bases, the main objective was to perform a systematic comparative analysis of INs in the subcortical regions of different animal species (characterized by different brain size, cortical gyrification, lifespan, ecological niche) in the most (possibly) similar way to that performed in the cerebral cortex in the previous PhD project. For this reason, we included two rodent species with highly different lifespan (mouse and naked mole rat, which live 1.5 and 30 years, respectively), and two primates with different features (marmoset, a non-human primate with a small and lissencephalic brain; and chimpanzee, a human primate with a larger, gyrencephalic brain). We also considered the rabbit, with small, lissencephalic brain, yet displaying high structural plasticity (Luzzati et al., 2003; 2006; Bonfanti and Ponti, 2008; Ponti et al., 2008), and two species of domestic animals (cat, sheep; hence including herbivores and carnivores) endowed with different brain size, yet clearly gyrencephalic.

To perform systematic qualitative and quantitative analysis in our 7 mammalian species (Human brain tissues were added in the methodological study; see Chapter 2) four specimens (4 entire hemispheres) for each animal species and age group were collected (see below the Method sections in each Chapter, especially Figure 4.1 in Chapter 4). Collecting such a material, trying to reduce the variables in type and time of fixation, postmortem interval, and ages, is not an easy task, also considering that some species are protected by international laws (see for example CITES regulations for chimpanzees). Overall, a total of 116 brains (24 in Part 1, 24 in Part 2, and 68 in Part 3), belonging to eight mammalian species and five orders, were analysed in this study (see Figures 1.11, 2.1 and 4.1; Tables 1.2, 2.3, 3.1 and 4.1, and Box 4).

To achieve the main goal, this PhD project have been developed on three different levels (for a brief description of the methodology employed for each level, see paragraph below, *Overall organization of the results*):

i) with our analysis on subcortical regions being performed on widely different species with different brains size, we hypothesized that such heterogeneity could influence the proper detection of common markers in brain structural plasticity (mainly DCX and Ki67-antigen). Scarce

information exists in literature regarding comparative detection of these markers, thus the first aim was to study to what extent different variables involved in comparative analysis carried out with immunocytochemistry may influence the detection of both DCX and Ki67-antigen, to set the best experimental condition for our comparative analysis.

ii) since in the literature the occurrence of DCX+ cells have been repeatedly reported to occur in subcortical regions of different mammals (with heterogeneous results and interpretations), we questioned both to what extent their occurrence is heterogeneous among different species and whether they are newly generated elements or INs. To do this, we studied the nature, distribution and amount of DCX+ cells in the amygdala of seven widely different mammals, from mice to primates.

iii) being the occurrence and rate of neurogenic processes affected by age progression, and considering different age groups in our comparative analyses, we systematically addressed the possible age-related changes of cINs in the piriform cortex of mice, from very young ages to aging.

Overall, the above three levels can be divided in five ***specific objectives:***

- *To set the appropriate conditions for detecting antigens for the study of INs in postmortem brains of widely different mammals (point i)*

- *To assess the possible occurrence of DCX+ neurons in the subcortical regions of widely different mammals (point ii)*

- *To assess their putative nature of "immature" neurons (non-dividing, DCX-expressing cells frozen in an immature state; point ii)*

- *To investigate the possible phylogenetic variation of subcortical INs, in order to discuss the results with current knowledge in the cerebral cortex (point ii)*

- *To investigate the possible age-related changes in the occurrence of cINs in the mouse piriform cortex, to compare the results with current knowledge in other animal species and brain regions (point iii)*

Overall organization of the results

The results of this PhD thesis have been split into three main parts, each conceived for distinct publications. At the time the thesis is delivered, parts 1 and 2 have been published (Ghibaudi et al., 2023a, *Int. J. Mol. Sci.*; Ghibaudi et al., 2023b, *Front. Cell. Neurosci.*). A third manuscript concerning part 3 will be submitted after the PhD, due to the total amount of work required for completing the mapping of the DCX+ immature neurons in both claustrum and amygdala, in 8 mammalian species (adding the horse as a very large-brained species, and further ages for some species).

In parallel with the above-mentioned Original Research articles, a Perspective Article (Ghibaudi and Bonfanti, 2022, *Front. Neurosci*.) and a Review Article (Bonfanti, La Rosa, Ghibaudi, Sherwood, *Brain Struct. Funct*.) have been published, to expand and discuss the hypothesis issued from this PhD work.

Part 1 (Chapter 2) – Different variables involved in comparative analysis of the mammalian brain were combined to understand their influence in the detection of two well-known markers of CNS structural plasticity: the cytoskeletal protein DCX, a marker of immaturity, and Ki67 antigen, a marker of cell division. Different commercial antibodies directed against these two markers were compared in six mammals (including humans) in four different brain regions: neocortex, paleocortex, SVZ and SGZ.

Part 2 (Chapter 3) – This part was dedicated at understanding the variation in number and features of cINs in the mouse paleocortex at different ages (from 1 to 15 months). The study was directed to understand whether a general age-related decrease known to affect plasticity also extends to cINs, and whether it is comparable to that described for adult neurogenesis. The data will be compared with those found in the study of INs in the subcortical regions (part 3).

Part 3 (Chapter 4) – In this part, the analysis of the occurrence, distribution, and amount of DCX+ and Ki67+ cells in the amygdala of 7 widely different mammalian species was done. The aim was to understand whether amygdalar DCX+ cells also underwent a phylogenetic variation, and if they can be considered either newly generated cells or INs (by performing coexpression analysis with Ki67 antigen, and by studying the spatial distribution/age-related changes of the two cell populations). The data obtained will be compared to those found in the previous PhD project, showing phylogenetic variation of cINs in the cerebral cortex of mammals.

Each part is discussed separately at the end of the Chapters, while a more general discussion is provided in Chapter 5.

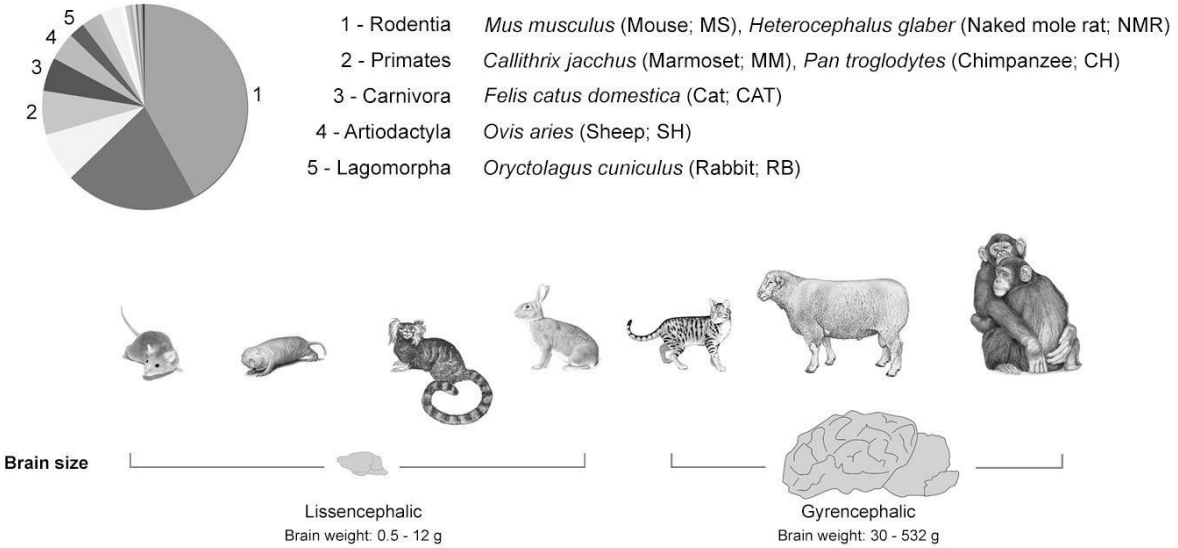


Figure 1.11 - Animal species considered in this thesis (humans are not included here since we only analyzed some brain tissues, not entire hemispheres). Seven mammalian species belonging to five different orders were considered. From left to right: mouse, naked mole rat, marmoset, rabbit (mostly small-brained and lissencephalic), cat, sheep, chimpanzee (mostly gyrencephalic; see also Table 1.2).

Table 1.2 - Main information the animal species considered in this study. Further detail can be found in the *Identity cards* section and in each *Materials and Methods* sections in each Chapter.

Scientific name	Common name	Brain weight	Gyrification index	Lifespan	Food habits
Mus musculus	Mouse	0,4 g	1.03	1.5 y	Omnivorous
Heterocephalus glaber	NMR (Naked mole rat)	0.5 g	-	30 y	Herbivorous
Callithrix jacchus	Marmoset	7-10 g	1.18	10-16 y	Herbivorous
Pan troglodytes	Chimpanzee	384 g	2.31	60 y	Omnivorous
Oryctolagus cuniculus	Rabbit	1.5-2.5 g	1.15	9 y	Herbivours
Ovis aries	Sheep	120 g	2.29	22.8 y	Herbivorous
Felis catus domestica	Cat	25-30 g	1.6	15 y	Carnivorous

BOX 4 – Identity cards of the animal species involved in this thesis

***Mus musculus* [Mouse]**		
	**Lifespan:**	1.5 year
	Sexual maturity:	5 – 7 weeks
	Gestation period:	21 days
	Weight:	12 – 30 g
	Brain weight:	0.4 g
	Geographic Range:	Spread throughout the world by humans
	Ecological niche:	Close association with humans, cultivated fields, fencerows, wood areas
Order: Rodentia	**Food Habits:**	Omnivorous
Family: Muridae	**Behaviour:**	Territorial and colonial
Genus: Mus	**Communication:**	Chemical (pheromones) and acoustic
Species: *Mus musculus*	**Brain atlas:**	http://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas
	Source: http://animaldiversity.org/accounts/Mus_musculus/	

***Heterocephalus glaber* [Naked mole rat]**		
	**Lifespan:**	30 years
	Sexual maturity:	7.6 months (F) – 1 year (M)
	Gestation period:	70 days
	Weight:	30 – 80 g
	Brain weight:	0.5 g
	Geographic Range:	Eastern Africa (Ethiopia, Somalia and Kenia)
	Ecological niche:	Underground tunnels in grasslands and savannas
Order: Rodentia	**Food Habits:**	Herbivorous
Family: Bathyergydae	**Behaviour:**	Eusocial animals living in colonies composed by the queen, breeding males and non-breeders
Genus: Heterocephalus	**Communication:**	Tactile and acoustic
Species: *Heterocephalus glaber*	**Brain atlas:**	NA
	Source: http://animaldiversity.org/accounts/Heterocephalus_glaber/	

***Callithrix jacchus* [Common marmoset]**		
	**Lifespan:**	10 – 16 years
	Sexual maturity:	13 months (M) – 16 months (F)
	Gestation period:	5 months
	Weight:	300 – 360 g
	Brain weight:	7 – 10 g
	Geographic Range:	Brazil
	Ecological niche:	Different forest types, including plantations
Order: Primates	**Food Habits:**	Herbivorous, but sometimes they eat insects, spiders, small lizards, bird's eggs, nestlings, and frogs
Family: Cebidae	**Behaviour:**	Diurnal animals, generally live in groups
Genus: Callithrix	**Communication:**	Tactile and chemical
Species: *Callithrix jacchus*	**Brain atlas:**	http://neurosciencelibrary.org/specimens/primates/marmoset/index.html
	Source: http://animaldiversity.org/accounts/Callithrix_jacchus/	

***Pan troglodytes* [Chimpanzee]**		
	**Lifespan:**	60 years
	Sexual maturity:	10 – 13 years (F) / 12 – 15 years (M)
	Gestation period:	7 – 8.5 months
	Weight:	26 – 70 kg
	Brain weight:	384 g
	Geographic Range:	Central Africa
	Ecological niche:	Typically tropical rainforest, but also found in forest-savana mosaic, mountain forest at elevation up to 2750 m and savanna
Order: Primates	**Food Habits:**	Omnivorous
Family: Hominidaes	**Behaviour:**	Highly social, with long memory. Not strictly territorial and diurnal animals
Genus: Pan	**Communication:**	Visual, tactile, acoustic and chemical
Species: *Pan troglodytes*	**Brain atlas:**	http://neurosciencelibrary.org/specimens/primates/chimp/index.html
	Source: http://animaldiversity.org/accounts/Pan_troglodytes/	

***Oryctolagus cuniculus* [Rabbit]**		
 **Order:** Lagomorpha **Family:** Leporidae **Genus:** Oryctolagus **Species:** *Oryctolagus cuniculus*	**Lifespan:**	9 years
	Sexual maturity:	8 months
	Gestation period:	1 month
	Weight:	1.5 – 2.5 g
	Brain weight:	12 g
	Geographic Range:	Every continent except Asia and Antartica
	Ecological niche:	Dry areas near sea level with soft, sandy soil, brushy fields, cultivated land and forest
	Food Habits:	Herbivorous
	Behaviour:	Gregarious, live in colony with clear dominance hierarchies
	Communication:	Visual, tactile, chemical and acoustic
	Brain atlas:	http://neurosciencelibrary.org/specimens/lagomorpha/domesticrabbit/index.html
	Source: http://animaldiversity.org/accounts/Oryctolagus_cuniculus/	

***Ovis aries* [Sheep]**		
 **Order:** Cetartiodactyla **Family:** Bovidae **Genus:** Ovis **Species:** *Ovis aries*	**Lifespan:**	22.8 years
	Sexual maturity:	1.5 year (F) – 2.5 years (M)
	Gestation period:	5 months
	Weight:	20 – 200 kg
	Brain weight:	120 g
	Geographic Range:	Worldwide
	Ecological niche:	wide variety of habitats ranging from temperate mountain forests to deserts
	Food Habits:	Herbivorous
	Behaviour:	Social flocking animals
	Communication:	Visual, tactile, chemical and acoustic
	Brain atlas:	https://msu.edu/~brains/brains/sheep/index.html
	Source: http://animaldiversity.org/accounts/Ovis_aries/	

***Felis catus domestica* [Cat]**		
 **Order:** Carnivora **Family:** Felidae **Genus:** Felis **Species:** *Felis catus domestica*	**Lifespan:**	15 years
	Sexual maturity:	6 months (F) – 8 months (M)
	Gestation period:	50 – 67 days
	Weight:	4 – 5.4 kg
	Brain weight:	25 – 30 g
	Geographic Range:	Every continent except Antartica
	Ecological niche:	Urban, suburban, agricultural area
	Food Habits:	Carnivorous
	Behaviour:	Solitary with dominance hierarchies and highly territorial
	Communication:	Visual, tactile (vibrissae), acoustic and chemical
	Brain atlas:	http://neurosciencelibrary.org/specimens/carnivora/cat/index.html
	Source: https://animaldiversity.org/accounts/Felis_catus/	

CHAPTER 2

DCX and Ki67 antigen staining in the brain of mammals: dealing with interspecies differences in postmortem tissues

Specific questions

Are there external/internal factors that may influence the detection of our main markers of interest (DCX; Ki-67 antigen) in the brain of widely different mammals?

Which is the relationship between different variables, including fixation protocol, antibody specificity, and real interspecific differences in the occurrence/distribution of antigens in postmortem brains?

2.1 Introduction

Developmental neurobiology is characterized by complex, highly dynamic processes which persist in the adult brain and allow extended assembly/modulation of the neural circuits (structural plasticity), including the addition of new neurons (adult neurogenesis). Recent progress in the field revealed increasingly complex landscapes involving different populations of "young", undifferentiated neurons of different origin (Ghibaudi and Bonfanti, 2022). The new twists concern different populations of immature neuronal precursors belonging to canonical and non-canonical neurogenic processes, taking place both inside and outside of the neurogenic sites (Bonfanti and Peretto, 2011; Bond et al., 2015; Feliciano et al., 2015; Kempermann, 2015; Lim and Alvarez-Buylla, 2016; Piumatti et al., 2018; Rotheneichner et al., 2018; Sorrells et al., 2019; La Rosa et al., 2020b; Bonfanti and Charvet, 2021; Bonfanti and Seki, 2021; Benedetti and Couillard-Despres, 2022; Page et al., 2022). At least two populations of young, immature neurons co-exist in the postnatal/adult brain: i) newly born neurons generated from active division of adult neural stem cells, mainly hosted in the canonical neurogenic sites (the subventricular zone of the lateral ventricle and the dentate gyrus of the hippocampus) (Bond et al., 2015; Kempermann 2015; Lim and Alvarez-Buylla, 2016), and ii) non-newly born "immature" or "dormant" neurons, which form during embryogenesis, then continuing to express markers of immaturity through adulthood (Gomez-Climent at al., 2008; Bonfanti and Nacher, 2012; König et al., 2016; Rotheneichner et al., 2018; Piumatti et al., 2018; Benedetti and Couillard-Despres, 2022; Alderman et al., 2022). The "dormant" neurons are located in brain regions not endowed with stem cell-driven neurogenesis, such as the cerebral cortex and amygdala (Bonfanti and Nacher, 2012; König et al., 2016; Piumatti et al., 2018; Bonfanti and Ghibaudi, 2022; Benedetti and Couillard-Despres, 2022; Alderman et al., 2022). These cell populations are not easy to distinguish since they share the same markers of immaturity during some phases of their life (La Rosa et al., 2020b; Bonfanti and Seki, 2021). Such

a distinction remained neglected for long time, leading to some misunderstandings in the interpretation of results and generating confusion in different types/sources of neurogenesis (Bonfanti and Seki, 2021; Ghibaudi and Bonfanti, 2022). The origin of the young neurons can be revealed by the presence/absence of co-expression with markers of cell division, or pulse-chase experiments with 5-Bromo-2′-deoxyuridine (BrdU) and its analogues (Kuhn and Cooper-Kuhn, 2007). Yet, another important element of confusion consists of interspecies differences, with remarkable variation in the occurrence and distribution of the abovementioned types of plasticity and immature cell populations across mammals (Amrein, 2015; Lipp and Bonfanti, 2016; Paredes et al., 2016b; Parolisi et al., 2018; Palazzo et al., 2018; Piumatti et al., 2018; La Rosa et al., 2020). This fact makes it necessary to extend the level of investigation to large-brained, gyrencephalic species, or directly to humans (Moreno-Jimenez et al., 2019; Sorrells et al., 2018,2019; Zhou et al., 2022). When dealing with large-sized gyrencephalic brains some technical/practical difficulties arise, concerning fixation (Moreno-Jimenez 2021; Sorrells et al., 2021) and ethical issues (Bianchi et al., 2018; Cozzi et al., 2020; Figure 1.9). Consequently, most work must be performed on postmortem brain tissues or intraoperative samples, thus putting limits to the experimental approach. For instance, the long-term tracing of the newly born elements (BrdU label-retaining cells) cannot be successfully used in humans or in mammalian species that are protected by international law (e.g., human primates and cetaceans). In alternative, cell division can be detected by immunocytochemistry in postmortem tissues, though limitedly to cells dividing at the exact time of animal death (e.g., Ki67 antigen; Kee et al., 2002). Additionally, some discrepancies emerging after comparing the results with those obtained in laboratory rodents can be linked to real interspecific differences of the biological processes themselves (Sanai et al., 2011; Lipp and Bonfanti, 2016; Parolisi et al., 2017; Sorrells et al., 2018; Seki et al., 2020; La Rosa et al., 2020a; Franjic et al., 2022) or to antibody specificity in the different mammals. Hence, the overall issue of analysing large, gyrencephalic brains is more complex than simply fixation, which overlap with natural interspecies variation and/or different specificity of antibodies across phylogeny, the latter two aspects being often neglected.

As shown in Figure 1.9, elements of complexity can be summarised as follows:

i) some markers of immaturity previously considered as specific for newly born neurons (e.g., doublecortin - DCX - and polysialylated form of N-CAM - PSA-NCAM), are also expressed by wide populations of non-newly generated “immature” neurons (La Rosa et al., 2020b; Bonfanti and Seki, 2021);

Table 2.1 Most common antibodies used to detect DCX in different animal species.

Host and source	**References**	**Animal species** (common name)
Santa Cruz[1] *(goat)*	Fasemore et al., 2018	Galago; Lemur; Potto
	Chawana et al., 2020	Egyptian fruit bat
	La Rosa et al., 2020	12 mammals (from mouse to chimpanzee)
	Kirby et al., 2012	Rat
	Fudge et al., 2012	Crab-eating macaque; Southern pig-tailed macaque
	Zhang et al., 2009	Rhesus macaque
	Flor-Garcìa et al., 2020	Human
	Sorrells et al., 2018	Human; Rhesus macaque
	Tobin et al., 2019	Human
	Boekhoorn et al., 2006	Human
	Liu et al., 2020	Rhesus macaque
	Marlatt et al., 2011	Common marmoset
	Parolisi et al., 2017	Dolphin
	La Rosa et al., 2018	Dolphin; Sheep
	Moreno-Jiménez et al., 2019	Human
	Jin et al., 2006	Human
	Crews et al., 2010	Human; Mouse
	Knoth et al., 2010	Human
	Wang et al., 2011	Human; Rhesus macaque
	Gomez-Nicola et al., 2014	Human; Mice
	Ekonomou et al., 2015	Human
	Dennis et al., 2016	Human
	Galàn et al., 2017	Human
	Liu et al., 2008	Human
	Ponti et al., 2006	Rabbit
	Kunze et al., 2015	Mouse
	Cai et al., 2009	Human; Rhesus macaque; Cat
	Verwer et al., 2007	Human
	Bloch et al., 2011	Human; Cynomolgus monkey; African green monkey
	Li et al., 2023	Human
Abcam *(rabbit)*	Piumatti et al., 2018	Sheep
	Jhaveri et al., 2018	Mouse
	Flor-Garcìa et al., 2020	Human
	Sorrells et al., 2018	Human; Rhesus macaque
	Cipriani et al., 2018	Human
	Tobin et al., 2019	Human
	Liu et al., 2020	Rhesus macaque
	Parolisi et al., 2017	Dolphin
	La Rosa et al., 2018	Dolphin; Sheep
	Wang et al., 2011	Human; Rhesus macaque
	Nogueira et al., 2014	Human
	Perry et al., 2012	Human
	Bloch et al., 2011	Human; Cynomolgus monkey; African green monkey
	Cai et al., 2009	Human; Rhesus macaque; Cat
Millipore *(guinea pig)*	Akter et al., 2020	Common marmoset
	Benedetti et al., 2019	Mouse
	Jhaveri et al., 2018	Mouse
	Flor-Garcìa et al., 2020	Human
	Sorrells et al., 2018	Human; Rhesus macaque
	Sorrells et al., 2019	Human
	Cipriani et al., 2018	Human
	Gomez-Nicola et al., 2014	Human; Mice
	Paredes et al., 2016a	Human
	Kunze et al., 2015	Mouse
	Bloch et al., 2011	Human; Cynomolgus monkey; African green monkey
	Alderman et al., 2022	Human; Mouse
Cell Signaling Technology *(rabbit)*	Sorrells et al., 2018	Human; Rhesus macaque
	Liu et al., 2008	Human
	Maheu et al., 2015	Human
	Paredes et al., 2016a	Human
	Sorrells et al., 2019	Human
	Martì-Mengual et al., 2013	Human; Squirrel monkey; Cat
	Alderman et al., 2022	Human; Mouse
	Coviello et al., 2022	Human

Table 2.2 Most common antibodies used to detect Ki67 in different animal species.

Host and source	References	Animal species
Leica-Novocastra[2] *(rabbit)*	Fasemore et al., 2018	Galago; Lemur; Potto
	Akter et al., 2020	Common marmoset
	Chawana et al., 2020	Egyptian fruit bat
	La Rosa et al., 2020	12 mammals (from mouse to chimpanzee)
	Jhaveri et al., 2018	Mouse
	Sorrells et al., 2018	Human; Rhesus macaque
	Sorrells et al., 2019	Human
	Tobin et al., 2019	Human
	Boekhoorn et al., 2006	Human
	Quiñones-Hinojosa et al., 2006	Human
	Fahrner et al., 2007	Human
	Parolisi et al., 2017	Dolphin
	La Rosa et al., 2018	Dolphin; Sheep
	Martì-Mengual et al., 2013	Human; Squirrel monkey; Cat
BD Pharmingen *(mouse)*	La Rosa et al., 2020	12 mammals (from mouse to chimpanzee)
	Sorrells et al., 2018	Human; Rhesus macaque
	Sorrells et al., 2019	Human
	La Rosa et al., 2018	Dolphin; Sheep
Abcam *(rabbit)*	Gomez-Nicola et al., 2014	Human; Mice
	Allen et al., 2016	Human
	Cipriani et al., 2018	Human

[1]Out of production (stock in the Turin lab); [2]Out of production

ii) both the rate of postnatal neurogenesis and the occurrence/distribution of immature neurons remarkably vary among mammals (Paredes et al., 2016b; Parolisi et al., 2018; La Rosa et al., 2020a; Duque et al., 2022; Figure 1.9B);

iii); when directly comparing brains of widely different size, belonging to animal species rising technical/ethical issues, some variables/approaches cannot be completely standardized (e.g., type and time of fixation, postmortem interval; Figure 1.9C);

iv) the study of such variations requires systematic, comparable approaches to actually match brain tissues belonging to widely different animal species, size and ages (La Rosa et al., 2020a; Figure 2.1).

Several antibodies from different manufacturers were employed over the years to detect the most popular antigens linked to structural plasticity/immaturity (e.g., DCX; Table 2.1) and cell division (e.g., Ki67 antigen; Table 2.2).

Therefore, heterogeneous results were reported by different authors by using various methods and antibodies in each species, in a manner that makes it difficult to really compare data (La Rosa et al., 2020a). Although the focus has been put on quantification methods (Zhao and van Praag., 2020), real interspecies differences in the occurrence/distribution of antigens as well as antibody specificity can play a role. Here, we tried to combine some of the abovementioned variables in six

mammals, including humans (Figure 2.1), with a twofold aim: i) to map the best performance of antibodies raised against the most used markers for neuronal immaturity (DCX) and cell division (Ki-67 antigen) in each species, and ii) to reach a comparable landscape for these markers across species. The screening was performed on four selected brain regions in which the occurrence of DCX and Ki67 staining is well known, two of them hosting non-newly generated "immature" neurons (paleocortex and neocortex) and other two hosting newly born neurons (hippocampus and subventricular zone; Figures 2.1 and 2.2). The study has been conceived on two levels: a) detection of DCX and Ki67 antigen immunoreactivity by testing seven commercial antibodies in five mammals (including lissencephalic and gyrencephalic species), by considering the abovementioned brain regions (Figure 2.2); b) detection of DCX in the human brain cerebral cortex in combination with its mRNA by using the RNAscope technique (Figure 2.2).

In spite of an obvious interest for visualising DCX in the human brain, the detection of this antigen in cerebral cortex of primates has been controversial, spanning from claiming its occurrence in most cortical layers (Bloch et al., 2011) to its very low level due to non-specific staining (Liu et al., 2020). Due to the complexity of the approach (seven antibodies tested in four neuroanatomical regions obtained from 24 brains of six widely different species) only qualitative aspects were considered, including cases of occurrence/absence of staining, background, or obvious non-specific staining (Figure 2.3).

2.2 Materials and Methods

Brain sample. Brains used in this study were collected from various institutions and tissue banks, all provided by the necessary authorizations. All experiments were conducted in accordance with current EU and Italian laws (for additional details on the animals used in this study see Table 2.3). Three young adult mice were analysed. Perfusion was performed under anesthesia (i.p. injection of a mixture of ketamine, 100 mg/kg, Ketavet, Bayern, Leverkusen, Germany; xylazine, 5 mg/kg; Rompun, Bayer, Milan, Italy; authorization of the Italian Ministry of Health and the Bioethical Committee of the University of Turin; code 813/2018-PR, courtesy of Serena Bovetti) and brains were postfixed for 4 hours.

Three young-adult female rabbits were used. Animals were deeply anesthetized (ketamine 100 mg/kg - Ketavet, - and xylazine 33 mg/ kg body weight - Rompun) and perfused with fixative (Italian Ministry of Health, authorization n. 66/99-A). Tissues were postfixed for 6 hours.

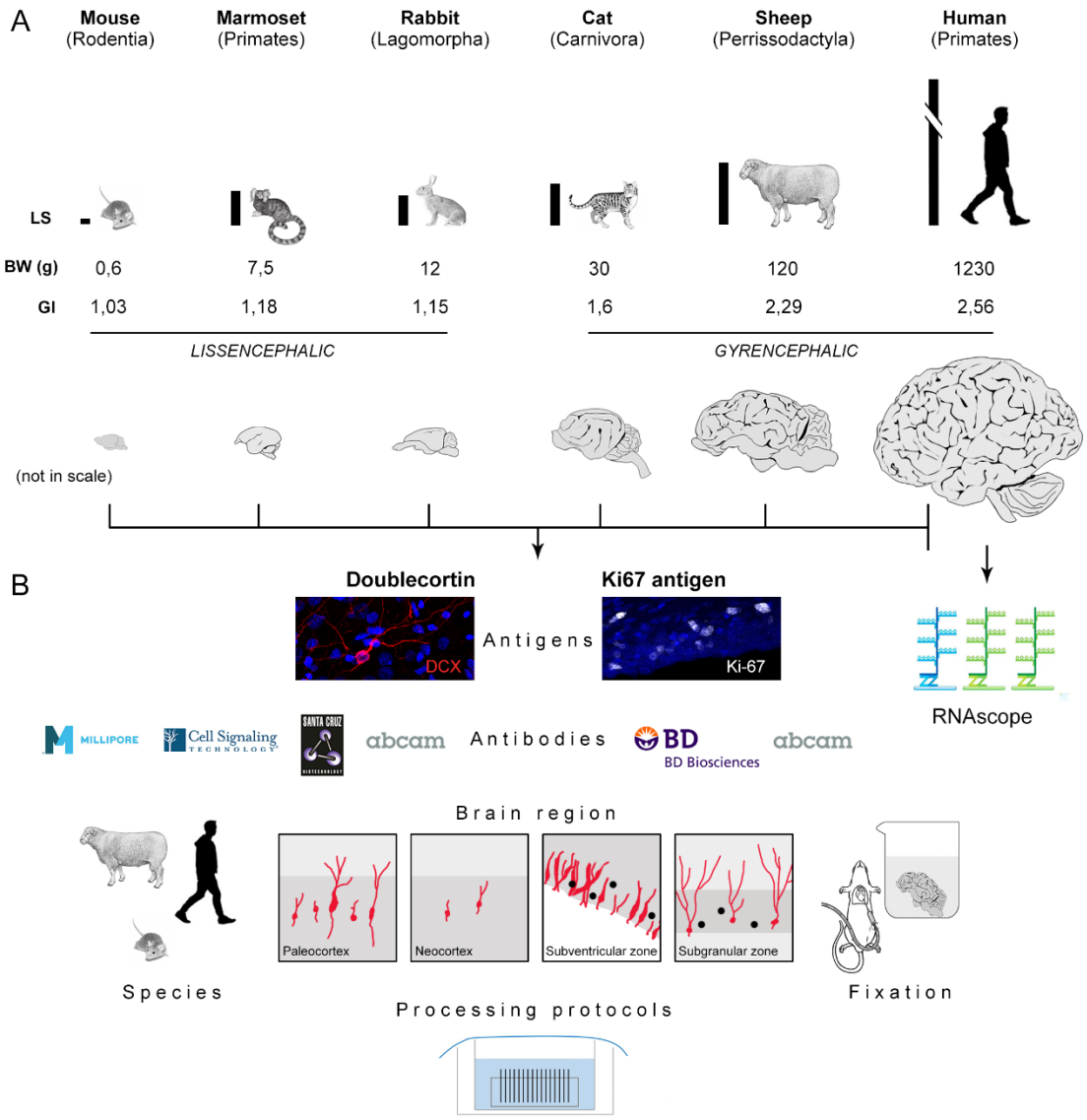


Figure 2.1 - Experimental plan. (**a**) Animal species considered in this study, widely varying for brain size (BW, brain weight, in grams), gyrencephaly (GI, gyrification index), and lifespan (LS). (**b**) Schematic summary of the variables investigated; four brain regions are considered (squares: red cells, DCX; black dots, dividing nuclei).

Three adult marmoset brains were extracted 1 hour after death and post-fixed for 3 months. The exact ages of the animals are unknown; they were aged as adults by experienced veterinarians.

Three young-adult cat brains were extracted post-mortem (the PMI was less than 1 hour), fixed and kept in the fixative solution for a 1 month.

Three young-adult sheep brains were perfused through both carotid arteries with 2 L of 1%sodium nitrite in phosphate buffer saline, followed by 4 L of ice-cold 4% paraformaldehyde solution in

0,1 M phosphate buffer, pH 7.4. The brains were then dissected out, cut into blocks, and postfixed in the same fixative for 48 h. Three young-adult sheep brains were extracted post-mortem (the PMI was less than 1 hour), fixed and kept in fixative for 1 month.

Human intraoperative brain samples were collected from the Neurosurgery Unit of the Humanitas Hospital during selected surgeries for brain tumor. A portion of healthy/perilesioned tissue of temporal lobe cortex resected for surgical reasons (considered free-of-diseases from clinical and pathological evaluation), were washed in cold NaCl 0.9% solution and directly (within 1 hour) fixed in PFA 4% (ethical approvement by IRCCS Humanitas prot. Nr. 400/19). Three different donors, have been used for this study (Table 2.4).

Postmortem human brain tissues were obtained from the Human Brain Collection Core (HBCC), National Institute of Mental Health Intramural Research Program (NIMH-IRP), with permission from the legal next-of-kin according to the National Institutes of Health Institutional Review Board and ethical guidelines under protocol 17-M-N073. Cases were obtained from the Offices of the Chief Medical Examiner of the District of Columbia. Clinical characterization, neuropathological screening, and toxicological analyses were performed as previously described (Deep-Soboslay et al., 2005; Lipska et al., 2006) (Table 2.4).

Table 2.3 Brain samples used in this study

Species	**Source**	**Specimens**	**Age**	**Fixation**	**Fixative**	**PMI**
Mouse	(a)	3	3 months	Perfusion	4% PFA	None
Marmoset	(b)		Adult	Immersion	4% PFA (15% picric acid)	1 hour
Rabbit	(a)		3.5 years	Perfusion	4% PFA	None
Cat	(c)		1.5 years	Immersion	4% buffered formalin	1 hour
Sheep	(d)		2 years	Perfusion	4% PFA	None
	(c)		5 years	Immersion	4% buffered formalin	1 hour
Human (see Table 4)	(e)		Adult (postmortem)	Immersion	4% buffered formalin	16-23 hours
	(f)		Adult (intraoperative)		4% PFA	None

Source: (a) Neuroscience Institute Cavalieri Ottolenghi (NICO); (b) Institute of Anatomy – University of Zurich; (c) Department of Comparative Biomedicine and Food Science – University of Padova; (d) INRA research center – Nouzilly, France; (e) United States National
Institute of Health (NIH) - Bethesda, Maryland, USA; (f) University of Milan

Table 2.4 Origin of human samples

Intraoperative tissues					
ID	**Fixation**	**Age**	**Sex**	**PSI**	**Cause of surgery**
226012	4% PFA	81	Male	< 1 h	Diffuse high-grade glioma
201032	4% PFA	67	Female	< 2 h	Gliosarcoma grade IV
130079	4% PFA	79	Male	< 2 h	Glioblastoma grade IV
Postmortem tissues					
ID	**Fixation**	**Age**	**Sex**	**PMI**	**Cause of death**
1271	4% buffered formalin	46,6	Male	16 h	Thrombosis
1164	4% buffered formalin	48,1	Female	16 h	Homicide
1122	4% buffered formalin	48,6	Male	23 h	Cardiomegaly

Tissue processing for histology. The whole brain hemispheres were cut into coronal slabs (1-2 cm thick). The slabs were washed in a phosphate buffer (PB) 0.1 M solution, pH 7.4, for 24-72 hours (on the basis of brain size) and then cryoprotected in sucrose solutions of gradually increasing concentration up to 30% in PB 0.1 M. Then they were frozen by immersion in liquid nitrogen-chilled isopentane at -80°C. Before sectioning, they were kept at -20°C for at least 5 hours (time depending on the basis of brain size) and then cut into 40 μm thick coronal sections using a cryostat. Free-floating sections were then collected and stored in cryoprotectant solution at -20 °C until staining.

Human intraoperative brain samples collected during surgery (2-4 cm size; Figure 2.2C2) were fixed on PFA 4% for 72-96 hours (depending on sample size), washed in PBS 1X and maintained in 30% sucrose solution in PBS 1X at 4°C. One single coronal slice (0.5 cm thick) was embedded in optimal cutting temperature compound and cut into 40 μm thick coronal sections by using a cryostat. Slices were mounted on SuperFrost plus slides (Fisher Scientific - Epredia™TM SuperFrost Plus© Gold) and stored at -20°C until use.

Comparable neuroanatomy. Correspondent coronal brain sections were identified in the different animal species (mouse, marmoset, rabbit, cat, sheep) in order to include the four regions to be analysed: hippocampal dentate gyrus and forebrain subventricular zone (as internal positive controls for Ki67 antigen), paleocortex (piriform cortex) and parietal neocortex (as brain area hosting DCX+ non-newly born immature neurons in non-rodent species) (Figure 2.2A,B). Two cryostat sections, 40 μm thick, cut at different anterior-posterior coronal levels (Figure 2.2A,B) were analysed for each animal, for DCX and Ki67 antigen immunocytochemistry with all antibodies (Table 2.5).

Human intraoperative samples were cut out from the temporal lobe (Figure 2.2C'). Human postmortem tissues were obtained from coronal slices (1 cm thick) including the parietal and temporal cortex (Figure 2.2C'').

Immunofluorescence protocol. For immunofluorescence staining, sections were rinsed in PBS 0.01 M, pH 7.4, then immersed in appropriate blocking solution (1–3% Bovine Serum Albumin, 2% Normal Donkey Serum, 1–2% Triton X-100 in 0.01M PBS, pH 7.4) for 90 min at RT. Then the sections were incubated for 48 hr at 4°C with primary antibodies (see Table 3), and subsequently with appropriate solutions of secondary antibodies for 4 hr at RT: Cyanine 3 (Cy3)-conjugated anti-goat (1:400; Jackson ImmunoResearch, West Grove, PA - 705-165-147), Cyanine 3 (Cy3)-conjugated anti-rabbit (1:400; Jackson ImmunoResearch, West Grove, PA - 711-165-152), Cyanine 3 (Cy3)-conjugated anti-guinea pig (1:400; Jackson ImmunoResearch, West Grove, PA - 706-165-148), Cyanine 3 (Cy3)-conjugated anti-mouse (1:400; Jackson ImmunoResearch, West Grove, PA - 715-165-150), Alexa 647-conjugated anti-mouse (1:400; Jackson ImmunoResearch, West Grove, PA - 715-605-151), Alexa 647-conjugated anti-rabbit (1:400; Jackson ImmunoResearch, West Grove, PA 711-605-152). Immunostained sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, KPL, Gaithersburg, Maryland USA) and mounted with MOWIOL 4–88 (Calbiochem, Lajolla,CA). All staining protocols were performed with and without antigen retrieval treatment, to exclude signal alteration due to a possible masking of the epitopes linked to fixation. When performed, antigen retrieval was done using citrate buffer at 90°C for 5 min (only for Ki67 nuclear staining of marmoset and sheep tissues fixed by immersion, it was extended to 30 minutes; Figure 2.5). Neither signal amplification nor tissue autofluorescence elimination protocols were applied to avoid signal alteration.

Table 2.5 Primary antibodies used in this study

Antigen	Host	Type	Code	Raised against	Dilution	Source
DCX	goat	polyclonal	SC8066	Peptide mapping at the C-terminus of Doublecortin of human origin	1:1000	Santa Cruz Biotechnology
	mouse	monoclonal	SC271390	Aminoacids 123-402 mapping at the C-terminus of Doublecortin of human origin		
	rabbit	polyclonal	ab18723	Synthetic peptide conjugated to KLH derived from within residues 300 to the C-terminus of Human Doublecortin		Abcam
			4604	A peptide corresponding to human doublecortin		Cell Signaling
	guinea pig		ab2253	Epitope within 17 amino acids from the C-terminal region		Merck Millipore
Ki-67	rabbit		ab15580	Synthetic peptide	1:500	Abcam
	mouse	monoclonal	550609	Human Ki-67		BD Pharmingen

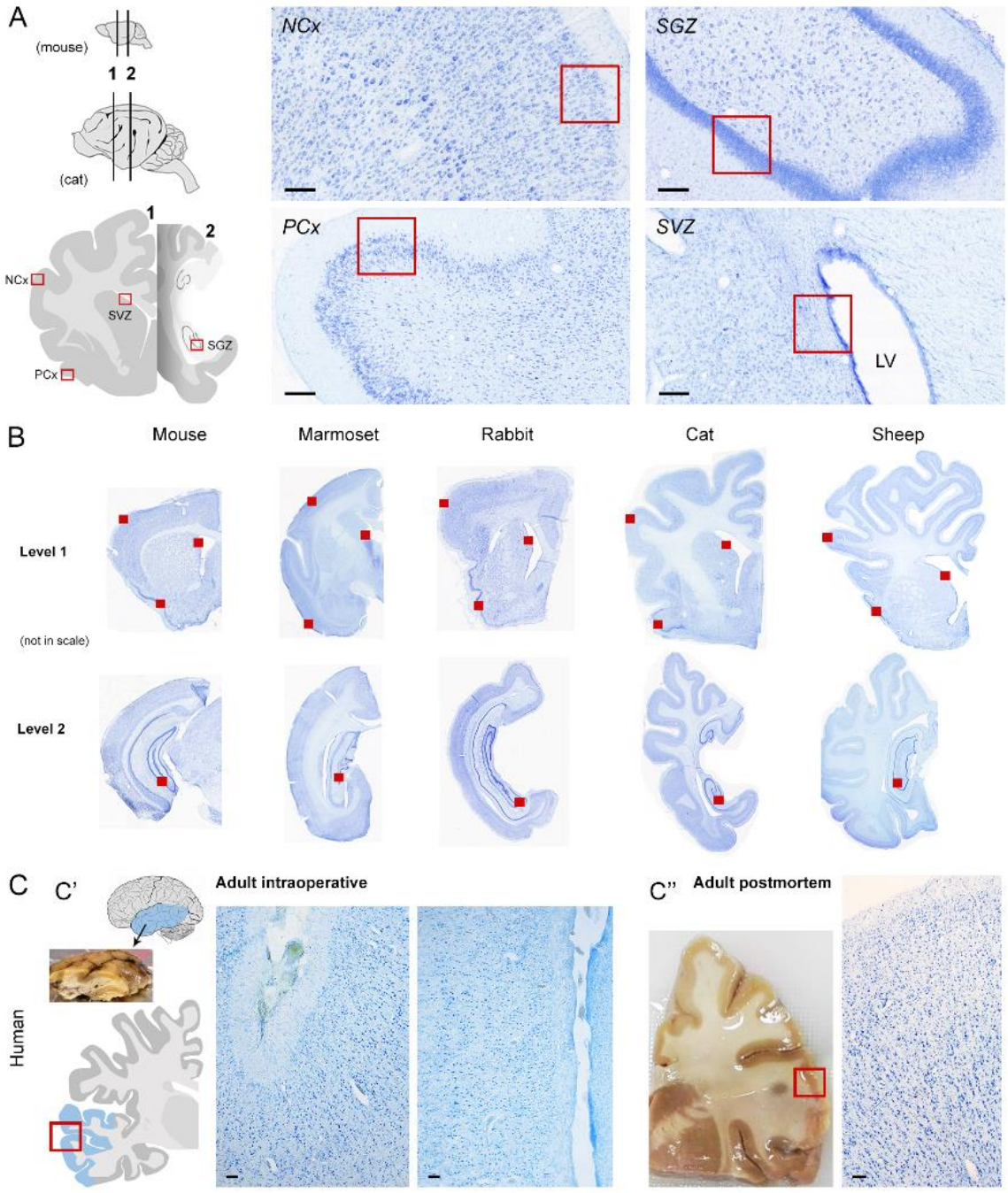


Figure 2.2 - Anatomy of the brain regions studied in part 1. (A) Four different regions were considered (SVZ, subventricular zone; SGZ, sub-granular zone of the hippocampal dentate gyrus; NCx, neocortex; PCx, paleocortex; indicated by red boxes in (A,C), and red squares in (B)) at two different anterior–posterior, coronal levels (level 1: crossing the SVZ; level 2: crossing the hippocampus) in five mammalian species. (B) Whole coronal sections cut at the two brain levels, stained with toluidine blue and scanned with slidescanner Axioscan (Zaiss; Oberkochen, Germany). (C) Human brain tissues were obtained from intraoperative (C′) and post-mortem (C″) specimens corresponding to the temporal lobe (light blue). Scale bars: (A,C), 100 µm.

All antibodies used in this study were predicted to work on mouse brain tissue (as stated by their datasheet). Thus, to confirm the quality of our antibodies before conducting the experiment, this species was used as a control. All antibodies gave a staining consistent with previous data, in all regions of interest (see Results). Moreover, to exclude possible aspecific staining due to fluorescent secondary antibodies, primary antibody omission experiments were performed by replicating the immunofluorescence protocol (see above) without the primary antibody incubation. This resulted in a complete absence of staining.

RNAscope (in collaboration with Prof. Francesco Bifari, University of Milan). Fixed frozen intraoperative human brain samples were sectioned coronally at 20µm on a cryostat (Leica CM1860) and mounted on SuperFrost Plus slides. RNA scope was performed following the purchaser instructions. Briefly, following washing in PBS1X for 5 minutes, slides were baked 30 minutes at 60°C in the HybEZ™ Oven and post-fixed by PFA 4% immersion for 3 hours. Thereafter, sections were treated with ascending series of ethanol and left to dry after the last 100% ethanol step. Slices were pretreated with hydrogen peroxide for 10 minutes, put at 98-102°C in target retrieval solution for 10 minutes and treated with protease plus at 40°C for 30 minutes in the HybEZ™ Oven. After pretreatment, the single-plex, chromogenic RNAscope assay (RNAscope™ 2.5 HD Assay – RED Cat No. 322360) was performed using human DCX probe (Cat No.489551), human Ubiquitine probe (Cat No. 31004) as positive control and DapB probe (Cat No. 310043) as negative control. Probes hybridization was performed at 40°C for 2 hours in the HybEZ™ Oven and then the signal was amplified using multiple amplifier probes. For the detection of signal, fast red chromogen was used.
Following RNAscope assay, immunofluorescence was performed to combine RNA and protein analysis. After the chromogen, the slides were washed in PBS-T (0.3% Triton in PBS 1X) and incubated in Blocking solution (2% BSA and 0.25% Triton in PBS 1X) for 1 hour. DCX (Cat No. ab18723 or ab2253) or GFAP (Cat No. ab53554) primary antibody was incubated overnight at 4°. The following day the donkey anti-rabbit IgG Secondary Antibody, Alexa Fluor™ (Thermofisher, Cat No. A31573 or Cat No. A32849) was added for 1 hour and the nuclei stained with DAPI (1:1000, KPL, Gaithersburg, Maryland USA) (Highet et al., 2021; Ciarpella et al., 2021).

Image Processing. Images were collected using a Nikon Eclipse 90i confocal microscope (Nikon, Melville, NY) or Zeiss Axio Observer.Z1/7 (Zeiss, Oberkochen, Germany). All images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA) and ImageJ version 1.53t

(Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). Adjustments to color, contrast, and brightness were avoided.

2.3 Results

As shown in Figure 2.3, we focused on the quality of staining defined by four parameters, spanning from a clear and clean staining of the specific cell populations to the absence of immunocytochemical signal. Results obtained from the comparative analysis in mouse, marmoset, rabbit, cat and sheep are reported in Figures 2.4-2.5 and Tables 2.6 and 2.7. Full results obtained with and without antigen retrieval are reported in Tables 2.6 and 2.7, whereas confocal images are provided for citrate treatment only (Figures 2.4a,2.4b, and 2.5). The set of results concerning the detection of DCX in the human cerebral cortex is reported in Table 2.8 and Figure 2.7.

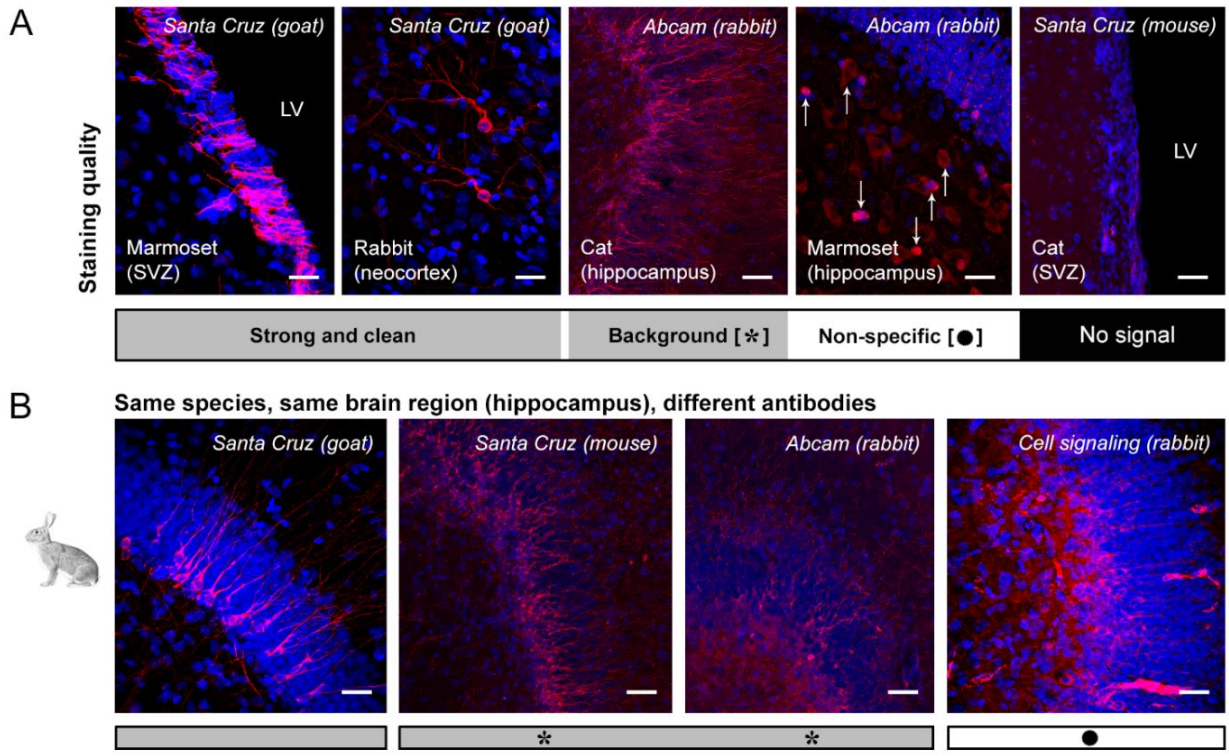


Figure 2.3 - Type and quality of immunostaining obtained with different antibodies in different animal species and brain regions. (a) Four types of staining were considered, including a clear and clean staining without background noise, a specific staining bleary with background, an aspecific staining (with or without background; including artifacts, or staining associated with other structures, e.g. blood vessels, astrocytes, other neurons), and the absence of signal. Bottom: legend with symbols reported in Tables 2.6, 2.7 and 2.8. (b), Some examples considering substantial differences depending on the different antibodies used. LV, lateral ventricle. Scale bars: 30 µm.

Comparative immunostaining in the brain of five mammals

Tissues analysed in this study belong to widely different mammals endowed with different brain size and gyrencephaly (Figures 2.1 and 2.2); for this reason, they did not undergo the same type of fixation (Table 2.3). We tried to obtain maximal homogeneity for heterogeneous tissues, in terms of fixation and postmortem interval, as previously reported in a study involving 12 mammalian species (La Rosa et al., 2020a). Nevertheless (as described in the Introduction, and also in view of the specific aims of the present study), we included specimens fixed by perfusion (mouse, rabbit, sheep), immersion (cat, marmoset, sheep, human), postmortem tissues and intraoperative samples (human). Considering sheep, both perfused and immersed brains were collected, in order to extend our comparison. As a result, a substantial ability to detect the two antigens in all animal species was observed (out of 115 immunocytochemical staining samples performed for DCX with 5 minutes (5') citrate treatment, 89 gave a positive signal while only 26 gave no signal or non-specific staining; see Table 2.6a), with no difference among the three specimens analysed in each species. Nevertheless, substantial differences in the occurrence/type of staining were also observed (purple areas in Tables 2.6b and 2.7b). By observing the distribution of the staining rates (success and failure, the latter including the absence of signal and a non-specific staining), they are not strictly linked to the animal species or the type of fixation, rather to the use of different antibodies (in some cases, also to real interspecies differences; Figure 2.6A).

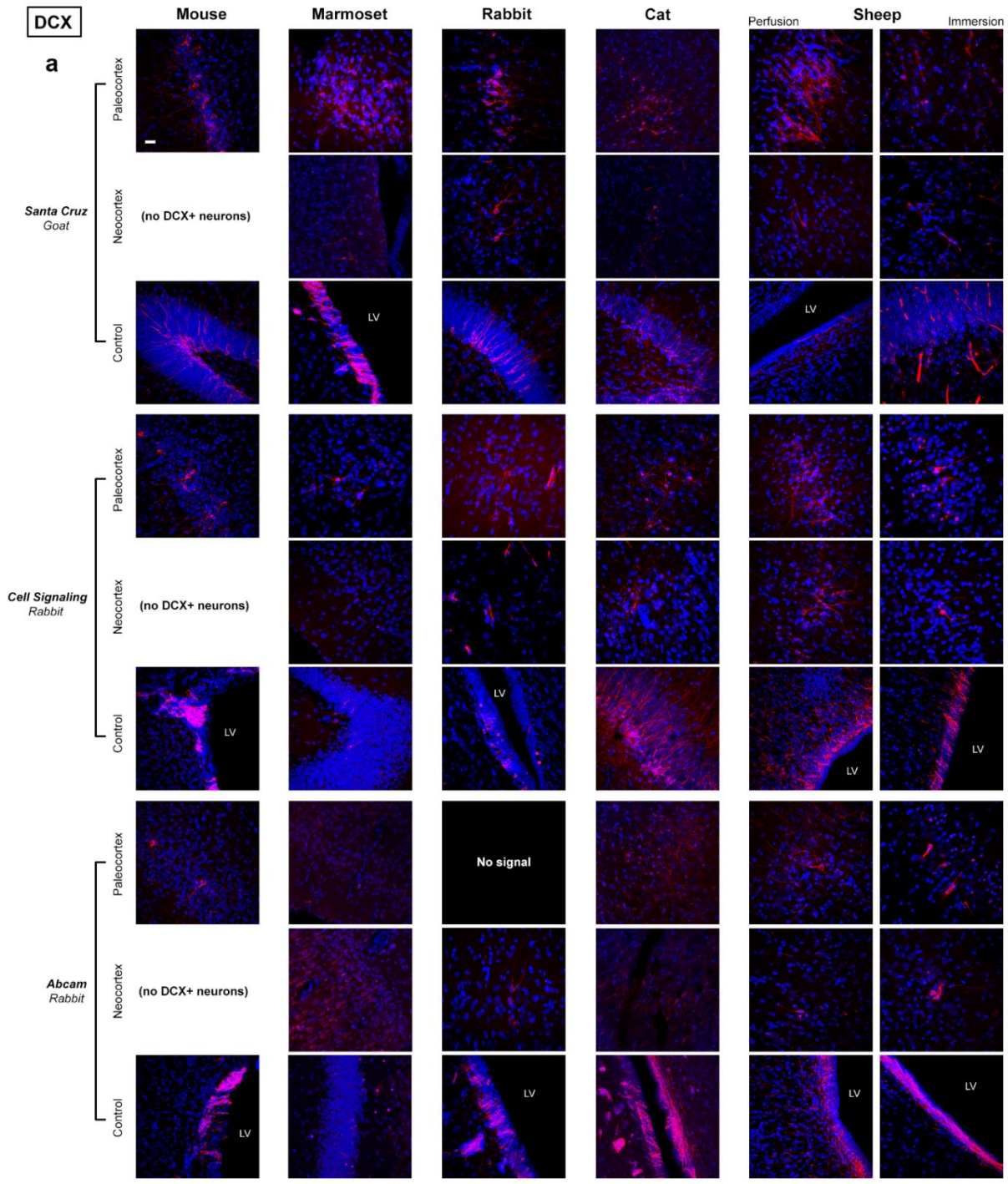


Figure 2.4 a (see legend in the next page)

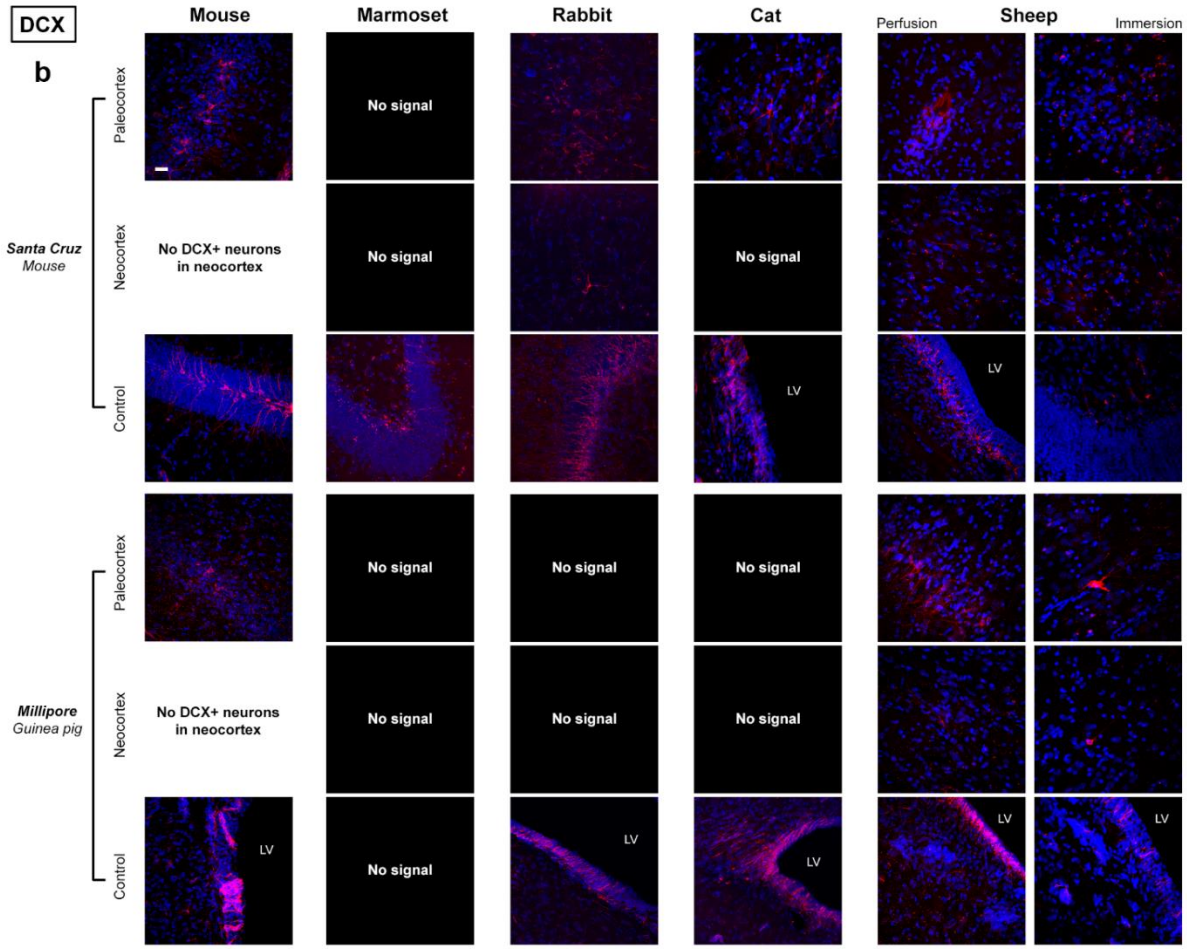


Figure 2.4 a, b - Representative confocal images of DCX detection (in red) in different mammals for five different antibodies. All specimens are counterstained with DAPI. All photographs have been performed at the same magnification (scale bar: 30 µm). Control is represented by one of the two neurogenic sites, either SVZ or hippocampus. LV, lateral ventricle.

Table 2.6 - (a) Occurrence and quality of staining for DCX. (b) Merge (with and without citrate treatment).

(**a**)

Citrate Treatment (5 min)

Antibodies	Regions	Mouse	Marmoset	Rabbit	Cat	Sheep (perfusion)	Sheep (immersion)
Santa Cruz (goat)	**pc**						●
	nc						●
	svz						●
	sgz					*	●
Cell Signalling (rabbit)	**pc**			*	*	*	*
	nc		*	*	*	*	
	svz		*				
	sgz		*	*	*	*	*
Abcam (rabbit)	**pc**	*	●	*	*	*	*
	nc		●	*	*	*	
	svz		●				
	sgz	*	●		*	*	*
Santa Cruz (mouse)	**pc**	*		*		*	●
	nc			*	*	*	●
	svz		*				●
	sgz		*	*			●
Millipore (guinea pig)	**pc**	*		*	*	*	
	nc			*	*	*	
	svz				*	*	
	sgz	*		*	*	*	*

No Citrate

Antibodies	Regions	Mouse	Marmoset	Rabbit	Cat	Sheep (perfusion)	Sheep (immersion)
Santa Cruz (goat)	**pc**				*	*	●
	nc		*		*	*	●
	svz					*	●
	sgz		*		*	*	●
Cell Signalling (rabbit)	**pc**			*	*	*	●
	nc		*	*	*	*	●
	svz		*	*			●
	sgz		*	*	*	*	●
Abcam (rabbit)	**pc**	*	●	*	*	*	
	nc		●	*	*	*	
	svz		●				
	sgz		●	*	*	*	*
Santa Cruz (mouse)	**pc**	*	*	*	*		
	nc		*	*	*		●
	svz	*	*	*	*		
	sgz	*	*	*	*		
Millipore (guinea pig)	**pc**	*	*	*	*	*	
	nc		*	*	*	*	
	svz	*	*	*	*		
	sgz	*	●	●	*	*	

(**b**)

Antibodies	Regions	Mouse	Marmoset	Rabbit	Cat	Sheep (perfusion)	Sheep (immersion)
Santa Cruz (goat)	**pc**				**(5′)**	**(5′)**	
	nc		**(5′)**		**(5′)**	**(5′)**	
	svz					**(5′)**	
	sgz		**(5′)**		**(5′)**		
Cell Signalling	**pc**						**only 5′ C**
	nc						**only 5′ C**

(rabbit)	**svz**		**(5′)**	**(5′)**			**only 5′ C**
	sgz		**(5′)**				**only 5′ C**
Abcam (rabbit)	**pc**			**without C**			**without C**
	nc		**without C**				
	svz						
	sgz	**without C**		**(5′)**			
Santa Cruz (mouse)	**pc**	**only 5′ C**		**only 5′ C**	**only 5′ C**	**only 5′ C**	
	nc			**only 5′ C**		**only 5′ C**	
	svz		**only 5′ C**	**(5′)**	**(5′)**	**only 5′ C**	
	sgz	**(5′)**	**only 5′ C**		**only 5′ C**		
Millipore (guinea pig)	**pc**	**(5′)**					
	nc						
	svz	**(5′)**		**(5′)**		**without C**	
	sgz					**without C**	

Subtable **(a)**: Absence of staining due to real interspecies difference (e.g., no cell population containg DCX in the mouse neocortex); Clear and clean immunoreaction; * Immunoreaction with background; ● Immunoreaction with non-specific staining; * No signal with background; ● No signal with non-specific staining; No signal; Subtable **(b)**: MERGE of results obtained with and without 5′ Citrate: Same results; Different results; (5′) better with Citrate; only (5′) C signal only with Citrate; without C signal only without Citrate.

Regional differences

Some recurrent regional differences depending on the neuroanatomical area investigated were observed for DCX. A clear and clean staining was generally detectable in the SVZ (21 positivities out of 30, with 8 cases of background or non-specificity), whereas the occurrence of background or non-specific staining was more frequent in the SGZ (15) and cerebral cortex layer II (23). These differences, observed in different brain regions of the same animal species, do not seem linked to antibody specificity, rather to neuroanatomical features and to the cell populations involved (see also Discussion). Also, the impact of the whole staining in the microscope field can be different depending on the structures detected: the SVZ hosts masses of neuroblasts forming chains enriched in DCX, whereas isolated neurons at different maturational stages are detectable in the dentate gyrus, even more diluted in space and less immature in the neocortex.

The detection of Ki67 antigen was more homogeneous with respect to the region investigated, mainly because the analysis was restricted to neurogenic sites (Figure 2.5 and Table 2.7). The rate of failure and the occurrence of background noise were slightly constant in both regions. Being Ki67 a nuclear antigen, can be hardly reached by antibodies in tissue fixed by immersion; for this reason, it requires longer citrate treatment to unmask antigenicity (Figure 2.5).

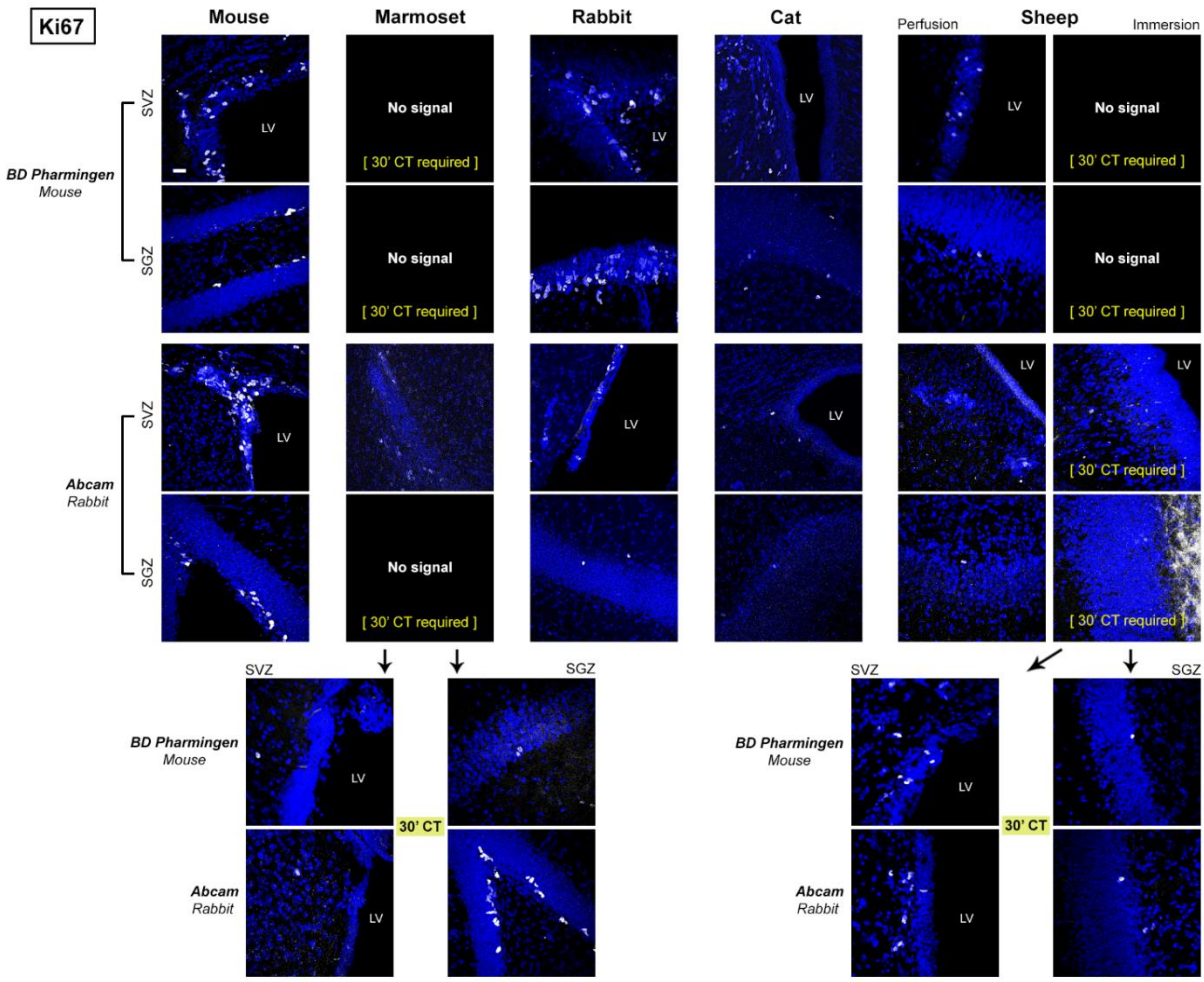


Figure 2.5 - Representative confocal images of Ki67 antigen detection (in white) in different mammals for two different antibodies in the two neurogenic sites (forebrain SVZ and hippocampal SGZ). All specimens are counterstained with DAPI. All photographs have been performed at the same magnification (scale bar: 30 μm). In marmoset, [30' CT] indicates that the antigen can be detected only after 30 minutes of citrate buffer treatment. LV, lateral ventricle.

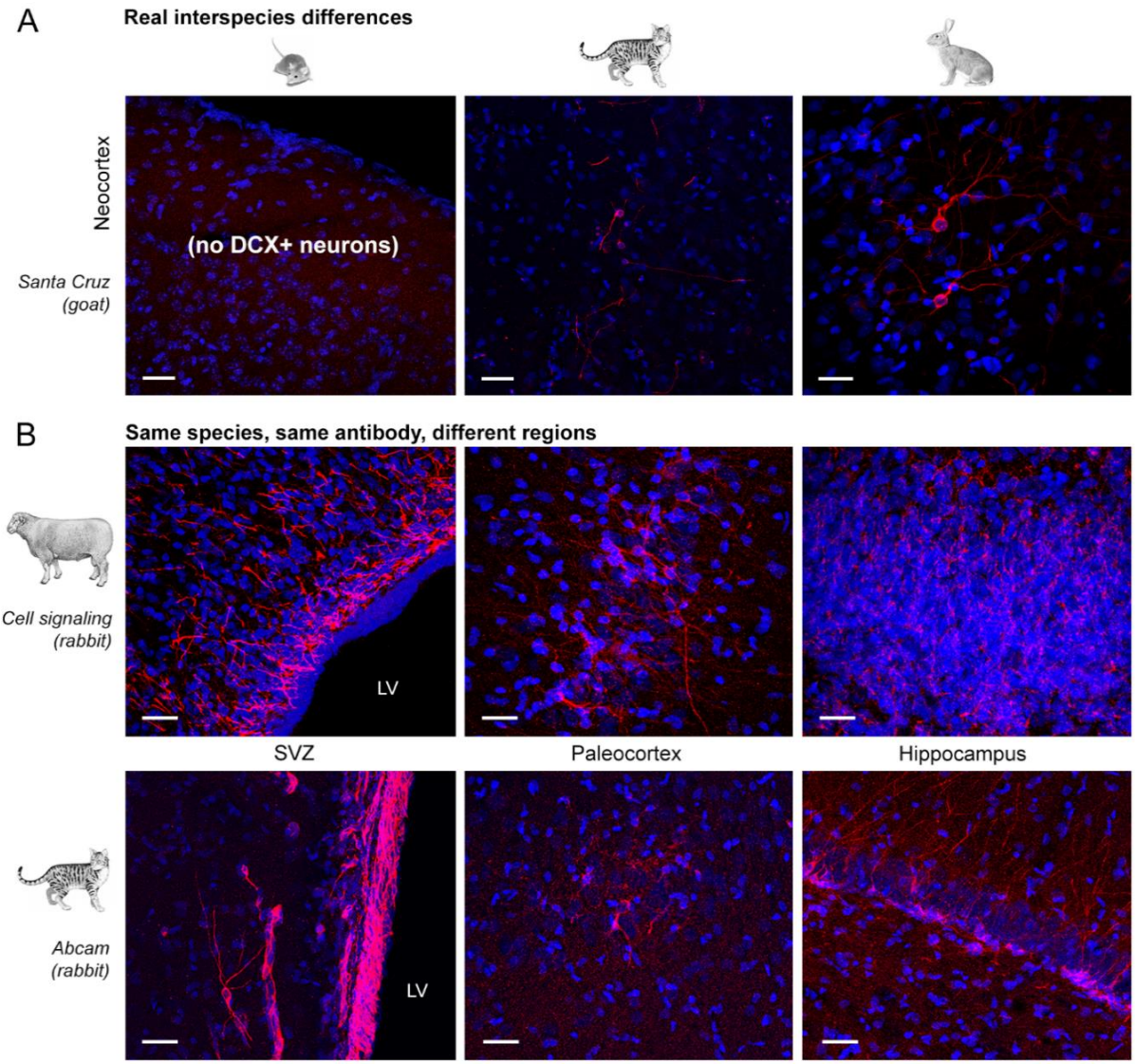


Figure 2.6 - Variation in the type and quality of immunostaining obtained in different animal species (a) and in different brain regions (b). (a) In comparative studies the detection DCX+ cell populations can yeld different results depending on real interspecies differences, e.g., the absence of DCX+, immature neurons in the mouse neocortex (La Rosa et al., 2020a). (b) Even in the same species, using the same antibody, different results can be found depending on the brain region. In most cases, the SVZ bordering the lateral ventricle (LV) stains far more clean and clear with respect to parenchymal regions such as the cortex and the hippocampus (sheep and cat). Note, in sheep, that SVZ and cortex stain for the expected neuronal populations while a non-specific staining is detectable in the hippocampus. Scale bar: 30 µm.

Table 2.7 - (**a**) Occurrence and quality of staining for Ki67 antigen. (**b**) Merge (with and without citrate treatment).

(**a**)

Citrate Treatment (5 min)							
Antibodies	**Regions**	**Mouse**	**Marmoset**	**Rabbit**	**Cat**	**Sheep** (perfusion)	**Sheep** (immersion)
BD Pharmingen (mouse)	**svz**		*				*
	sgz						*
Abcam (rabbit)	**svz**		*		*	*	*
	sgz		*		*	*	●

No Citrate							
Antibodies	**Regions**	**Mouse**	**Marmoset**	**Rabbit**	**Cat**	(perfusion)	(immersion)
BD Pharmingen (mouse)	**svz**	*	*		*		*
	sgz	●	*		*		●
Abcam (rabbit)	**svz**		*	●	*	*	*
	sgz		*			*	*

(**b**)

Antibodies	**Regions**	**Mouse**	**Marmoset**	**Rabbit**	**Cat**	**Sheep** (perfusion)	**Sheep** (immersion)
BD Pharmingen (mouse)	**svz**	**(5′)**			**(5′)**	**only 5′ C**	
	sgz	**only 5′ C**			**only 5′ C**	**only 5′ C**	
Abcam (rabbit)	**svz**		**only 5′ C**	**(5′)**			**only 5′ C**
	sgz			**only 5′ C**	**only 5′ C**		**only 5′ C**

Subtable **(a)**: Absence of staining due to real interspecies difference (e.g., no cell population containg DCX in the mouse neocortex); Clear and clean immunoreaction; * Immunoreaction with background; ● Immunoreaction with non-specific staining; * No signal with background; ● No signal with non-specific staining; No signal; Subtable **(b)**: MERGE of results obtained with and without 5′ Citrate: Same results; Different results; (5′) better with Citrate; only (5′) C signal only with Citrate; without C signal only without Citrate

DCX detection in the human cerebral cortex

In the human cerebral cortex, the picture appeared different from that observed in the other mammals, since only one antibody was properly working. Results obtained in humans are reported in Table 2.8 and Figure 2.7. Analyses were restricted to the neocortex, both from postmortem tissues and intraoperative samples, in the latter integrated with RNAscope analysis in order to assess the spatial expression of RNA molecules with cellular specificity for DCX (Figure 2.8).

Table 2.8 - Occurrence and quality of staining for DCX in human brain tissue.

Antibody	**Region**	**Immuno**	**RNAscope**
Santa Cruz (goat)	Cerebral cortex (temporal)	●	-
Cell Signalling (rabbit)		●	-
Abcam (rabbit)		*	Co-expression in some DCX+ neurons in layer II-III
Santa Cruz (mouse)		●	No co-expression in DCX+ neurons (non-specific staining)
Millipore (guinea pig)		●	No co-expression in DCX+ neurons (non-specific staining)

* Immunoreaction with background; ● Immunoreaction with non-specific staining; ● No signal with non-specific staining.

All antibodies raised against DCX and used on the other mammals were tested immunocytochemically on coronal cryostat sections of human temporal cortex (Figure 2.7A). Among the five antibodies employed, only the Abcam rabbit and the Millipore guinea pig revealed neuronal-like cells (mostly localised in the superficial cortical layers II and III), the other antibodies giving no signal and/or a non-specific punctate reaction (Table 2.8 and Figure 2.7A). The detection of DCX+ neuronal-like cells was rare and revealed elongated cell bodies whose staining extended only in a short part of a process, as expected in human brains of old individuals (ranging from 67 to 81 years in our study; Table 2.4) (Srikandarajah et al., 2009; Cai et al., 2009; Coviello et al., 2022; Li et al., 2023). Other cell bodies located in different layers and showing a faint staining resembling autofluorescence were also observed. To check whether the immunostaining was specifically associated with DCX-expressing cells, an in-situ hybridization with RNA probe (RNAscope) was performed in double staining with Abcam, Millipore and Santa Cruz anti-DCX antibodies (Figure 2.8). Only in the case of Abcam antibody a coexpression was detected, revealing a subpopulation of DCX-expressing neurons in layers II and III (Figure 2.8A and Table 2.8).

Postmortem, heavily formalin-fixed human tissues were also used, to make a comparison. In these tissues, by using the Abcam antibody some unipolar/bipolar neurons were detectable in the cortical layer II (Figure 2.7B, top), yet, also interlaminar astrocytes of the layer I (Falcone et al., 2019) were heavily stained (Figure 2.7B, bottom). To further validate DCX expression in these cells, we combined RNAscope analysis for DCX gene expression with immunocytochemistry for the astrocytic marker GFAP and found no $GFAP^+$ astrocytes co-expressing DCX mRNA (Figure 2.8C). This result, together with the absence GFAP+/DCX+ detection in intraoperative samples, indicate that GFAP+/DCX+ double positive cells detected in the heavily formalin-fixed human tissues are likely due to non-specific staining.

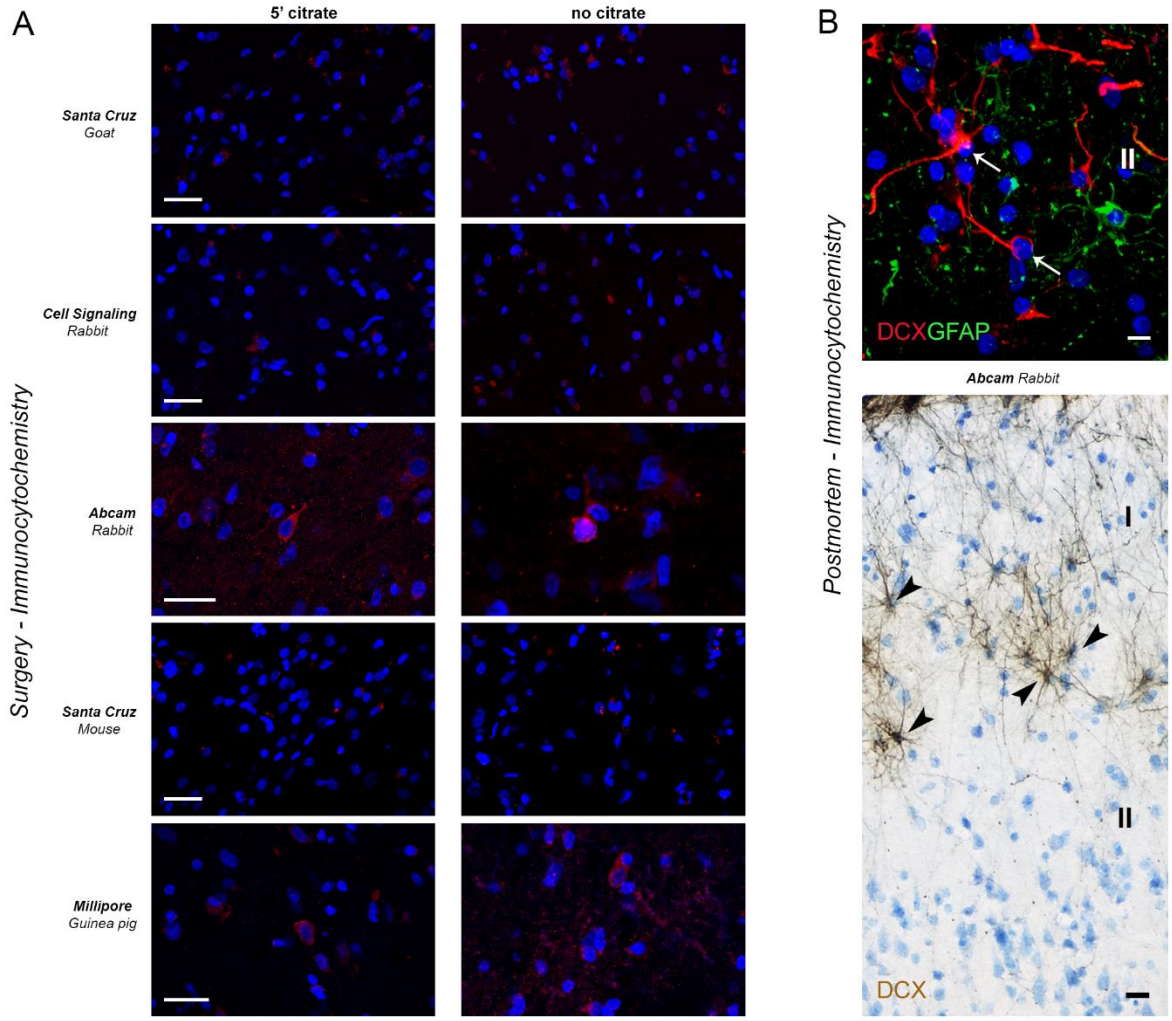


Figure 2.7 - Detection of DCX in the human neocortex by immunocytochemistry and RNAscope. (a) Immunocytochemical staining for DCX by using different antibodies, both with and without Citrate buffer treatment. Confocal images taken in cortical layer II-III (I, II, cortical layers). (b) Immuno-cytchemistry on postmortem brain tissue with Abcam primary antibody reveals some unipo-lar/bipolar neurons (arrows) in layer II, but also a non-specific staining on interlaminar astrocytes in layer I (arrowheads; see C). (c) RNAscope technique in association with anti-DCX Abcam primary antibody confirms that some labeled cells in layer II-III actually are DCX-expressing neurons (white arrows); some cells positive only for the RNA-probe do not express the protein (red arrows); bottom, another double-labeled cell at higher magnification. (c'), No coexpression of DCX-RNAprobe (red arrows) with GFAP+ astrocytes (white arrowheads) was detected. (c'') No coexpression of DCX-RNAprobe (red arrows) with anti-DCX Santa Cruz and Millipore. Scale bars: (a),(b), 20 µm; (c)-(c''), 10 µm.

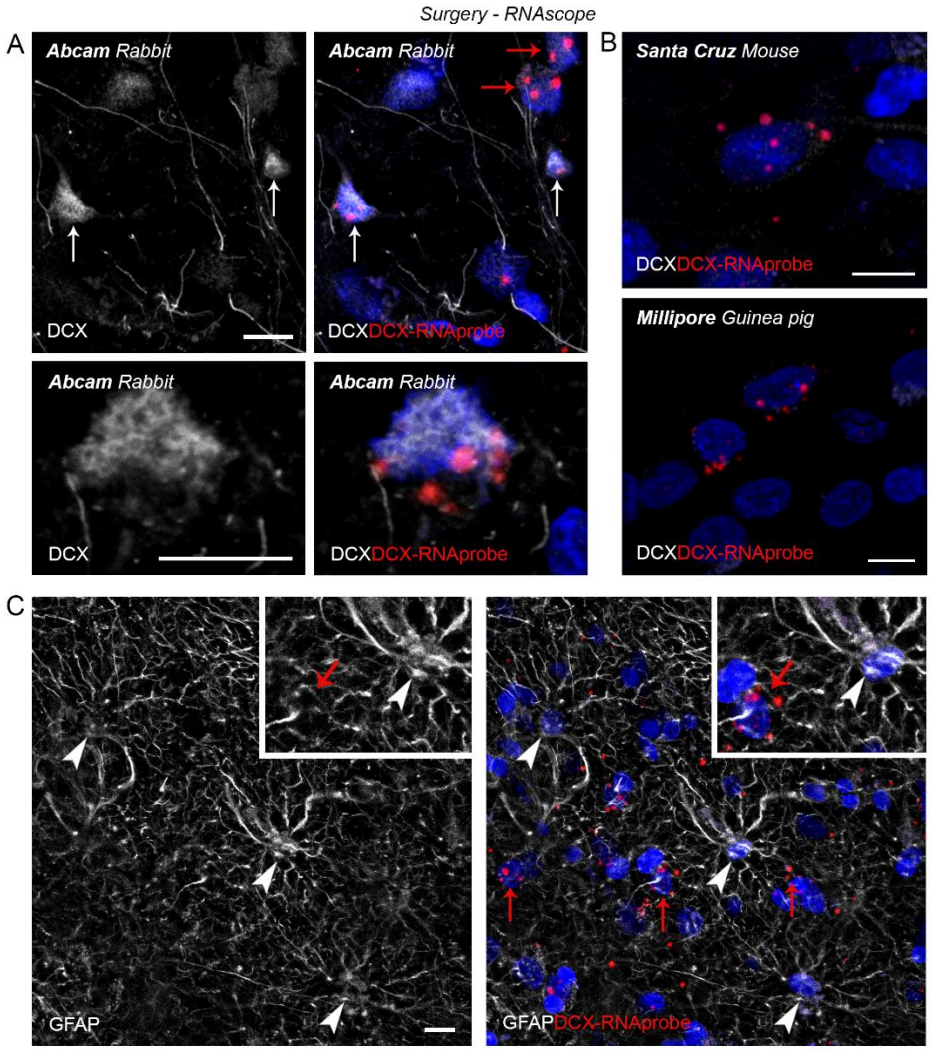


Figure 2.8 - Detection of DCX in the human neocortex by RNAscope (experiments carried out by Alessia Amenta and Francesco Bifari, University of Milan). (A) RNAscope technique in association with anti-DCX Abcam primary antibody confirms that some labelled cells in layer II-III actually are DCX-expressing cells (white arrows); some cells positive only for the RNA-probe do not express the protein (red arrows); bottom, higher magnification of a double-labelled cell. (B) No co-expression of DCX-RNAprobe (red arrows) with anti-DCX Santa Cruz and Millipore. (C), No co-expression of DCX-RNAprobe (red arrows) with GFAP+ astrocytes (white arrowheads) was detected. Scale bars: 10 µm.

2.4 Discussion

Cell marker immunocytochemical detection is an important tool in developmental neurobiology, helping to define different cell populations on the basis of their phenotype and/or maturational stages. The reliability of antibodies used to detect such markers is obviously a prominent aspect, and acquires special complexity in comparative studies involving widely different mammalian species characterized by different brain size and inherent difficulties in obtaining fresh, well-fixed

material (Figure 2.1). When protected animal species or humans are involved, the post mortem interval, as well as the type of fixation, cannot be the same as in laboratory rodents. Consequently, even when several animal species are considered in the same study and thus processed by using the same methods, the original conditions of the tissues cannot be exactly the same (Patzke et al., 2015; van Dijk et al., 2016; La Rosa et al., 2020a). Conversely, when a single animal species is investigated to find the best conditions to detect antigens in that brain tissue (a most frequent event in the literature), comparison with other species can be tricky.

On these bases, we performed here a systematic testing of different commercial antibodies raised against two widely used markers of structural plasticity on different brain regions (neurogenic and non-neurogenic) of five mammalian species and on the cerebral cortex of humans, in search of similarities and possible substantial, qualitative differences. The first aim was to establish a screening on the most commonly used antibodies, to check whether they can be considered specific for all species, or otherwise, to map which antibodies do not work in some of them, in order to obtain a panel to be used in future comparative studies, in the view of reaching a "comparable" picture.

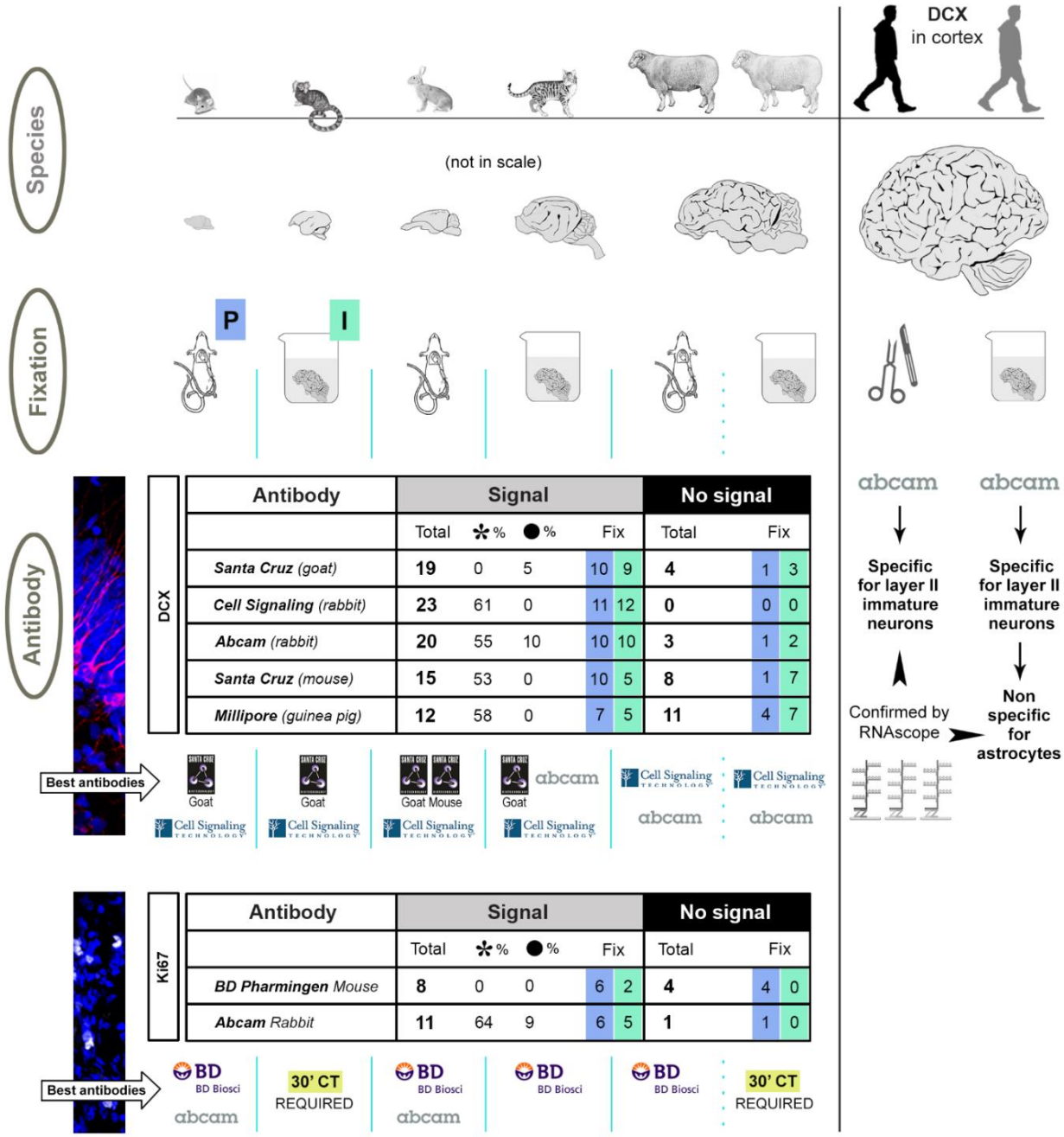
Variables affecting the occurrence/quality of staining

The present study involved seven antibodies raised against two antigens, detected in four brain regions of five mammalian species, as well as in the cerebral cortex of humans (Figure 2.2). Our results revealed that, beside a substantial prevalence of positive immunostainings, remarkable differences can exist when performing comparative analyses in mammals endowed with widely different brains (Figures 2.3-2.8). In general, by considering the data obtained here and those collected from the current literature (Tables 2.1 and 2.2), the cytoskeletal protein DCX and Ki67 antigen appear to be well conserved through phylogeny and particularly resistant to fixation. As an extreme example, both antigens were detectable in internal positive controls of dolphin cerebella (external germinal layer at early postnatal stages) that were collected with a relatively long post-mortem delay (varying between 18 and 40 hours), and kept in fixative (4% buffered formalin) for months, even years (Parolisi et al., 2015,2017). Nevertheless, variation in the occurrence and/or quality of staining is frequently reported in comparative studies (references in Tables 2.1 and 2.2). Accordingly, while the detection of the two antigens was mostly successful in the present study, we also show some remarkably different results depending on the animal species investigated and the antibody employed. The most significant evidence emerging from our comparative analysis is the finding that DCX and Ki67 antigen can be successfully detected by

using different antibodies in different animal species, in spite of various types/degrees of fixation of the different brains studied (Figure 2.9). Hence, taking for granted that an appropriate tissue fixation is always required, the next step in comparative studies should be to find the right antibody(ies) tailored for the species under investigation. Our results indicate that the right antibodies can be successful in a relatively wide range of different types of fixation (Figure 2.9).

To a lesser extent, differences were observed depending on the brain region investigated. Regional neuroanatomy can affect the quality of fixation and staining. The SVZ, lining the ventricular cavity, can be easily reached by the fixative, especially when using a tissue immersion procedure; then SVZ and SGZ neurogenic sites are enriched in neuroblasts which are filled in with DCX, whereas some of the "immature" neurons of the cortical layer II can show a lower content of DCX protein, due to their state of immature cells (thus, in a more advanced maturational stage than neuroblasts; see for example La Rosa et al., 2019). This seems to be the case of cortical immature neurons in the brain of old animals (Xiong et al., 2008; Cai et al., 2009) and adult/old human individuals (Srikandarajah et al., 2009; Li et al., 2023).

In some cases, when dealing with comparison between species, the absence of staining can be simply due to interspecies differences. We now know that some DCX+ cell populations can be present or not in different species depending on the brain region or animal age. Here we show the example of the layer II cortical "immature" neurons, which are present in the neocortex of non-rodent mammals, being absent in mice (Figures 2.4 and 2.6). This can appear trivial, yet the wide use of laboratory rodents as an almost exclusive animal model for biomedical research has induced many scientists to expect that results coming from other animal species should replicate what previously shown in mice (Faykoo-Martinez et al., 2017; La Rosa et al., 2018). Comparative studies are revealing that remarkable differences can exist concerning the occurrence/rate/location of various forms of brain structural plasticity, especially regarding the different origin of the DCX+ "young" neurons (La Rosa et al., 2020; Ghibaudi and Bonfanti, 2022). While differences/similarities concerning stem cell-driven adult neurogenesis in rodents and humans is currently controversial (Moreno-Jimenez 2021; Sorrells et al., 2021), substantial interspecies differences are known to exist for the non-newly born, immature neurons of the cerebral cortex (Piumatti et al., 2018; La Rosa et al., 2020a; Ghibaudi and Bonfanti, 2022). For these reasons, a more complete mapping of structural plasticity processes across mammals, brain regions and ages is needed. This can be done addressing the whole issue of interspecies differences, tissue collection/fixation, and antibody specificity taking into account that in some cases results can be different from what has been established in rodents.



DCX	Antibody	Signal					No signal		
		Total	✻%	●%	Fix		Total	Fix	
	Santa Cruz *(goat)*	**19**	0	5	10	9	**4**	1	3
	Cell Signaling *(rabbit)*	**23**	61	0	11	12	**0**	0	0
	Abcam *(rabbit)*	**20**	55	10	10	10	**3**	1	2
	Santa Cruz *(mouse)*	**15**	53	0	10	5	**8**	1	7
	Millipore *(guinea pig)*	**12**	58	0	7	5	**11**	4	7

Ki67	Antibody	Signal					No signal		
		Total	✻%	●%	Fix		Total	Fix	
	BD Pharmingen *Mouse*	**8**	0	0	6	2	**4**	4	0
	Abcam *Rabbit*	**11**	64	9	6	5	**1**	1	0

Figure 2.9 - Schematic summary of the main results and conclusions. The three main variables considered in the study are reported in grey ovals on the left, including the animal species (characterized by widely different brain sizes), the type of fixation (perfusion: P, blue squares; immersion: I, green squares), and the commercial antibodies tested for DCX and Ki67 antigen. In the tables, the numbers of staining samples are reported (analysed in the four brain regions of the five mammalian species, each corresponding to two cryostat sections from three animals, treated with 5′ citrate), for a total of 115 staining samples for DCX (120 including the mouse neocortex lacking DCX+ cells), and 24 for Ki67. On the whole, a total of 89 staining samples for DCX made a positive signal, while 26 did not. For Ki67, there were 19 with positive staining, and 5 negative. The "No signal" column was considered to also include non-specific staining (i.e., non-successful staining). It is worth noting that the number of specimens fixed with perfusion or immersion (reported in blue and green squares, respectively) showing successful staining were almost equally distributed, especially for DCX. In the case of Ki67, we showed that some specimens fixed by immersion require longer time of citrate treatment to reveal staining (see Figure 2.5). The best-performing antibodies for each animal species are reported in tables below. Also in this case, the result is mostly independent from fixation (see for example the same outcome for DCX staining in perfused and immersed sheep brains), and rather linked to the association between animal species and antigen considered. In humans, only the Abcam antibody delivered a satisfactory result; on intraoperative specimens, the specificity of the staining was confirmed by RNAscope analysis (see Figure 2.8), also revealing a non-specific staining on astrocytes in the post-mortem tissue.

Detecting DCX in the human cerebral cortex

In humans, the study was restricted to the neocortex, a region of uttermost importance in cognition (Roth and Dicke, 2005), as well as in pathology (Vinters, 2015), that has been recently shown to host a population of "immature" DCX+ neurons (Coviello et al., 2022; Li et al., 2023), substantially absent in mice (La Rosa et al., 2020a). DCX detection in the human cerebral cortex, and more in general in the cortex of primates, has been controversial since long time. For instance, some authors reported a widespread occurrence of this cytoskeletal protein in most cortical layers of the macaque cerebral cortex (Bloch et al., 2011), while in a recent report, carried out on 9-10-year-old macaques with western blot analysis, immunocytochemistry and antigen adsorption controls (Liu et al., 2020), it was concluded that some antibodies might give non-specific staining, its presence resulting far more spatially restricted. For this reason, our analysis in the human cortex was extended to an RNAscope assay to check the visualization of RNA molecules in individual cells, thus revealing gene expression beyond the simple protein transcription. Our results substantially confirm the Liu et al. findings, since only one of the five antibodies tested by immunocytochemistry (the Abcam antibody) did produce a staining coexpressing with the RNAprobe (Figure 2.7 and 2.8) and was restricted to a small population of cortical layer II-III neurons.

Comparative antibody performance

One of the main problems in comparative research is the absence of systematic, comparable analyses among widely different mammals. Most studies on brain plasticity and neurogenesis were carried out on very standardized animal models, such as the laboratory rodents (Faykoo-Martinez et al., 2017; La Rosa and Bonfanti, 2018). As a result, the gap between our knowledge in rodents and humans remains widely unexplored, hampering the identification of real phylogenetic variations that might be useful for the correct interpretation of data obtained in mice and for a proper translation (Faykoo-Martinez et al., 2017; Brenowitz and Zakon, 2015). Nonetheless, most comparative studies consider either a single animal species or a small group of them, while analysing different species with the same method/approach in a study is rare (e.g., Patzke et al., 2015; van Dijk et al., 2016; La Rosa et al., 2020a). Finally, in studies using immunocytochemical markers on postmortem tissues, several antibodies raised against the same antigen are often reported in the Methods section without stating which of them gave a successful staining or in which condition/treatment.

A clear result emerging from the present comparative study is the lack of an "optimal" antibody for all the animal species considered. Overall, there is not an animal species among those considered (apart from mouse) in which all antibodies are working (Tables 2.6 and 2.7). On the other hand, the antigens were always detectable in each species with one or more antibodies (what can be considered as further indication of specific staining). Those giving the best results in each species are reported in Figure 2.9 below tables for DCX and Ki67. Especially for DCX, the association between animal species and successful staining appears to shift from mouse to humans, indicating that substantial differences must be considered along phylogeny. Of importance, most of the tested antibodies worked well in small-brained mammals whereas the possibility for successful, specific staining progressively decreased towards humans. As reported above, the type of fixation did not impact a lot in the whole picture, and most cases of lower rate of success due to tissue immersion procedure can be resolved with unmasking treatments.

CHAPTER 3

Age-related changes in layer II immature neurons of the murine piriform cortex

Specific questions

Does the number of piriform cortex cINs vary (or is maintained) during the mouse lifespan?

How the answer obtained from the first question fits (or not) with the temporal pattern of canonical adult neurogenesis?

3.1 Introduction

The mammalian brain is a highly complex machine needing stability (Frotscher, 1992), and hardly capable of renewing its neuronal elements, especially in mammals (Bonfanti, 2011; Bonfanti, 2013; Semënov, 2021). These features are less strict during youth, thanks to the existence of remarkable brain plasticity, yet they can become a problem with aging (Ben Abdallah et al., 2010; Grady, 2012; Snyder, 2019). Brain structural plasticity can occur in different forms, from synaptic changes (formation/elimination of synaptic contacts) to the genesis of new neurons, referred to as adult neurogenesis (Bond et al., 2015; Lim and Alvarez-Buylla, 2016; La Rosa et al., 2020a; Bonfanti and Charvet, 2021). It is well known that some forms of plasticity, especially the striking structural changes involving persistent neurogenesis, progressively decline as the animal age progresses (Ben Abdallah et al., 2010; Sanai et al., 2011; Snyder, 2019; Bonfanti and Charvet, 2021; Duque et al., 2022; Bond et al., 2022). In stem cell-driven adult neurogenesis, the decrease is due to stem cell depletion and/or transition to a quiescent state. In the last few years, a counterintuitive example of "neurogenesis without division" has been shown to exist (Gómez-Climent et al., 2008; Klempin et al., 2011; Bonfanti and Nacher, 2012; König et al., 2016; La Rosa et al., 2020a; Bonfanti and Seki, 2021). It consists of prenatally generated neurons (Gómez-Climent et al., 2008; Piumatti et al., 2018) which stop their maturation for long periods, being capable to restart it during adulthood to eventually integrate into neural circuits (Rotheneichner et al., 2018; Benedetti et al., 2020). These immature "standby" or "dormant" neurons are here referred to as cortical immature neurons (cINs). cINs are present in the cerebral cortex, a brain region not endowed with stem cell-driven neurogenesis and characterized by high cognitive functions. The recent demonstration that cINs are more abundant and more widely distributed in large-brained, gyrencephalic mammals with respect to rodents (La Rosa et al., 2020a), suggests that they represent an evolutionary choice to grant new neurons as a sort of brain reserve in animal species endowed with high computational/cognitive capabilities linked to expanded neocortex

(Palazzo et al., 2018; La Rosa et al., 2019; La Rosa et al., 2020a; Benedetti and Couillard-Despres, 2022).

Although the currently available information is still fragmentary, it appears that the number of layer II cINs varies across animal ages (Xiong et al., 2008; Cai et al., 2009; Piumatti et al., 2018; Rotheneichner et al., 2018; Ai et al., 2021; La Rosa et al., 2020a; Li et al., 2023). A general reduction in the extent of brain structural plasticity is known to affect all species, from non-mammalian vertebrates to mammals (Bonfanti, 2011), likely linked to lifespan and to a role in refinement of neural circuitries during brain growth and maturation (Snyder, 2019; Ben Abdallah et al., 2010; Encinas et al., 2011; Sanai et al., 2011; Luo et al., 2006; Semënov, 2019,2021; Bonfanti and Charvet, 2021; Bond et al., 2022). For mammalian adult neurogenesis, such a reduction has been carefully described (Luo et al., 2006; Ben Abdallah et al., 2010) and is considered to be associated with a decrease in cell division (Semënov, 2021), as a consequence of progressive exhaustion/depletion/quiescence of neural stem cells (Encinas et al., 2011; Urbán et al., 2019).

The question remains open whether also the cINs, which are not dependent on stem cell division, might follow a similar pattern of reduction or whether they might be considered as a reservoir of young, undifferentiated neurons in the adult/aging brain (König et al., 2016; La Rosa et al., 2019; Benedetti and Couillard-Despres, 2022). To answer this question, we investigated the amount of DCX^+ cINs in the mouse paleocortex at six different ages, from postnatal month 1 to the old age of 15 months (Figure 3.1A), at three anterior-posterior levels of the piriform cortex (Figure 3.1B). Cell density (linear density: number of cells/mm in the cortical layer II perimeter) and percentage of type 1 (small, unipolar/bipolar, highly immature neuronal precursors) and type 2 cINs (large, ramified, less immature "complex" cells; see Figure 3.3B') were considered (Figure 3.1C). Furthermore, we studied the coexpression of DCX with other markers for maturity/immaturity, such as the anti-adhesive form of the Neural Cell Adhesion Molecule N-CAM (PSA-NCAM; Bonfanti, 2006), whose expression is known to maintain immaturity in the piriform cortex cINs (Coviello et al., 2021), and NeuN, a marker of postmitotic neurons starting differentiation (Mullen et al., 1992), in search for possible variation at the different ages.

3.2 Materials and Methods

Animals and tissue processing

Animal experiments were performed in agreement 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", according to protocol number 785_2019PR, following the Institutional guidelines of IBBC/CNR and the approval of the Ethical Committee. Mice C57/BL6 (Charles River Laboratories, RRID:MGI:3696370) were housed in cages with corncob bedding in conventional animal facility. All animals were maintained under standard laboratory conditions with an artificial 12 hr light/dark cycle (lights on at 7:00 a.m. and off at 7:00 p.m.), temperature controller (22 ± 2°C) and humidity maintenance (55 ± 5%). Mice took a standard diet with access to food and water ad libitum. For our experiments, only male mice were used.

Animals aged 1, 3, 5, 7, 12, 15 months (4 animals for each age; Figure 3.1A and Table 3.1) were deeply anesthetized and perfused with a 4% solution of paraformaldehyde (PFA) dissolved in 0,01 M PBS. After cardiac perfusion the brains were isolated, post-fixed overnight in PFA at 4°C and equilibrated in 30 % sucrose. Brains were embedded in Tissue-Tek OCT (Sakura, Alphen an den Rijn, The Netherlands), cut by cryostat at −25 ◦C in 40 μm thick coronal, serial free-floating sections.

Table 3.1 Mouse specimens and ages used in this study

Species	**Source**	**Specimens**	**Age** (months)	**Fixation procedure**	**Fixative**	**PMI**
Mouse	(a)	4	1	Perfusion (IC)	4% PFA	None
			3			
			5			
			7			
			12			
			15			

IC, intra-cardiac. Source: (a) National Research Council, Rome, Italy

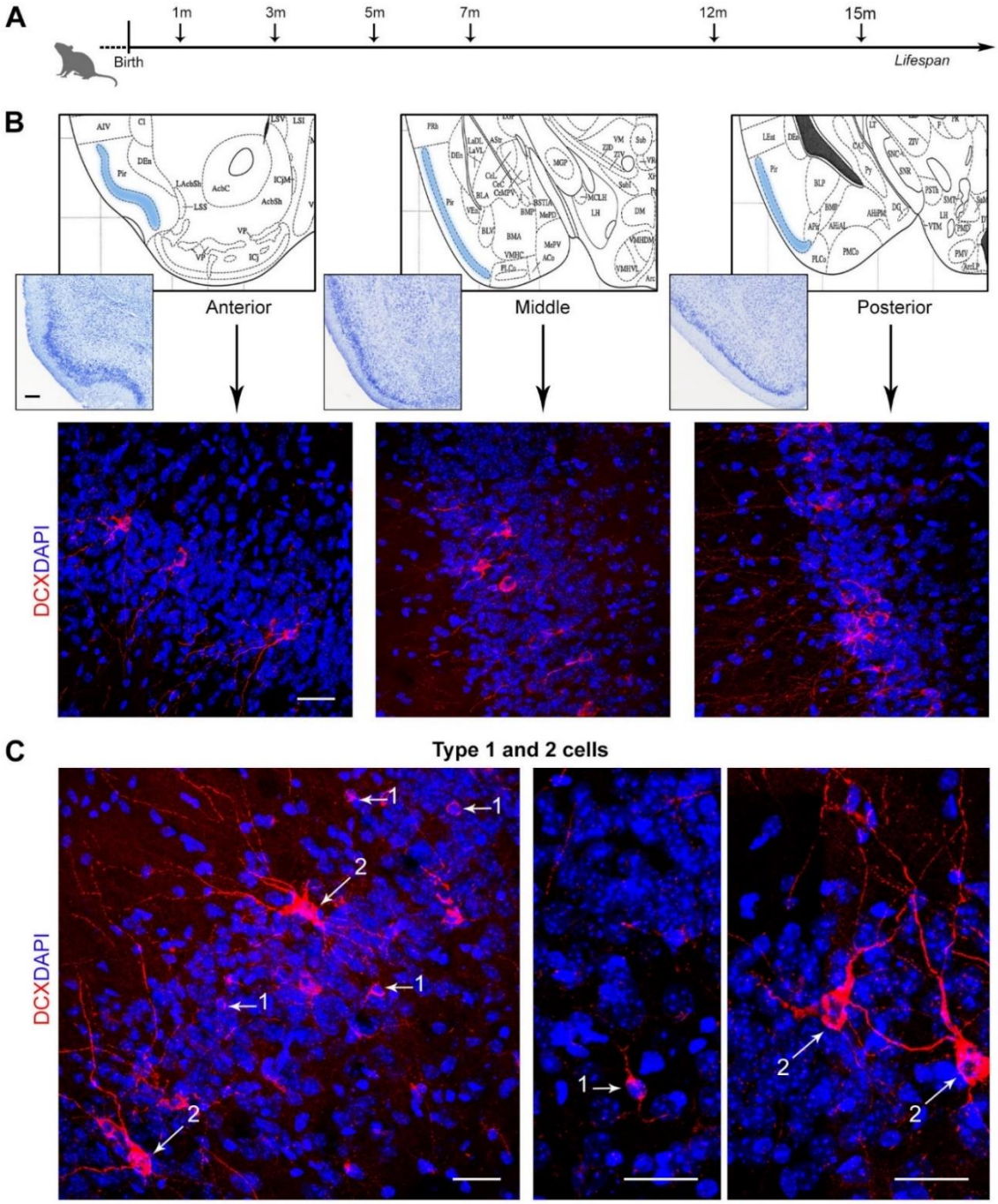


Figure 3.1 - Analysis of layer II cortical immature neurons (cINs) of the mouse piriform cortex at different ages. (A) Schematic representation of the age groups investigated in the present study. (b, top) Three anterior-posterior levels of the piriform cortex were considered (represented from coronal views of the Paxinos atlas of the mouse brain; Paxinos and Franklin, 2019): anterior, Interaural 4,98 – Bregma 1,18; middle, 2,22 – 1,58; posterior, 1,26 – 2,54. (B, bottom) Representative confocal images of each piriform cortex region (3 month-old mouse), stained for doublecortin (DCX, red) and counterstained with DAPI. (C) Confocal images of DCX^+ type 1 and type 2 cells, representing respectively small, unipolar/bipolar neuronal precursors and large, ramified "complex" cells. Scale bars: 200 µm (histology); 30 µm (immunofluorescence).

Immunofluorescence for doublecortin (DCX), polysialylated Neural Cell Adhesion Molecule (PSA-NCAM), NeuN, and NG2.

The entire anterior-to-posterior length of the piriform cortex, easily identifiable in toluidine blue-stained sections (Figure 3.1B), extended for approximately 5 mm at all ages; 120 coronal sections (40 µm thick) were considered in each animal (4,8 mm piriform cortex length; see Table 3.3). Three different series of slices were collected: rostral sections (approximately from Bregma + 2.46 to Bregma – 0.34, Paxinos and Franklin, 2019), middle sections (approximately from Bregma - 0.46 to Bregma – 2.18), and caudal sections (approximately from Bregma – 2.30 to Bregma – 2.80; Figure 3.1B). For immunofluorescence analysis, sections were then stained by using fluorescent methods. After permeabilization with 0.3% Triton X-100 in PBS, the sections were incubated with 3% normal donkey serum in PBS for 16–18 h with primary antibodies (Table 3.2): 1:800 goat polyclonal antibody against doublecortin (DCX) (Santa Cruz Biotechnology, Inc. Cat# sc-8066); 1:700 mouse anti-PSA-NCAM (Millipore, Bellerica, MA - MAB5324), 1:300 mouse anti-NeuN (1:300, Millipore, Bellerica, MA - MAB377), and 1:200 rabbit anti-NG2 (Neuron Glia antigen 2; Millipore, Bellerica, MA – AB5320). Secondary antibody used to visualize the antigen were 1:200 donkey anti-goat Cy2-conjugated (Jackson ImmunoResearch Cat# 705-225-147), Alexa 647-conjugated anti-mouse (1:400; Jackson ImmunoResearch, West Grove, PA - 715-605-151), and Alexa 488-conjugated anti-rabbit (1:400; Jackson ImmunoResearch, West Grove, PA - 711-545-152). Nuclei were observed incubating sections with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, KPL, Gaithersburg, Maryland USA) and mounted with MOWIOL 4–88 (Calbiochem, Lajolla,CA).

Table 3.2 Primary antibodies used in this study

Antigen	**Host**	**Type**	**Code**	**Raised against**	**Dilution**	**Source**
DCX	goat	polyclonal	SC8066	Epitope within the last 50 c-terminal amino acids	1:1000	Santa Cruz Biotechnology
PSA-NCAM	mouse	monoclonal	MAB5324	Viable Meningococcus group B (strain 355)	1:700	Sigma Aldrich
NeuN			MAB377	Purified cell nuclei from mouse brain	1:300	
NG2	rabbit	polyclonal	AB5320	Recombinant rat Chondroitin sulfate proteoglycan 4 containing amino acids 30 to 2225	1:200	

Image processing and cell quantification

Images were collected using a Nikon Eclipse 90i confocal microscope (Nikon, Melville, NY). All images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA) and ImageJ version 1.53t (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA).

DCX^+ cell quantification was performed on three immunofluorescence-stained sections/animal (one/each series - rostral, middle, and caudal - located in the middle of the series; 12 sections/age; total: 72 sections), in which 40x confocal fields were acquired in order to represent the entire ventral-to-dorsal length of the piriform cortex in each section (4 to 8 confocal fields/section, depending on the length and orientation of the piriform cortex, Figure 3.2; total number of confocal fields analyzed: 419). DCX^+ cell counting was performed directly by an experienced operator, using "Cell Counter" Plugin of ImageJ software. In each section, confocal field (objective: 40x; corresponding to 318,215 µm x 318,215 µm) were acquired along the layer II of the paleocortex (see above). Then, the total perimeter of paleocortical layer II was traced using the "Straight line" tool of ImageJ (Figure 3.2C) and all DCX^+ cells along its length were counted in each confocal acquisition (linear density = number of cells/mm). In the same sections, the morphology of cINs was evaluated and the number of type 1 and type 2 cells (identified on the basis of their cell soma size: < 9, type 1 cells; ≥ 9, type 2 cells) was counted using different markers selected from the Cell Counter toolbar in ImageJ software. The cell soma size (diameter) was obtained by evaluating the width orthogonal to main axis. Cells cut on the superior surface of the stack were not considered, to avoid overcounting.

Counting of DCX/PSA-NCAM, DCX/NeuN, and DCX/NG2 double staining was performed on the three-month-old and the fifteen-month-old animals. Three 40x confocal fields were considered along the ventral-to-dorsal extension of the piriform cortex, in each of two coronal sections/animal (corresponding to middle and caudal regions, which are the most enriched in DCX^+ cells); a total of 353 (DCX/PSA-NCAM) and 252 (DCX/NeuN) coexpressing cells were counted. Similarly, counting of DCX and NG2 possible coexpression was performed as for the above-mentioned markers (on three 40x confocal fields along the ventral-to-dorsal extension of the piriform cortex, in each of two coronal sections/animal corresponding to middle and caudal regions); a total of 215 DCX^+ cells were counted.

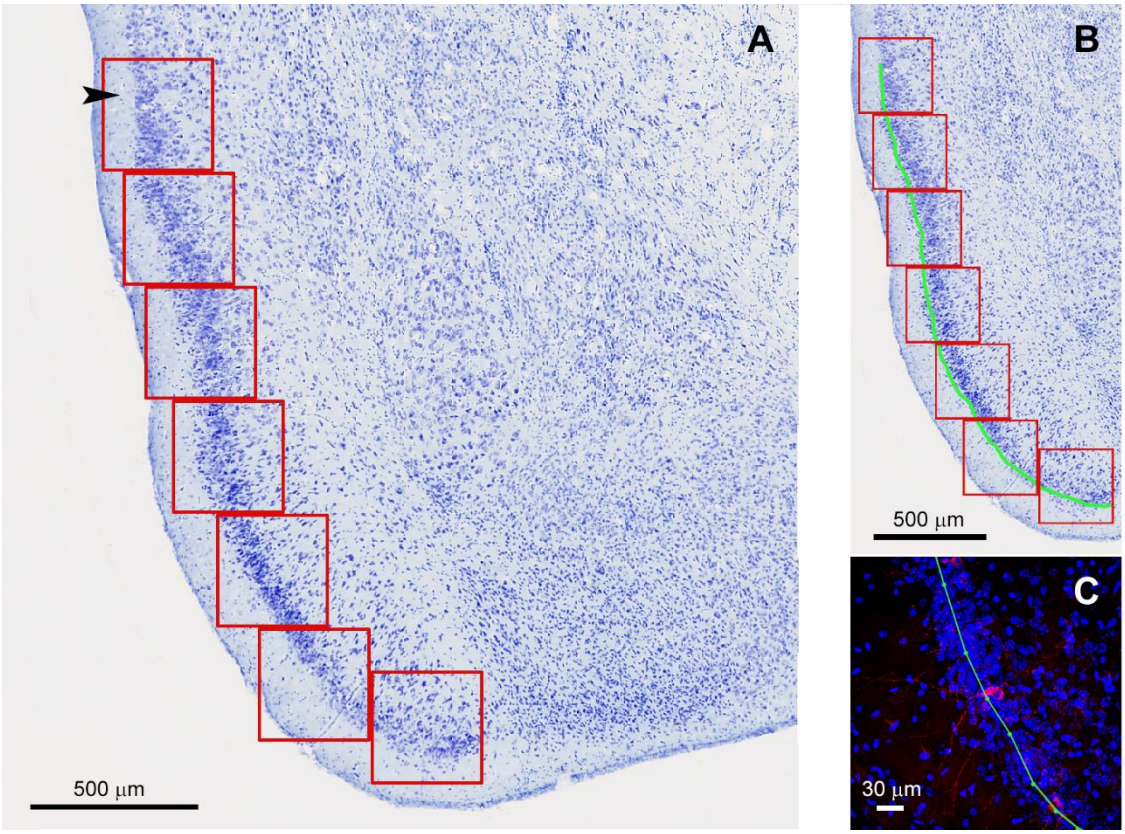


Figure 3.2 – Counting method in each coronal section of the piriform cortex (at anterior, middle, and posterior sites). (A) Adjacent confocal fields (318,215 x 318,215 µm wide; here represented in red on a toluidine blue-stained section of the middle piriform cortex at 7 months of age) were used to cover the entire ventral-dorsal extension of the piriform cortex (allocortex, but also recognizable at the confocal microscope by the high density of DAPI+ nuclei).
(B) The total length (ventral-to-dorsal) of the piriform cortex in each section was obtained by tracing the length of cortical layer II in each confocal field on imageJ (green line, C). Arrowhead: allocortex/isocortex transition.

Statistical analysis

All graphs and statistical analyses were performed using GraphPad Prism Software (San Diego California, USA) using different nonparametric tests: Mann-Whitney test, Kruskal-Wallis test with Dunn's multiple comparison post test and Two-way ANOVA with Bonferroni post-hoc test. $p<0.05$ was considered as statistically significant. Median was used as a central measure.

3.3 Results

Immunocytochemical detection and morphology of DCX$^+$ cells

Immunocytochemistry for DCX was carried out by using one of the best performing antibodies for this antigen in mice (Ghibaudi et al., 2023a). As expected, different populations of DCX$^+$ cells were detectable at typical locations previously described in the mouse brain, including the subventricular zone of the lateral ventricles (SVZ; Brown et al., 2003; see below and Figure 3.4A), the dentate gyrus of the hippocampus (subgranular zone, SGZ; Brown et al., 2003; see below and Figure 3.4A), and the layer II of the piriform cortex (Nacher et al., 2001; Bonfanti and Nacher, 2012; Luzzati et al., 2009; Figure 3.1). In the piriform cortex, two morphological types of DCX$^+$ cells were detectable in layer II at the limit with layer III, as previously described (Piumatti et al., 2018; La Rosa et al., 2020a): type 1 cells, characterized by a small cell soma (diameter 5-8 µm) and very simple cell process ramification (unipolar or bipolar), and type 2 cells, with larger cell soma (diameter 8-18 µm) and complex apical dendrites (Figures 3.1C and 3.3B'). Type 1 cells, usually far more abundant than type 2 (Piumatti et al., 2018; La Rosa et al., 2020a), are known to be highly immature elements, while type 2 cells do represent more mature and morphologically more complex forms (Rotheneichner et al., 2018; Benedetti et al., 2020; Benedetti and Couillard-Despres, 2022).

Counting of total DCX$^+$ cells, and type 1 / type 2 cells, in the piriform cortex

Total counting of layer II DCX$^+$ cells in the whole piriform cortex, and selective counting in each anterior-middle-posterior part were performed on confocal images using ImageJ software (Figure 3.3C). Linear density was used considering that cINs are arranged in a monolayer-like row within the piriform cortex (see La Rosa et al., 2020a). Animals of six different adult ages were analysed and a strong decrease in the linear density was observed comparing 1-month-old to 12- and 15-months-old mice (nonparametric Mann Whitney test, *$p < 0.05$; **$p < 0.01$, Figure 3.3C') and comparing 3-months-old animals to 15-months-old ones (nonparametric Mann Whitney test, $p<0.05$). Considering the percentage of decrease of DCX$^+$ cells in piriform cortex layer II starting from 1-month-old animals, a dramatic decrease was evident between 5 and 7-months (see Table 3.5). Then, we calculated the mean value of total DCX$^+$ cells in the ventral-to-dorsal extension of the piriform cortex in each section (average value considering rostral, middle, and caudal regions), and multiplied this value for the number of sections of the whole anterior-to-posterior extension (120), to estimate the total number of DCX$^+$ cells/hemisphere at each age. Such amount ranged approximately from 18.000 cells at 1-3 months to 1.700 cells at 15 months (Table 3.3).

To evaluate whether the distribution of DCX^+ immature neurons might be heterogeneous through the anterior-posterior extension of the piriform cortex, the linear density obtained in three regions (anterior, middle, posterior; Figure 3.1B) was considered. Two-way ANOVA with Bonferroni post-hoc tests found no big differences among these regions at all the ages considered, apart from a slightly higher amount in middle and caudal regions with respect to rostral (Figure 3.3C'').
Type 1/type 2 abundance (expressed in percentage) is represented in pie charts in Figure 3.3B. A decrease of large type 2 (complex) cells with respect to small type 1 (simple) cells was evident with age progression, reaching a minimum at 12 months (Figure 3.3B)

Table 3.3 - Estimation of total piriform cortex length (anterior-to-posterior) and total DCX+ cells in the piriform cortex (one hemisphere)

Age	**Piriform cortex coronal sections in a whole hemisphere**	**Section thickness**	**Total piriform cortex length (anterior-to-posterior)**	**Total DCX+ cells/section (mean from rostral, middle, and caudal sections)**	**Estimation of total DCX+ cells in piriform cortex (one hemisphere)**
1m	**120**	**40 µm**	**4,8 mm** (120x40: 4800 µm)	**151,75**	**18.210** (151,75x120)
3m				**152,25**	**18.270** (152,25x120)
5m				**90,25**	**10.830** (90,25x120)
7m				**37,5**	**4.500** (37,5x120)
12m				**23,5**	**2.820** (23,5x120)
15m				**14**	**1.680** (14x120)

Table 3.4 - Percentage of DCX+ cells over piriform cortex whole neuronal cell population

Age	**Estimation of total DCX+ cells (see Table S1)**	**Estimation of neuronal number in the three month-old mouse piriform cortex** (Srinivasan & Steven, 2017)	**% of DCX+ cells**
1m	18.210	532.617 cells	3%
3m	18.270		3%
5m	10.830		2%
7m	4.500		1%
12m	2.820		0,5%
15m	1.680		0,3%

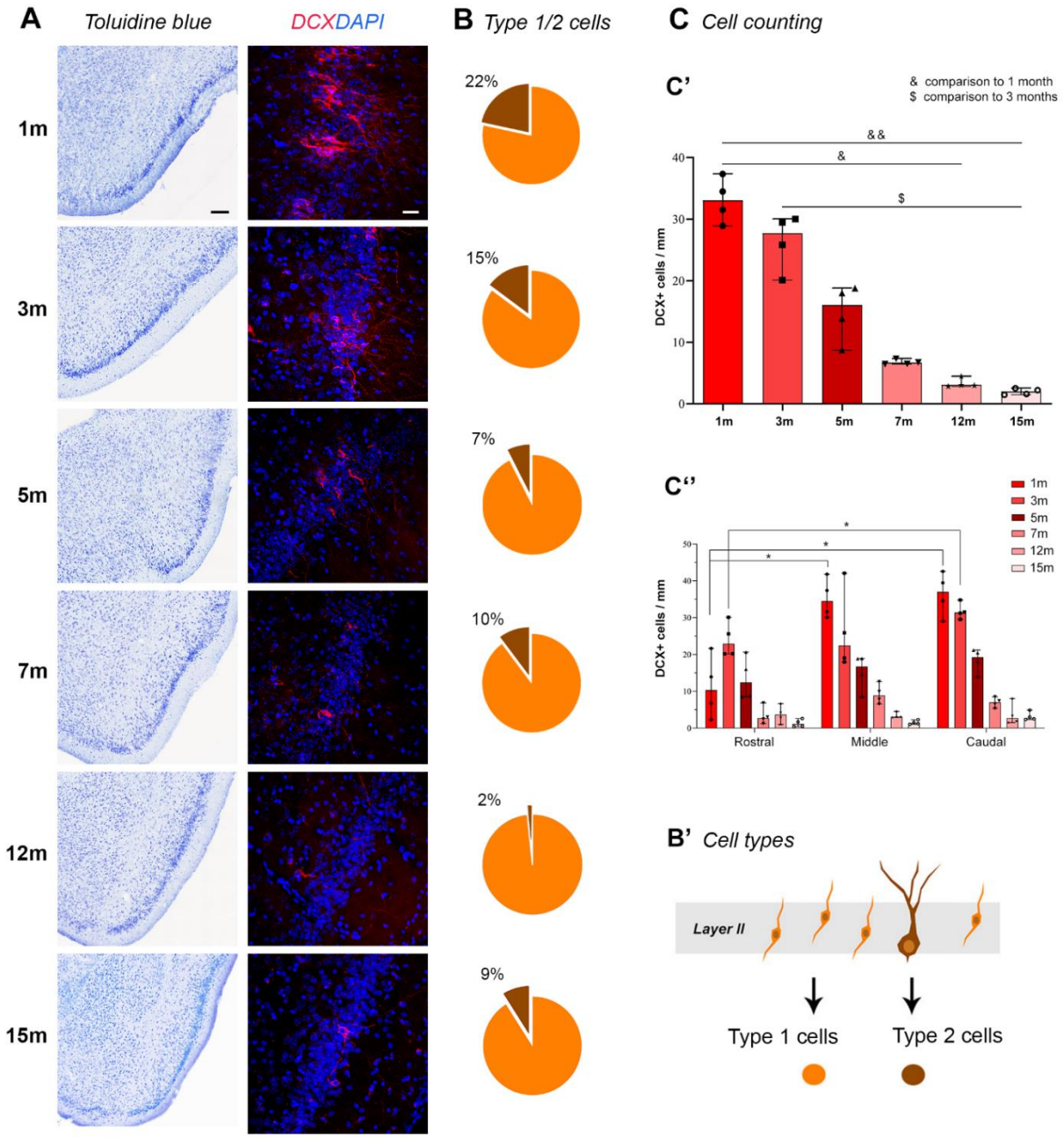


Figure 3.3. - Detection and counting of DCX$^+$ cells in the piriform cortex at different ages. (A) representative photographs from histological staining of the piriform cortex (caudal domain) and confocal images of DCX staining at all ages considered. Note the age-dependent progressive reduction in cINs. (B) Pie charts indicating the percentages of type 1 (yellow) and type 2 cells (brown); note the progressive decrease of type 2 "complex" cells with respect to the type 1, highly immature cells. (B') Schematic representation of the type 1 (yellow) and type 2 cells (brown) corresponding to different morphologies and different maturational stages of the cINs. (C) Results of the cell counting carried out on ImageJ software; (C') total DCX$^+$ cell counting; (C'') counting within the rostral, middle, and caudal domain of the piriform cortex. Scale bars: 200 µm (histology); 30 µm (immunofluorescence).

DCX/PSA-NCAM and DCX/NeuN coexpression at different ages

The cytoskeletal protein DCX and the membrane-bound, anti-adhesive molecule PSA-NCAM are known to be generally coexpressed in cortical immature neurons (Rotheneichner et al., 2018; Bonfanti and Seki, 2021; Coviello et al., 2021), both being downregulated with maturation (Rotheneichner et al., 2018). On the other hand, NeuN an RNA-binding protein expressed by postmitotic neurons that reached a high degree of differentiation (Mullen et al., 1992), can identify most types of mature neurons and is expressed by many type 2 cINs (Piumatti et al., 2018). On these bases, we explored whether such coexpression is stable in the mouse piriform cortex at different ages, by focusing on young (3 months old) and aged (15 months) animals (Figure 3.4). The analysis revealed coexpression of the two markers in immature cells of the piriform cortex, involving all morphological types: type 1 cells, type 2 cells and transitional forms (cells with soma size intermediate between type 1 and type 2 cells, and moderate dendritic ramification; Piumatti et al., 2018), at all the ages investigated (yellow arrowheads in Figure 3.4). In type 1 DCX^+ cells, PSA-NCAM was present on both cell soma and processes, while in type 2 cells and transitional forms PSA-NCAM was distributed more heterogeneously (being present either on both the cell soma and process, only on the cell soma or only on processes, possibly reflecting different maturational stages). Occasionally, some type 2 DCX^+ cells were devoid of PSA-NCAM, likely corresponding to more mature forms (having lost PSA-NCAM expression) (red arrowheads in Figure 3.4). Rare cells single-stained for PSA-NCAM, not expressing DCX were observed. These elements might be a scarce type of interneurons (Gómez-Climent et al., 2011; Figure 3.4A, green arrowheads). The coexpression of DCX and PSA-NCAM was always consistent and strong in the newly generated cells of the neurogenic sites (Figure 3.4A, top), as previously described (La Rosa et al., 2019).

In DCX/NeuN double staining, coexpression was frequently observed in type 2 cells with mature morphologies, both at 3 and 15 months (Figure 3.4A, bottom). Type 2 cells expressing only DCX were also detected both at 3 and 15 months (red arrowhead in Figure 3.4A, bottom), likely reflecting more immature forms which will express NeuN later (e.g., transitional forms coexpressing PSA-NCAM in the correspondent double staining). Counting of DCX/PSA-NCAM and DCX/NeuN double staining are reported as pie charts and percentages in Figure 3.4A. While coexpression with PSA-NCAM remained substantially unvaried from 3 to 15 months of age, coexpression with NeuN increased with age, more than doubling at 15 months.

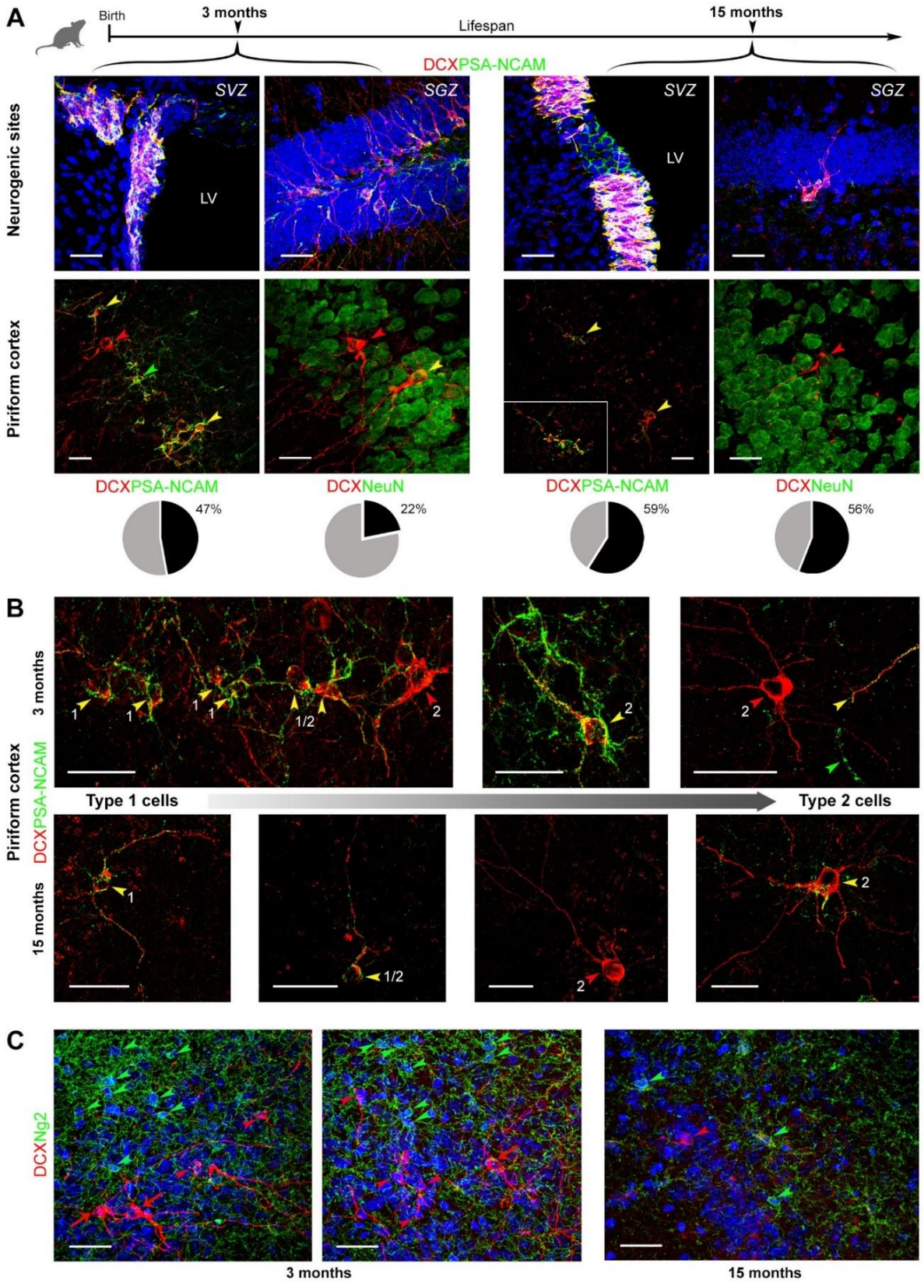

A
Birth
3 months
Lifespan
15 months
DCXPSA-NCAM
Neurogenic sites
SVZ
LV
SGZ
SVZ
LV
SGZ
Piriform cortex
DCXPSA-NCAM
47%
DCXNeuN
22%
DCXPSA-NCAM
59%
DCXNeuN
56%
B
3 months
Piriform cortex
DCXPSA-NCAM
Type 1 cells
Type 2 cells
15 months
C
DCXNg2
3 months
15 months

Figure 3.4 - Coexpression of DCX and markers for maturity/immaturity in cINs at different ages (3 and 15 months). A, coexpression of DCX and PSA-NCAM (top, in neurogenic sites; bottom left, in piriform cortex), and DCX and NeuN (bottom right, in piriform cortex) in young and old mice. Below each double staining image in the piriform cortex, pie charts indicate the percentage of marker coexpression (grey, single-stained DCX^+ cells; black, double-stained cells, with PSA-NCAM or NeuN respectively). B, coexpression of DCX and PSA-NCAM in cells with different morphology reminiscent of different degrees of maturation (the smaller, unipolar/bipolar type 1 and larger, ramified type 2 cells represent the extremes). Arrowheads: red, DCX^+ cells; green, $PSA\text{-}NCAM^+$ cells; yellow, cells coexpressing the two markers. Overall, all types of cells and combinations of staining are detectable at different ages, and in different maturational stages of the cells. The coexpression of markers for immaturity (DCX and PSA-NCAM) in cell soma and processes is frequent in type 1, small cells, even at 15 months (see higher magnification in the inset). Features linked to maturity (DCX staining without PSA-NCAM or DCX/NeuN coexpression are detectable in type 2, large cells. C, Double staining with anti-DCX and anti-NG2 antibodies. No coexpression of the two markers was ever found, neither in young (three-months-old) nor in old (fifteen-months-old) animals. Green arrowheads, $NG2^+$ cells; red arrowheads, DCX^+ type 1 neurons; red arrows, DCX^+ type 2 neurons. Scale bars: 30 μm.

Since previous studies using transgenic reporter mouse suggested that some oligodendrocyte precursors may express DCX (Rotheneichner et al., 2018), we checked for possible coexpression of DCX and the Neuron Glial antigen 2 (NG2) by performing double staining at 3 and 15 months of age. However, in our system, we did not find any cells coexpressing DCX and NG2 across the piriform cortex, at young or at old age (Figure 3.4C). In addition, all cells single stained either for DCX or for NG2 displayed clearly distinct morphologies, typical of type 1 and type 2 immature neurons and multipolar oligodendrocyte progenitor cells, respectively (Figure 3.4C). Thereby, the entire population of DCX^+ cells described and quantified in this study accounts for *bona fide* Type 1 and Type 2 cINs.

3.4 Conclusions and Discussion

The recent identification of DCX^+ cells in the piriform cortex layer II as non-newborn, "immature" or "dormant" neurons (history reviewed in Bonfanti and Seki, 2021), has introduced a possibility to place new, functional neurons in postnatal/adult neural circuits in the absence of stem cell division (Benedetti and Couillard-Despres, 2022), in parallel with the well-known process of stem cell-driven neurogenesis occurring in the brain neurogenic sites (SVZ and SGZ; Bond et al., 2015; Lim and Alvarez-Buylla, 2016; La Rosa et al., 2020b). Of interest, this "neurogenesis without division" does occur in the cerebral cortex, namely a region devoid of active stem cells/stem cell

niches, which is of uttermost importance for high-order cognitive functions (Roth and Dicke, 2005), and known to be affected by many forms of dementia/neurodegenerative pathologies (Vinters, 2015). With these premises, questions arise about how these "young" neurons are temporally distributed across the lifespan and whether they can represent a sort of "reservoir" for the adult/aging brain (König et al., 2016; La Rosa et al., 2019). Or, alternatively, whether their occurrence might follow a progressive reduction, similarly to what observed for other forms of plasticity (Bonfanti, 2011; Paredes et al., 2016b; Bonfanti and Charvet, 2021; Parolisi et al., 2018; Snyder, 2019). Here, by analyzing the number, morphology, and immunocytochemical features of cINs in the mouse piriform cortex from postnatal to old stages, we asked whether this cell population either undergoes a progressive decrease in number or remains stable, and how the pattern might be related to the well-known decrease of adult neurogenesis (Ben Abdallah et al., 2010).

Occurrence of layer II cortical "immature" neurons progressively decreases across lifespan

Our findings show that occurrence of DCX^+ cINs is very high during juvenile stages, declining at young-adult ages to reach lower levels with aging (Figure 3.3). Since these cINs do not depend on stem cell activity (they are produced during embryogenesis and then persist as immature, "dormant" elements in postnatal and adult ages, Rotheneichner et al., 2018; Benedetti et al., 2020; reviewed in La Rosa et al., 2019; Benedetti and Couillard-Despres, 2022), their progressive reduction can be explained by slow maturation and subsequent integration into the layer II of the piriform cortex, occurring at different ages and involving the loss of DCX expression (Benedetti and Couillard-Despres, 2022). Hence, unlike neurogenic processes, which produce less neurons with increasing ages (Luo et al., 2006; Ben Abdallah et al., 2010; Encinas et al., 2011; Figure 3.5B), the "disappearing" cINs do persist as mature neurons, which are no more visible by DCX immunocytochemistry, due to their maturation (schematic representation in Figure 3.5A'). Such "apparent disappearance", corresponding to maturation and integration, has been well demonstrated using a DCX-CreERT2/Flox-EGFP transgenic mouse, in which the immature cells can be visualized with green fluorescent protein, and followed through time after the loss of their DCX-expression (Zhang et al., 2010; Rotheneichner et al., 2018; Benedetti and Couillard-Despres, 2022). In this transgenic model, the vast majority of the cINs were found to accomplish their maturation through the animal life, the cells lost through aging being negligible (Rotheneichner et al., 2018). In other words, the apparent disappearance of the DCX^+ cells in the piriform cortex

observed at increasing ages, consists of an activation of previously inactive, "dormant" elements, thus maintaining the cell population. Here, we estimated the cell population of immature elements hosted in the piriform cortex in about 18.000 cells/hemisphere at 1 and 3 months of age, then progressively dropping with age, and leaving about 1.700 cells at 15 months (see Table 3.3 and 3.4). These numbers represent respectively 3% and 0,3% of the total amount of neurons in the piriform cortex (considered to be around 500.000 in the three-month-old mouse; Srinivasan and Stevens, 2017), which can potentially act as a reserve/enhancer for cortical plasticity. The fact that the number of sections considered in the counting was not high (three sections/animal) may explain the variability observed between animals, what could represent a limit of this study. Yet, the entire ventral-to-dorsal extension of the piriform cortex was considered in each section, and significant decrease of the number of DCX^+ cells was found at different ages, despite interindividual variability.

No big differences in the amount of cINs were observed by comparing the three anterior-posterior subregions of the piriform cortex at different ages, apart from a slight prevalence of DCX^+ cells in middle and caudal regions with respect to the rostral one (Figure 3.1B and 3.3C''), indicating that the total number of cINs at each age and their age-related decrease can be considered a general feature of the piriform cortex. We did such a distinction since a recent study showed that piriform cortex connectivity is spatially structured in triadic circuit motifs along its anterior-posterior axis (Chen et al., 2022). Single olfactory bulb neurons targeting a particular location along the anterior-posterior axis of piriform cortex also project to matched, functionally distinct, extra-piriform targets (e.g., anterior olfactory nucleus, amygdala, or lateral entorhinal cortex), and neurons in the piriform cortex complete the triad, also projecting to the same extra-piriform target (Chen et al., 2022). Our results suggest that plasticity potentially involving the cINs of all these circuits undergo the same age-related changes across the lifespan.

The general decrease observed here for the cINs show similarities with that of hippocampal adult neurogenesis (Ben Abdallah et al., 2010; see Figure 3.5A,B and related section, below) and, to a lesser extent, olfactory bulb/subventricular zone (Luo et al., 2006), thus suggesting that all these forms of plasticity play important roles mostly at juvenile ages.

Variation in cell types and marker coexpression linked to different maturational stages

The two cell morphologies indicated as type 1 and type 2 cells are known to represent the extremes of the maturational process of cINs during the period of DCX expression (Figures 3.1C and 3.5B').

Such maturation has been well characterized using the DCX-CreERT2/Flox-EGFP transgenic mouse, in which the immature, DCX-expressing cells can be followed across time with green fluorescent protein, until full maturation (and consequent loss of DCX staining) and functional integration into the layer II circuits (Rotheneichner et al., 2018; Benedetti et al., 2020). Of course, each brain section immunocytochemically stained with markers of maturity/immaturity (e.g., NeuN, DCX, PSA-NCAM) can include a gradient of maturational forms between the two extremes (see Figures 3.1 and 3.4B). In the temporal window of DCX expression, the small, unipolar-bipolar type 1 elements are the most immature DCX^+ neuronal precursors that, after progressive maturation increase their soma size and grow a ramified dendritic harborization, becoming the type 2 "complex cells" (Piumatti et al., 2018; Rotheneichner et al., 2018; La Rosa et al., 2020a; Benedetti and Couillard-Despres, 2022; Figure 3.4B). Here, we counted the cells falling into the category of type 1 and type 2 morphologies and their ratio was analysed at each age (Figure 3.3B). As expected (see Piumatti et al., 2018; La Rosa et al., 2020a), type 1 cells were prevalent at all the ages, yet the percentage of type 2 cells was decreasing with age (from 1 to 12 months), with a small increase at 15 months (Figure 3.3B). This pattern, along with that reported above for total DCX^+ cells, suggests that a small amount of highly immature (type 1) cells might represent a reservoir for plasticity in the adult/aging brain.

Two additional markers were used in association with DCX to assess the neuronal maturational stage of the cINs, as described in Piumatti et al. (2018) and Coviello et al. (2021) (Figure 3.4): PSA-NCAM, a low-adhesive form of N-CAM widely present in neurons during brain development and expressed by cells retaining plasticity during adulthood (Hoffman et al., 1983; Bonfanti, 2006; Bonfanti and Nacher, 2012); NeuN, expressed by postmitotic neurons starting differentiation (Mullen et al., 1992), which can identify most types of mature neurons (Gusel'nikova and Korzhevskiy, 2015), and is expressed in type 2 cINs (Piumatti et al., 2018; referred to as "complex cells" in Rotheneichner et al., 2018).

The analysis carried out on DCX/PSA-NCAM double staining revealed coexpression of the two markers in most immature cells of the piriform cortex, involving all morphological types: type 1 cells, type 2 cells and transitional forms, at all the ages investigated (Figure 3.4B). Only few DCX^+ type 2 cells were devoid of PSA-NCAM, likely corresponding to the most complex forms, close to the accomplishment of their full maturation (Figure 3.4B). Accordingly, it was recently demonstrated that PSA-NCAM depletion promotes maturation of cINs in rodent paleocortex (Coviello et al., 2021), thus indicating that loss of PSA-NCAM can precede loss of DCX.

Concerning the heterogeneous coexpression of PSA-NCAM and DCX in cell soma and/or cell processes, the heterogeneity may therefore reflect different stages of maturation. Counting of the

DCX/PSA-NCAM double-stained cells at 3 and 15 months of age revealed substantially similar values of coexpression (pie charts in Figure 3.4A), indicating that a population of highly plastic neurons is maintained through ages.

On the other hand, the increase in the number of DCX^+ cells coexpressing NeuN (more than doubling at 15 months; pie charts in Figure 3.4A) might be caused by a slowing down of the maturational process with increasing age, resulting in some cells being "blocked" in a "late immature" state. Under such assumption, one could also explain the decrease of type 2 cells with respect to type 1 cells in older animals. Namely, the age-related decrease in the percentage of type 2 cells would be justified by a dwindling, yet maintained, population of ever-immature type 1 cell (small reservoir of highly plastic cells), on one hand, and by some type 2 cells slowing down their maturation with age, on the other.

Comparison with adult hippocampal neurogenesis

The findings obtained in the present study on the cIN age-related variation in the piriform cortex appear quite similar to those reported for the neurogenic process of the hippocampal dentate gyrus in C57BL/6 mice from 1 to 9 months (Ben Abdallah et al., 2010), since both processes undergo substantial reduction with increasing age (Figure 3.5A,B). It is clear that cINs and hippocampal adult neurogenesis are different processes, which are not fully comparable; nevertheless, they both represent forms of neurogenesis, in terms of addition of new neurons. Moreover, they both show a progressive exhaustion of available cell types: of cells retaining immaturity in the former, and of actively dividing stem cells and their product in the latter (Figure 3.5A' and B'). Here we focus on the time period extension in which they are still detectable, because they show interesting differences beside a common trend of age-related decline. Our results reveal a strong decrease of cINs between 5 and 7-months, while data from Ben Abdallah et al. in the hippocampus show this decrease already starting from 1-3 months (Figure 3.5A,B and Table 3.5).

In addition, by considering the percentage of decrease in DCX^+ cells from their initial occurrence at 1 month to subsequent ages, it is only 19% in the piriform cortex against 97% in the hippocampus at 3 months, and 394% against 1094%, respectively, at 7 months. In the piriform cortex of older animals, cINs reach a value comparable to that of the hippocampus at 9 months, only at 15 months (Table 3.5), indicating a longer persistence of cINs with respect to cells involved in adult neurogenesis.

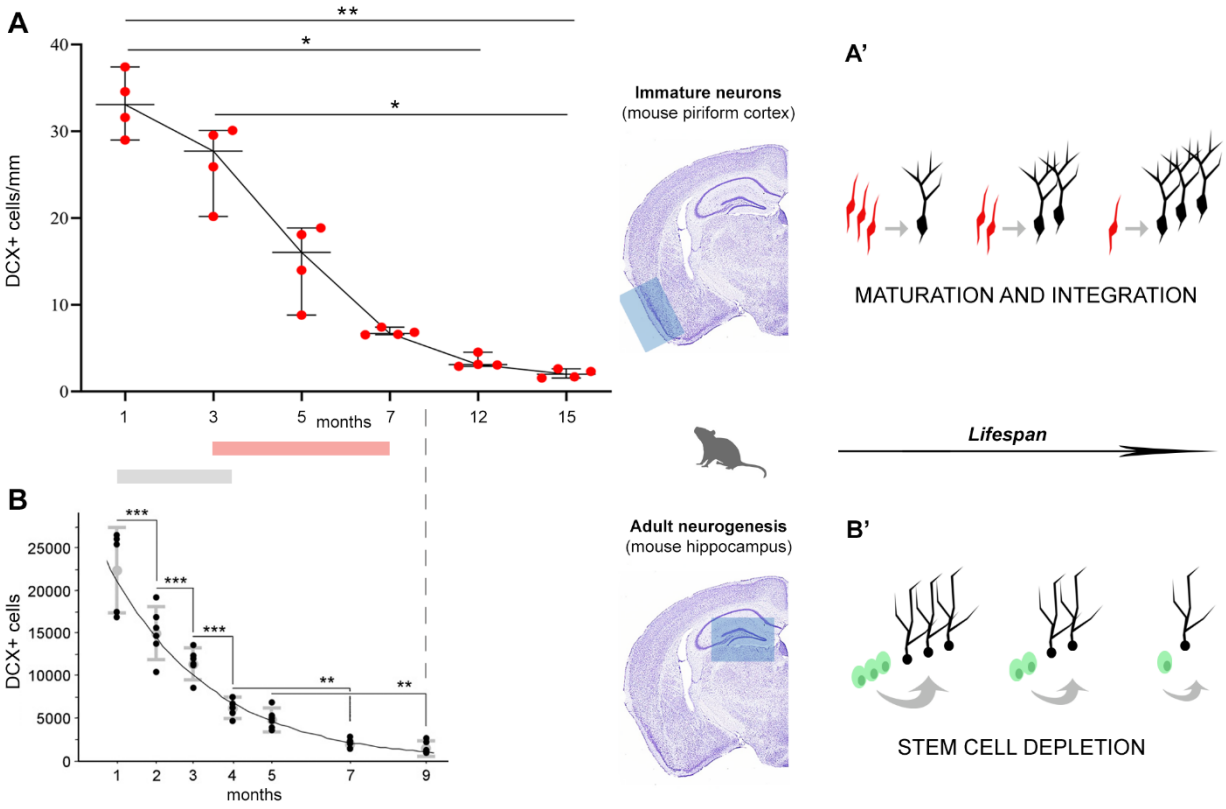


Figure 3.5 - Age-related decrease of different populations of DCX$^+$ cells in mice (A,B), and in comparison with other mammals (C). (A,B) Comparison between non-newly born, immature neurons of the piriform cortex (cINs; A) and newly born neurons of the hippocampal dentate gyrus (B). (A) Data obtained in the present study demonstrating the decrease of cINs during life (*$p < 0.05$; **$p < 0.01$); (B) panel reproducing Figure 5 in Ben Abdallah et al. (2010; modified with permission from Elsevier), showing the decrease with age of DCX$^+$ cell numbers in the mouse dentate gyrus (**$p < 0.01$; ***$p < 0.0001$). Grey and pink bars indicate the period in which the main drop occurs.
(A',B') Schematic representation of the two distinct processes, both leading to integration of new functional elements (black neurons), either starting from cINs (red, in the cortex; A') or from stem cell division (green, in the neurogenic sites; B'). Both processes undergo progressive age-dependent reduction, yet, for different reasons. The apparent decrease of immature neurons (a reduction in the number of DCX$^+$ cells) does correspond to a maintenance of the population through the maturing cells. On the other hand, in neurogenic processes the reduction really affects the production of new neurons, due to stem cell depletion/quiescence.

These data suggest that exhaustion of the cIN reservoir does occur in a more diluted way with respect to adult neurogenesis. As a matter of fact, in the 15-month-old mouse piriform cortex there are still 1.680 DCX$^+$ cells, namely a similar or higher amount with respect to those present in the hippocampus six months before (about 1.300 at 9 months; Ben Abdallah et al., 2010).

Table 3.5 - Percentages of DCX^+ cells age-related reduction of cortical immature neurons (present work; Figure 3.5A) and hippocampal adult neurogenesis (Ben Abdallah et al., 2010; see Figure 3.5B). m, months; bold, comparable ages.

Age comparison	Piriform cortex	Dentate gyrus
1m vs 2m	-	-50%
1m vs 3m	**-19%**	**-97%**
1m vs 4m	-	-261%
1m vs 5m	**-106%**	**-374%**
1m vs 7m	**-394%**	**-1094%**
1m vs 9m	-	-1568%
1m vs 12m	-972%	-
1m vs 15m	-1560%	-

Of course, our data and data collected from Ben Abdallah are heterogeneous. First of all, our quantification of DCX^+ cells was performed using a direct cell counting on ImageJ software instead of stereological methods with Stereoinvestigator, as done by Ben Abdallah et al. (2010). We chose this method because of the low number of cINs: counting with stereological methods are recommended when a considerably larger number of particles per individual are present (700-1000; Herculano-Houzel et al., 2015). Moreover, the ages considered in these two studies are only partially overlapping (Figure 3.5A,B): differences in cIN abundance is only significant when comparing 1-month-old to 12- and 15-months-old mice, hence no closer ages (e.g., 2, 4, 9 months) were considered; on the other side, hippocampal adult neurogenesis highlights significant differences starting from the first months of life, requiring the analysis of closer ages.

Overall, the decrease of cINs during young/adult stages can fit with the general view of structural plasticity as a process which is prevalent during youth, to allow the refinement of brain circuits on the basis of experience (in mouse, mainly linked to olfactory experience). Yet, a more moderate decrease of cINs with respect to adult neurogenesis, can leave a "small reserve" of young elements even during adult/senior stages. A fact that seems even more evident in large-brained mammals (see below).

Conclusions

Recent findings revealed that brain structural plasticity can occur in different forms varying with age, brain region, and species, thus stressing the importance of defining plasticity along temporal and spatial dimensions (Bonfanti and Charvet, 2021). Stem cell-driven adult neurogenesis is a

developmental process particularly useful during juvenile stages of the animal lifespan (Ben Abdallah et al., 2010; Sanai et al., 2011; Sorrells et al., 2018; Snyder, 2019; Bond et al., 2022). It supports the postnatal growth of the brain and the refinement of its neural circuits based on experience (Semënov, 2019; Seki, 2020; Kempermann et al., 2022; Bogado Lopes et al., 2023), but undergoes a progressive depletion of neural stem cells and/or entry in a quiescent state during adulthood (Encinas et al., 2011; Urbán et al., 2019). As such, it is different from the continuous cell renewal maintaining homeostasis in other stem cell systems of the body through the entire lifespan (Semënov, 2019).

Findings of the present study, along with recent comparative data obtained in large-brained, long-living mammals, do suggest that cINs can behave conversely, by maintaining their presence when the stem cell-driven neurogenesis has started to decline. In this process, interspecies differences are important: stem cell-driven neurogenesis and cINs appear to behave similarly in rodents but differently in large-brained species, suggesting a positive selection of cINs across evolution, as a preferential form of plasticity in large-brained mammals (Palazzo et al., 2018; La Rosa et al., 2020a; Ghibaudi and Bonfanti, 2022). Their independence from stem cell division can be an advantage, likely at the basis of the evolutionary choices that supported their widespread occurrence in the neocortex. Furthermore, in longevous species there might have been a selective pressure to develop a pool of long-lasting cINs in the expanded cortical areas, while the same pressure did not apply for the neurogenic niche pool.

In conclusion, considering the temporal dimension of plasticity in mammals, it is possible that the situation of stem cell-driven neurogenesis in young-adult rodents is more similar to human childhood (Bond et al., 2022), whereas for cortical immature neurons the process might be extended/diluted in time (La Rosa et al., 2019, 2020a; Li et al., 2023), providing a “small reserve of young neurons” even at advanced ages.

CHAPTER 4

Immature neurons in the subcortical regions of widely different mammals: the amygdala

Specific questions

Do INs occur in subcortical regions?
Does their relative amount undergo variation among widely different mammals?
Do subcortical IN amount vary at different animal ages?

Are there proliferating cell populations in the amygdala of the mammalian species considered?
Do the dividing nuclei in the amygdala coexpress DCX?
Which is their density and topographical distribution?
Are they oligodendrocyte progenitor cells?

Do the subcortical region volumes change in relation to brain size, species or age?
Is there any relationship with the occurrence/amount/distribution of INs?

4.1 Introduction

The search for young, undifferentiated neurons in the adult brain as a potential reservoir of plasticity, for lifelong neural circuit refinement and, possibly, repair after damage or disease, is one of the major goals of the Neurosciences (reviewed in Bao and Song, 2018; Bonfanti and Charvet, 2021; Ghibaudi and Bonfanti, 2022). The discovery of adult neurogenesis, a process in which new neurons are produced starting from actively dividing neural stem cells, has been a breakthrough in neurodevelopmental biology (Doetsch et al., 1999; Kempermann et al., 2004; Bond et al., 2015; Lim and Alvarez-Buylla, 2016). In parallel, a number of comparative studies carried out in various non-rodent mammalian species (including humans) started to indicate that stem cell-driven adult neurogenesis can remarkably vary among mammals as to its features, location, rate and persistence through age, with a general decrease/age-related dilution from small-brained to large-brained species (Barker et al., 2011; Sanai et al., 2011; Amrein, 2015; Paredes et al., 2016b; Parolisi et al., 2018), though some controversies still remain in humans (Sorrells et al., 2021; Moreno-Jiménez et al., 2021). In the last few years, a population of non-newly generated, "immature" neurons, which are generated prenatally, then remaining in an immature state for long time before full maturation and integration in the neural circuits, has been demonstrated to exist in the cerebral cortex (cortical immature neurons, cINs; Gomez-Climent et al., 2008; Luzzati et al.,

2009; Rotheneichner et al., 2018; Benedetti et al., 2020; reviewed in Bonfanti and Nacher, 2012; König et al., 2016; Bonfanti and Seki, 2021; see also Chapter 3). Hence, at least two "young" neuronal populations which share the same markers of immaturity in some phases of their life, despite their different features and origin, coexist in the adult brain at different locations: newly born cells in the neurogenic sites and "immature" or "dormant", pre-natally generated cells in the cerebral cortex (La Rosa et al., 2020b; Ghibaudi and Bonfanti, 2022; Benedetti and Couillard-Déspres, 2022; Figure 1.8). Across the years, several studies reported the existence of neurons showing similar features of immaturity (e.g., expression of the cytoskeletal protein doublecortin; DCX; Gleeson et al., 1999; Brown et al., 2003) in subcortical brain regions, including amygdala, claustrum, and white matter (Bernier et al., 2002; Fudge et al., 2012; Jhaveri et al., 2018; Chareyron et al., 2021; Marlatt et al., 2011; Marti-Mengual et al., 2013; Piumatti et al., 2018; Sorrells et al., 2019; Zhang et al., 2009). Most of these studies were carried out in non-rodent mammals, suggesting the existence of interspecies variations. Due to their marker expression, these cell populations were frequently considered as a possible product of non-canonical (parenchymal) neurogenesis (reviewed in Ghibaudi and Bonfanti, 2022), though definitive proof for their division was elusive (Bonfanti and Peretto, 2011; Feliciano et al., 2015; Piumatti et al., 2018; Sorrells et al., 2019; Chareyron et al., 2021). At present, a complex, fragmentary picture exists, because of different methods/approaches employed, different species considered, and various interpretations of the results from different researchers (Ghibaudi and Bonfanti, 2022). On the whole, it is suggested that "young" neurons can persist in a more widespread manner than previously thought, independently from to the highly- restricted stem cell niches (Feliciano et al., 2015; Ghibaudi and Bonfanti, 2022). Thus, unlike the "sharp" situation that appears to occur in neurogenic sites and cerebral cortex (newly born versus pre-natally generated cells, respectively), the distinction is far less evident at subcortical locations, raising multiple possibilities for the DCX+ cells: either newly generated neurons or "immature" neurons, or a mixed population of proliferating and non-proliferating (immature) cells. Accordingly, hereafter the DCX+ cells in the subcortical regions will be referred to as *immature-appearing neurons* (Figure 1.8 and 4.1).

A previous study carried out in the cerebral cortex with qualitative and quantitative analyses revealed that the cortical immature neurons (cINs), showed remarkable interspecies differences in widely different mammals, with increase in their occurrence, topographical extension, and amount (cell density) from small-brained to large-brained species (La Rosa et al., 2020a). Current data suggest that a similar interspecies variation might be present in subcortical regions (Piumatti et al., 2018; Sorrells et al., 2019).

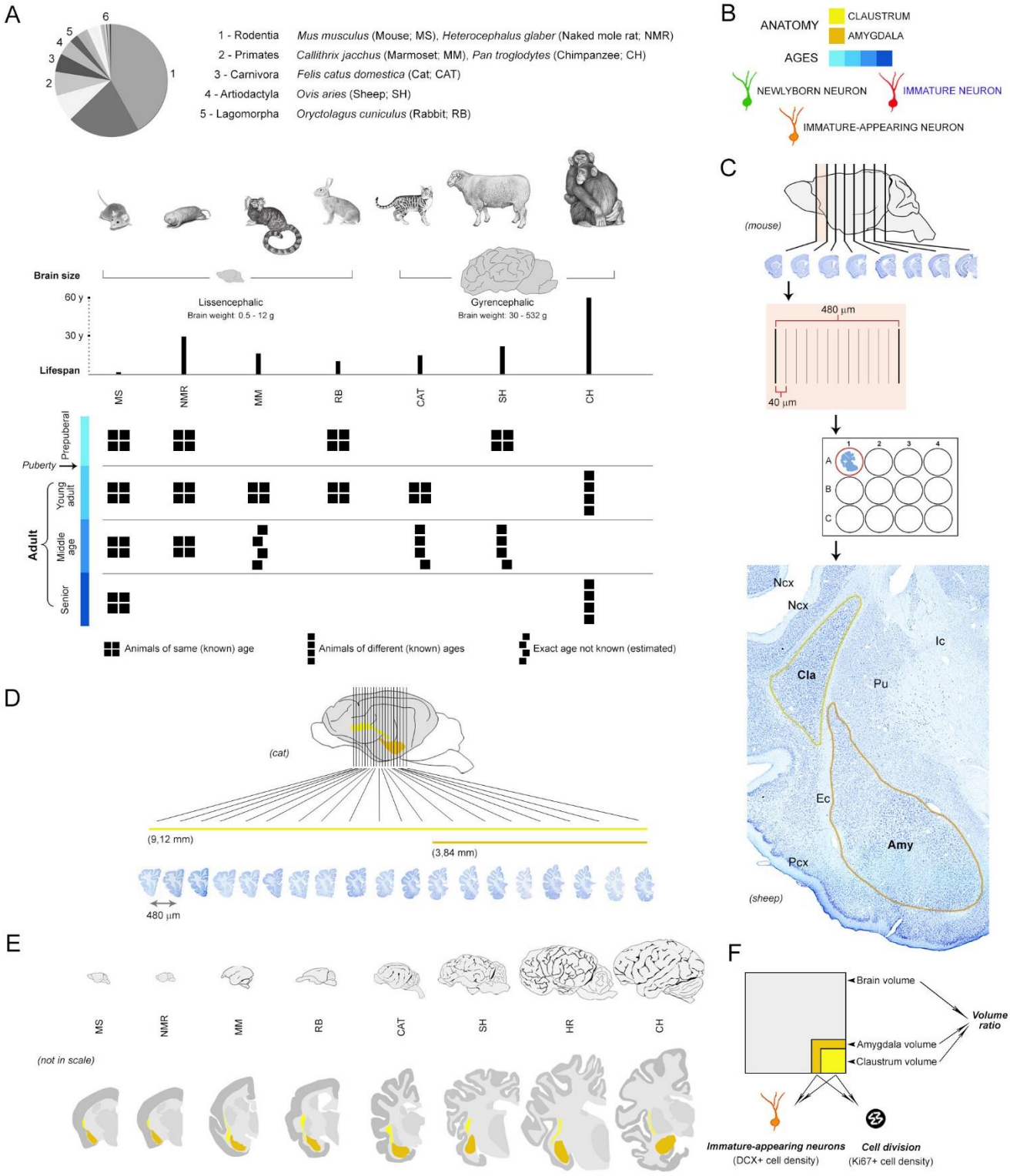

A
1 - Rodentia
2 - Primates
3 - Carnivora
4 - Artiodactyla
5 - Lagomorpha
Mus musculus (Mouse; MS), Heterocephalus glaber (Naked mole rat; NMR)
Callithrix jacchus (Marmoset; MM), Pan troglodytes (Chimpanzee; CH)
Felis catus domestica (Cat; CAT)
Ovis aries (Sheep; SH)
Oryctolagus cuniculus (Rabbit; RB)
Brain size
Lissencephalic
Brain weight: 0.5 - 12 g
Gyrencephalic
Brain weight: 30 - 532 g
60 y
30 y
Lifespan
MS
NMR
MM
RB
CAT
SH
CH
Prepuberal
Puberty
Young adult
Adult
Middle age
Senior
Animals of same (known) age
Animals of different (known) ages
Exact age not known (estimated)
B
ANATOMY
CLAUSTRUM
AMYGDALA
AGES
NEWLYBORN NEURON
IMMATURE NEURON
IMMATURE-APPEARING NEURON
C
(mouse)
480 µm
40 µm
Ncx
Ncx
Ic
Cla
Pu
Ec
Amy
Pcx
(sheep)
D
(cat)
(9,12 mm)
(3,84 mm)
480 µm
E
MS
NMR
MM
RB
CAT
SH
HR
CH
(not in scale)
F
Brain volume
Amygdala volume
Claustrum volume
Volume ratio
Immature-appearing neurons
(DCX+ cell density)
Cell division
(Ki67+ cell density)

Figure 4.1 - Sample of species, ages, and comparable brain regions (established on coronal levels) of the mammals used in this study. A, top, mammalian species and orders (scientific name, common name – used hereafter – and abbreviation) with special reference to their brain size and lifespan. A, bottom, different ages considered for each species; all groups are composed of four individuals. B, Color code. C, Brain tissue processing adopted to obtain comparable data in all species considered: serial coronal sections 40 µm thick of the entire hemisphere of each animal species were placed in multi-well plates in order to have a pace of 480 µm in each well to analyse the anterior-posterior extension of amygdala and claustrum, followed by staining of sections and segmentation of subcortical regions based on histology (final drawings represented in E). Different steps of the processing of mouse, sheep, and cat brain are represented here as an example. D, a different number of sections were obtained in each species depending on the brain size and consequent extension of the two subcortical regions. F, by using the comparable method described above, volumes of the amygdala, claustrum, and whole hemisphere were calculated in each species. Then, counting of DCX+ cells (immature-appearing neurons) and Ki67+, dividing cells were performed in the two subcortical regions.

Here we addressed three open points concerning the subcortical DCX+ cell populations: i) whether they are dividing or not; ii) whether they show phylogenetic variation in mammals; and iii) whether their occurrence/amount does vary with age. In the current thesis both claustrum and amygdala will be considered under a qualitative profile, while quantification will be performed on amygdala only (namely, the region in which the DCX+ cells appear most abundant). We addressed these issues at different ages in seven mammalian species that widely differ for their neuroanatomy (brain size, gyrencephaly, encephalization) and other life history and socioecological features (lifespan, habitat, food habit). DCX+ cells and dividing (Ki67+) cells were investigated as to their occurrence, morphological and phenotypic types, amount and topographical distribution, and results were put in a phylogenetic analysis perspective.

4.2 Materials and Methods

4.2.1 Brain tissues and legal permission

Most of the brains of the different animal species used in this study were collected from various institutions and tissue banks (Table 4.1), all provided by the necessary authorizations (see below description of each group of animals).

Callithrix jacchus (Marmoset) brains were provided by the University of Zurich, Switzerland and from the Department of Anthropology, George Washington University, Washington DC, USA. *Pan troglodytes* (Chimpanzee) come from the Department of Anthropology, George Washington

University, Washington DC, USA. *Felis catus domestica* (Cat) from the Mediterranean Marine Mammal Tissue Bank (MMMTB) of the University of Padova, Italy. *Ovis aries* (Sheep) both from MMMTB of the University of Padova, Italy and from the INRA research center, Nouzilly, France, *Heterocephalus glaber* (NMR) from the School of Biological & Chemical Sciences, Queen Mary University of London, London. *Mus musculus* (Mouse) brains from the Neuroscience Institute Cavalieri Ottolenghi (NICO) animal facility (Orbassano, Turin; Italy).

Adult marmoset (*C. jacchus*) brains were obtained from the Veterinary service of the University of Zurich. They were extracted post-mortem and fixed by immersion in 4% PFA with 15% picric acid (PA). Brains were separated into hemispheres and post-fixed for 24 h in PFA-PA. The exact ages of the animals were unknown; they were aged as adults (categorizable in the middle age life stage) by experienced veterinarian with the following criteria: the closure of the femoral and humeral epiphyseal plate, the body weight, the forearm length and sexual maturity (evidence of lactation or pregnancy in female and testis size in male; Gatome et al., 2010). Young adult marmoset from the George Washington University were provided by the Texas Biomedical Research Institute and the Trinity College (USA): they were collected at the time of necropsy following euthanasia. None of the brains included in this study showed gross abnormalities or pathology on veterinary inspection. All brains were immersion-fixed in 10% buffered formalin immediately at necropsy. After a 5-day period of fixation, brains were transferred into a 0.1 M phosphate buffered saline (PBS, pH 7.4) solution containing 0.1% sodium azide and stored at 4° C. All procedures were approved by the Institutional Animal Care and Use Committee at The George Washington University, and follow the ethical guidelines outlined by the American Society of Primatologists.

Chimpanzee (*P. troglodytes*) brains were provided by the National Chimpanzee Brain Resource (USA): they were collected post-mortem from Association of Zoos and Aquariums, maintained in accordance with each institution's animal care guidelines and fixed by immersion in 10% formalin (Schenker et al., 2010). After 10-14 days the brain was transferred in 0.1 M phosphate buffered saline (PBS) with 0.1% sodium azide solution and stored at 4°C. Experiments were conducted following the international guiding principles for biomedical research involving animals developed by the Council for International Organizations of Medical Sciences (CIOMS) and were also in compliance with the laws, regulations, and policies of the "Animal welfare assurance for humane care and use of laboratory animals," permit number A5761-01 approved by the Office of Laboratory Animal Welfare (OLAW) of the National Institutes of Health, USA (Huang et al. 2015).

Cat (*F. catus domestica*) and sheep (*O. aries*) brains were provided from University of Padova, Department of Comparative Biomedicine and Food Science. Tissue samples preserved in the MMMTB are distributed to qualified research centers worldwide, following a specific documented request, according to national and international regulations on protected species (CITES). These brains, obtained post-mortem, were fixed by immersion in 4% buffered formalin and kept in the fixative solution for a few months.

Sheep (*O. aries* - breed: Ile de France) were raised at the Institut National de la Recherche Agronomique (INRA; Nouzilly, Indre et Loire, France; ethical permissions are reported in Brus et al., 2013). In this study, 8 adult ewes (2, 8 and 10 year old) and 4 young ewes (4 month old) were involved. Young ewes were housed together in a large pen. Adult ewes were housed in an individual pen during pregnancy (2x1 m). They were perfused through both carotid arteries with 2 L of 1% sodium nitrite in phosphate buffer saline, followed by 4 L of ice-cold 4% paraformaldehyde solution in 0.1 M phosphate buffer at pH 7.4. The brains were then dissected out, cut into blocks and post-fixed in the same fixative for 48 h.

Naked mole rats (*H. glaber*): four prepuberal (2 months old), four young adult (2 years old), four middle age (10 years old) animals were intracardially perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 (courtesy of Dr. Chris G. Faulkes, London, UK). Brains were then postfixed overnight and sent to our lab.

Rabbit (*O. cuniculus*) brains come from a stock at the NICO animal facility used in a previous report (Ponti et al., 2008). Four young (3,5 months old) and four adult (3 years old) female New Zealand White were used. Animals were deeply anesthetized (ketamine 100 mg/kg - Ketavet, Bayern, Leverkusen, Germany - and xylazine 33 mg/ kg body weight - Rompun; Bayer, Milan, Italy) and perfused intracardially with a heparinized saline solution followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Brains were then postfixed for 6 hours. All experiments were in accordance with the European Communities Council Directive of 24 November 1986 (86-609 EU) and the Italian law for the care and use of experimental animals (DL.vo 116/92). All procedures carried out in this study were approved by the Italian Ministry of Health (8 october 2009) and the Bioethical Committee of the University of Turin (Crociara et al., 2013).

For mouse brain (*M. musculus*), C57BL/6 mice raised at the NICO facility (courtesy of Serena Bovetti and Charles River Laboratories, RRID:MGI:3696370) were used: 10 days, 3, 6 and 9 months old animals (P10-P270) were analysed. Transcardial perfusion was performed under

anesthesia (hypothermia up to P7; i.p. injection of a mixture of ketamine, 100 mg/kg, Ketavet, Bayern, Leverkusen, Germany; xylazine, 5 mg/kg; Rompun).

Table 4.1 - Animals used in the study of subcortical regions.

Species	Source	Specimens	Age	Life stage	Fixation	Fixative	PMI
Mouse	(d)	4	10 days	Prepuberal	Immersion	4% PFA	A few minutes
			3 months	Young adult	Perfusion (IC)		None
			6 months	Middle age			
			9 months	Aged			
Naked mole rat	(f)		2 months	Prepuberal	Immersion		A few minutes
			2 years	Young adult	Perfusion (IC)		None
			10 years	Middle age			
Marmoset	(h)		2.5 years	Young adult	Immersion	10% formalin	A few minutes
	(a)		Adult	Middle age		4% PAF 15% picric acid	1 hour
Rabbit	(d)		3 months	Prepuberal	Perfusion	4% PFA	None
			3 years	Middle age			
Cat	(b)		1.5 years	Young adult	Immersion	4% formalin	1 hour
			6-7 years	Middle age			
Sheep	(e)		4 months	Prepuberal	Perfusion (CA)	4% PFA	None
			8-10 years	Middle age	Immersion	10% formalin	20 minutes
Chimpanzee	(c)		19-27 years	Young adult	Immersion	10% formalin	14 hours
			40-48 years	Aged			

CA, carotid artery; IC, intra-cardiac. Source: (a) Institute of Anatomy – University of Zurich; (b) Department of Comparative Biomedicine and Food Science – University of Padova; (c) National Chimpanzee Brain Resource – USA; (d) Neuroscience Institute Cavalieri Ottolenghi (NICO); (e) INRA research center – Nouzilly, France; (f) School of Biological & Chemical Sciences, Queen Mary University of London, London; (g); George Washington University, Washington DC (h).

4.2.2 Tissue processing for histology and immunohistochemistry

The whole brain hemispheres were cut into coronal slices (1-2 cm thick). The slices were washed in a phosphate buffer (PB) 0.1 M solution, pH 7.4, for 24-72 hours (on the basis of brain size) and then cryoprotected in sucrose solutions of gradually increasing concentration up to 30% in PB 0.1 M. Then, they were frozen by immersion in liquid nitrogen-chilled isopentane at -80°C. Before sectioning, they were kept at -20°C for at least 5 hours (time depending on the basis of brain size) and then cut into 40 μm thick coronal sections using a cryostat. Free-floating sections were then collected and stored in cryoprotectant solution at -20 °C until staining.

Cryostat sections were used for both histological staining procedures (aimed at defining the overall neuroanatomy and boundaries of the regions of interest), and immunocytochemical detection of

specific markers (Table 4.2). Histological analyses were performed on Toluidine blue stained sections. For immunohistochemistry, two different protocols of indirect staining were used: peroxidase or immunofluorescence techniques. For 3,3'-diaminobenzidine (DAB) immunohistochemistry, free-floating sections were rinsed in PBS 0.01 M, pH 7.4. Antigen retrieval was performed using citric acid at 90°C for 5-45 minutes. After further washing in PBS 0.01 M, pH 7.4, the sections were immersed in appropriate blocking solution (1-3% Bovine Serum Albumin, 2% Normal Horse Serum, 0,2-2%Triton X-100 in 0.01M PBS, pH 7.4) for 90 minutes at RT. Following, sections were incubated with primary antibodies for 48 hours at 4°C (Table 4.2). After washing in PBS 0.01 M, pH 7.4, sections were incubated for 2 hours at RT with biotinylated secondary antibodies (Anti-goat, made in horse, 1:250; Anti-mouse made in horse, 1:250; Anti-rabbit made in horse, 1:250, Vector Laboratories). Then, sections were washed with PBS 0.01 M, pH 7.4 and incubated in avidin–biotin–peroxidase complex (Vectastain ABC Elite kit; Vector Laboratories, Burlingame, CA 94010) for 1 hour at RT. The reaction was detected with DAB, as chromogen, in TRIS-HCl 50 mM, pH 7.6, containing 0,025% hydrogen peroxide for few minutes and then washed in PBS 0.01 M, pH 7.4. Sections were counterstained with Toluidine blue staining, mounted with NeoMount Mountant (Sigma-Aldrich, 1090160500) and coverslipped.

For immunofluorescence staining, free-floating sections were rinsed in PBS 0.01 M, pH 7.4. Antigen retrieval was performed using citric acid at 90°C for 5-30 minutes. After further washes in PBS 0.01 M, pH 7.4, sections were immersed in appropriate blocking solution (1-3% Bovine Serum Albumin, 2% Normal Donkey Serum, 1-2% Triton X-100 in 0.01M PBS, pH 7.4) for 90 minutes at RT. Then the sections were incubated for 48 hours at 4°C with primary antibodies, and subsequently with appropriate solutions of secondary antibodies: Alexa 488-conjugated anti-mouse (1:400; Jackson ImmunoResearch, West Grove, PA), Alexa 488-conjugated anti-rabbit (1:400; Jackson ImmunoResearch, West Grove, PA), cyanine 3 (Cy3)-conjugated anti-goat (1:400; Jackson ImmunoResearch, West Grove, PA), Alexa 647-conjugated anti-mouse (1:400; Jackson ImmunoResearch, West Grove, PA), antibodies for 4 hours at RT. Immunostained sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1:1000, KPL, Gaithersburg, Maryland USA) and mounted with MOWIOL 4-88 (Calbiochem, Lajolla,CA).

Table 4.2 – Primary antibodies used in the study of subcortical regions.

Antigen	Host	Type	Code	Raised against	Dilution	Source
DCX	goat	polyclonal	SC8066	Epitope within the last 50 c-terminal amino acids	1:1000	Santa Cruz Biotechnology
Ki-67	rabbit		ab15580	Synthetic peptide	1:500	Abcam
	mouse	monoclonal	550609	Human Ki-67		BD Pharmingen
SOX10	rabbit	polyclonal	383A-7	NA	1:1000	Sigma Aldrich

4.2.3. Comparable neuroanatomy of the mammalian species considered

All mammalian brains evaluated here differ in terms of brain size, weight, gyrencephaly index and overall neuroanatomical organization. Together with these interspecies differences, some other variables were present, mostly due to tissue processing: brains of the same species can differ for tissue shrinkage or cryostat cutting (e.g., temperature during cutting might affect section thickness). Thus, each specimen for each animal species can show slightly different numbers of coronal sections covering the entire hemisphere (and consequently the amygdala). To counteract this variable, a fixed number of serial coronal sections covering both the whole hemisphere and the amygdala was considered in each species, acting as follows: the total number of sections obtained for each specimen/species were compared, and the specimen with the lower number of sections was used as a reference to reach the same number of sections in all specimens. To do this, sections at the very beginning and at the very end of the brain were excluded. The same was done for the amygdala, by excluding sections at the very beginning and very end of the structure. In this way, sections were all comparable among all specimen in a single species.

4.2.4. Cell counting and volume measurement

Volume measurement. To measure the volume of the amygdala, serial coronal sections at 480 μm interval from each other (1 section out of 12 total sections; see Table 4.3 for number of sections employed for the analysis) were collected covering the entire region in each species (3 specimen/species at each life stage).

Sections were stained with Toluidine blue to highlight the overall amygdala neuroanatomy (Figure 4.1). Then, the stained sections were scanned using Axio Scan (Zeiss; Oberkochen, Germany) and the amygdala areas for each specimen were measured using the “Contour Line” tool of ZEN Blue

Software (Zeiss; Oberkochen, Germany). Last, the volume was calculated in each specimen by using the following formula:

Average area of amygdala X Section Thickness (0,04 mm) X Number of sections covering the entire amygdala in a single specimen (Table 4.4)

Cell counting. To perform the quantification analysis of both DCX+ cells and Ki67+ nuclei, serial sections at 480 μm interval from each other were collected covering the whole amygdala in each species (4 specimen/species at each life stage; Figure 4.1). The counting was done using Neurolucida software (MicroBrightfield, Colchester, VT) as follows: a trained operator performed the tracing of the amygdala area in each section using the "Contour" tool of Neurolucida and all positive stained cells herein (objective: 20x magnification) were counted with the marker toolbar (Figure 4.2) to obtain the DCX+, or Ki67+ cells density/mm^2. Cells cut on the superior surface of the section were not considered, to avoid overcounting. In Table 4.3 are listed the numbers of sections considered for each species. A total of 68 brains and 2580 cryostat sections were analyzed.

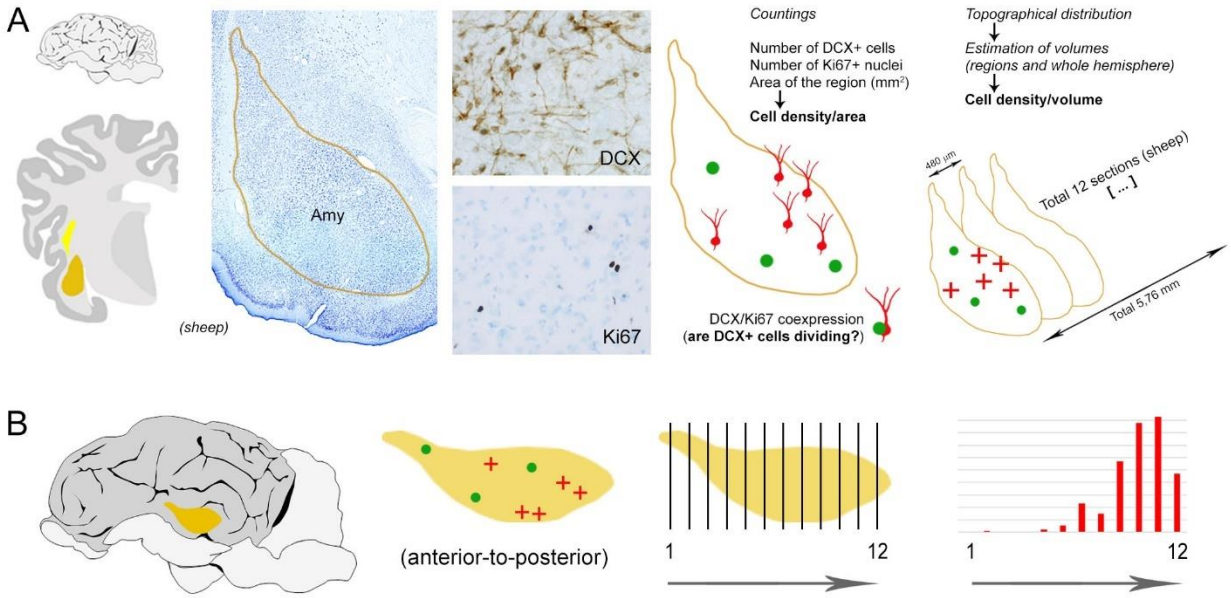


Figure 4.2 - Establishment of subcortical brain region areas for counting of DCX+ cells and Ki67+ nuclei. A, after segmentation of the amygdala (see Figure 4.1), DCX+ cells (immature-appearing neurons; red crosses) and Ki67+ nuclei (dividing cells; green dots) were counted on Neurolucida software, to obtain cell density/area of the entire region of interest. Bidimensional (on single coronal sections) and anterior-to-posterior topographical distribution of immunoreactive elements (B) was studied.

Table 4.3 - Number of coronal sections employed for volume/cell quantification analysis and estimation of total sections in the amygdala.

Species	Coronal sections cut in the entire amygdala	Coronal sections considered for volume analysis and cell counting in the amygdala
Mouse PP	36	3
Mouse YA		
Mouse MA		
Mouse AG		
NMR PP		
NMR YA		
NMR MA		
Marmoset YA	60	5
Marmoset MA		
Rabbit PP	84	7
Rabbit YA	96	8
Cat YA	96	8
Cat MA	84	7
Sheep PP	132	11
Sheep MA	180	15
Chimpanzee YA	144	12
Chimpanzee AG		

PP – Prepuberal; YA – Young Adult; MA – Middle Age; AG - Aged

4.2.5. Image acquisition and processing

Images were collected using a Nikon Eclipse 90i microscope (Nikon, Melville, NY) connected to a color CCD Camera, a Leica TCS SP5, Leica Microsystems, Wetzlar, Germany and a Nikon Eclipse 90i confocal microscope (Nikon, Melville, NY). For volume analysis, Zeiss Axio Scan.Z1 and Zen Blue software were used (Zeiss; Oberkochen, Germany). For quantitative analysis of DCX+ and Ki67+ cells, Neurolucida software (MicroBrightfield, Colchester, VT) was used.

All images were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA) and ImageJ version 1.50b (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA). Only general adjustments to color, contrast, and brightness were made.

4.2.6. Statistical analysis

All graphs and relative statistical analysis were performed using GraphPad Prism Software (San Diego California, USA) using different nonparametric tests: Mann-Whitney test, Kruskal-Wallis test with Dunn's multiple comparison post test. $p < 0.05$ was considered statistically significant. Median are used as data central measure.

4.3 Results

NOTE: the final aim of this part of the project is to provide a complete mapping of DCX+ cells and Ki67+ dividing cells in both claustrum and amygdala of 8 widely different mammalian species (including four young-adult and four middle-aged horses, in addition to the 68 brains analyzed here). Due to the high amount of time required to complete the analysis (results of this study will be published after the end of the three-year PhD), here will be presented and discussed the results concerning the amygdala in 7 mammals (only qualitative data obtained in the claustrum are reported).

To perform the study, the best performing antibodies to detect DCX and Ki67 antigen in all specimens of the different mammals were selected (Ghibaudi et al., 2023a; see Chapter 2). In spite of slight differences in postmortem interval, fixation procedure, and fixation time (Table 4.1), no substantial variation was observed in the quality and intensity of DCX or Ki67 staining (Figures 2.4 a,b, and 4.5).
In addition to subcortical regions, other brain regions from the same animals, including the forebrain SVZ, the hippocampal SGZ, the neocortex and the piriform cortex, where used as internal positive (neurogenic sites) or negative (cerebral cortex) controls (Figure 4.11A).

4.3.1 Analysis of amygdala volume

Given the great heterogeneity in terms of brain size and gyrencephaly across the mammalian species involved in this study, measurements of amygdala volume were performed, to understand if (and how) it changes in widely different mammals. The results are first presented at young-adult age (being all the species involved in this study comparable at this life stage), and then extended to other ages (prepuberal, middle age and aged). Later, the same will be reported for DCX+ and the Ki67+ cell analysis (see sections 4.3.2 and 4.3.3).

Analysis in young adult animals. As expected, by comparing all the widely different mammals involved in the study, the amygdala volume increases with increasing brain size (Figure 4.3), and a remarkable difference was present between mouse and chimpanzee (Kruskal-Wallis test, $p<0.05$; Figure 4.3 and Table 4.4). This information will be used for the volumetric cell density of immature neurons in the amygdala (see Chapter 5).

Comparison with other ages. To check whether the volume of amygdala changes during the animal lifespan, the analysis was extended to other available (comparable) ages: young adult and middle age in mouse, naked mole rat, marmoset and cat. The results indicate that while rodents did not show substantial changes in amygdala volume, both marmoset and cat show a slight increase in its volume with aging (not significantly; Mann-Whitney test, $p<0.05$; Figure 4.3; Table 4.4).

The same was later confirmed by extending the analysis to other species and ages: in all non-rodent species, a mild increase in the amygdala volume is present (except for the chimpanzee), though not significant (Mann-Whitney test, $p<0.05$; Figure 4.3; Table 4.4). In general, the amygdala volumes did not undergo significant changes with aging.

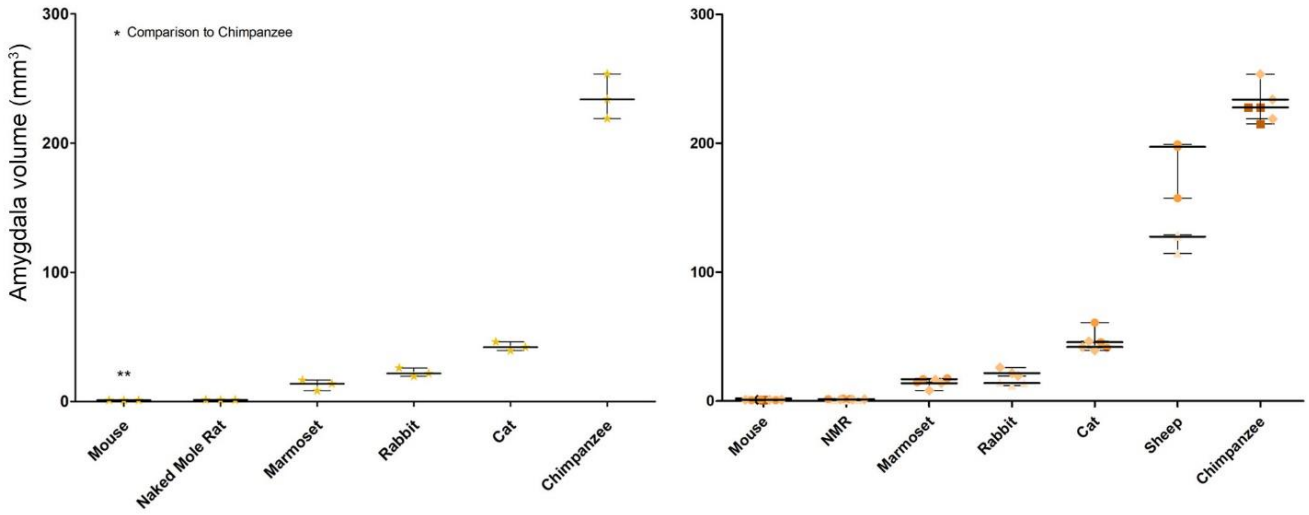


Figure 4.3 – Volume estimation of the amygdala in the mammalian species considered. Left, total volume at young-adult stage; the volume increases with increasing brain size. Right, comparison at different ages (see Figure 4.1 for ages considered in different species); note that increase of volume is higher when the prepuberal stage is considered (sheep), while substantial stabilization is observed during adulthood (and aging, e.g., chimpanzee).

Table 4.4 - Amygdala volumes (median from 3 specimen/species) in all species and ages analyzed

Species	**Age**	**Amygdala volume (mm^3)**
Mouse	P10	1,980
	3 months	0,974
	6 months	0,772
	9 months	0,920
Naked mole rat	2 months	0,708
	2 years	1,253
	10 years	1,610
Marmoset	2.5 years	13,809
	Adult	17,059
Rabbit	3 monhts	13,949
	3 years	21,716
Cat	1.5 years	42,075
	6-7 years	45,594
Sheep	4 months	127,625
	8-10 years	197,310
Chimpanzee	19-27 years	233,920
	40-48 years	227,809

4.3.1 Morphology, spatial organization, and topographical distribution of DCX+ cells in the amygdala of different mammals

DCX+ cells were found in the amygdala and claustrum of all the species studied, at all ages considered (Figures 4.4 and 4.5). Only in adult mice, DCX+ cells were occasionally found, being not present in all sections. This is consistent with the results of Alderman et al. in their preprint (2022), wherein DCX+ cells were investigated in the mouse amygdala through different ages (P7 to P60), showing a sharp decline through adolescence, reaching almost zero in the 2-month-old animals.

Morphology. At least three different morphological cell types were detectable (Figure 4.4A), which were reminiscent of those previously reported in the cerebral cortex (type 1, small cell soma, bipolar; type 2, large cell soma with ramified dendrites; La Rosa et al., 2020). Type 1 cells were very similar to the correspondent type in the cortex and present in both amygdala and claustrum (cell soma size range: 3-8 μm), while type 2 cells were split into two subtypes (Figure 4.4A): type 2a (bipolar, with a ramified dendrite, present in both subcortical regions; range 8-12 μm) and type 2b with a stellate morphology (larger soma and ramified dendrites with multipolar appearance, only found in the amygdala; range 12-18 μm).

This aspect strongly suggests that type 2b in the amygdala do correspond to the “complex cells” of the cortex, namely the maturing principal cells (pyramidal neurons) of the piriform cortex layer II (Rotheneichner et al., 2018; Benedetti et al., 2020; Benedetti and Couillard-Despres, 2022).

Indeed, type 2b cells resemble the Class I neurons, which represent the principal mature cell type of the amygdala (Sah et al., 2003; McDonald 1982), thus confirming that they may be in an advanced stage of maturation.

Spatial organization. In the amygdala of rodents (mouse, naked mole rat), the DCX+ cells were rare, not present in all sections, and in the form of isolated cells, while in other species their mutual organization also included clusters, networks and a combination of both (Figure 4.4B), with networks being the most abundant type of cells aggregation.

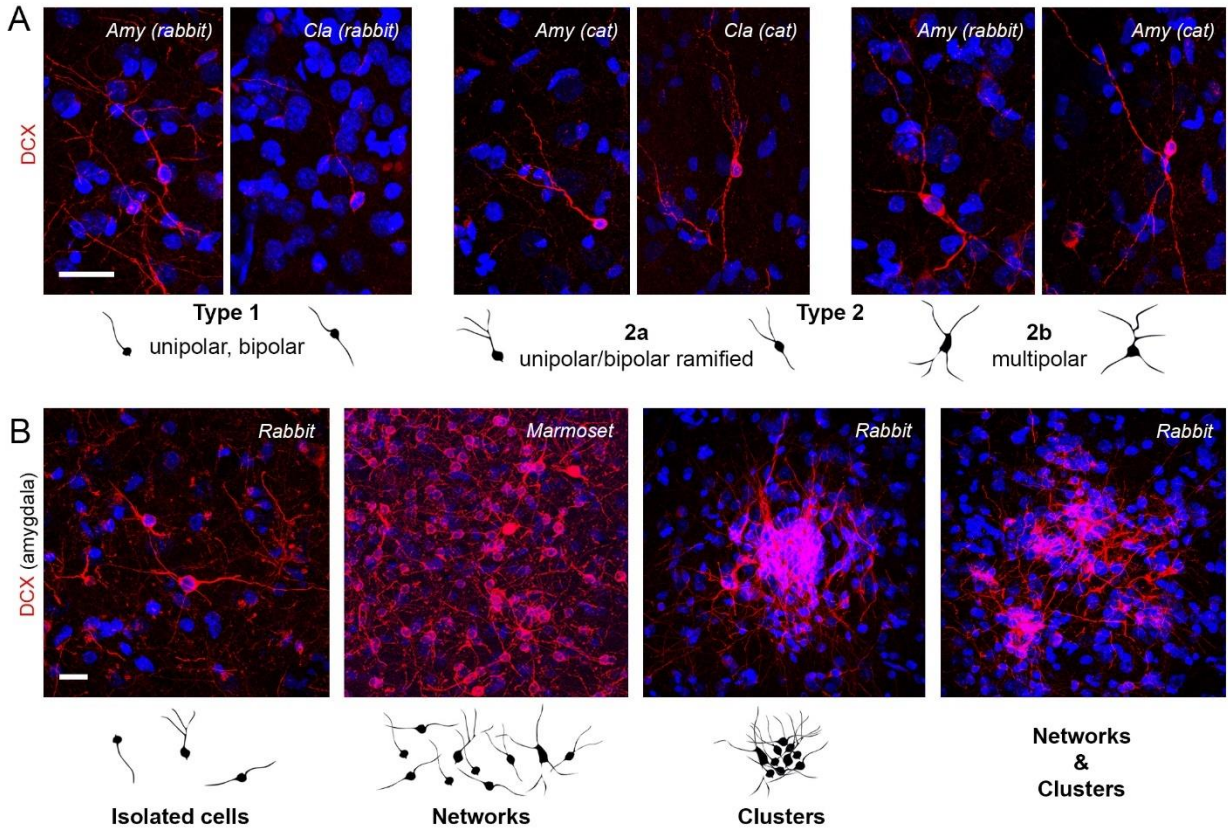


Figure 4.4 – Morphologically-recognizable cell types and overall distribution of the DCX+ neurons in the amygdala and claustrum of mammals. A, three main cell types were found: two characterized by simpler ramification (unipolar, bipolar) were detectable in both regions, and one more complex cell type (multipolar) only in the amygdala. B, at least three types of cell distribution/aggregation were observed, from isolated cells to tightly packed clusters. The prevalent distribution of the DCX+ cell populations was in the form of extended networks. Scale bars: 30µm.

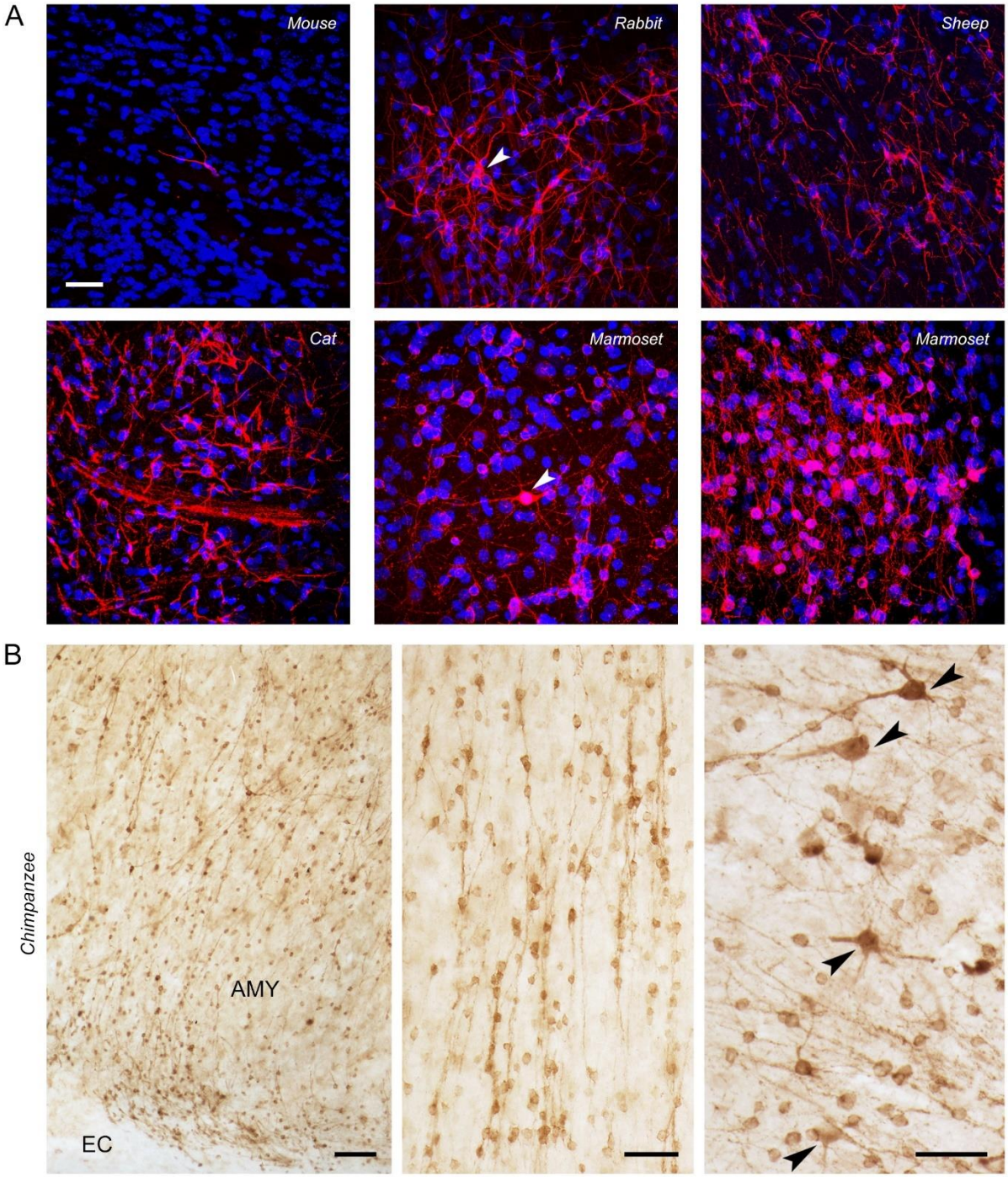


Figure 4.5 – Representative images of the occurrence and distribution of DCX+ cells in the amygdala of different mammals. A, confocal fields after immunofluorescence staining; B, light microscopy on avidin-biotin complex immunocytochemistry. AMY, amygdala; EC, external capsule; arrowheads, type 3 cells. Scale bars: A, 30µm; B, 100µm (left); 50µm (middle, right)

Topographical distribution. The possible widespread or prevalent location of the DCX+ cells within the amygdala was analyzed in the entire anterior-to posterior and medial-to-lateral/ventral-to-dorsal extension of the region, in all species considered (Figure 4.6 and 4.7). The anterior-to-posterior distribution was obtained after counting of the cells (see below), by generating histograms of the total number of cells for each of the coronal sections considered (Figure 4.7 left; red). Such distribution showed remarkable interspecies variation. Particularly in gyrencephalic species, the DCX+ cells were mainly grouped in different compartments: mostly posterior in cat and sheep, mostly in the middle in primates (Figure 4.6; Figure 4.7; left). To identify the different amygdala subnuclei, available atlas were used: Interactive Atlas Viewer – Allen Mouse Brain Atlas (mouse, naked mole rat); Paxinos et al., 2012 (marmoset); Scheider et al., 2018 (rabbit); Martì-Mengual et al., 2013; Krettek and Price, 1978 (cat); Meurisse et al., 2009 (sheep); Barger et al., 2012 (chimpanzee). In rodents, the scarce DCX+ cells detectable were mainly located in the basolateral and basomedial nuclei, occasionally invading the cortical nucleus of the amygdala. In non-rodent species, DCX+ cells were also extending in the lateral nucleus, however remaining prominent in both the basolateral and basomedial complexes. Interestingly, marmoset and chimpanzee not only have networks of DCX+ cells in these nuclei, but they were also remarkably present in the paralaminar nucleus, a region relatively expanded in primate amygdala compared to other species (DeCampo and Fudge, 2012). No DCX+ cells were ever found in the medial and central nuclei of the amygdala in both rodent and non-rodent species (Figure 4.7; middle).

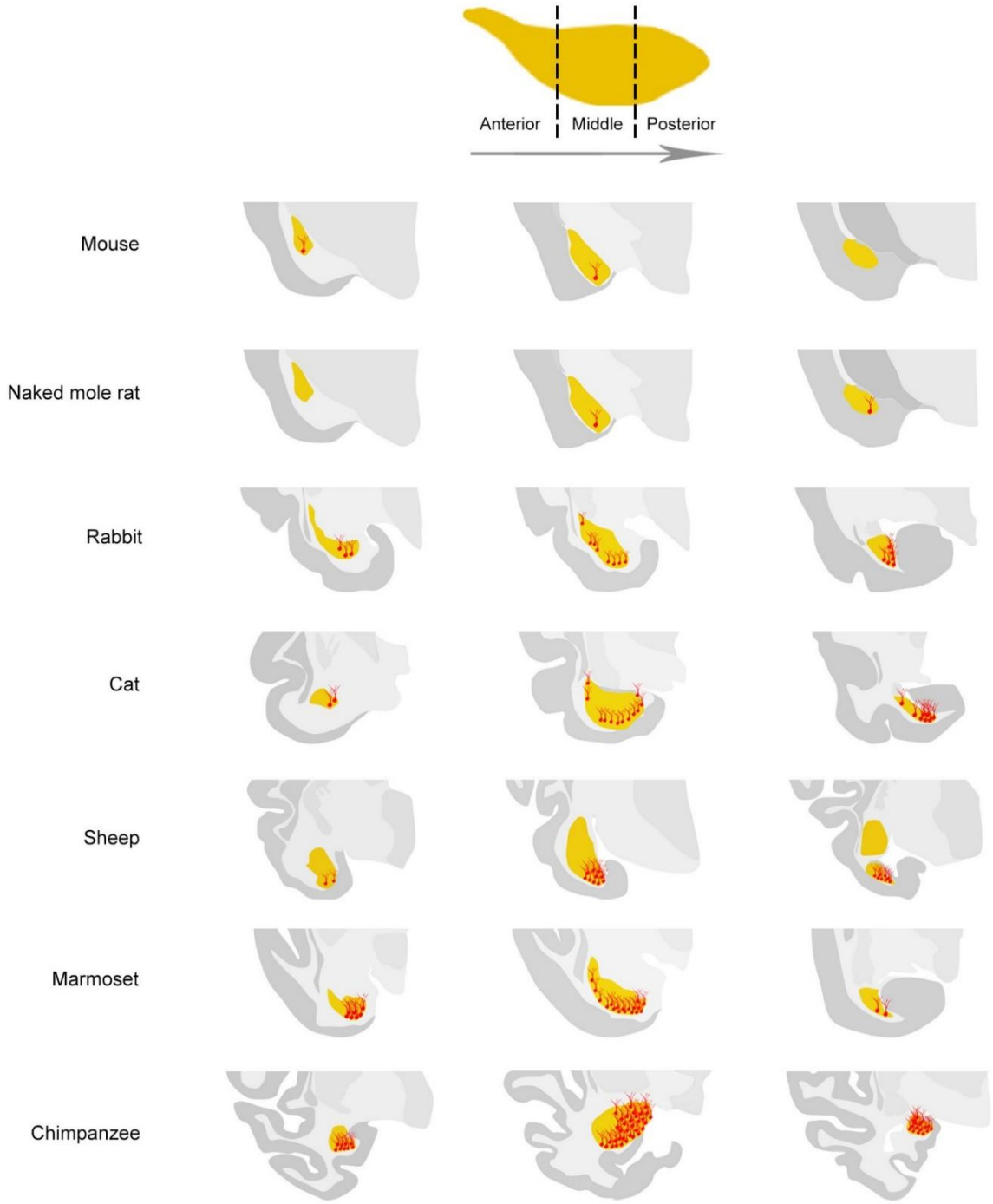


Figure 4.6 – Topographical distribution of the DCX+ cells (red neurons) in coronal sections of the amygdala (yellow) of different mammals. The anterior-to-posterior extension of the amygdala has been split in three representative parts: anterior, middle, and posterior. See Figure 4.7 for amygdalar nuclei subdivision and quantitative aspects.

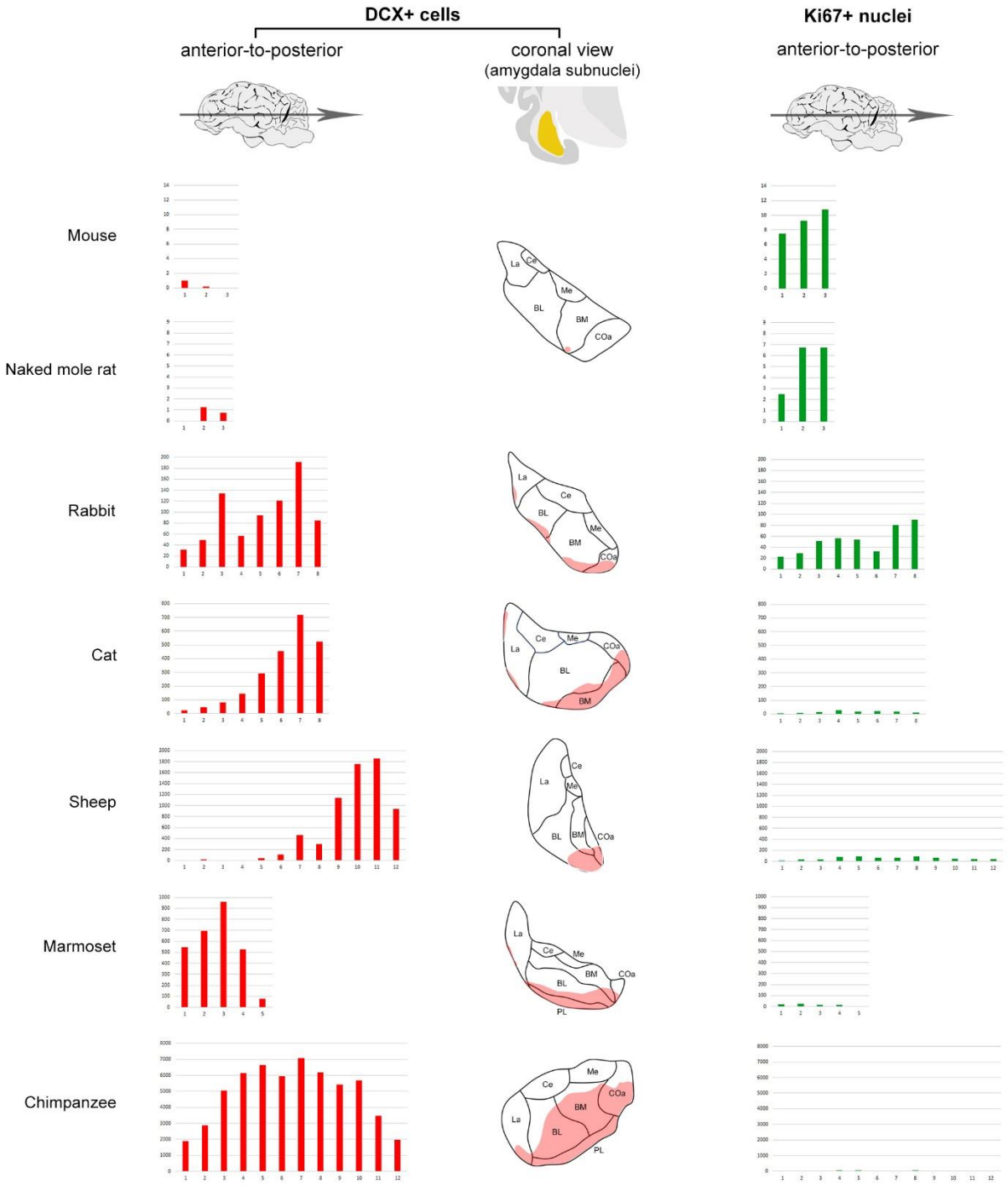


Figure 4.7 – Visual representation of the anterior-to-posterior topographical distribution of DCX+ cells (red) and dividing cells (Ki67+, green) in the amygdala of different mammals. Histograms report the total number of cells in each coronal section spaced 480 µm through the amygdala; see Figure 4.3). Note the highly heterogeneous anterior-posterior distribution of the DCX+ cells in gyrencephalic species while the proliferating cells appear quite uniform. In the middle, pink areas indicate the subnuclei of the amygdala hosting the DCX+ cells. La, lateral nucleus; Ce, central nucleus; Me, medial nucleus; BL basolateral nucleus; BM, basomedial nucleus; PL, paralaminar nucleus; COA, cortical amygdalar area. From: Allen Brain Atlas (mouse), Schenider et al., 2018 (rabbit), PMartì-Mengual et al., 2013, Krettek and Price, 1978 (cat), Meurisse et al., 2009 (sheep), Paxinos et al., 2012 (marmoset), Barger et al., 2012 (chimpanzee).

4.3.2 Quantitative analysis of DCX+ cells in the amygdala of different mammals

The number of DCX+ cells per mm^2 of amygdala area was assessed in serial coronal sections varying in number depending on the animal species (because of the different length of the structure), and at different ages (Figure 4.1 and Table 4.3). Density/area was measured to have a comparable value in all species, in spite of their heterogeneous arrangement within the amygdala (see Figures 4.6 and 4.7). The total count of DCX+ cells was performed in each coronal section with Neurolucida software, to establish also the exact anatomical location of the DCX+ cells in amygdala in each species (Figure 4.2). Given the non-homogeneous distribution of DCX+ cells in the amygdala, a stereological method for quantification was excluded in favor of a direct cell counting, both to avoid an incorrect quantification of cells and to make the analysis as close as possible (and thus fully comparable) to that previously performed in the cerebral cortex (La Rosa et al., 2020a).

Analysis in young-adult animals. At young-adult stage, all non-rodent species (marmoset, rabbit, cat, chimpanzee; sheep was not included in this panel since at present we have three out of four brains at this age) showed a higher density of DCX+ cells in the amygdala with respect to rodents (mouse, naked mole rat). Among non-rodent species, both primates (marmoset and chimpanzee) showed the highest density of DCX+ cells, the chimpanzees showing a statistically significant difference with respect to mouse and naked mole rat (nonparametric Kruskal-Wallis test, $p<0.05$; Figure 4.8A, left and Table 4.5).

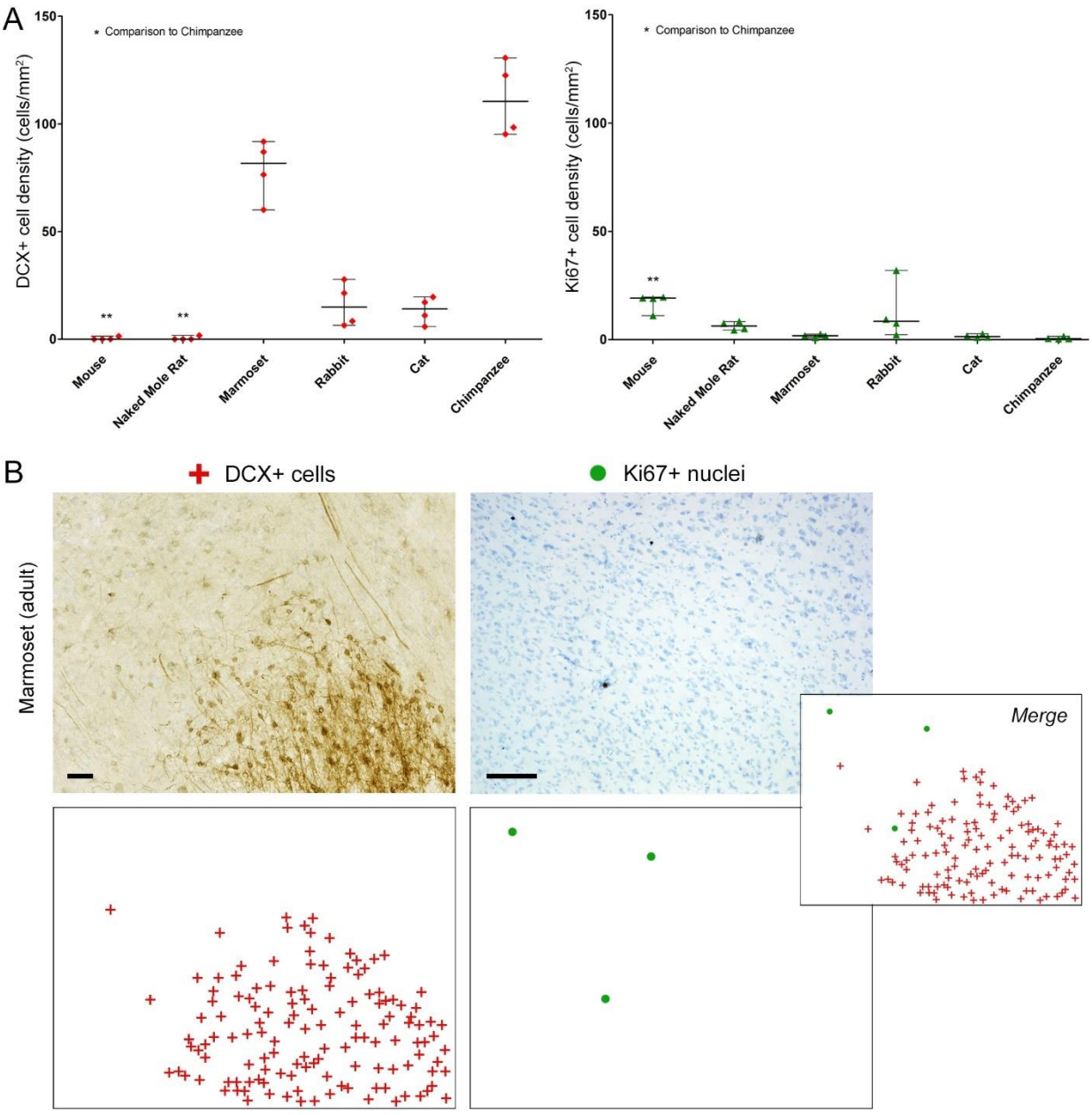


Figure 4.8 – A, densities/area of DCX+ (left) and Ki67+ cells (right) in young-adult animal groups. Counting of DCX+ cells revealed phylogenetic variation for the immature-appearing neurons, especially in primates with respect to rodents; on the other hand, counting of Ki-67+ nuclei revealed a substantially stable rate of cell division, apart from mouse Vs. chimpanzee, the latter being very low. The very low rate of cell division found in the primates, in contrast with their high densities of DCX+ cells (note the contrast between the density of dividing nuclei and DCX+ cells in chimpanzee), does indicate that these cells are far from being newly born neurons, rather non-dividing immature neurons. Further proof for such a nature consists of the very different topographical distribution of the DCX+ cells and the Ki67+ nuclei (B): in corresponding fields of adjacent coronal sections (here is an example in the amygdala of adult marmoset) the DCX+ cells (red +) are mostly grouped to form networks within compartments of the amygdala while the dividing cells (green dots) are uniformly scattered in the whole area. Scale bar: 100µm.

Comparison with other ages. To check whether the amount of DCX+ cells can change during the animal lifespan, the analysis was extended to other available, comparable ages: young adult and middle age in mouse, naked mole rat, marmoset and cat, and young-adult and aged in mouse and chimpanzees (Figure 4.1). In rodents, the amount of DCX+ cells remained low at both ages, while in marmoset a decrease is present between young-adult and middle-aged specimens. A slight increase in density of DCX+ cells is present in middle-aged cats compared to young-adults, yet these changes are not statistically significant, thus indicating a substantial stabilization (nonparametric Mann-Whitney test, $p<0.05$; Figure 4.9B; left). Comparing mouse and chimpanzees, despite remarkable interspecies differences in cell density, the DCX+ cell populations remain substantially unchanged at different life stages, even between widely distant ages (e.g., young-adult and aged; Figure 4.10; top and Table 4.5).

The same analysis was extended to the other species at all ages available (to show an overview of the DCX+ cells density through each animal group lifespan, the life stage being not comparable in all species considered). In mouse and sheep, a significant decrease in the amount of DCX+ cells is present between prepuberal and adult stages (nonparametric Mann-Whitney test, $p<0.05$). In the other species (except for cats) a decrease is present at older ages, but not significant (Figure 4.9A; left and Table 4.5).

Taken together, these results suggest that the amygdala of non-rodent species (especially primates) may rely on abundant populations of DCX+ cells, whose amount can decrease around puberty, then reaching a stabilization in adulthood.

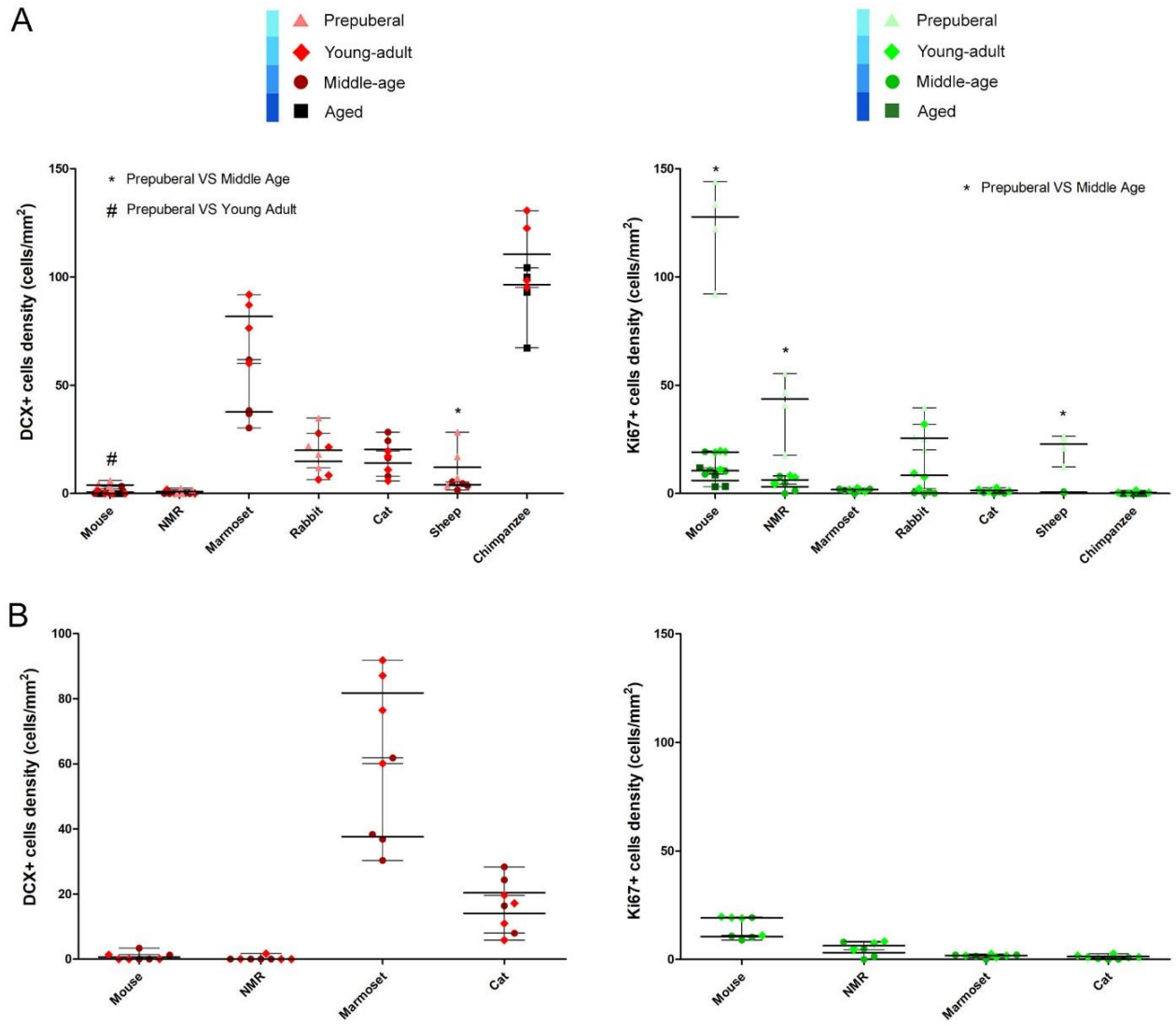


Figure 4.9 – Comparison of quantitative data (density of DCX+ cells and Ki67+ cells) at different ages. A, all ages considered (see Figure 4.1 for association between species and ages); note that statistically significant difference is only emerging when prepuberal stages are involved. B, comparison in four animal species at young-adult and middle-age stages. Note that during adulthood there are no statistically significant differences, indicating a sort of stabilization in the amount of both immature neurons and proliferating cells.

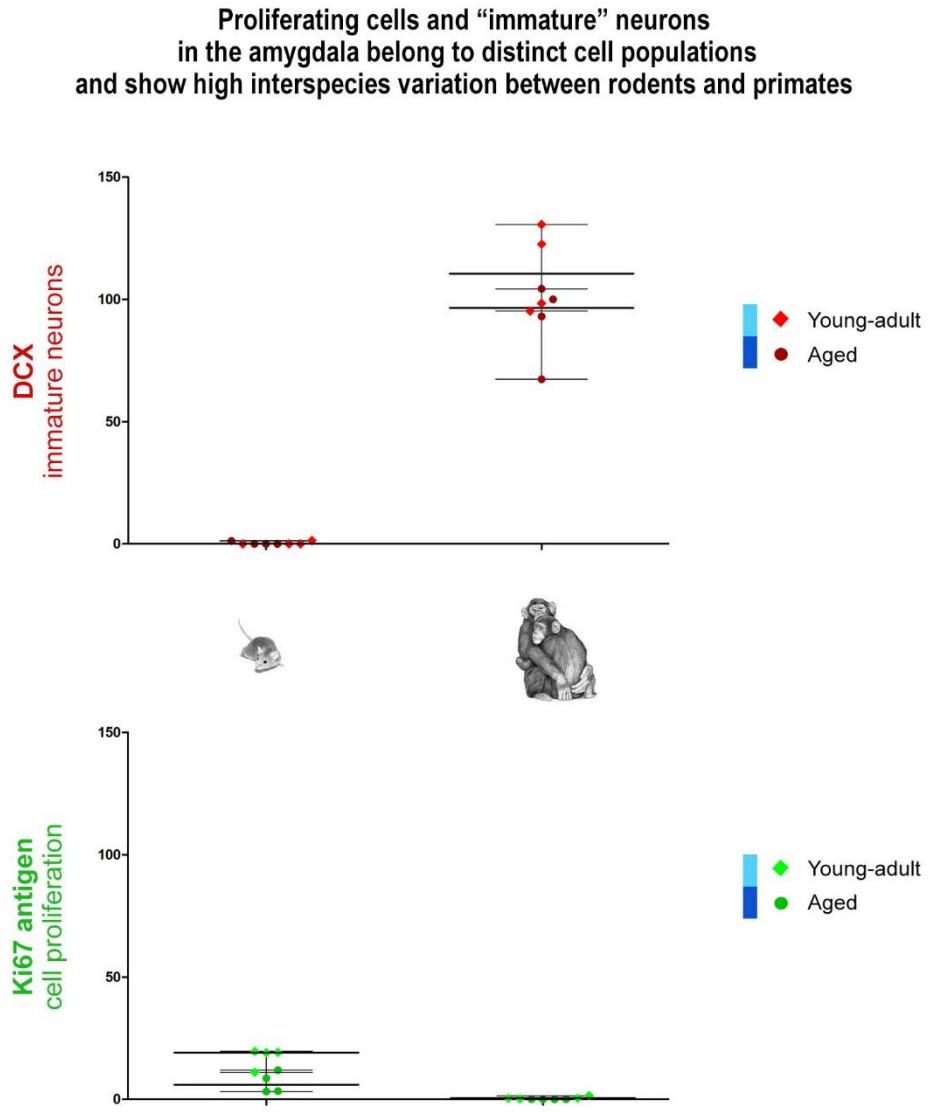


Figure 4.10 – Quantitative data concerning mouse and chimpanzee, representing two extremes under different points of view: i) chimpanzee, among the species studied here, is the primate more similar to humans, while mouse is the most used animal model in biomedical research; ii) DCX+ cells are extremely rare in the amygdala of mice while very abundant in chimpanzee; yet, in both cases they do not substantially vary at different adult ages, including advanced stages; iii) on the other hand, cell proliferation is higher in the amygdala of mice than in chimpanzees. These data suggest that immature neurons, though scarce in rodents, might play a role in plasticity of primates through adulthood; such interspecies differences can have an impact in translation and in the interpretation of preclinical data coming from research carried out on laboratory rodents (see Chapter 5 for discussion).

Table 4.5 – DCX+ and Ki67+ cell densities (median from 4 specimen/species) in all species and ages analyzed

Species	Age	DCX+ cells/mm^2	Ki67+ cells/mm^2
Mouse	P10	3,942	127,790
	3 months	0	19,128
	6 months	0,622	10,550
	9 months	0	6,005
Naked mole rat	2 months	0,851	43,677
	2 years	0	6,262
	10 years	0	3,101
Marmoset	2.5 years	81,763	1,774
	Adult	37,591	1,778
Rabbit	3 monhts	19,967	25,574
	3 years	14,889	8,449
Cat	1.5 years	14,076	1,392
	6-7 years	20,367	0,248
Sheep	4 months	12,467	22,825
	8-10 years	4,073	0,527
Chimpanzee	19-27 years	110,467	0,428
	40-48 years	96,526	0,062

4.3.3 Quantitative analysis of Ki67+ nuclei in the amygdala of different mammals

A quantification of Ki67+ nuclei in amygdala was performed in all species and all ages considered, taking into account their number (density/area), topographical location and distribution, to be compared with those of the DCX+ cell population.

Analysis in young-adult animals. In contrast with data obtained for the DCX+ cell population, Ki67+ cell density was high in rodents while sporadic in non-rodent species. This difference was significant in mouse with respect to chimpanzee (nonparametric Kruskal-Wallis test, $p<0.05$; Figure 4.8A, right and Table 4.5).

Comparison with other ages. As done for DCX+ cell density, a counting of the Ki67+ nuclei was performed in mouse, naked mole rat, marmoset and cat at different ages (young-adult and middle-age) and between mouse and chimpanzee at young-adult and aged life stages. The analysis revealed levels of cell proliferation that did not change significantly, remaining low in adulthood both in rodents and non-rodent species (nonparametric Mann-Whitney test, $p<0.05$; Figure 4.9B; right and Table 4.5). Interestingly, mouse and chimpanzee substantially differ in terms of cell proliferation, the former having a higher amount of Ki67+ nuclei at young-adult stage, then

decreasing with age. On the other hand, the levels of cell division remain very low in chimpanzee at both young-adult and aged life stages (Figure 4.10; bottom and Table 4.5).
Extending the analysis to other species and ages, a significant decrease in Ki67+ cell density is evident between prepuberal and adult stage in both rodents (mouse, naked mole rat) and in non-rodent species (sheep; nonparametric Mann-Whitney test, $p<0.05$; Figure 4.9A; right and Table 4.5).
These results indicate that rodents have a higher amount of cell proliferation in the amygdala with respect to non-rodent species (showing an opposite trend with respect to that observed for DCX+ cells), while no substantial changes in the amount of Ki67+ cells are detectable during adulthood in both groups, thus suggesting that DCX+ and Ki67+ cells actually might belong to distinct populations. Hence, the next step was to check whether the two antigens can be coexpressed in the amygdala.

4.3.4 Search for Ki67/DCX coexpression

To assess whether some of the DCX+ cells in the amygdala might be newly born, a search for possible coexpression of Ki67 antigen and DCX was performed (analysis carried out at different ages in marmoset, rabbit cat and sheep; Figure 4.11A; bottom).
To understand whether the rare Ki67+ nuclei in the amygdala colocalize with DCX+ cells, double immunofluorescence staining for the two markers was analyzed at the confocal level (Figure 4.11). No coexpression was ever detected in all experiments, suggesting that the DCX+ neurons and the proliferating cells do belong to distinct cell populations (Figure 4.11A; bottom). In Ki67-stained specimens, the immunopositive nuclei did not follow the topographical distribution of DCX+ cells, being randomly distributed in the entire anterior-to-posterior and medial-to-lateral/ventral-to-dorsal extension of the region, in all species considered (Figure 4.7; right, and Figure 4.8B). In addition, they frequently appeared as “doublets” (Figure 4.11B), namely the typical aspect displayed by the dividing oligodentrocyte progenitor cells (OPC; Boda et al., 2015), which are known to represent the larger population of proliferative cells in the brain parenchyma (Boda and Buffo, 2010; Semënov, 2021; see below for coexpression analysis of OPC markers).

4.3.5 Search for Ki67/SOX10 coexpression

To find other candidates for the parenchymal proliferating cells, we looked for a marker of OPCs. Although NG2 (Neuron Glia Antigen 2) is considered the ideal molecular marker for the

identification of early developing OPCs (see Box1), its expression cannot be easily detected in postmortem tissues, which are usually too heavily fixed (Rivers et al., 2008). As a matter of fact, we had poor results in our mammalian tissues, while the marker worked well in perfused mice brains (see Figure 3.4C in Chapter 3). In alternative, the pan-oligodendrocyte marker SOX10 was employed in double staining with Ki67 antigen, and indeed, several coexpressing cells were observed in all the specimens examined (Figure 4.11B).

Overall, these results indicate that the decrease in the level of cell proliferation through ages is not accompanied by a same decrease in the amount of DCX+ cells, and appears to be independent from such cell population, thus suggesting that the latter belongs to a pool of "immature" neurons rather than to a neurogenic process (see general discussion in Chapter 5).

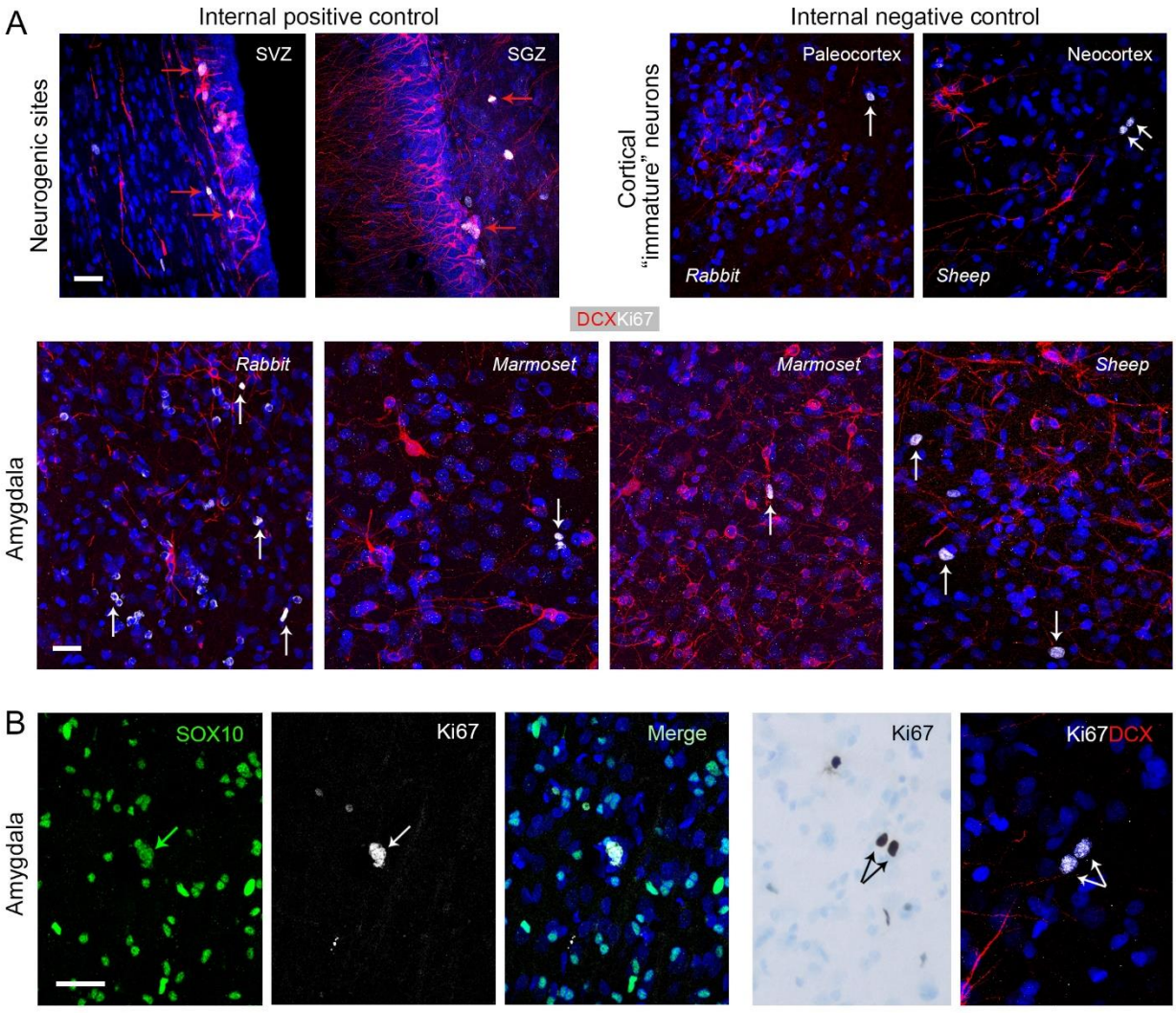


Figure 4.11 – Analysis of cell proliferation in the subcortical regions hosting the DCX+ cells (immature-appearing neurons). A, local cell division detected with Ki67 antigen (top; internal positive/negative controls), and search for DCX/Ki67 double staining in subcortical regions (bottom). B, Double staining with Ki67 antigen and the oligodendroglial marker SOX10, showing coexpression in proliferating cells (left), and detection of typical "doublets" (right; see text). Scale bars: 30 µm

---

4.4 Conclusions and Discussion

Main conclusions. Coming back to the specific questions asked at the beginning of the study, it can be concluded that the DCX+ immature-appearing neurons occur in subcortical regions (amygdala) of widely different mammals, their amount varying remarkably among species, with the extremes being represented by rodents (very low numbers/density) and primates (very high numbers/density). Their amount slightly changes at different ages, mainly around puberty, then remaining substantially stable through adulthood and aging.

As to the nature of these DCX+ cells, they can be considered as "immature" neurons (INs) rather than newly born elements since they were never found to coexpress the marker for cell division Ki67 antigen, also displaying a series of features (marker expression, topographical distribution, interspecies variation and variation with age) that strongly indicate the existence of two distinct populations: the INs and a widespread population of proliferating (parenchymal) SOX10+ glial cells.

For a more general discussion of these data in the context of brain plasticity (with special reference to comparison with cortical immature neurons – cINs – and with canonical adult neurogenesis), see Chapter 5.

Possible functional implications. As to the functional role of the amygdalar INs, it remains currently far from clear. One possibility is that INs in subcortical regions may be involved in a late form of development. In humans, most INs in the amygdalar paralaminar nucleus become mature excitatory neurons during adolescence (Sorrells et al., 2019), namely in a phase of essential formative years in terms of social interactions and emotional processing, in which amygdala may undergo changes in its structure and connectivity (Campbell et al., 2021; Azad et al., 2021; Avino et al., 2018). Similarly, we observed a significant decrease in the amount of INs between prepuberal and adult stages in our specimens (mouse and sheep). Yet, at present we did not investigate the phenotype/fate of these cells. The basolateral complex (hosting most of the INs in

the amygdala; Jhaveri et al., 2018; Chareyron et al., 2021; Marlatt et al., 2011; Marti-Mengual et al., 2013; Sorrells et al., 2019; and data from this thesis) is functionally connected to the medial temporal lobe memory system with afferents to hippocampus and perirhinal cortex (Petrovich et al., 2001), and is involved in learning and memory formation, by processing emotionally salient experiences (LeDoux 2003). Moreover, extensive connections are present within and between the different nuclei of the amygdaloid complex (Sah et al., 2003). This aspect could support the view of a slow integration of INs shaped by external cues (such as emotions), in a sort of "experience-induced" plasticity that could play a role in processing amygdala-mediated emotional information.

In addition, recent data demonstrated that some of the INs in the lateral, basal and paralaminar nuclei of macaque amygdala appear to differentiate into mature neurons after hippocampal lesion (Chareyron et al., 2016; Chareyron et al., 2021), suggesting that they may be involved in a form of "lesion-induced" plasticity that could respond to damage into brain regions functionally connected to the amygdala. However, as pointed out by the authors, an abrupt increase of mature neurons may also have detrimental effect on the proper functioning of amygdala (Chareyron et al., 2021), thus this explanation requires further investigation.

However, it has to be underlined that there is no evidence that amygdalar INs structurally and/or functionally integrate in the neural network once they reach full maturation (as it has been shown for their cortical counterpart in the mouse piriform cortex; Rotheneichner et al., 2018; Benedetti et al., 2019). Further research is needed to unravel these important aspects, yet another hypothesis should be left open, based on the results of this thesis: the possibility that final integration might not be the fate for most of these "immature" cells. By looking at the numbers of amygdalar INs in gyrencephalic species at different ages (especially in chimpanzees), it is evident that the DCX+ cells do not decrease a lot during adulthood, and very high numbers are still present in aging. This behavior is very different from that of cINs in the mouse piriform cortex, which undergo substantial decrease during youth and adulthood (see Chapter 3), and which are known to progressively mature and integrate (what explains their exhaustion).
Of course, with the current knowledge, it is not easy to find a possible role for a large population of "young neurons" that maintain their immaturity for so long time. Further discussion on this topic will be provided in Chapter 5.

CHAPTER 5

Discussion

5.1 Why investigating comparative aspects of the "immature" neurons?

Non-newly generated "immature" or "dormant" neurons are gaining interest in the field of brain structural plasticity, as an alternative form of neurogenesis delayed in time (neurogenesis without division; La Rosa et al., 2020b; Bonfanti and Seki, 2021; Benedetti and Couillard-Despres, 2022). In this thesis, the work previously started in other PhD projects was continued trying to understand whether these neurons might be present in brain extracortical areas, such as the subcortical regions, and whether they can vary among different mammals as it has been shown for the cortex (La Rosa et al., 2020; Palazzo et al., 2018; La Rosa and Bonfanti 2021). Though the role/function of the INs is still utterly obscure, our lab made this comparative choice based on a simple consideration: in the field of canonical adult neurogenesis, a lot of resources have been put in place in the last 30 years trying to understand the role (both physiological and possibly in brain repair) of this biological process; a task that was addressed by investigating almost exclusively laboratory rodents. Then, only in recent years (following the publication of more than 13,000 papers on this topic), a comparative approach considering other mammals, including the study of human brain tissues, has started to reveal that neurogenic processes can be highly reduced, if not absent, in adult brains of long living, gyrencephalic mammals (Sanai et al., 2011; Patzke et al., 2015; Sorrells et al., 2018; Parolisi et al., 2017;2018; Cipriani et al., 2018). In this context, starting from some observations regarding the occurrence/anatomical distribution of putative "immature" neurons in non-rodent species (Luzzati et al., 2009; Cai et al., 2009; Zhang et al., 2009; Varea et al., 2011), our lab decided to map these cells in widely different mammals, in different brain regions and ages, to investigate whether also this kind of plasticity might follow an evolutionary trend from small-brained (mostly lissencephalic and short-living) to large-brained (mostly gyrencephalic and long-living) mammals.

Placed within a PhD in Veterinary Sciences, and in a context of comparative medicine (Mobasheri, 2015; La Rosa and Bonfanti, 2018), the work of the present thesis was directed at investigating possible heterogeneity (cell types, amount, spatial and topographical distribution, age-related variation) of INs across widely different mammalian species, by using the most comparable approach despite difficulties in gathering a certain number of relatively fresh, well-fixed, complete brains (one hemisphere/individual) from each animal species and ages considered. Due to obvious limitations and to the time available (three years), this work can be considered as an advancement, yet not a completion, of the whole mapping of these cell populations in the mammalian brain. Nevertheless, by adding comparative data concerning the subcortical INs to those already available for the cortex, would provide insights on the whole landscape of brain neurogenic plasticity in

different mammals, thus revealing whether a sort of trade-off between forms of plasticity might exist, as a consequence of evolutionary choices (see Section 5.5).

In addition to interspecies (phylogenetic) variation, the present work was conceived also to address possible variation with the animal age. This point was considered since it is well known that different kinds of plasticity (especially adult neurogenesis) undergo substantial reduction across the animal lifespan, with a marked drop around puberty (Ben Abdallah et al., 2010; Sanai et al., 2011; Sorrells et al., 2018; Bonfanti and Charvet, 2021). Since the nervous systems of different mammals do follow different postnatal developmental patterns (Finlay and Darlington, 1995; Workman et al., 2013), the impact on brain plasticity can be species-specific (Charvet and Finlay, 2018; Bonfanti and Charvet, 2021). One of the hypotheses pursued in this thesis, is the possibility that different types of plasticity (e.g., canonical adult neurogenesis Vs. INs) can follow different temporal patterns.

5.2 Dealing with postmortem brains of non-rodent, large-brained mammals

After the recent discovery that non-newly generated, "immature" or "dormant" neuronal populations can be present in non-neurogenic brain regions (cerebral cortex and amygdala), and that these neurons are particularly abundant and widespread in large-brained species, the need for reliable cell markers to correctly identify the "young" neurons across mammals is gaining more and more importance. Since we are pursuing a general mapping of these cells in different animal species (from mouse to chimpanzee) and in different brain regions (cortical and subcortical) there is a need for "comparable methods" in order to obtain "comparable results", a goal that is often neglected, being different laboratories mostly working on one-two species at a time, using their own method and sampling. In this context, we planned to perform a series of studies across the years by using the same approach, and we felt the need to systematically check our widely different brain tissues for two immunocytochemical markers currently used in the study of structural plasticity.

Overall, our methodological study (Chapter 2; Ghibaudi et al., 2023a) showed that substantial differences in the occurrence, quality and specificity of immunocytochemical staining for DCX and Ki67 antigen can exist when comparing different mammalian species whose brain tissues are treated with different antibodies. Real interspecies variation in the occurrence of antigens and high species specificity of different antibodies (and, to a lesser extent, regional differences) can impact even more than the type/degree of fixation. Hence, the overall issue of analysing large,

gyrencephalic brains is more complex than simply fixation, which overlap with natural interspecies variation and/or different specificity of antibodies across phylogeny, the latter two aspects being often underestimated.

Our results confirmed that special troubles and non-specific staining can be particularly frequent when studying DCX in the cerebral cortex of primates and humans using most of the currently available antibodies. This might be explained by the simple fact that most of the tools currently available have been developed for research in laboratory rodents. To overcome such an impasse, there is urgent need to develop antibodies tailored for, and tested on, large-brained species, including humans. An additional reason for such a need is the simple fact that the best-performing antibody against DCX across the animal species studied here has revealed to be the goat Santa Cruz polyclonal serum (see Chapter 2), that is now out of production since several years. We used a large stock of this antibody still present in our lab and ample enough to complete the analyses on the subcortical regions. Yet, for future studies this product must be replaced.

Despite any efforts aimed at normalizing multiple variables, when comparing brains of different size (from 0,5 to 380 grams in our study) and belonging to species widely different as to their availability (an example for all: the different possibility to perform complex experiments in mice or chimpanzees), tissues cannot be processed with the same procedures. Yet, despite these problems, we obtained substantially homogeneous results in all species examined (e.g., quality of tissue morphology and quality of immunostaining). In parallel, the quantitative results obtained by studying the INs in large-brained mammals gave relatively high standard deviations observed among individuals of the same group of animals. In some cases, this can be due to the fact that ages were different within the same age group, yet variability was frequent even in animals of the same age. We interpret such variability as a sign of individuality, likely linked to environmental influences. It is perfectly reasonable that also INs can be affected by environment and lifestyle, similarly to other forms of plasticity (an hypothesis that must be verified in a project on its own; see the future perspective Section, below). Anyway, in our studies aimed at assessing the possible interspecies variation (La Rosa et al., 2020; this thesis), this aspect was not a problem, since the interspecies differences in the number of INs observed between rodents and non-rodent mammals (both in cortex and amygdala), were overcoming one or two orders of magnitude.

5.3 Does the nature of subcortical DCX+ cells match with non-dividing "immature" neurons?

In previous reports describing DCX+ cells in the amygdala, some authors considered them as potentially newly generated (see Ghibaudi and Bonfanti 2022 for review and discussion of this topic). In this thesis, multiple aspects of these neurons were characterized, including their morphology, number, density, topographical distribution, interspecific and age-related variation, as well as their relationship with the proliferating cells present in the same region. By putting together the results obtained, it is possible to make an hypothesis about the nature/origin of this cell population. Here, following, some elements converging in our assumption are listed (summarized in Figure 5.1):

1) Specific features of the DCX+ cells and their relationship with the proliferating cell population:

a) although expressing the cytoskeletal protein DCX, usually associated to neuronal immaturity and neurogenesis, these cells never coexpress the marker of cell division Ki67 antigen, thus being not dividing elements;

b) the spatial distribution of these cells is largely different from that of the proliferating cells, being grouped in some compartments of the amygdala while the Ki67+ nuclei are more widespread, being reminiscent of a OPC sparse population;

c) while the amount of DCX+ cells show remarkable interspecies variation, the dividing cells occur in a more uniform pattern across mammals; this is particularly evident in gyrencephalic mammals, in which the DCX+ cells are very abundant;

d) by comparing mouse and chimpanzee, widely differing for their brain size, gyrencephaly, and lifespan, it is evident that mice have a few DCX+ cells and higher proliferation rate, while an inverse situation is present in primates;

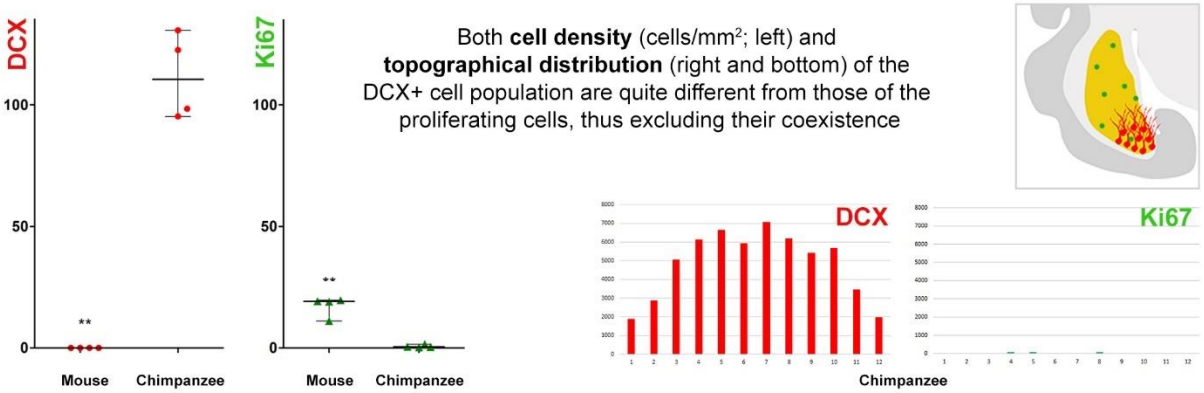
2) Similarities with the well characterized population of cortical "immature" neurons (cINs) of the mouse piriform cortex and large-brained mammal neocortex:

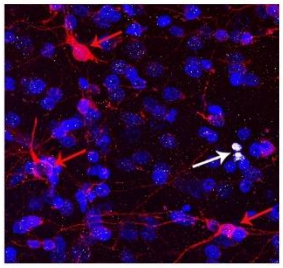
e) Ki67+ nuclei can be found to coexpress the OPC marker SOX10, thus suggesting that the parenchymal proliferating cells belong to a glial cell population;

f) the general morphology of the DCX+ cells (small, bipolar cells and large, ramified cells) is very similar to that described for the two different stages of maturation of the cortical immature neurons (type 1 and type 2 cells);

Converging elements of proof indicating the "immature neuron" nature for the DCX+ cells in the amygdala

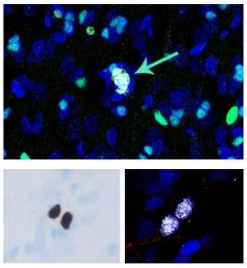
FROM THIS WORK



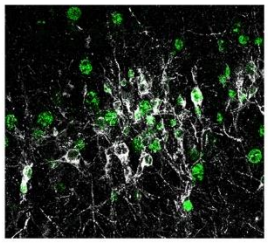


No coexpression was ever found between DCX and Ki67 antigen in the amydgala of all animal species investigated (left),

while coexpression can be found between **Ki67 antigen and SOX10**, and **"doublets"** of proliferating cells are frequent (right)



FROM LITERATURE



BrdU (green) / **DCX** (white) coexpression in the neocortex of lambs born from sheep injected with the tymidine analogue at two months pregnancy (Piumatti et al., 2018)

This paper demonstrates the prenatal origin of DCX+ cells in paleocortex, neocortex, amygdala, and claustrum of a gyrencephalic mammal (sheep)

Alderman et al.
2022 *BioRxiv* (preprint)

This paper demonstrates the prenatal origin of DCX+ cells in the amygdala, and their subsequent migration to the piriform cortex, in mice

Figure 5.1 – Several aspects emerging from the current study do converge in suggesting an "immature", non-dividing nature of the DCX+ cells existing in the amygdala (top). Even in postmortem tissues of mammalian species not allowing (or making it extremely complex to be performed) experiments of pulse-chase analysis (e.g., with BrdU injections), the results obtained concerning the density and topographical distribution of the DCX+ cell population Vs. the Ki67+ dividing cells, do suggest that they belong to two distinct compartments. This is confirmed by the absence of coexpression between the two antigens after immunofluorescence double-staining and confocal analysis (a results confirmed in all species and ages studied here) and by the frequent detection of coexpression between Ki67 antigen and the oligodendrocyte marker SOX10. These results are also in agreement with currently available reports from the literature (bottom), in which direct proof of the prenatal origin of these cells were obtained.

---

g) while canonical adult neurogenesis follows a trend of reduction from rodents to large-brained mammals, the DCX+ cells of the amygdala follow an inverse trend, more similar to that described for INs in the cortex;

3) Data reported in the literature:

h) in experiments using pregnant sheep treated with BrdU injections, with subsequent analysis of the lamb's brains, we previously showed that most of the DCX+ neurons in the cerebral cortex (including neocortex), as well as some in subcortical regions (amygdala and claustrum), were generated prenatally (Piumatti et al., 2018).

i) by performing BrdU injections in pregnant mice between E12.5 and E16.5, then analyzing the percentage and density of DCX+/NeuN+ cells coexpressing BrdU in pups at P21, Alderman et al. in their preprint (2022) demonstrated that DCX+ cells in the paralaminar nucleus of the mouse amygdala are born prenatally (between E12.5 and E14.5).

5.4 Comparing amygdalar and cortical INs: how important is the "adult neurogenesis without division" for large-brained mammals?

The results obtained in the present study of amygdalar INs revealed a picture quite similar to that previously found in the cerebral cortex (La Rosa et al., 2020a). Nevertheless, while in the cortex the prevalence of INs seems more distributed among the large-brained, gyrencephalic species, in the amygdala these cells are strikingly abundant in primates (Figure 5.2 and Tables 5.1, 5.2). In both cases, the INs are extremely rare (if not absent, such as in the neocortex) in rodents. This

pattern would confirm a “preference” for INs with respect to canonical adult neurogenesis in large-brained, long-living species (Palazzo et al., 2018).

Overall, it is evident that different types of neurogenic processes (with and without cell division) are mainly distributed in selected brain regions: canonical neurogenic sites (with cell division), cortex, amygdala (without cell division). Why do large-brained, long-living mammals host large populations of INs in cortical and subcortical regions? A possible answer is that in those animal species both cortex (especially neocortex) and amygdala have gained higher importance for the survival of individuals compared with olfaction and hippocampal functions.

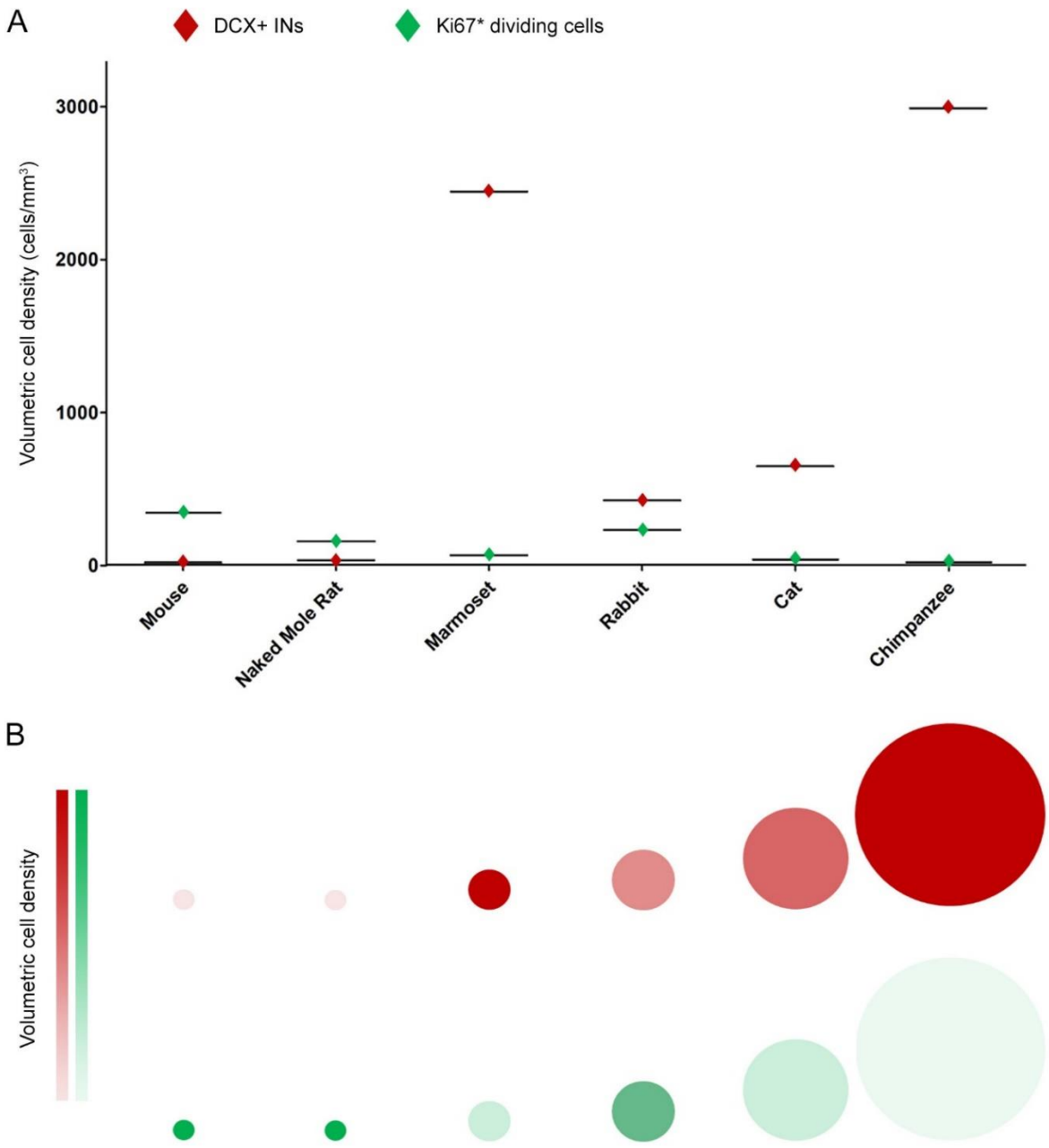


Figure 5.2 – Volumetric densities considering the total number of DCX+ "immature" neurons (INs; red) and Ki67+ dividing cells (green) in the total volume of the amygdala of different mammals. A, data expressed in cells/mm^3; note that values referring to dividing cells are higher than those of INs in rodents, while the situation is inverted in gyrencephalic species. B, schematic representation considering both the absolute volume of the amygdala in different species (the size of the dots is reminiscent of the real size of the amygdala, but not in scale; the real volume being 200-fold higher in chimpanzee than in mouse) and the different cell densities expressed as gradients of shades (same colors as in A); note the inversion between rodents and primates.

Table 5.1 – Estimation of the total number of DCX+ cells in the amygdala of different mammals (one hemisphere).

Species	Coronal serial sections cut in the entire amygdala	Coronal sections considered for cell counting	Average number of DCX+ cells in a coronal section of the amygdala (mean from all sections considered for counting)	Estimation of total DCX+ cells in amygdala
Mouse PP	36	3	5,4	**194,4** (5,4x36)
Mouse YA			0,4	**14,4** (0,4X36)
Mouse MA			0,6	**21,6** (0,6x36)
Mouse AG			0,25	**9** (0,25x36)
NMR PP			0,58	**20,88** (0,58x36)
NMR YA			1	**36** (1x36)
NMR MA			0	**0** (0x36)
Marmoset YA	60	5	561	**33.660** (561x60)
Marmoset MA			398	**23.880** (398x60)
Rabbit PP	84	7	113,3	**9.517,2** (113,3x84)
Rabbit YA	96	8	95	**9.120** (95x96)
Cat YA	96	8	285	**27.360** (285x96)
Cat MA	84	7	439,3	**36.901,2** (439,3x84)
Sheep PP	132	11	998,8	**131.841,6** (998,8x132)
Sheep MA	180	15	518,1	**93.258** (518,1x180)
Chimpanzee YA	144	12	4857,6	**699.494,4** (4857,6x144)
Chimpanzee AG			3291,7	**474.004,8** (3291,7x144)

Table 5.2 – Estimation of the total number of Ki67+ cells in the amygdala of different mammals (one hemisphere).

Species	Coronal serial sections cut in the entire amygdala	Coronal sections considered for cell counting	Average number of Ki67+ cells in a coronal section of the amygdala (mean from all sections considered for counting)	Estimation of total Ki67+ cells in amygdala
Mouse PP	36	3	260,6	**9.381,6** (260,6x36)
Mouse YA			9,1	**327,6** (9,1x36)
Mouse MA			8,5	**306** (8,5x36)
Mouse AG			2,8	**100,8** (2,8x36)
NMR PP			16,3	**586,8** (16,3x36)
NMR YA			5,3	**190,8** (5,3x36)
NMR MA			1,4	**50,4** (1,4x36)
Marmoset YA	60	5	14,9	**894** (14,9x36)
Marmoset MA			7,7	**462** (7,7x36)
Rabbit PP	84	7	94,7	**7.954,8** (94,7x36)
Rabbit YA	96	8	52,1	**5.001,6** (52,1x36)
Cat YA	96	8	15,2	**1.459,2** (15,2x36)
Cat MA	84	7	3,7	**310,8** (3,7x36)
Sheep PP	132	11	442,9	**58.462,8** (442,9x36)
Sheep MA	180	15	18,8	**3.384** (18,8x36)
Chimpanzee YA	144	12	27	**3.888** (27x36)
Chimpanzee AG	144		3,6	**518,4** (3,6x36)

PP, prepuberal; YA, young-adult; MA, middle age; AG, aged

In other words, a trade-off between different types of neurogenic plasticity might have occurred through evolution, reducing the importance of stem cell-driven neurogenesis and increasing the

occurrence of INs in some species (e.g., primates), while doing the opposite in rodents (Figure 5.3; see Bonfanti et al., 2023; for this hypothesis applied to the cerebral cortex, see below: Section 5.5).

Another aspect emerging from the present thesis and confirming a trend described for the previous study on the cortex (La Rosa et al., 2020a) is the substantial maintenance of the IN cell populations during the animal lifespan, again a feature present in primates but not in rodents (for further discussion of these temporal patterns, see Section 5.6).

On these bases, another hypothesis can be made: stem cell-driven neurogenesis (SVZ and SGZ neurogenic niches) is a prevalently juvenile process, aimed at sculpting the hippocampus and olfactory bulb circuits while the animal is exploring the world (environment) in order to get its "vision of the world", to be used for living in that environment as an adult (Cushman et al., 2021). In short-living mammals (e.g., mice), also the INs of the paleocortex (the only reserve of INs in these animals) follow a similar pattern. On the other hand, in primates the life expectancy after puberty (and after the period of brain growth and sculpting) is very long, attaining at more than 50 years in chimpanzees. Hence, after having "built up" their brain and their vision of the world during youth, these species would need plasticity for long time in brain regions that are strategic for their survival, namely those processing the highest cognitive functions (cortex) and the intricate system of emotions (amygdala), both linked to their complex social behavior.

5.5 Hypotheses on a possible evolutionary trade-off between different types of neurogenic plasticity

Based on the evidence of phylogenetic variation in stem cell-driven neurogenesis and non-dividing "immature" neurons, it is likely that evolutionary pressures associated with ecological niche or neurodevelopmental constraints have led to the selection of different types of plasticity in various species and brain regions. This suggests a "trade-off", which refers to a situation where compromise occurs between two or more traits that offer distinct benefits but cannot be fully optimized concurrently. Such compromises can arise due to limited resources or energy that must be allocated among competing demands, or due to anatomical or developmental limitations (Heldstab et al., 2022).

The concept of a trade-off implies that the balance of resource allocation can shift between different options without necessarily indicating an exclusive commitment to one over the other (van Noordwijdek and Jong, 1986).

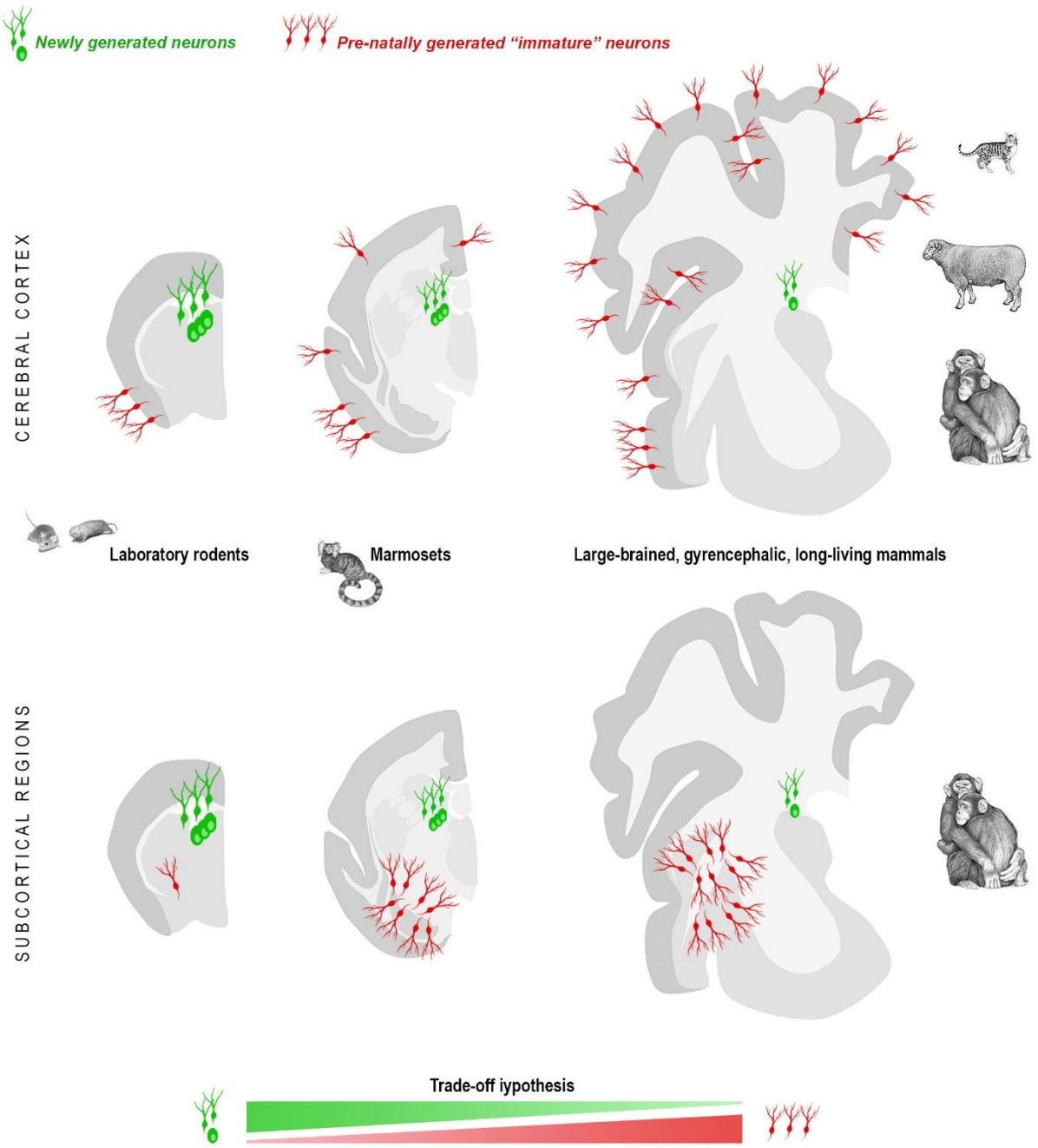


Figure 5.3 – Summary of the anatomical distribution/total amount of different neurogenic processes in the mammalian brain. A prevalence of immature neurons in the cortex of large-brained species and in subcortical regions of primates appear to follow an opposite trend with respect to stem cell-driven neurogenesis (here represented schematically for both the main neurogenic sites – canonical neurogenesis) that is prevalent in rodents. The mapping of newly generated and non-newly generated (immature) neurons across widely different mammals does suggest the existence of a trade-off in different types of neurogenic plasticity (see also Bonfanti et al., 2023).

Consequently, evolutionary processes might favor specific forms of plasticity in certain species or brain regions, depending on the ecological pressures and functional demands they face.
We hypothesize that trade-offs play a crucial role in shaping the evolutionary trajectory of neural plasticity and contribute to the remarkable diversity observed across species.

Brain size and balance in the allocation of resources

It is important to consider the factors that influence the occurrence of trade-offs in evolutionary biology. Limited resources, such as energy or nutrients, pose constraints on an organism's ability to optimize multiple traits simultaneously. These constraints can lead to balance the allocation of resources so that one trait may come at the expense of another. For example, in the context of neurogenesis, maintaining a larger pool of stem cells for continuous regeneration may come at the cost of other energy-demanding processes, such as enhancing synaptic plasticity or cognitive functions (Walton et al., 2012).

Additionally, anatomical constraints can also contribute to trade-offs. The physical structure and organization of an organism's brain can impose limitations on the optimization of multiple traits. For instance, brain regions with limited space or specialized functions may prioritize specific forms of plasticity that are most beneficial for their ecological niche, while compromising on others (Charvet and Finlay, 2018).

Nevertheless, it is important to note that evolution, as a process, is not linear, progressive, or predictable. While it operates through natural selection and the accumulation of advantageous traits over time, it also encompasses elements of randomness through neutral drift and contingent exceptions that defy straightforward explanations. The interplay of genetic variation, environmental factors, and chance events introduces a level of unpredictability (e.g., bats, small-brained mammals with reduced adult neurogenesis, or naked mole rats, long-living rodents with abundant adult neurogenesis; Amrein et al., 2007; Penz et al., 2015). However, amid this complexity, certain trends and patterns can be identified. These trends are governed by the balance of energetic allocation and developmental constraints, ultimately shaping variation in brain structure across species and the capacity for different forms of plasticity.

In the case of neurogenic plasticity, the following variables are relevant: i) the type of plasticity (e.g., stem cell-driven neurogenesis and non-dividing immature neurons); ii) the anatomical region hosting plasticity and linked to specific functions (e.g., canonical neurogenic site linked to

olfaction and cerebral cortex linked to high-order computational capabilities); iii) the phylogenetic lineage of the species and their brain size.

Adult neurogenesis in large mammal brains is subject to various energetic costs and developmental constraints. The biosynthetic process of generating new neurons requires substantial metabolic resources, including glucose and oxygen, which can impose a significant burden on the energy budget of the brain (Bauernfeind and Babbitt, 2020). Large mammal brains may face challenges in allocating sufficient resources for neurogenesis while maintaining other essential functions. Additionally, the developmental constraints associated with large brain size can limit the spatial and temporal availability of neurogenic niches, where new neurons are generated (Patzke et al., 2015; Charvet and Finlay, 2018; Martinez-Cerdeno et al., 2018; Duque and Spector, 2019). This may restrict the extent and duration of adult neurogenesis in large mammals.
By contrast, the prenatally generated, non-dividing cortical "immature" neurons, which do not require stem/progenitor cells to occur as undifferentiated elements within the mature cortex, are far more abundant and widespread in large-brained mammals (La Rosa et al., 2020a), likely representing a "low energy cost", alternative form of neurogenic plasticity.

From olfaction to neocortex: the hypothesis of navigation adapted to plasticity

Recent theories propose that the origin of the neocortex in early mammals resulted from behavioral adaptations related to olfaction-mediated goal-directed and navigational behaviors, accompanied by integrated sensory map development, which in turn resulted in developmental changes in the distribution of cells and the formation of circuits in the telencephalon (Aboitiz et al., 2003; Aboitiz and Montiel, 2015). Early mammals likely adopted a nocturnal and burrowing lifestyle, utilizing internal cues such as proprioceptive information in conjunction with sensory inputs from the olfactory and somatosensory systems for spatial navigation. In such conditions, orientation was predominantly based on one-dimensional maps that encoded sequences of events in a time series. These early mammals are thought to have heavily relied on their sense of smell, leading to an expansion of the olfactory bulb and olfactory cortex as brain size increased. Accordingly, selective pressures led to the emergence of an interface between olfactory-hippocampal networks, integrating somatosensory information for navigation (Kaas, 2019).

As mammals diversified and occupied new ecological niches, including diurnal environments for some species, vision and audition provided additional information regarding distance and location.

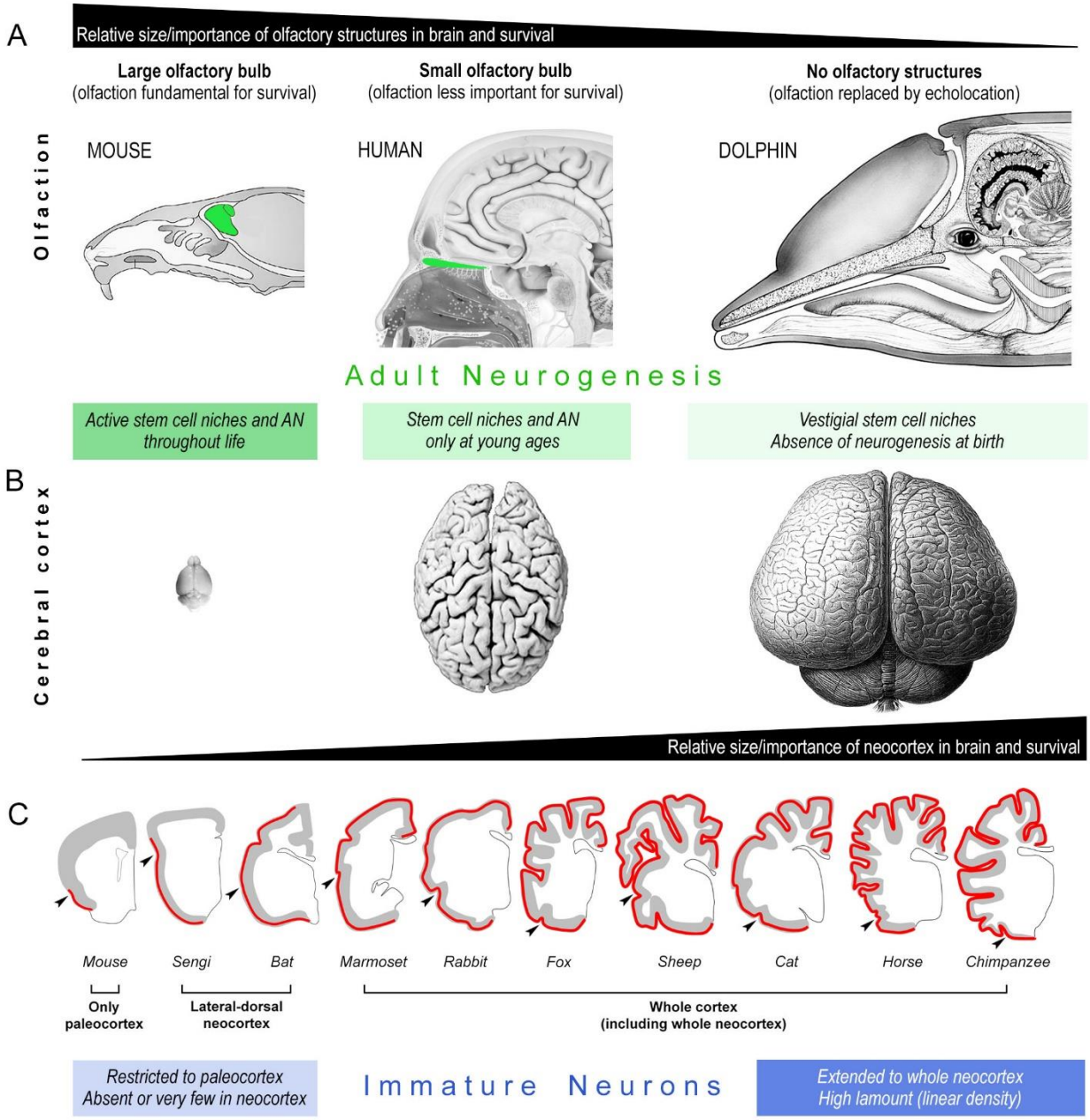


Figure 5.4 Anatomical and functional aspects at the basis of the evolutionary trade-off hypothesis to explain the relative occurrence between different types of brain structural plasticity (adult neurogenesis and cortical immature neurons) in mammals. A,B, different importance of certain brain regions/functions for navigation and survival: small-brained rodents rely mostly on olfaction, whereas large-brained, gyrencephalic species rely mostly on widely expanded cerebral cortex (neocortex). C, immature neurons are more widespread (in the cortical mantle) and abundant (in terms of cell density) in large-brained, gyrencephalic species with respect to rodents. They would have been favored by evolution to place a process of "neurogenesis without division" (i.e., the addition of new functional neurons) in brain regions not endowed with stem cell-driven neurogenesis (e.g., cerebral cortex). D, the prevalence of immature neurons in the cortex of large-brained species and of stem cell-driven neurogenesis in the neurogenic sites of rodents suggests a trade-off in different types of neurogenic plasticity. Modified from Bonfanti et al., 2023).

These senses are vital for generating accurate two-dimensional and time-independent spatial maps, providing more detailed information relevant to navigation (Buzsáki, 2005; Eichenbaum, 2014). Over time, the expanding neocortex played an increasingly prominent role in the formation of multimodal association networks and map-like representations of space (Aboitiz and Montiel, 2015).

The current diversity of brain structure in mammals is extraordinary. Large-brained mammals with gyrencephalic brains often exhibit reduced olfactory bulb size or complete absence of olfactory bulbs, as observed in dolphins (Figure 5.4A; references in Parolisi et al., 2018). In contrast, smaller-brained mammals, including most rodents, possess prominent olfactory structures and relatively smooth neocortices. This anatomical variation reflects functional adaptations, with rodents heavily relying on olfaction, while larger mammals exploit the computational capabilities of their expanded neocortical circuits. The neocortex, characterized by six layers, undergoes remarkable elaboration in large-brained mammals with greater differentiation of specialization fields that are important for sensorimotor integration and cognitive functions (Englund and Krubitzer, 2022). Large-brained species with highly folded neocortices, such as primates, exhibit reduced dependence on olfaction, with their behavioral complexity predominantly linked to an extensively expanded neocortical mantle. This difference in reliance on olfaction and neocortical development could explain the lower levels of neurogenesis observed in these species, which primarily occur during the postnatal and juvenile stages to shape neural circuits through experiential learning (Semënov, 2019; Kempermann, 2019; Cushman et al., 2021). Consequently, a possible adaptation in large-brained, long-living mammals is the selection of non-dividing immature neurons (cINs) as a mechanism to provide a form of neurogenic plasticity in layer II of the cerebral cortex (as discussed in La Rosa et al., 2020a). This adaptation becomes essential as cerebral cortex lacks stem cells or neurogenic sites but is crucial for their survival.

5.6 Age-related reduction of cINs in mice compared with data available in large-brained mammals and humans: a link with lifespan?

Comparative studies carried out in mammals revealed the existence of remarkable interspecies differences in the distribution and total amount of cINs, with greater abundance of these cells in large-brained, gyrencephalic species (Piumatti et al., 2018; La Rosa et al., 2020a), including humans (Li et al, 2023). Another difference seems to be linked to lifespan: in large-brained, long-living species the cINs do persist at adult/old stages, not showing the same decrease observed in

mice (Figure 5.5, middle left). In chimpanzees of young-adult and senior ages (19-27 and 40-48 years old, respectively; La Rosa et al., 2020a), as well as in sheep of young-adult and middle ages (2 and 8-10 years old, respectively; La Rosa et al., 2020a), no substantial reduction was observed in the amount of cINs within the piriform cortex (Figure 5.5, middle) and moderate reduction was seen in the neocortex (La Rosa et al., 2020a). A possible trend of "stabilization" in the amount of cINs in large-brained, long-living species, is supported by the highly similar pattern obtained by comparing young-adult with middle age stages in sheep, with young-adult and senior stages in chimpanzee (Figure 5.5, middle right), indicating a long-lasting maintenance of the DCX+ cell population in mammals with longer lifespan. In humans, no comparable quantification is available at present, yet the cINs can be observed in aged individuals, being mostly maintained in the temporal lobe (Coviello et al., 2022, age range: 5-62 years; Li et al., 2023, age range: 6 months-100 years).

An even higher stabilization through age has been suggested for DCX+ neurons in subcortical regions of a gyrencephalic mammal: the sheep (Piumatti et al., 2018). On these bases, the current thesis expanded our knowledge on INs in the amygdala, by systematically investigating their possible variation in widely different mammals. We confirmed a sharp prevalence of amygdalar INs in gyrencephalic species, with particular abundance in primates, also confirming the substantial stabilization of the DCX+ cell population through ages (Figure 5.5, bottom). On the other hand, the systematic study of cIN age-related variation in the mouse piriform cortex (Chapter 3) revealed a progressive decrease in the occurrence of these cells through the young-adult life stages, similar to that previously described for adult neurogenesis (Figure 5.5, top). On the whole, it appears evident that amygdalar INs represent a neuronal cell population with similarities in "nature" with its cortical counterpart, but also differing under the temporal pattern profile. In other words, a sort of hierarchy in temporal variation seems to exist: canonical adult neurogenesis remarkably drops around puberty, cINs decrease more slowly (especially in large-brained mammals), and subcortical INs are more stable during time (especially in large-brained mammals and especially in chimpanzees), even during aging (Figure 5.6).

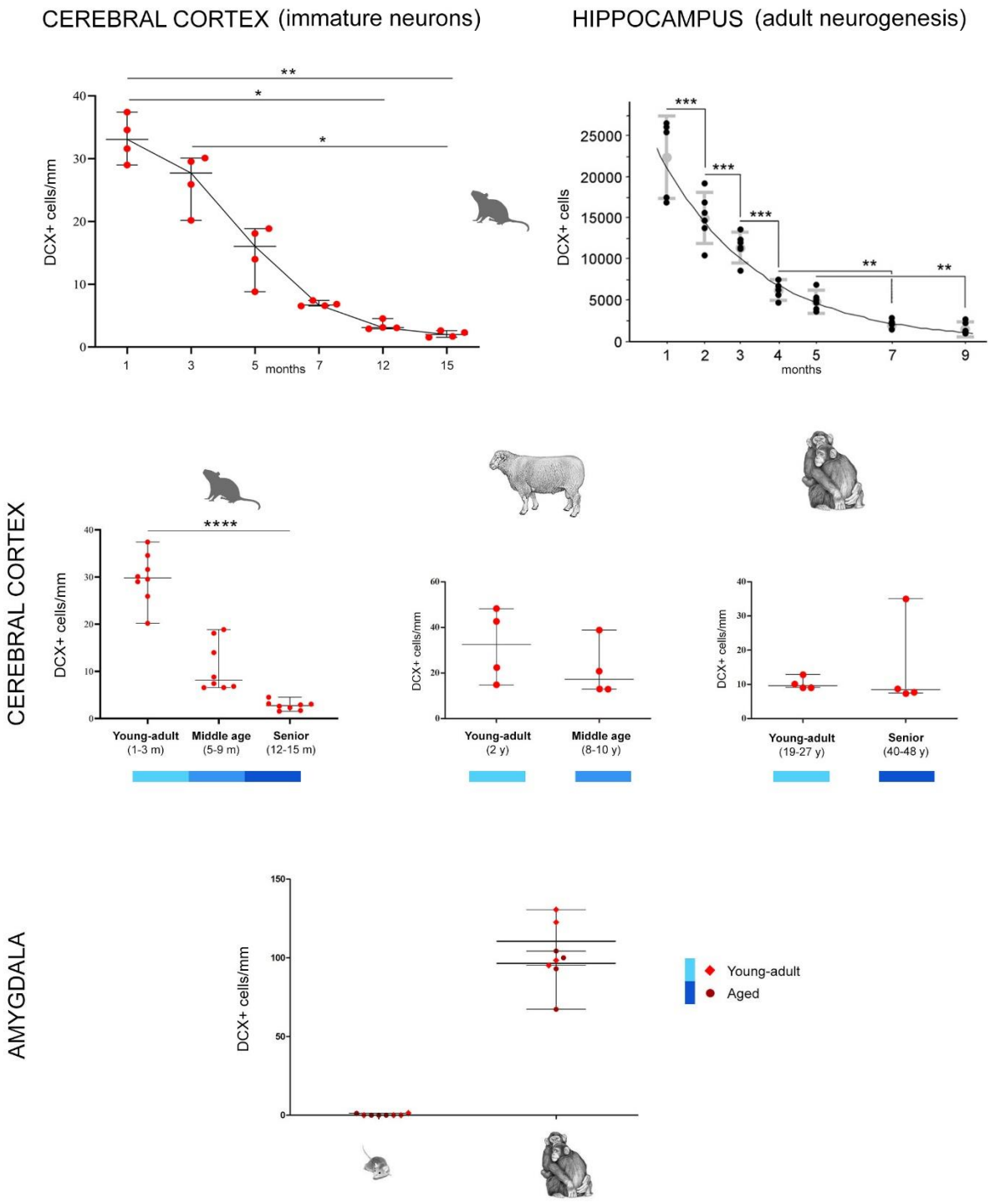


Figure 5.5 Comparing the temporal extension of different neurogenic processes in widely different mammals. Top, left: data obtained in the present study (Chapter 3) demonstrating the age-related decrease of cINs in mouse and its comparison with adult neurogenesis in mouse dentate gyrus (top, right). Middle, DCX+ immature neuron amount (cortical layer II linear density) in the paleocortex of mammals, at different ages. Despite the substantial decrease observed in rodents (left; n = 8 for each life stage), a substantial maintenance characterizes the gyrencephalic mammals, such as sheep and chimpanzee (row data from La Rosa et al., 2020b; eLife Sciences Publ Ltd; n = 4 for each life stage); a similar pattern was detected for INs in the amygdala (bottom; see Chapter 4).

Overall, the currently available results indicate that mammalian species characterized by small, lissencephalic brain, short lifespan and rapid development of the nervous system (such as the laboratory mouse) are endowed with a small population of cINs, which is restricted to the paleocortex and follows a temporal pattern of reduction very similar to that of stem cell-driven adult neurogenesis. By contrast, large-brained, long-living species, which have far higher amount and more widespread distribution of cINs (including the entire neocortex) seem to maintain longer these populations of "young" neurons across adulthood and aging, suggesting a slowing down of their maturation/integration through the lifespan. The idea that cINs might represent a "cognitive reserve" for the cerebral cortex in long-living species (Palazzo et al., 2018; La Rosa et al., 2019) remains a stimulating hypothesis, nevertheless further studies systematically considering different ages in primates and humans are needed.

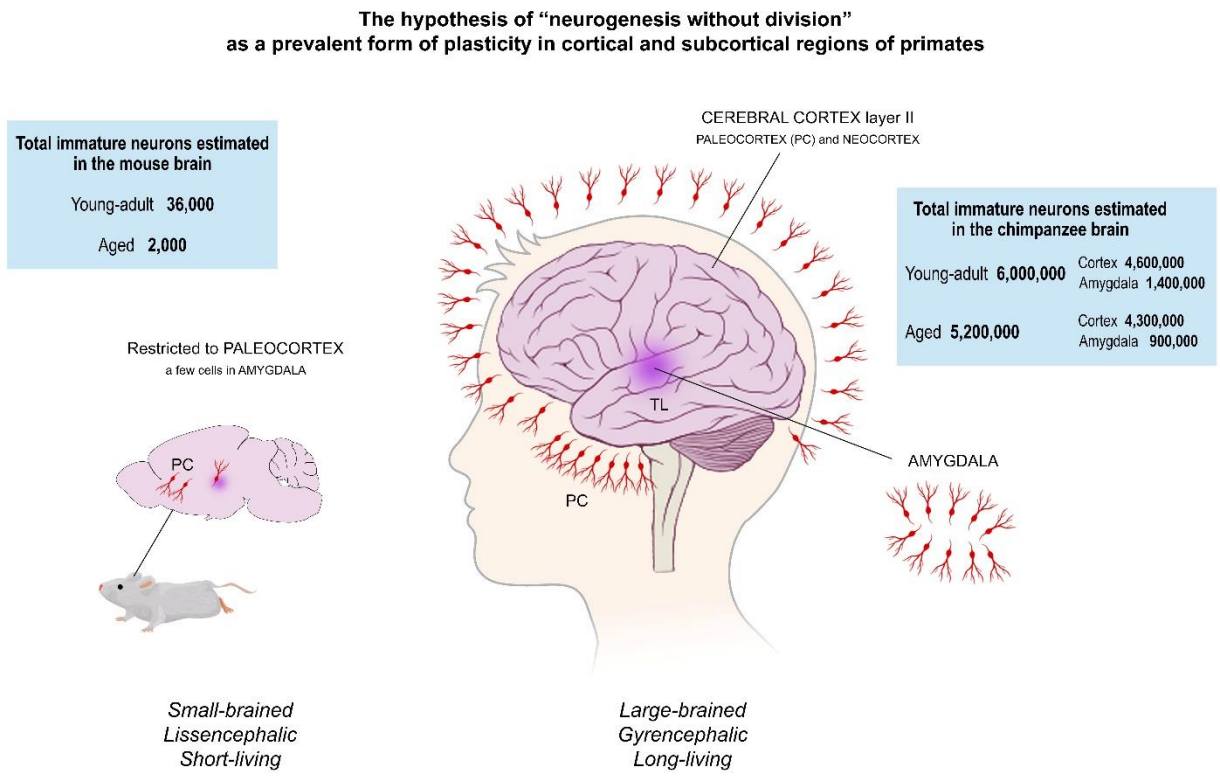


Figure 5.6 – The existence of a trade-off between stem cell-driven neurogenesis and non-dividing "immature" neurons (INs) in mammals differing for their brain size, gyrencephaly, and lifespan, with high prevalence of INs in primates (see Figure 5.3), does open the hypothesis for a wide reserve of "neurogenesis without division" in cortical and subcortical regions of these species, including the widely expanded neocortex of primates and humans. This idea, though based on solid comparative studies, should be considered at present as a hypothesis (for its current limits, see text).

A final word should be written here to underline the limits still existing in our knowledge: first, we still lack an experimental proof that the DCX+, immature cells described in the neocortex of multiple species actually correspond to prenatally generated cells (among gyrencephalic species we have such proof only in sheep; Piumatti et al., 2018); second, we lack the proof that they can subsequently mature and integrate into the cortical circuits (as it has shown in the piriform cortex of transgenic mice; Rotheneichner et al., 2018; Benedetti et al., 2020). Third, it has not yet fully established the prenatal origin of the subcortical INs in large brained mammals, although some indications came from BrdU experiments carried out in pregnant sheep (Piumatti et al., 2018) and the absence of coexpression with proliferating cell markers described in this thesis also supports this idea.

5.7 Possible fall-out on translation

Biomedical research, including the neurosciences, is largely conducted on laboratory animal models, mostly mice and rats (Brenowitz and Zakon, 2015; Bolker, 2012,2017; Faykoo-Martinez et al., 2017; La Rosa and Bonfanti, 2018; Cozzi et al., 2020). Comparative studies using different mammalian species represent a small fraction of current research, although interest in the neurobiology of non-rodent mammals, including large-brained species and humans, has been increasing (Figure 5.7). One of the reasons surely is due to recent findings highlighting remarkable interspecies differences in the occurrence, extension, and rate of neural plastic processes (this thesis). Comparative studies can help to better understand the possible trade-off that occurred during evolution between different types of plasticity, thus providing a more comprehensive picture of these processes in mammals to avoid confusion and misinterpretation coming from the exclusive use of rodents as animal models (Lipp and Bonfanti, 2016; Faykoo-Martinez et al., 2017). Do these differences have consequences for cognition, learning, capacity to recover from injury, or some other function? According to Jessica Bolker (2012) “disparities between mice and humans may help to explain why the millions of dollars spent on basic research have yielded frustratingly few clinical advances”. Now we know that disparities between reparative and physiological (homeostatic) plasticity, as well as between adult neurogenesis and “immature” (dormant) neurons, may contribute to explain difficulties in translation. These aspects might explain the general failure of most clinical trials approved for testing therapeutic approaches for neurological disorders and based on stem cells/regenerative medicine. Out of nearly 500 trials activated during the last 30 years for the most common neurological disorders (injuries of brain, spinal cord, and peripheral nerves, stroke, multiple sclerosis, and brain tumors), no substantial

results led to final approval of the stem cell treatments tested. By analysing the data, some points repeatedly emerge: a low clinical efficacy in most cases, also described as “a gap between pre-clinical studies with positive results and clinical trials with the shortage of efficacy”, “there is a fairly noticeable gap between preclinical studies and actual clinical trials“ and “it is common that results that were obtained at the preclinical stages do not correspond to those achieved in clinics” (Namiot et al., 2022).

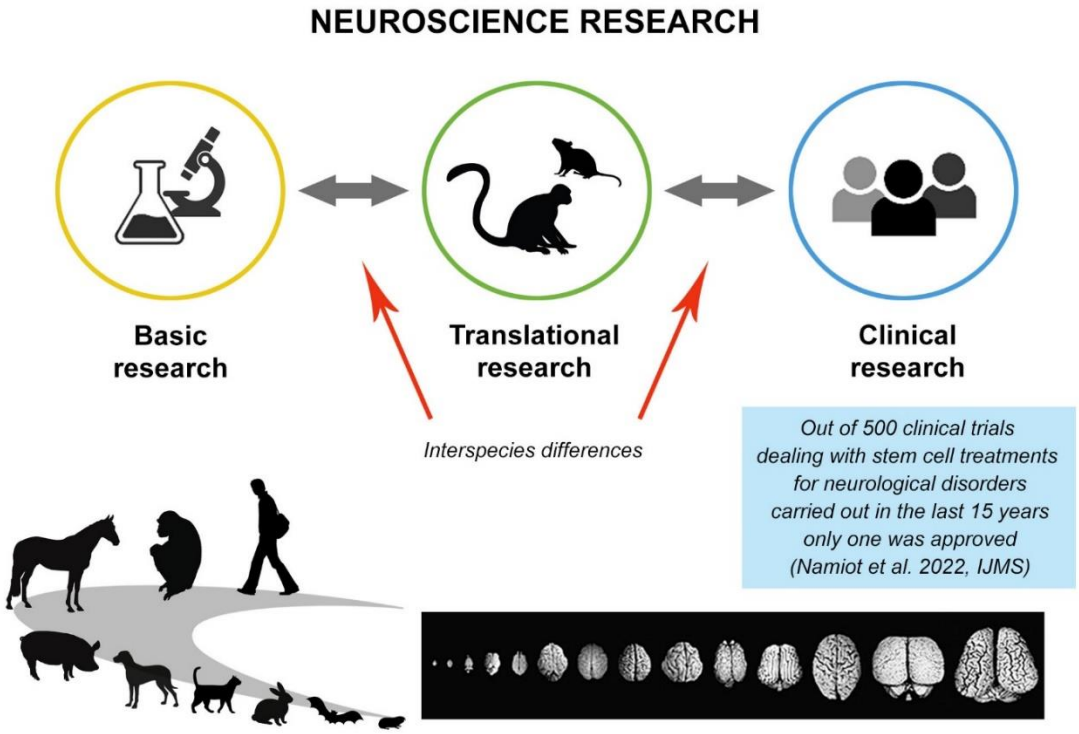


Figure 5.7 – Translational implications. Basic research is conducted mostly on laboratory rodents, then results are expected to be translated to humans. In the last few years, several studies started to address the differences existing between rodents and humans in the neurosciences, concerning neuroanatomy, neurophysiology, and neural plasticity. In this thesis some aspects concerning the neurogenic processes were addressed, particularly considering their two forms (stem cell-driven adult neurogenesis and non-dividing “immature” neurons) and the possible trade-off between them. The remarkable differences that are emerging from the studies in comparative neuroplasticity may explain the high level of failure of clinical trials aimed at assessing the efficacy of stem cell treatment for neurological disorders (regenerative medicine), since most trials are based on results obtained in animal models, mostly mice (see Namiot et al., 2022; but also, Bolker, 2012,2017; Brenowitz and Zakon, 2015; Faykoo-Martinez et al., 2017; La Rosa and Bonfanti, 2018; Cozzi et al., 2020).

In our view, the current situation strongly suggest that the problem resides somewhere in the basic research. The fact that clinical trials are frequently approved based on preclinical data which are mostly obtained on laboratory rodents, might be a logic explanation in view of the remarkable differences existing between mice and humans in terms of brain plasticity (differences that have started to be viewed only in recent years).
Whatever the evolutionary reason, the differences in brain structural plasticity among animal species do exist, are remarkable, and indicate a gain in widespread adaptive plasticity at the expenses of loss in reparative and regenerative capability across species. This diversity may potentially frustrate therapeutic translation from animal models, but there is reason to be optimistic that the comparative perspective will bring exciting breakthroughs in our understanding of the role of plasticity in driving postnatal brain development and maintaining a healthy and efficient brain throughout life.

5.8 Future perspectives

The topic of "immature" or "dormant" neurons (INs) is still at its beginnings. Despite recent insights obtained concerning their prenatal origin (in rodents and sheep), ability for awakening, maturation, full integration during adulthood (in transgenic mice), and phylogenetic variation in mammals (La Rosa et al., 2020 and this thesis), most fundamental questions remain unanswered. The present thesis, by adding some other small pieces in the puzzle, confirmed that INs might have important roles in large-brained, long-living mammals, opening new translational perspectives.

Here, following, are some examples of unresolved issues:

a) Since INs can remain in a "dormant" state for a long time, future studies should primarily focus on the cellular mechanisms underlying the blocking of their maturational process (likely very early during embryogenesis), and then those responsible for their awakening. This might be achieved by studying transgenic models in which genes known to be expressed by INs are downregulated (an example comes from the study by Coviello et al., which performed PSA-NCAM depletion in the mouse piriform cortex), or by performing selective lesions in brain regions known to be connected to the cortex and the amygdala, to understand whether they may exert a "control" over their immature state.

b) While cINs in the mouse piriform cortex can complete their maturation and functionally integrate into the brain circuitry, it is far from clear whether the same happens for neocortical INs of gyrencephalic mammals, and for amygdalar INs. While it is likely that cortical cells do behave

similarly both in paleo and neocortex, the substantial stabilization of high numbers of INs during adult and aged stages would suggest other outcomes. Thus, future studies should consider that, beside common mechanisms (e.g., quiescence and awakening), some functional aspects (fate, outcome, physiological function, reaction to lesion) might differ in different IN cell populations, depending on the brain region.

c) Another important and unanswered question concerns the existence/absence of synaptic contacts between INs and the surrounding neuropil. Preliminary data published by Gomez-Climent et al. (2008) using electron microscopy, suggest that mouse cINs in the piriform cortex have only a few synapses on their surface (if not at all, the number/occurrence of synapses likely depending on the degree of immaturity of the cell type considered), what is reasonable for a "dormant", non-active neuron. Yet, this situation seems to defy one of the last existing dogmas in the neurosciences: the fact that neurons which cannot establish contacts, and thus "being not working", are fated to die. Hence, the question is: which strategies have adopted the INs to remain alive for long periods (even years) in their unconnected state? In some ultrastructural images, Gomez-Climent et al. showed that IN cell membranes appear to be ensheathed by astrocytic lamellae. Do the astrocytes play a role in the "isolation" of dormant neurons from the mature neuropil? Are there special astrocytic cells in the cortical layer II? What is the pattern of synapses regarding the subcortical INs?

d) An obvious question also concerns the functional role these neurons can play in the cortical or amygdalar neural circuits. Their fate is strictly linked to this aspect. Which kind of neurons they become after integrating in the preexisting circuits? At present there are data suggesting they become glutamatergic excitatory neurons (Rotheneichner et al., 2018; Sorrells et al., 2019; Alderman et al., 2022).[4] Nevertheless, as described in other systems (Luzzati et al., 2014), they might have a transient existence, especially in the amygdala, in relation to point (b).

e) Under a comparative profile, widening the analysis to other species would be useful to confirm/explore phylogenetic trends adopted in mammals. Future studies should include a high number of large-brained species, possibly using the same sampling procedures, quantification method, and taking into consideration the suggestions for the detection of DCX and Ki67-antigen (Ghibaudi et al., 2023a; Chapter 2 of this thesis). As to the specific experimental plan of this PhD thesis, the work will be implemented by adding horse brains (to investigate a large-brained mammal) and the quantitative analysis on the claustrum of all species. Of course, studies in human

[4] In our analysis we did not yet address this issue, and this is surely a limit of our work.

brains are also needed. At present we know the presence of INs in most of the human cerebral cortex and in the amygdala, at different ages (Sorrells et al., 2019; Coviello et al., 2022; Li et al., 2023), though we do not have comparable data on their density with respect to other species. Although some authors do believe that research should be performed only on mice and humans, the knowledge accumulated from phylogenetically different species can give a vision of how specific biological processes or cell populations changed/adapted during evolution. Such knowledge can be important for correctly interpreting mouse and human data, for understanding the nervous systems as a transformative event during animal evolution, and for driving/instructing future research.
In this context, it is worth mentioning that future comparative studies of the INs should be extended to transcriptomic analyses aimed at profiling cell types, thus going beyond the limits of the immuncytochemical marker detection.

f) In theory, the persistence of a reservoir of INs at later ages in gyrencephalic mammals might be enriched by processes of dematuration or astrocyte dedifferentiation, namely biological transformations that have been shown to occur after tissue inflammation, neuronal hyperexcitation, or lesion (Hagihara et al., 2019).

g) Both in cortex and amygdala, it would be worth investigating whether such a plastic reserve of immature cells can be modulated in different conditions/by different stimuli, possibly starting from those which have been recognized to affect adult neurogenesis (e.g., physical activity, enriched environment, stress, lesion, inflammation, or specific pathological states). Such a modulation would likely affect the speed/slowness of IN maturation and/or elicit/block their awakening. Nevertheless, in the current absence of data, it is far from clear what is better for the brain: either to use as early as possible all INs available to appropriately refine the growing brain, or to keep a reserve on these cells in view of later ages?

In conclusion, the study of INs is at its dawn. It could represent an emerging field in brain structural plasticity, useful to complete the picture of neurogenic process heterogeneity, to unravel possibilities to exploit these cells for translational purposes, and to provide explanations for currently unresolved issues.

REFERENCES

Abrous DN, Montaron MF, Petry KG, Rougon G, Darnaudery 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.

Aboitiz F, Morales D, Montiel J (2003) The evolutionary origin of the mammalian isocortex: towards an integrated developmental and functional approach. *Behav Brain Sci* 26: 535–552.

Aboitiz F (2011) Genetic and developmental homology in amniote brains. Toward conciliating radical views of brain evolution. *Brain Res Bull* 84: 125–136.

Aboitiz F, Zamorano F (2013) Neural progenitors, patterning and ecology in neocortical origins. *Front Neuroanat* 7:38.

Aboitiz F, Montiel JF (2015) Olfaction, navigation, and the origin of isocortex. *Front Neurosci* 9: 402.

Ai J, Luo R, Tu T, Yang C, Jiang J, Zhang B, Bi R, Tu E, Yao Y, Yan X (2021) Doublecortin-expressing neurons in chinese tree shrew forebrain exhibit mixed rodent and primate-like topographic characteristics. *Front Neuroanat* 15:727883.

Aimone JB, Li Y, Lee SW, Clemenson GD, Deng W, Gage FH (2014) Regulation and function of adult neurogenesis: from genes to cognition. *Physiol Rev* 94, 1991-1026.

Akter M, Kaneko N, Herranz-Pérez V, Nakamura S, Oishi H, Garcìa-Verdugo JM, Sawamoto K (2020) Dynamic changes in the neurogenic potential in the ventricu-lar-subventricular zone of common marmoset during postnatal brain development. *Cereb Cortex* 30(7):4092-4109.

Alderman PJ, Saxon D, Torrijos-Saiz LI, Sharief M, Biagiotti SW, Page CE, Melamed A, Kuo CT, Garcia-Verdugo J-M, Herranz-Perez V et al. (2022) Mouse paralaminar amygdala excitatory neurons migrate and mature during adolescence. *bioRxiv* 2022.09.23.509244.

Allen, KM, Fung SJ, Weickert CS (2016) Cell proliferation is reduced in the hippocampus in schizophrenia. *Aust N Z J Psychiatry* 50(5):473-80.

Altman J., Das G.D. (1965) Post-natal origin of microneurones in the rat brain. *Nature* 207, 953–956.

Alunni A, Bally-Cuif L (2016) A comparative view of regenerative neurogenesis in vertebrates. Development 143:741-753

Alvarez-Buylla A, García-Verdugo JM, Tramontin AD (2001) A unified hypothesis on the lineage of neural stem cells. *Nat Rev Neurosci* 2(4):287-293.

Ampofo E, Schmitt BM, Menger MD, Lashke MW (2017) The regulatory mechanisms of NG2/CSPG4 expression. *Cell Mol Biol Lett* 22: 4.

Amrein I, Slomianka L, Poletaeva II, Bologova NV, Lipp HP (2004) Marked species and age-dependent differences in cell proliferation and neurogenesis in the hippocampus of wild-living rodents. *Hippocampus* 14, 1000-1010.

Amrein I, Dechmann DK, Winter Y, Lipp HP (2007) Absent or low rate of adult neurogenesis in the hippocampus of bats (Chiroptera). *PLoS One* 2(5):e455.

Amrein I (2015) Adult hippocampal neurogenesis in natural populations of mammals. *Cold Spring Harb Perspect Biol* 7(5). pii: a021295.

Aniol VA (2016) The effects of prolonged formalin fixation on immunohistochemical staining of PCNA and Dcx proteins in the mouse hippocampus. *Neurochem J* 10, 244–247.

Arellano JI, Harding B, Thomas JL (2018) Adult human hippocampus: no new neurons in sight. *Cereb Cortex* 28(7):2479-2481.

Avino TA, Barger N, Vargas MV, Carlson EL, Amaral DG, Bauman MD, Schumann CM (2018) Neuron numbers increase in the human amygdala from birth to adulthood, but not in autism. *Proc Natl Acad Sci U S A* 115(14):3710-3715.

Azad N, Cabeen RT, Sepehrband F, Kim R, Campbell CE, Lynch K, Tyszka JM, Herting MM (2021) Microstructural properties within the amygdala and affiliated white matter tracts across adolescence. *Neuroimage* 243:118489.

Bao H, Song J (2018) Treating brain disorders by targeting adult neural stem cells. *Trends Mol Med* pii: S1471-4914(18)30187-4.

Barger N, Stefanacci L, Schumann CM, Sherwood CC, Annese J, Allman JM, Buckwalter JA, Hof PR, Semendeferi K (2012) Neuronal populations in the basolateral nuclei of the amygdala are differentially increased in humans compared with apes: A stereological study. *J Comp Neurol* 520(13): 3035–3054.

Barker JM, Boonstra R, Wojtowicz JM (2011) From pattern to purpose: How comparative studies contribute to understanding the function of adult neurogenesis. *Eur J Neurosci* 34, 963-977

Barker RA, Götz M, Parmar M (2018) New approaches for brain repair - from rescue to reprogramming. *Nature* 557: 329-334.

Batailler M, Droguerre M, Baroncini M, Fontaine C, Prevot V, Migaud M (2013) DCX-expressing cells in the vicinity of the hypothalamic neurogenic niche: A comparative study between mouse, sheep, and human tissues. J Comp Neurol 522(8):1966-85.

Bauernfeind AL, Babbitt CC (2020) Metabolic changes in human brain evolution. *Evol Anthropol* 29:201-211

Ben Abdallah NM, Slomianka L, Vyssotski AL, Lipp HP (2010) Early age-related changes in adult hippocampal neurogenesis in C57 mice. *Neurobiol Aging* 31: 151-161.

Benedetti B, Dannehl D, König R, Coviello S, Kreutzer C, Zaunmair P, Jakubecova D, Weiger TM, Aigner L, Nacher J, Engelhardt M, Couillard-Després S (2020) Functional integration of neuronal precursors in the adult murine piriform cortex. *Cereb Cortex* 30(3): 1499-1515.

Benedetti B, Couillard-Després S (2022) Why would the brain need dormant neuronal precursors? *Front Neurosci* 16:877167

Berardi N, Sale A, Maffei L (2015) Brain structural and functional development: genetics and experience. *Dev Med Child Neurol* 57 Suppl 2:4-9.

Bergmann O, Spalding KL, Frisén J (2015) Adult Neurogenesis in Humans. *Cold Spring Harb Perspect Biol* 7(7):a018994.

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.

Bianchi DW, Cooper JA, Gordon JA, Heemskerk J, Hodes R, Koob GF, Koroshetz WJ, Shurtleff D, Sieving PA, Volkow ND, Churchill JD, Ramos KM (2018) Neuro-ethics for the national institutes of health BRAIN initiative. *J Neurosci* 38, 10583–10585.

Bloch J, Kaeser M, Sadeghi Y, Rouiller EM, Redmond DE Jr, Brunet JF (2011) Doublecortin-positive cells in the adult primate cerebral cortex and possible role in brain plasticity and development. *J Comp Neurol* 519, 775–789.

Boda E, Buffo A (2010) Glial cells in non-germinal territories: insights into their stem/progenitor properties in the intact and injured nervous tissue. *Arch Ital Biol* 148(2):119-136.

Boda E, Di Maria S, Rosa P, Taylor V, Abbracchio MP, Buffo A (2015) Early phenotypic asymmetry of sister oligodendrocyte progenitor cells after mitosis and its modulation by aging and extrinsic factors. *Glia* 63(2):271-86.

Boekhoorn K, Joels M, Lucassen PJ (2006) Increased proliferation reflects glial and vascular-associated changes, but not neurogenesis in the presenile Alzheimer hippocampus. *Neurobiol Dis* 24(1):1-14.

Bogado Lopes J, Senko AN, Bahnsen K, Geisler D, Kim E, Bernanos M, Cash D, Ehrlich S, Vernon AC, and Kempermann G (2023) Individual behavioral trajectories shape whole-brain connectivity in mice. *eLife* 16;12:e80379.

Bolker JA (2012) There's more to life than rats and flies. *Nature* 491, 31-33.

Bolker JA (2017) Animal models in translational research: Rosetta stone or stumbling block? *Bioessays* 39(12).

Bond AM, Ming G, Song H (2015) Adult mammalian neural stem cells and neurogenesis: five decades later. *Cell Stem Cell* 17:385-395.

Bond AM, Ming Gl, and Song H. (2022) What is the relationship between hippocampal neurogenesis across different stages of the lifespan? *Front Neurosci* 16:891713.

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(2):419-436.

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.

Bonfanti L (2006) PSA-NCAM in mammalian structural plasticity and neurogenesis. *Prog Neurobiol* 80, 129–164.

Bonfanti L, Peretto P (2007) Radial glial origin of the adult neural stem cells in the subventricular zone. *Prog Neurobiol* 83(1):24-36.

Bonfanti L, Ponti G (2008) Adult mammalian neurogenesis and the New Zealand white rabbit. *Vet J* 175, 310–331.

Bonfanti L, Theodosis DT (2009) Polysialic acid and activity-dependent synapse remodeling. *Cell Adh Migr* 3(1):43-50.

Bonfanti L (2011) From hydra regeneration to human brain structural plasticity: a long trip through narrowing roads. *ScientificWorldJournal* 11, pp.1270–1299.

Bonfanti L (2016) Adult neurogenesis 50 years later: Limits and opportunities in mammals. *Front. Neurosci.* 10:44

Bonfanti L, Peretto P (2011) Adult neurogenesis in mammals - a theme with many variations. *Eur J Neurosci* 34(6), 930–950.

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), 1–15.

Bonfanti L (2013) The (Real) neurogenic/gliogenic potential of the postnatal and adult brain parenchyma. *ISRN Neuroscience* 2013:354136.

Bonfanti L (2016) Adult Neurogenesis 50 Years Later: Limits and Opportunities in Mammals. *Front Neurosci* 10:44.

Bonfanti L, Amrein I (2018) Editorial: Adult neurogenesis: Beyond rats and mice. *Front Neurosci* 12:904.

Bonfanti L, Charvet CJ (2021) Brain plasticity in humans and model systems: Advances, challenges, and future directions. *Int J Mol Sci* 22, 9358.

Bonfanti L, Seki T (2021) The PSA-NCAM-positive "immature" neurons: An old discovery providingnew vistas on brain structural plasticity. *Cells* 10, 2542.

Bonfanti L, La Rosa C, Ghibaudi M, Sherwood CC (2023) Adult neurogenesis and "immature" neurons in mammals: An evolutionary trade-off in plasticity? *Brain Struct Funct* (epub).

Braak H, Braak E (1982) Neuronal types in the claustrum of man. *Anat Embryol (Berl)* 163(4):447.

Brenowitz EA, Zakon HH (2015) Emerging from the bottleneck: benefits of the comparative approach to modern neuroscience. *Trends Neurosci* 38(5):273-278.

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.

Bruguier H, Suarez R, Manger P, Hoerder-Suabedissen A, Shelton AM, Oliver DK, Packer AM, Ferran JL, Garcìa-Moreno F, Puelles L, Molnàr Z (2020) In search of common developmental and evolutionary origin of the claustrum and subplate. *J Comp Neurol* 528(17):2956-2977.

Brus M, Meurisse M, Gheusi G, Keller M, Lledo P, Levy F (2013) Dynamics of olfactory and hippocampal neurogenesis in adult sheep. *J Comp Neurol* 521:169–188.

Bunk EC, Stelzer S, Hermann S, Schäfers S, Schlatt S, Schwamborn JC (2011) Cellular organization of adult neurogenesis in the Common Marmoset. *Aging Cell* 10(1):28-38.

Burgess HA, Reiner O (2000) Doublecortin-like kinase is associated with microtubules in neuronal growth cones. *Mol Cell Neurosci* 16(5):529-541.

Burgin CJ, Collela JP, Kahn PL, Upham NS (2018) How many species of mammals are there? *J Mammal* 99:1-14

Buzsáki G (2005) Theta rhythm of navigation: link between path integration and landmark navigation, episodic and semantic memory. *Hippocampus* 15, 827-840

Cai Y, Xiong K, Chu Y, Luo DW, Luo XG, Yuan XY, Struble RG, Clough RW, Spencer DD, Williamson A, Kordower JH, Patrylo PR, Yan XX (2009) Doublecortin expression in adult cat and primate cerebral cortex relates to immature neurons that develop into GABAergic subgroups. *Exp Neurol* 216, 342–356.

Cajal RS (1928) Degeneration and regeneration of the nervous system. *Hafner*, New York, NY, USA.

Campbell CE, Mezher AF, Eckel SP, Tyszka JM, Pauli WM, Nagel BJ, Herting MM (2021) Restructuring of amygdala subregion apportion across adolescence. Dev Cogn Neurosci 48:100883.

Chareyron LJ, Amaral DG, Lavenex P (2016) Selective lesion of the hippocampus increases the differentiation of immature neurons in the monkey amygdala. *Proc Natl Acad Sci U S A* 113(50):14420-14425

Chareyron LJ, Banta Lavenex P, Amaral DG, Lavenex P (2021) Life and death of immature neurons in the juvenile and adult primate amygdala. *Int J Mol Sci* 22:6691.

Charvet CJ, Finlay BL (2018) Comparing adult hippocampal neurogenesis across species: Translating time to predict the tempo in humans. *Front Neurosci* 12:706.

Chawana R, Patzke N, Alagaili AN, Bennett NC, Mohammed OB, Kaswera-Kyamakya C, Gilissen E, Ihunwo AO, Pettigrew JD, Manger PR (2016) The distribution of Ki-67 and doublecortin immunopositive cells in the brains of three microchiropteran species, Hipposideros fuliginosus, Triaenops persicus, and Asellia tridens. *Anat Rec* 299(11):1548-1560.

Chen JL, Nedivi E (2010) Neuronal structural remodeling: is it all about access? *Curr Opin Neurobiol*, 20, 557–562.

Chen Y, Chen X, Baserdem B, Zhan H, Li Y, Davis MB, Kebschull JM, Zador AM, Koulakov AA, and Albeanu DF (2022) High-throughput sequencing of single neuron projections reveals spatial organization in the olfactory cortex. *Cell* 185(22):4117-4134.e28.

Ciarpella F, Zamfir RG, Campanelli A, Ren E, Pedrotti G, Bottani E, Borioli A, Caron D, Di Chio M, Dolci S, Ahtiainen A, Malpeli G, Malerba G, Bardoni R, Fumagalli G, Hyttinen J, Bifari F, Palazzolo G, Panuccio G, Curia G, Decimo I (2021) Murine cerebral organoids develop network of functional neurons and hippocampal brain region identity. *iScience* 24(12):103438.

Cipriani S, Ferrer A, Arinica I, Kovacs G, Verney C, Nardelli J, Khung S, Delezoide AL, Milenkovic I, Rasika S, Manivet P, Benifla JL, Deriot N, Gressens P, Adle-Biassette H (2018) Hippocampal radial glial subtypes and their neurogenic potential in human fetuses and healthy and Alzheimer disease adults. *Cereb Cortex* 28: 2458-2478.

Citri A, Malenka RC (2008) Synaptic plasticity: multiple forms, functions, and mechanisms. *Neuropsychopharmacology* 33(1): 18-41.

Coviello S, Benedetti B, Jakubecova D, Belles M, Klimczak P, Gramuntell Y, Couillard-Despres S, and Nacher J (2021) PSA depletion induces the differentiation of immature neurons in the piriform cortex of adult mice. *Int J Mol Sci 22*, 5733.

Coviello S, Gramuntell Y, Klimczak P, Varea E, Blasco-Ibañez JM, Crespo C, Gutierrez A, Nacher J (2022) Phenotype and distribution of immature neurons in the human cerebral cortex layer II. *Front Neuroanat* 16, 851432.

Cozzi B, Bonfanti L, Canali E, Minero M (2020) Brain waste: The neglect of animal brains. *Front Neuroanat* 14: 573934.

Crews L, Adame A, Patrick C, DeLaney A, Pham E, Rockenstein E, Hansen L, Masliah E (2010) Increased BMP6 levels in the brains of Alzheimer's disease patients and APP transgenic mice are accompanied by impaired neurogenesis. *J Neurosci* 30(37):12252-62.

Crick FC, Koch C (2005) What is the function of the claustrum? *Phil Trans R Soc B* 360, 1271-1279.

Crociara P, Parolisi R, Conte D, Fumagalli M, Bonfanti L (2013) Cellular and molecular characterization of multipolar Map5-expressing cells: a subset of newly generated, stage-specific parenchymal cells in the mammalian central nervous system. *PLoS One* 7;8(5):e63258.

Cushman JD, Drew MR, Krasne FB (2021) The environmental sculpting hypothesis of juvenile and adult hippocampal neurogenesis. *Prog Neurobiol* 199:101961.

Davidson RJ, McEwen BS (2012) Social influences on neuroplasticity: stress and interventions to promote well-being. *Nat Neurosci* 15(5):689-95.

De Campo DM, Fudge JL (2012) Where and what is the paralaminar nucleus? A review on a unique and frequently overlooked area of the primate amygdala. *Neurosci Biobehav Rev* 36: 520-535.

De Olmos JS, Beltramino CA, Alheid G (2004) Amygdala and extended amygdala of the rat: A cytoarchitectonical, fibroarchitectonical, and chemoarchitectonical survey. *Chapter 19 in "The rat nervous system", G. Paxinos Elsevier Academic Press (2004),* pp. 509-603.

Deep-Soboslay A, Akil M, Martin CE, Bigelow LB, Herman MM, Hyde TM, Klein-man JE (2005) Reliability of psychiatric diagnosis in postmortem research. *Biol Psychiatry* 57(1):96-101

Dennis CV, Suh LS, Rodriguez ML, Kril JJ, Sutherland GT (2016) Human adult neurogenesis across the ages: An immunohistochemical study. *Neuropathol Appl Neurobiol* 42(7):621-638.

Doetsch F, Caillé I, Lim DA, García-Verdugo JM, Alvarez-Buylla A (1999) Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* 97(6):703-16.

Duque A, Arellano JI, Rakic P (2022) An assessment of the existence of adult neurogenesis in humans and value of its rodent models for neuropsychiatric diseases. *Mol Psychiatr* 27(1):377-382.

Duque A, Spector R (2019) A balanced evaluation of the evidence for adult neurogenesis in humans: implication for neuropsychiatric disorders. *Brain Struct Funct* 224: 2281-2295.

Eichenbaum H (2014) Time cells in the hippocampus: a new dimension for mapping memories. *Nat Rev Neurosci* 15,732-744.

Ekonomou A, Savva GM, Brayne C, Forster G, Francis PT, Johnson M, Perry EK, At-tems J, Somani A, Minger SL, Ballared CG (2015) Stage-specific changes in neurogenic and gli-al markers in Alzheimer's disease. *Biol Psychiatry* 77(8):711-9.

Encinas JM, Michurina TV, Peunova N, Park J-H, Tordo J, Peterson DA, Fishell G, Koulakov A, Enikolopov G (2011) Division-coupled astrocytic differentiation and age-related depletion of neural stem cells in the adult hippocampus. *Cell Stem Cell* 8, 566-579.

Englund M, Krubitzer L (2022) Phenotypic alterations in cortical organization and connectivity across different time scales. *Brain Behav Evol* 97: 108-120.

Fahrner A, Kann G, Flubacher A, Heinrich C, Freiman TM, Zentner J, Frotscher M, Haas CA (2007) Granule cell dispersion is not accompanied by enhanced neurogenesis in tem-poral lobe epilepsy patients. *Exp Neurol* 203(2):320-32.

Falcone C, Wolf-Ochoa M, Amina S, Hong T, Vakilzadeh G, Hopkins WD, Hof PR, Sherwood CC, Manger PR, Noctor SC, Martínez-Cerdeño V (2019) Cortical interlaminar astrocytes across the the-rian mammal radiation. *J Comp Neurol* 1–21.

Fasemore TM, Patzke N, Kaswera-Kyamakya C, Gilissen E, Manger PR, Ihunwo AO (2018) The distribution of Ki-67 and doublecortin-immunopositive cells in the brains of three strepsirrhine primates: Galago demidoff, Perodicticus potto, and Lemur catta. *Neuroscience* 372:46-57.

Faykoo-Martinez M, Toor I and Holmes MM (2017) Solving the neurogenesis puzzle: looking for pieces outside the traditional box. *Front Neurosci* 11: 505.

Feliciano DM, Bordey A, Bonfanti L (2015) Noncanonical sites of adult neurogenesis in the mammalian brain. Cold Spring Harb Perspect Biol 18;7(10):a018846.

Finlay BL, Darlington RB (1995) Linked regularities in the development and evolution of mammalian brains. *Science* 268(5217):1578-84.

Flor-García M, Terreros-Roncal J, Moreno-Jimènez EP, Avila J, Ràbano A, Llores-Martìn M (2020) Unraveling human adult hippocampal neurogenesis. *Nat Protoc* 15(2):668-693.

Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, McConnell SK, Berwald-Netter Y, Denoulet P, Chelly J (1999) Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron* 23, 247–256.

Franjic D, Skarica M, Ma S, Arellano JI, Tebbenkamp ATN, Choi J, Xu C, Li Q, Morozov YM, Andrijevic D, Vrselja Z, Spajic A, Santpere G, Li M, Zhang S, Liu Y, Spurrier J, Zhang L, Gudelj I, Rapan L, Takahashi H, Huttner A, Fan R, Strittmatter SM, Sousa AMM, Rakic P, Sestan N (2022) Transcriptomic taxonomy and neurogenic trajectories of adult human, macaque, and pig hippocampal and entorhinal cells. *Neuron* 110(3):452-469.e14.

Frotscher M (1992) Specificity of interneuronal connections. *Ann. Anat.* 174, 377-382.

Fung SJ, Joshi D, Allen KM, Sivagnanasundaram S, Rothmond DA, Saunders R, Noble PL, Webster MJ, Shannon Weickert CS (2011) Developmental patterns of doublecortin expression and white matter neuron density in the postnatal primate prefrontal cortex and schizophrenia. *PLoS One* 6(9):e25194.

Galàn L, Gòmez-Pinedo U, Guerrero A, Garcìa-Verdugo JM, Matìas-Guiu J (2017) Amyo-trophic lateral sclerosis modifies progenitor neural proliferation in adult classic neurogenic brain niches. *BMC Neurol* 17(1):173.

Ganz J, Brand M (2016) Adult neurogenesis in fish. *Cold Spring Harb Perspect Biol* 8(7).

Gatome CW, Slomianka L, Mwangi DK, Lipp HP, Amrein I (2010) The entorhinal cortex of the Megachiroptera: a comparative study of Wahlberg's epauletted fruit bat and the straw-coloured fruit bat. *Brain Struct Funct* 214(4):375-393.

Ghibaudi M, Amenta A, Agosti M, Riva M, Graïc J-M, Bifari F, Bonfanti L (2023a) Consistency and variation in doublecortin and Ki67 antigen detection in the brain tissue of different mammals, including humans. *Int J Mol Sci* 24, 2514.

Ghibaudi M, Bonfanti L (2022) How widespread are the "young" neurons of the mammalian brain? *Front Neurosci* 16:918616.

Ghibaudi M, Marchetti N, Vergnano E, La Rosa C, Benedetti B, Couillard-Despres S, Farioli-Vecchioli S and Bonfanti L (2023b) Age-related changes in layer II immature neurons of the murine piriform cortex. *Front Cell Neurosci* 17:1205173.

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, Nacher J (2008) A population of prenatally generated cells in the rat paleocortex maintains an immature neuronal pheno- type into adulthood. *Cereb Cortex* 18, 2229–2240.

Gómez-Climent MA, Guirado R, Castillo-Gomez E, Varea E, Gutierrez-Mecinas M, Gilabert-Juan J, Garcìa-Mompò C, Vidueira S, Sanchez-Mataredona D, Hernàndez S, Blasco-Ibanez J.M, Crespo C, Rutishauser U, Schachner M, Nacher J (2011) The polysialylated form of the Neural Cell Adhesion Molecule (PSA-NCAM) is expressed in a subpopulation of mature cortical interneurons characterized by reduced structural features and connectivity. *Cereb Cortex* 21, 1028-1041.

Gomez-Nicola D, Suzzi S, Vargas-Caballero M, Fransen NL, Al-Malki H, Cebrian-Silla A, Garcia-Verdugo JM, Riecken K, Fehse B, Perry VH (2014) Temporal dynamics of hippocampal neurogenesis in chronic neurodegeneration. *Brain* 137(Pt 8):2312-28.

Gonçalves JT, Schafer ST, Gage FH (2016) Adult neurogenesis in the hippocampus: from stem cells to behavior. *Cell* 167(4):897-914.

Götz M, Nakafuku M, Petrik D (2016) Neurogenesis in the developing and adult brain similarities and key differences. *Cold Spring Harb Perspect Biol* 8(7).

Grzimek B (1990) General Introduction. In: B Grzimek ed. *Grzimek's Encyclopedia of Mammals*, Vol. 1, 1st Edition. Pp. 4-5. New York: Mcgraw-Hill.

Grady C (2012) The cognitive neuroscience of ageing. *Nat Rev Neurosci* 13(7):491-505.

Gritti A, Parati EA, Cova L, Frolichsthal P, Galli R, Wanke E, Faravelli L, Morassutti DJ, Roisen F, Nickel DD, Vescovi AL (1996) Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J Neurosci* 16:1091–1100.

Gusel'nikova VV, Korzhevskiy DE (2015) Neun as a neuronal nuclear antigen and neuron differentiation marker. *Acta Naturae* 7(2):42-47.

Hagihara H, Murano T, Ohira K, Miwa M, Nakamura K, Miyakawa T (2019) Expression of progenitor cell/immature neuron markers does not present definitive evidence for adult neurogenesis. *Mol Brain*, 12, 108.

Hauser T, Klaus F, Lipp HP, Amrein I (2009) No effect of running and laboratory housing on adult hippocampal neurogenesis in wild caught long-tailed woodmouse. *BMC Neurosci*. 10:43.

Heldstab SA, Isler K, Graber SM, Schuppli C, van Schaik CP (2022) The economics of brain size evolution in vertebrates. *Curr Biol* 32(12): R697-R708.

Herculano-Houzel S, Catania K, Manger PR, Kaas JH (2015) Mammalian brains are made of these: A dataset of the numbers and densities of neuronal and nonneuronal cells in the brain of Glires, Primates, Scandentia, Eulipotyphlans, Afrotherians and Artiodactyls, and their relationship with body mass. *Brain Behav Evol* 86(3-4):145-63.

Hitchman E, Hodgkinson C, Roberts D, Ashton G, Yunus Z, Byers R, Ward T, Womack C, Dive C (2011) Effect of prolonged formalin fixation on immunohistochemical staining for the proliferation marker Ki67. *Histopathology* 59(6):1261-3.

Highet B, Anekal PV, Ryan B, Murray H, Coppieters N, Dieriks BV, Singh-Bains MK, Mehrabi NF, Faull RLM, Dragunow M, Curtis MA (2021) fISHing with immunohistochemis-try for housekeeping gene changes in Alzheimer's disease using an automated quantitative analysis workflow. *J Neurochem* 157(4):1270-1283.

Hoffman S, Edelman, GM (1983) Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc Natl Acad Sci USA* 80, 5762–5766.

Holtmaat A, Svoboda K (2009) Experience-dependent structural synaptic plasticity in the mammalian brain. *Nat Rev Neurosci* 10(9):647-658.

Horner PJ, Power AE, Kempermann G, Kuhn HG, Palmer TD, Winkler J, Gage FH (2000) Proliferation and differentiation of progenitor cells throughout the intact adult rat spinal cord. *J Neurosci*, 20(6):2218-2228.

Huang S, Slomianka L, Farmer AJ, Kharlamova AV, Gulevich RG, Herbeck YE, Trut LN, Wolfer DP, Amrein I (2015) Selection for tameness, a key behavioral trait of domestication, increases adult hippocampal neurogenesis in foxes. *Hippocampus* 25(8):963-975.

Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R (2008) Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. *Nat Neurosci* 11(10):1153-1161.

Inta D, Alfonso J, Von Engelhardt J, Kreuzberg MM, Meyer AH, Van Hooft JA, Monyer H (2008) Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone. *Proc Natl Acad Sci USA* 105(52):20994-20999.

Jackson J, Smith JB, Lee AK (2020) The Anatomy and Physiology of Claustrum-Cortex Interactions. *Annu Rev Neurosci* 43:231-247.

Jhaveri D, Tedoldi A, Hunt S, Sullivan R, Watts N, Power J et al (2018) Evidence for newly generated interneurons in the basolateral amygdala of adult mice. *Mol Psychiatr* 23, 521-532.
Jin K, Wang X, Xie L, Mao X, Zhu W, Wang Y, Shen J, Mao Y, Banwait S, Greenberg, DA (2006) Evidence for stroke-induced neurogenesis in the human brain. *Proc Natl Acad Sci U S A* 103(35):13198-202.

Kaas JH (2019) The origin and evolution of neocortex: From early mammals to modern humans. *Prog Brain Res* 250: 61-81.

Kast RJ & Levitt P (2019) Precision in the development of neocortical architecture : From progenitors to cortical networks. *Prog Neurobiol* 175, 77–95.

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* 30;115(1):97-105.

Keifer J, Summers CH (2016) Putting the "biology" back into "neurobiology": The strength of diversity in animal model systems for neuroscience research. *Front Syst Neurosci* 22;10:69.

Kempermann G, Jessberger S, Steiner B, Kronenberg G (2004) Milestones of neuronal development in the adult hippocampus. *Trends Neurosci* 27(8):447-452.

Kempermann G, Song H, Gage FH (2015) Neurogenesis in the adult hippocampus. *Cold Spring Harb Perspect Biol* 7(9):a018812.

Kempermann G (2019) Environmental enrichment, new neurons and the neurobiology of individuality. *Nat Rev Neurosci* 20(4): 235-245.

Kempermann G, Bogado Lopes J, Zocher S, Schilling S, Ehret F, Garthe A, Karasinsky A, Brandmaier AM, Lindenberger U, Winter Y, Overall RW (2022) The individuality paradigm: Automated longitudinal activity tracking of large cohorts of genetically identical mice in an enriched environment. *Neurobiol Dis* 175:105916.

Kirby ED, Friedman AA, Covarrubias D, Ying C, Sun WG, Goosens KA, Sapolsky RM, Kaufer D (2021) Basolateral amygdala regulation of adult hippocampal neurogenesis and fear-related activation of newborn neurons. *Mol Psychiatry* 17(5):527-36.

Klaus F, Amrein I (2012) Running in laboratory and wild rodents: differences in context sensitivity and plasticity of hippocampal neurogenesis. *Behav Brain Res* 227, 363–370.

Klempin F, Kronenmberg 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, 1–10.

Koizumi H, Higginbotham H, Poon T, Tanaka T, Brinkman BC, Gleeson JG (2006) Doublecortin maintains bipolar shape and nuclear translocation during migration in the adult forebrain. *Nat Neurosci* 9, 779–786.

Kokoeva MV, Yin H, Flier JS (2005) Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. Science 310(5748):679-83.

König R, Benedetti B, Rotheneichner P, O' Sullivan A, Kreutzer C, Belles M, Nacher J, Weiger TM, Aigner L, 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? *Front Biol* 11:193–213.

Kornack DR, Rakic P (1999) Continuation of neurogenesis in the hippocampus of the adult macaque monkey. *Proc Natl Acad Sci USA* 96(10):5768-5773.

Kowianski P, Dziewiatkowski J, Kowianska J, Morys J (1999) Comparative anatomy of the claustrum in selected species: a morphometric analysis. *Brain Behav Evol* 53:44–54.

Knoth R, Singec I, Ditter M, Pantazis G, Capetian P, Meyer RP, Horvat V, Volk B, Kempermann G (2010) Murine features of neurogenesis in the human hippocampus across the lifespan from 0 to 100 years. *PLoS One* 5(1):e8809.

Krettek JE, Price JL (1978) A description of the amygdaloid complex in the rat and cat with observations on intra-amygdaloid axonal connections. *J Comp Neurol* 178(2):255-80

Kyritsis N, Kizil C, Zocher S, Kroehne V, Kaslin J, Freudenreich D, Iltzsche A, Brand M (2012) Acute inflammation initiates the regenerative response in the adult zebrafish brain. *Science* 338: 1353-1356.

Kuhn HG, Cooper-Kuhn CM (2007) Bromodeoxyuridine and the detection of neurogenesis. *Curr Pharm Biotech* 8, 127-131.

Kunze A, Achilles A, Keiner S, Witte OW, Redecker C (2015) Two distinct populations of doublecortin-positive cells in the perilesional zone of cortical infarcts. *BMC Neurosci* 2015, 16:20.

Küspert M, Hammer A, Bösl MR, Wegner M (2011) Olig2 regulates Sox10 expression in oligodendrocyte precursors through an evolutionary conserved distal enhancer. *Nucleic Acids Res* 39(4): 1280–1293.

La Rosa C, Parolisi R, Palazzo O, Lévy F, Meurisse M, Bonfanti L (2018) Clusters of DCX+ cells "trapped" in the subcortical white matter of early postnatal Cetartiodactyla (Tursiops truncatus, Stenella coeruloalba and Ovis aries). *Brain Struct Funct* 223: 3613-3632.

La Rosa C, Bonfanti L (2018) Brain plasticity in mammals: An example for the role of comparative medicine in the neurosciences. *Front Vet Sci* 5:274.

La Rosa C, Ghibaudi M, Bonfanti L (2019) Newly generated and non-newly generated "immature" neurons in the mammalian brain: A possible reservoir of young cells to prevent brain ageing and disease? *J Clin Med* 8, 685.

La Rosa C, Cavallo F, Pecora A, Chincarini M, Ala U, Faulkes CG, Nacher J, Cozzi B, Sherwood CC, Amrein I, Bonfanti L (2020a) Phylogenetic variation in cortical layer II immature neuron reservoir of mammals. *eLife* 9:e55456.

La Rosa C, Parolisi R, Bonfanti L (2020b) Brain structural plasticity: From adult neurogenesis to immature neurons. *Front Neurosci* 14: 75.

La Rosa C, Bonfanti L (2021) Searching for alternatives to brain regeneration. *Neural Reg Res* 16(11): 2198-2199.

LeDeoux J (2003) The emotional brain, fear, and the amygdala. Cell Mol Neurobiol 23(4-5):727-38.

Le Magueresse C, Alfonso A, Khodosevich K, Arroyo Martin AA, Bark C, Monyer H (2011) 'Small axonless neurons': postnatally generated neocortical interneurons with delayed functional maturation. *J Neurosci* 31:16731–16747.

Leuner B, Glasper ER, Mirescu C (2007) A critical time for new neurons in the adult hippocampus. *J Neurosci* 27(22):5845-6.

Leuner B, Gould E (2010) Structural plasticity and hippocampal function. *Annu Rev Psychol* 61:111-40, C1-3.

Li Y-N, Hu D-D, Cai X-L, Wang Y, Yang C, Jiang J, Zhang Q-L, Tu T, Wang X-S, Wang H, et al. (2023) Doublecortin expressing neurons in human cerebral cortex layer II and amygdala from infancy to 100 year-old. *Mol Neurobiol* 60(6):3464-3485.

Lim DA, Alvarez-Buylla A (2016) The adult ventricular-subventricular zone (V-SVZ) and olfactory bulb (OB) neurogenesis. *Cold Spring Harb Perspect Biol* 8(5).

Lindvall O, Kokaia Z (2010) Stem cells in human neurodegenerative disorders-time for clinical translation? *J Clin Invest* 120(1):29-40.

Lipp HP, Bonfanti L (2016). Adult neurogenesis in mammals: variations and confusions. *Brain Behav Evol* 87, 205-211.

Lipska BK, Deep-Soboslay A, Weickert CS, Hyde TM, Martin CE, Herman MM, Kleinman JE (2006) Critical factors in gene expression in postmortem human brain: Focus on studies in schizophrenia. *Biol Psychiatry* (60)6:650-658.

Liu YWJ, Curtis MA, Gibbons HM, Mee EW, Bergin PS, Teoh HH, Connor B, Dragunow M, Faull RLM (2008) Doublecortin expression in the normal and epileptic adult human brain. *Eur J Neurosci* (11):2254-65.

Liu RX, Ma J, Wang B, Tian T, Guo N, Liu SJ (2020) No DCX-positive neurogenesis in the cerebral cortex of the adult primate. *Neural Regen Res* 15(7):1290-1299.

Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. *Science* 264(5162):1145-1148.

Lucassen PJ, Meerlo P, Naylor AS, Van Dam AM, Dayer AG, Fuchs E, Oomen CA, Czéh B (2010) Regulation of adult neurogenesis by stress, sleep disruption, exercise and inflammation: Implications for depression and antidepressant action. *Eur Neuropsychopharmacology* 20, 1–17.

Luo J, Daniels SB, Lennington JB, Notti RQ, Conover JC (2006) The aging neurogenic subventricular zone. *Aging Cell* 5, 139-152.

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. *Proc Natl Acad Sci USA* 100:13036–13041.

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.

Luzzati F, De Marchis S, Fasolo A, Peretto P (2006) Neurogenesis in the caudate nucleus of the adult rabbit. *J Neurosci* 26: 609-621.

Luzzati F, Nato G, Oboti L, Vigna E, Rolando C, Armentano M, Bonfanti L, Fasolo A, Peretto P (2014) Quiescent neuronal progenitors are activated in the juvenile guinea pig lateral striatum and give rise to transient neurons. *Development* 141(21):4065-75.

Maheu ME, Devorak J, Freibauer A, Davoli MA, Turecki G, Mechawar N (2015) Increased doublecortin (DCX) expression and incidence of DCX-immunoreactive multipolar cells in the subventricular zone-olfactory bulb system of suicides. *Front Neuroanat* 9:74.

Maheu ME, Sharma S, King G, Maddox SA, Wingo A, Lori A, Michopoulos V, Richardson Ressler KJ (2021) Amygdala DCX and blood Cdk14 are implicated as cross-species indicators of individual differences in fear, extinction, and resilience to trauma exposure. *Mol Psychiatry* 27(2): 956–966.

Manger PR, Cort J, Ebrahim N, Goodman A, Henning J, Karolia M, Rodrigues S, StrkaljG (2008) Is 21st century neuroscience too focussed on the rat/mouse model of brain function and dysfunction? *Front Neuroanat* 12;2:5.

Makrygianni EA, Chrousos GP (2023) Neural progenitor cells and the hypothalamus. *Cells* 12(14):1822

Marlatt MW, Philippens I, Manders E, Czéh B, Marian Joels M, Harm Krugers H, et al. (2011) Distinct structural plasticity in the hippocampus and amygdala of the middle-aged common marmoset (Callithrix jacchus). *Exp Neurol* 230, 291-301.

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

Martínez-Cerdeno V, García-Moreno F, Tosches MA, Csillag A, Manger PR, Molnár Z. (2018) Update on forebrain evolution: From neurogenesis to thermogenesis. *Semin Cell Dev Biol* 76, 15-22.

Martino G, Pluchino S, Bonfanti L, Schwartz M (2011) Brain regeneration in physiology and pathology: the immune signature driving therapeutic plasticity of neural stem cells. *Physiol Rev* 91:1281-1304.

Meurisse M, Chaillou E, Lévy F (2009) Afferent and efferent connections of the cortical and medial nuclei of the amygdala in sheep. *J Chem Neuroanat* 37(2):87-97

Migaud M, Betailler M, Segura S, Duittoz A, Franceschini I, Pillon D (2010) Emerging new sites for adult neurogenesis in the mammalian brain: a comparative study between the hypothalamus and the classical neurogenic zones. Eur J Neurosci 32(12):2042-52

Ming GL, Song H (2005) Adult neurogenesis in the mammalian central nervous system. *Annu Rev Neurosci* 28:223-250.

Mobasheri A (2015) Comparative medicine in the twenty-first century: where are we now and where do we go from here? *Front Vet Sci* 2:2.

Moreno-Jimenéz EP, Flor-Garcia M, Terreros-Roncal J, Rabano A, Cafini F, Pallas-Bazarra N, Avila J, Llorens-Martin M. (2019) Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease. Nat. Med 25, 554–560.

Moreno-Jiménez EP, Terreros-Roncal J, Flor-García M, Rábano A, Llorens-Martín M (2021) Evidences for adult hippocampal neurogenesis in humans. *J Neurosci* 41, 2541-2553.

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

Murphy KJ, Fox GB, Foley AG, Gallagher HC, O'Connell A, Griffin AM, Nau H, Regan CM (2001) Pentyl-4-yn-valproic acid enhances both spatial and avoidance learning and attenuates age-related NCAM-mediated neuroplastic decline within the rat medial temporal lobe. *J Neurochem* 78, 704–714.

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

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* 15;927(2):111-121.

Namiot ED, Niemi JVL, Chubarev VN, Tarasov VV, Schiöth HB. (2022) Stem cells in clinical trials on neurological disorders: Trends in stem cells origins, indications, and status of the clinical trials. *Int J Mol Sci*. 2022 23(19):11453.

Nishiyama A, Komitova M, Suzuki R, Zhu X (2009) Polydendrocytes (NG2 cells): multifunctional cells with lineage plasticity. *Nat Rev Neurosci* 10(1):9-22.

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstain AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. *Nature* 409, 714–720.

Nogueira AdB, Sogayar MC, Colquhoun A, Siqueira SA, Nogueira ArB, Marchiori PE, Teixeira MJ (2014) Existence of a potential neurogenic system in the adult human brain. *J Transl Med* 12:75.

Obernier K, Tong CK, Alvarez-Buylla A (2014) Restricted nature of adult neural stem cells: re-evaluation of their potential for brain repair. *Front Neurosci* 8:162.

Paavilainen L, Edvinsson A, Asplund A, Hober S, Kampf C, Pontèn F, Wester K (2010) The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. *J Histochem Cytochem* 58(3):237-46.

Page CE, Biagiotti SW, Alderman PJ, Sorrells SF (2022) Immature excitatory neurons in the amygdala come of age during puberty. *Dev Cogn Neurosci* 56, 101133.

Palazzo O, La Rosa C, Piumatti M, Bonfanti L (2018) Do large brains of long-living mammals prefer non-newly generated, immature neurons? *Neural Regen Res* 13: 633-634.

Paredes MF, James D, Gil-Perotin S, Kim H, Cotter JA, Ng C, Sandoval K, Rowitch DH, Xu D, McQuillen PS, Garcia-Verdugo J-M, Huang EJ, Alvarez-Buylla A (2016a) Extensive migration of young neurons into the infant human frontal lobe. Science 7;354(6308).

Paredes MF, Sorrells SF, Garcia-Verdugo JM, Alvarez-Buylla A (2016b) Brain size and limits to adult neurogenesis. *J Comp Neurol* 524:646-664.

Parolisi R, Peruffo A, Messina S, Panin M, Montelli S, Giurisato M, Cozzi B,Bonfanti L (2015) Forebrain neuroanatomy of the neonatal and juvenile dolphin (T.truncatus and S. coeruloalba). *Front Neuroanat* 6;9:140.

Parolisi R, Cozzi B, Bonfanti L (2017) Non-neurogenic SVZ-like niche in dolphins, mammals devoid of olfaction. *Brain Struct Funct* 222(6):2625-2639.

Parolisi R, Cozzi B, Bonfanti L (2018) Humans and dolphins: Decline and fall of adult neurogenesis. *Front Neurosci* 12:497.

Patzke N, Spocter MA, Karlsson KÆ, Bertelsen MF, Haagensen M, Chawana R, Streicher S, Kaswera C, Gilissen E, Alagaili AN, Mohammed OB, Reep RL, Bennett NC, Siegel JM, Ihunwo AO, Manger PR (2015) In contrast to many other mammals, cetaceans have relatively small hippocampi that appear to lack adult neurogenesis. *Brain Struct Funct* 220(1):361-83.

Paxinos G, Franklin K (2019) Paxinos and Franklin's the mouse brain in stereotaxic coordinates (5th Edition); *Elsevier*.

Paxinos G, Watson C, Petrides M, Rosa M, Tokuno H (2012) The marmoset brain in stereotaxic coordinates (1st Edition); *Elsevier*

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(17):6706-17.

Penz OK, Fuzik J, Kurek AB, Romanov R, Larson J, Park TJ, Harkany T, Keimpema E (2015) Protracted brain development in a rodent model of extreme longevity. *Sci Rep* 5:11592.

Pèrez-Martin M, Cifuentes M, Grondona JM, Lòpez-Avalos MD, Gòmez-Pinedo O, Garcìa-Verdugo JM, Fernàndez-Llebrez P (2010) IGF-I stimulates neurogenesis in the hypothalamus of adult rats. Eur J Neurosci 31(9):1533-48.

Perry EK, Johnson M, Ekonomou A, Perry RH, Ballard C, Attems J (2014) Neurogenic abnormalities in Alzheimer's disease differ between stages of neurogenesis and are partly related to cholinergic pathology. *Neurobiol Dis* 47(2):155-62.

Petrovich GD, Canteras NS, Swanson LW (2001) Combinatorial amygdalar inputs to hippocampal domains and hypothalamic behavior systems. Brain Res Brain Res Rev 38(1-2):247-89.

Piumatti M, Palazzo O, La Rosa C, Crociara P, Parolisi R, Luzzati F, Lévy F, Bonfanti L (2018) Non-newly generated, "immature" neurons in the sheep brain are not restricted to cerebral cortex. *J Neurosci* 38(4):826-842.

Ponti G, Aimar P, Bonfanti L (2006a) Cellular composition and cytoarchitecture of the rabbit subventricular zone (SVZ) and its extensions in the forebrain. *J Comp Neurol* 498:491–507.

Ponti G, Peretto P, Bonfanti L (2006b) A subpial, transitory germinal zone forms chains of neuronal precursors in the rabbit cerebellum. *Dev Biol* 294, 168–180.

Ponti G, Peretto P, Bonfanti L (2008) Genesis of neuronal and glial progenitors in the cerebellar cortex of peripuberal and adult rabbits. *PLoS One* 3(6):e2366.

Puelles L (2014) Development and Evolution of the Claustrum. *Chapter 4 in: The Claustrum, Smythies JR, Edelstein LR, Ramachandran VS (eds) San Diego:Academic Press* pp 119-176.

Quinones-Hinojosa A, Sanai N, Soriano-Navarro M, Gonzalez-Perez O, Mirzadeh Z, Gil-Perotin S, Romero-Rodriguez R, Berger MS, Garcia-Verdugo JM, Alvarez-Buylla A (2006) Cellular composition and cytoarchitecture of the adult human subventricular zone: a niche of neural stem cells. *J Comp Neurol* 494(3):415-34.

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

Riccio O, Murthy S, Szabo G, Vutskits L, Kiss JZ, Vitalis T, Lebrand C, Dayer AG (2012) New pool of cortical interneuron precursors in the early postnatal dorsal white matter. *Cereb Cortex* 22:86–98.

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(12):1392-401.

Roth G, Dicke U (2005) Evolution of the brain and intelligence. *Trends Cogn Sci* 9, 250-257.

Rotheneichner P, Belles M, Benedetti B, König R, Dannehl D, Kreutzer C, Zaunmair P, Engelhardt M, Aigner L, Nacher J, Couillard-Despres S (2018) Cellular plasticity in the adult murine piriform cortex: continuous maturation of dormant precursors into excitatory neurons. *Cereb Cortex* 28(7):2610-2621.

Równiak M, Sztein S, Robak A (2003) A comparative study of the mammalian amygdala: a Golgi study of the basolateral amygdala. *Folia Morphol (Warsz)* 62(4):331-9.

Sah P, Faber ESL, Lopez De Armentia M, Power J (2003) The amygdaloid complex: Anatomy and physiology. Physiol Rev 83(3):803-34.

Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai H-H, Wong M, Gupta N, Berger MS, Huang E, Garcia-Verdugo JM, Rowitch DH, Alvarez-Buylla A (2011) Corridors of migrating neurons in the human brain and their decline during infancy. *Nature* 478: 382-386.

Sapir T, Horesh D, Caspi M, Atlas R, Burgess H, Wolf SG, Francis F, Chelly J, Elbaum M, Pietrokovski S, Reiner O (2000) Doublecortin mutations cluster in evolutionarily conserved functional domains. *Hum Mol Genet* 9, 703-712.

Schenker NM, Hopkins WD, Spocter MA, Garrison AR, Stimpson CD, Erwin JM, Hof PR, Sherwood CC (2010) Broca's area homologue in chimpanzees (*Pan troglodytes*): probabilistic mapping, asymmetry, and comparison to humans. *Cereb Cortex* 20(3):730-742.

Schneider NY, Datiche F, Coureaud G (2018) Brain anatomy of the 4-day-old European rabbit. *J Anat* 232(5):747-767.

Scholzen T, Gerdes J (2000) The Ki-67 protein: from the known and the unknown. *J Cell Physiol* 182(3):311-322.

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.

Seki T, Hori T, Miyata H, Maehara M, Namba T (2019) Analysis of proliferating neuronal progenitors and immature neurons in the human hippocampus surgically removed from control and epileptic patients. *Sci Rep* 9, 18194.

Seki T (2020) Understanding the real state of human adult hippocampal neurogenesis from studies of rodents and non-human primates. *Front Neurosci* 14: 839.

Semënov MV (2019) Adult hippocampal neurogenesis is a developmental process involved in cognitive development. *Front Neurosci* 13: 159.

Semënov MV (2021) Proliferative capacity of adult mouse brain. *Int J Mol Sci* 22, 3449.

Snyder JS (2019) Recalibrating the relevance of adult neurogenesis. Trends Neurosci 42, 164-178.

Sobecki M, Mrouj K, Camasses A, Parisis N, Nicolas E, Llères D, Gerbe F, Prieto S, Krasinska L, David A, Eguren M, Birling MC, Urbach S, Hem S, Déjardin J, Malumbres M, Jay P, Dulic V, Lafontaine DLj, Feil R, Fisher D (2016) The cell proliferation antigen Ki-67 organises heterochromatin. *Elife* 5:e13722.

Sorrells SF, Paredes MF, Cebrian-Silla A, Sandoval K, Qi D, Kelley KW, James D, Mayer S, Chang J, Auguste KI, Chang EF, Gutierrez AJ, Kriegstein AR, Mathern GW, Oldham MC, Huang EJ, Garcia-Verdugo JM, Yang Z, Alvarez-Buylla A (2018) Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. *Nature* 555, 377–381.

Sorrells SF, Paredes MF, Velmeshev D, Herranz-Pérez V, Sandoval K, Mayer S, Chang EF, Insausti R, Kriegstein AR, Rubenstein JL, Garcia-Verdugo JM, Huang EJ, Alvarez-Buylla A (2019) Immature excitatory neurons develop during adolescence in the human amygdala. *Nat Commun* 10(1): 2748.

Sorrells SF, Paredes MF, Zhang Z, Kang G, Pastor-Alonso O, Biagiotti S, Page CE, Sandoval K, Knox A, Connolly A, et al. (2021) Positive controls in adults and children support that very few, if any, new neurons are born in the adult human hippocampus. *J Neurosci* 41, 2554-2565.

Srikandarajah N, Martinian L, Sisodiya SM, Squier W, Blumcke I, Aronica E, Thom M (2009) Doublecortin expression in focal cortical dysplasia in epilepsy. *Epilepsia* 50(12):2619-28.

Srinivasan S, Stevens CF (2017) A quantitative description of the mouse piriform cortex. *bioRxiv* preprint.

Stallcup WB (2002) The NG2 proteoglycan: past insights and future prospects. *J Neurocytol* (6-7):423-35.

Swanson MEV, Murray HC, Oliver MH, Waldvogel HJ, Firth EC, Curtis MA (2017) Imposed running exercise does not alter cell proliferation in the neurogenic niches of young lambs. *J Anim Sci* 95: 4381-4390.

Takada N, Kucenas S, Appel B (2010) Sox10 is necessary for oligodendrocyte survival following axon wrapping. *Glia* 58(8): 996–1006.

Teeling EC, Madsen O, Van den Bussche RA, de Jong WW, Stanhope MJ, Springer MS (2002) Microbat paraphyly and the convergent evolution of a key innovation in Old World rhinolophoid microbats. *Proc Natl Acad Sci USA* 99(3):1431-1436.

Theodosis DT, Poulain DA, Oliet SH (2008) Activity-dependent structural and functional plasticity of astrocyte-neuron interactions. *Physiol Rev* 88:983–1008.

Tobin MK, Musaraca K, Disouky A, Shetti A, Bheri A, Honer WG, Kim N, Dawe RJ, Bennett DA, Arfanakis K, Lazarov O (2019) Human hippocampal neurogenesis persists in aged adults and Alzheimer's disease patients. *Cell Stem Cell* 974-982.e3.

Urbán N, Blomfield IM, Guillemot F (2019) Quiescence of adult mammalian neural stem cells: A highly regulated rest. Neuron 104, 834–848.

Van Dijk RM, Huang SH, Slomianka L, Amrein I (2016) Taxonomic separation of hippocampal networks: principal cell populations and adult neurogenesis. *Front Neuroanat* 10:22.

van Noordwijdk AJ, Jong G (1986) Acquisition and allocation of resources: Their influence on variation in life history tactics. *Am Nat* 128, 137-142.

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* 5, 808–818.

Varea E, Belles M, Vidueira S, 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.

Verwer RW, Sluiter AA, Balesar RA, Baayen JC, Noske DP, Dirven CM, Wouda J, Van Dam AM, Lucassen PJ, Swaab DF (2007) Mature astrocytes in the adult human neocortex express the early neuronal marker doublecortin. *Brain* 130, 3321–3335.

Vinters HV (2015) Emerging concepts in Alzheimer disease. *Annu Rev Pathol* 10, 291-319.

Vivar C, van Praag H (2017) Running changes the brain: the long and the short of it. *Physiology (Bethesda)* 32:410-424.

Walton NM, Shin R, Tajinda K, Heusner CL, Kogan JH, Miyake S, Chen Q, Tamura K, Matsumoto M (2012) Adult neurogenesis transiently generates oxidative stress. *PLoS One* 7(4):e35264.

Wang W, Wang M, Yang M, Zeng B, Qiu W, Ma Q, Jing X, Zhang Q, Wang B, Yin C, Zhang J, Ge Y, Lu Y, Ji W, Wu Q, Ma C, Wang X (2022) Transcriptome dynamics of hippocampal neurogenesis in macaques across the lifespan and aged humans. *Cell Res* 32(8):729-743.

Wilson DE, Reeder DM (2005) Mammal Species of the world: A taxonomic and geographic reference. *JHU Press, Volume 1.*

Workman AD, Charvet CJ, Clancy B, Darlington RB, Finlay BL (2013) Modeling transformations of neurodevelopmental sequences across mammalian species. *J Neurosci* 33, 7368–7383.

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.

Xu Y, Tamamaki M, Noda T, Kimura K, Itokazu Y, Matsumoto N, Dezawa M, Ide C (2005) Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. Exp Neurol 192(2):251-64.

Yoo S, Blackshaw S (2018) Regulation and function of neurogenesis in the adult mammalian hypothalamus. Prog Neurobiol 170:53-66.

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

Zhang J, Giesert F, Kloos K, Vogt Weisenhorn DM, 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.

Zhao X, van Praag H (2020) Steps towards standardized quantification of adult neurogenesis. *Nat Commun* 11, 4275.

Acknowledgements

This PhD has been an unforgettable journey and a rollercoaster of emotions: from the toughest days to those full of joy, these three-years could not have been the same without all the people that surrounded me inside and outside the lab.

First, I would like to thank my supervisor, Prof. Luca Bonfanti. In these three-years we faced a never-ending list of problems, including a pandemic, but in the end, we managed to get a lot of achievements (including future ones!). I would never be able to get through all of this without his help, total dedication to this research project and inspiring passion for science.

Beside my supervisor, I would like to thank all our collaborators involved in the project: First, I would like to express my gratitude to Prof. Chet Sherwood, which gave me the opportunity to analyze the chimpanzee and marmoset brains in his laboratory during my period abroad at the George Washington University. His kindness, help and the engaging discussions made my stay unforgettable. Alongside, I would like to thank all the people working in his lab, especially Imam, Elaine and Dustin: their support and friendship during my stay really helped me. I thank Prof. Francesco Bifari and Dr. Alessia Amenta which helped us analyzing brain tissues for our project; Prof. Stefano Farioli-Vecchioli, Prof. Sébastien Couillard-Després and Dr. Bruno Benedetti, which provided us brains and ideas for our study. Prof. Bruno Cozzi and Dr. Jean-Marie Graic, which provided us most of the tissues used, enduring our constant requests.

I would like to thank my family: my dad and mom for all their love and for supporting me in all my decisions in these years. I thank my sister Monica and Umberto for being always present and for helping me distracting from all my concerns. I thank Nonna Mela and all my cousins, uncles and aunts for all their love and support.

Most of all, I thank Ilaria for always being there, whenever I needed it and for putting up with me even in tough times. Without her love and support I would never be able to get through all these years and achieve this goal.

I would like to thank all the people that were with me at Neuroscience Institute Cavalieri Ottolenghi. I thank Chiara La Rosa: she taught me everything and her friendship, help and support were essential for me in these years. I thank all the people that passed through our lab, especially Chiara Olmeo, Elena Vergnano, Eleonora Pintauro, Nikita Telitsyn, Chiara Corrente and Alessandro Zanone. I thank Alessia Pattaro, which helped me in the last months of my PhD and I wish her to reach a lot of achievements in the next years. I thank all the colleagues and friends who made my experience at NICO unforgettable, especially Roberta Parolisi, Sara Bonzano, Brigitta Bonaldo, Nino Casile, Eriola Hoxha, Valentina Cerrato and Gabriela González.

I would like to thank all my friends which supported me in these years: Francesco for being still there after 25 years, Ludovica and Emanuele for their friendship and all the carefree evenings, Federico for all his support and for sharing with me the craziest moments during and after university, Matteo for sharing with me this three-year journey and Dejanira, Anna, Naiara, Diego, Davide and Francesca for all their support and love from high school to nowadays.