Universidade do Minho
Escola de Medicina

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Hilar hippocampal cytogenesis: *a new source of neural plasticity in the adult mammalian brain?*

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Hilar hippocampal cytogenesis: *a new source of neural plasticity in the adult mammalian brain?*

Dissertação de Mestrado
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Trabalho efetuado sob a orientação do
Doutor António Maria Restolho Mateus Pinheiro
e da
Professora Doutora Luísa Alexandra Meireles Pinto

setembro de 2019

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RESUMO

Citogénese no Hilus hippocampal: *nova fonte de plasticidade neuronal no cérebro de mamíferos adultos?*

A capacidade contínua de gerar novos neurónios e células gliais, processo denominado citogénese cerebral, é restrita a determinados locais do cérebro adulto. Tradicionalmente, este fenómeno ocorre em dois *locci* cerebrais: na zona subgranular (ZSG) do girus dentado (GD) do hipocampo e na zona subependimal. O GD tem sido classicamente implicado em importantes funções cognitivas como a aprendizagem e memóriae também participam em domínios emocionais. Apesar da citogénese cerebral adulta ser consensualmente aceite em roedores, a importância funcional da neurogénese adulta tem sido consideravelmente debatida. Mais recentemente, a discussão em torno deste tema reemergiu, em particular no cérebro humano.

Em íntima relação com o GD, a região hilar é uma das estruturas pertencentes à formação hipocampal mais complexas e celularmente diversificada, também esta com um papel importante para funções cognitivas de controlo hipocampal. Observações prévias, sugerem que também esta região hipocampal possa ser populada por células recém geradas no cérebro adulto. A informação sobre fenómenos citogénicos hilares no cérebro adulto mamífero é escassa, não existindo caracterização morfológica ou funcional das novas células no hilus. Assim, este trabalho engloba a primeira descrição neuroanatómica da citogénese hilar, complementada com o estudo da sua modulação por fatores extrínsecos em ratos adultos.

Os resultados demonstram que as novas células hilares adquirirem marcadores de neuroblastos, pese embora o facto de exibirem características citomorfológicas mais incipientes, quando comparadas com neuroblastos subgranulares. Ao contrário do que acontece na neurogénese da ZSG, os novos neurónios não expressam o marcador de maturação terminal NeuN. Uma fracção diminuta das novas células hilares, diz respeito a fenómenos astrogliogénicos, ubíquos no cérebro adulto. Apesar do número de novas células residentes no hilus ser significativamente inferior àquele da ZSG, esta região parece ser um importante nicho de plasticidade neural. A exposição a fatores de stress, atividade física e tratamento com fármacos psicotrópicos, modulam a citogénese hilar diferencialmente.

O trabalho desenvolvido nesta tese aumenta o conhecimento atual sobre a plasticidade estrutural do cérebro mamífero adulto, revisitando noções pré-estabelecidas sobre a distribuição regional e importância funcional dos nichos neuro- e gliogénicos pós-natais.

Palavras-chave: Citogénese Hilar, Hipocampo, Plasticidade Neuronal

ABSTRACT

Hilar hippocampal cytogenesis: *a new source of neural plasticity in the adult mammalian brain?*

The continuous capacity to generate new neuronal and glial cells, a process known as adult brain cytogenesis, persists in restricted niches of the adult mammalian brain. This phenomenon occurs traditionally in two regions: the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) and the periventricular subependymal zone. The dentate gyrus has been classically involved in important brain functions such as learning and memory. Beyond the participation in cognitive processing, the newborn neurons have been also shown to be important for several emotion domains. Despite the fact that brain cytogenesis in rodents is well accepted, the functional importance of adult neurogenesis in mammals has been a matter of much debate. Just recently, discussion around this fundamental subject of neurosciences was stirred up.

The dentate hilus is one of the most complex and cellular diverse sub-regions of the hippocampal formation with a central role in key cognitive hippocampal functions. Few descriptions exist of cytogenesis in the adult hilus. Moreover, the field lacks a morphological and functional characterization of hilar newborn cells. Hence this work provides an unprecedented neuroanatomical description of hilar cytogenesis complemented with insights on its extrinsic modulation, in the adult rat brain.

Results show that newborn hilar cells acquire neuroblasts' cell markers, although they present more incipient cytomorphological traits, when compared to their subgranular counterparts. Contrarily to SGZ neurogenesis, newborn hilar neurons do not express the final maturation marker, NeuN. A significantly small fraction of new hilar cells within the hilus reflect the astrogliogenic process, described to be ubiquitous in the adult brain. Even though the number of newborn cells in the hilus is lower than in the SGZ, that region emerges as an important niche of neural plasticity. Indeed, stress exposure, physical activity and treatment with psychotropic drugs differentially modulate hilar cytogenesis.

Work on this thesis improves current knowledge on structural plasticity of the adult mammalian brain, and revisits pre-established notions on the regional distribution and functional importance of post-natal neuro- and gliogenic niches.

Keywords: Hilar Cytogenesis, Hippocampus, Neural Plasticity

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Abbreviation List

B	
BrdU	5-Bromo-2'-Deoxyuridine
C	
CA	Cornu Ammonis
CNS	Central Nervous System
D	
DCX	Doublecortin
DG	Dentate Gyrus
E	
EPM	Elevated Plus Maze
G	
GCL	Granular Cell Layer
GFAP	Glial Fibrillary Acidic Protein
H	
HEGCs	Hilar Ectopic Granule Cells
HICAP	Hilar Commissural-associational pathway-related
HIPP	Hilar perforant path-associated
HPA	Hypothalamus-pituitary-adrenal
I	
IPC	Intermediate Progenitor Cells
M	
ML	Molecular Layer
N	
NMDA	Non-competitive N-methyl-D-aspartate
NSC	Neural Stem Cell
NSF	Novelty Suppressed Feeding
O	
OCT	Optimal Cutting Temperature compound
P	
PA	Physical Activity
PFA	Paraformaldehyde
PSA-NCAM	Polysialylated-neural cell adhesion molecule
R	

RGL	Radial glia-like
S	
SGZ	Subgranular Zone
SPT	Sucrose Preference Test
SNS	Sympathetic Nervous System
SVZ	Subventricular Zone
U	
uCMS	Unpredictable Chronic Mild Stress

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INTRODUCTION

Recognition that the central nervous system (CNS) has the ability to structurally, and thereby functionally, reconfigure itself throughout life, has widened the spectrum of research in the field of neurosciences. Such *plasticity* displayed by the post-natal brain opens new perspectives in the way science interprets how the environment modulates brain structure and function, but also on how degeneration of neuroplastic processes may precipitate disability and disease. Perhaps these two premises are the key propellants of research focused on unveiling the importance of brain plasticity in the post-natal brain. Discussion around this topic persists nowadays and has been recently heated up when prominent studies described conflicting data regarding the significance of the production of new neurons in the adult human brain, as I will later on discuss. Work on this thesis was conceived to be an *add on* to this discussion and to shed light on unanswered questions regarding the production of new cells in the adult mammalian brain.

1. Canonical distribution of adult cytogenesis in the mammalian brain

1.1 An overview on cytogenic regions

For many years, the brain was seen as a static structure. The traditional dogma claimed that proliferative processes were limited to the early stages of embryonic development (Bruel-Jungerman et al., 2007). For this reason, the number of cells in the brain were thought to be fixed after birth with the inability to generate new neurons and renew themselves (Taupin et al., 2002).

In the 1960s, this "*all you are born with, is what you get*" principle began to be questioned. In most mammalian brains, the production of new neurons is not limited to the prenatal period (Kuhn et al., 1996). In 1965, Joseph Altman presented the first description of the formation of new neurons in the adult rodent brain, a process named neurogenesis (Altman et al., 1996). However, this fact was not generally accepted until the middle of 1990s (Gross et al., 2000). At the time, neurogenesis was considered unlikely when considering the complexity of most neurons with highly branched dendrites and polysynaptic axonal combinations: in a mechanistic point of view, it was hard to conceive they would be able to re-enter cell cycle and divide (Gage et al., 2002). Beyond that, it was difficult to understand how new neurons could functionally integrate the pre-existing neural circuits without disrupting them (Gage et al., 2002).

Studies conducted decades after, showed how the neurogenic process was not restricted to the embryonic phase. A number of species: as for instance rabbit (Guéneau et al., 1982), guinea pig (Guidi

et al., 2005), primates (Gould et al., 1999) were able to produce new cells postnatally. Furthermore, using electron microscopy, Kaplan and Hinds, confirmed newly formed cells to have the structural properties of neurons, in the dentate gyrus of adult rats (Kaplan et al., 1977) (Fig. 1a and 1b). Follow up studies also verified the existence of neurogenesis in non-mammalian animals such as birds (Doetsch et al., 2001), reptiles, fish, or insects (Cayre et al., 2002).

The human brain was not different. In 1998, after the introduction of bromodeoxyuridine, a synthetic nucleotide analog used to detect proliferative cells, that labels DNA during the S phase (Fares et al., 2018), Eriksson and colleagues were the first to describe neurogenesis in the human adult brain (Eriksson et al., 1998). More recently, Kristy Spalding and Olaf Bergmann confirmed neurogenesis in adult brain by birth dating cells though ^{14}C levels (Altman et al., 1996). In the case of adult humans, adult neurogenesis is a much discussed topic recently, since two different teams claimed contrasting findings about the extension of neurogenesis in the adult human hippocampus (Sorrells et al., 2018, Boldrini et al., 2018).

A large bulk of evidence show how neurogenesis is greatly conserved throughout evolution (Kaslin et al., 2007). As such, since the beginning that it has been suggested to serve essential functions (Bruel-Jungerman et al.,2007).

These findings marked a breakthrough in neurosciences, challenging fundamental tenets of traditional dogma. The adult CNS system is susceptible to morphological and physiological alterations at several levels (Fares et al., 2018). Factors such as age, trauma and neurodegenerative diseases lead to loss of cognitive and motor abilities which are connected to loss of neurons (Bordiuk et al., 2004).

Presently, the vast majority of evidence gathered in this field allows to establish that new neurons continue to be added to the adult brain throughout life in the mammalian brain (Bruel-Jungerman et al.,2007). In mammalians, brain cytogenesis (a broader designation that stands for the formation of both new neurons and new astrocytes) has been reported to be limited to two canonical regions: in the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampal formation (Abrous et al., 2005, Jin et al., 2016). Both regions are remnants of the embryonic germinal layers (Feliciano et al., 2015). However, cytogenesis also occurs in non-canonical sites such as the neocortex, striatum, hypothalamus (Jin et al., 2016) and in the amygdala (Jhaveri et al., 2017); although less studied these scattered reports of additional cytogenic niches put forward one of two hypothesis: either we are still missing other brain milieus where production of new cells is possible, or we lack full comprehension on how newborn cells may migrate from canonical niches to *ectopic* regions.

Hence, studying adult brain cytogenesis is not only a fundamental scientific endeavor, but also an open door to new venues on how repair the brain and, possibly, on how to treat some neuropsychiatric diseases. The hippocampus is still the region to which more attention has been paid when studying the functional importance of newborn adult brain cells.

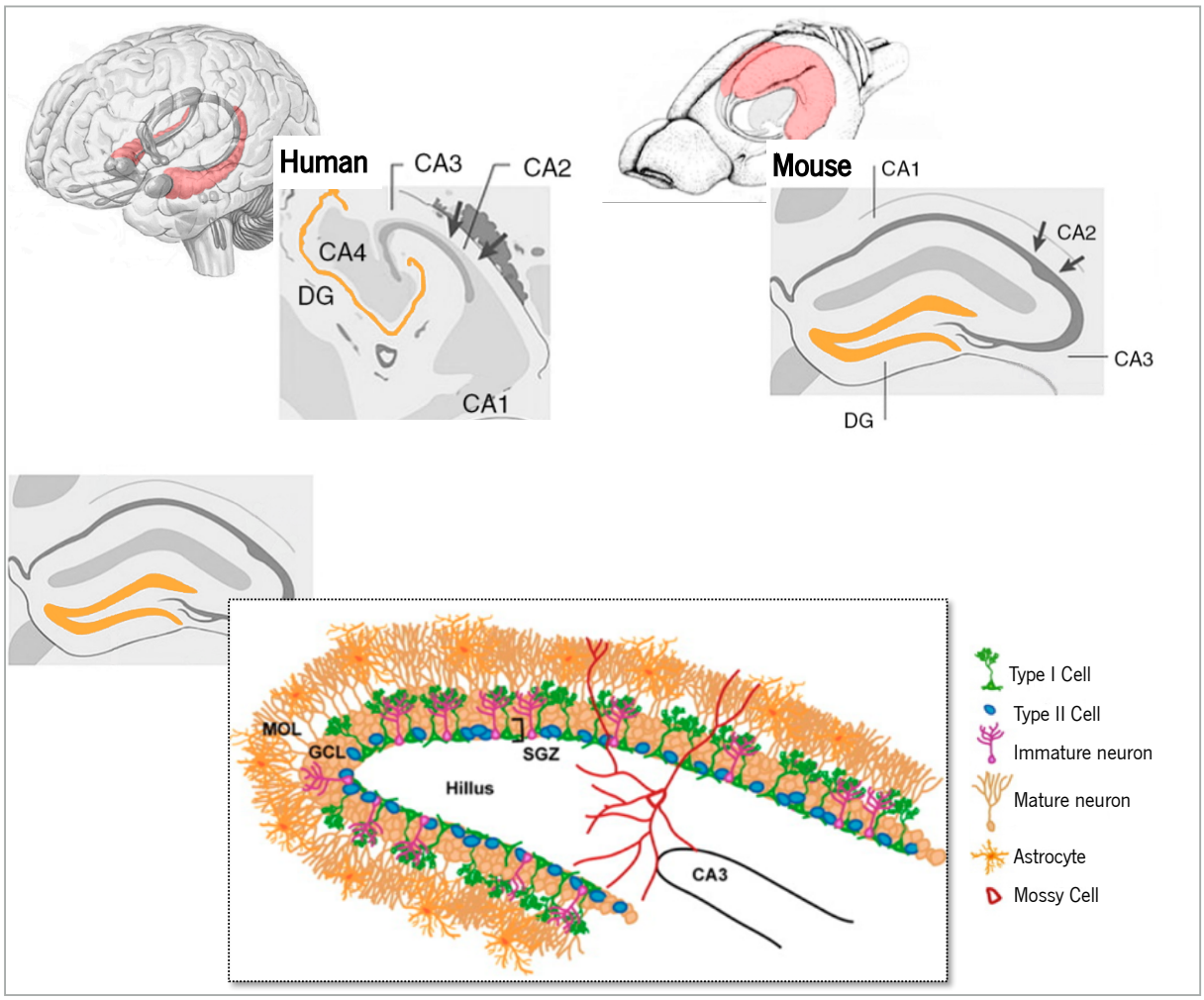


Figure 1 Structure of the Hippocampus and the Dentate Gyrus. a,b) Structural differences of the hippocampus (red) and the DG (yellow) between mammalians. c) Canonical characterization of the DG in rodent model. The subgranular zone is well described and it is composed by progenitor cells (type I cell and type II cells), immature and mature neurons, astrocytes and mossy cells.

1.2 On the hippocampal cytogenic niche

The hippocampus integrates the hippocampal formation which also includes the dentate gyrus, hippocampus, subiculum, presubiculum, parasubiculum and entorhinal cortex (Li et al.,2009). It belongs to the medial temporal lobe and is responsible for memory, learning and emotion-related processes (Bermudez-Hernandez et al., 2017). It displays an elongated structure with its septotemporal axis bending in a C-shaped manner from the septal nuclei rostro-dorsally to the temporal caudo-ventrally (Amaral et al., 1988, Amaral et al., 2007) (Fig 1a and 1b) and it is composed by the Cornu Ammonis which includes CA1, CA2 and CA3 (Giap et al., 2000).

The hippocampus is an extremely plastic region that may suffer alterations or even degeneration through diverse stimuli (Fares et al., 2018, Dhikav et al., 2012) and, therefore, has been reported to be affected in some neurological and psychiatric disorders (Dhikav et al., 2012).

The capacity to recall situations that have recently happened or that took place long time ago, is a unique and personalized experience which is also related with emotion stimuli. The hippocampus is an essential region for formation of long-term memories and memory consolidation (Li et al.,2009) and in the organization of them by a chronological order (Eichenbaum et al., 2014). Moreover, the hippocampus is involved in all aspects of declarative or explicit memory (Ekstrom et al., 2017,), spatio-temporal and contextual representations (Eichenbaum et al., 1999). The hippocampus is also important for the ability to distinguish between similar events in closely related episodes: episodic memory (Kassab et al., 2018).

Although the hippocampus clearly important to recall past events, it is highly selective in what information to store. Previous experience, the environment and the context itself and the emotion context are filters for the selection of which information to retain (McEwen et al., 1995), Richter-Levin et al., 2000). A dynamic interaction between the hippocampus and amygdala is essential for encoding of emotion experiences (Richter-Levin et al., 2000). This connection was suggested in 1937 by James Papez: it was suggested that the hippocampus was part of a circuit that supports emotion processes and it could control emotion (Papez, 1937).

In light of this "cognition versus emotion" dichotomy, it is important to note that the hippocampus is not a unitary structure. Instead, it can be divided into dorsal (septal pole) and ventral (temporal pole). The dorsal hippocampus is classically seen as the "cold" cognitive hippocampus. The ventral hippocampus is involved in emotional and affective processes (Moser et al., 1995), stress (Fanselow et al., 2010) and anxiety (Kheirbek et al., 2013), and it is known as the "hot" emotion-related hippocampus. Despite functional differences between these two sub-regions, they are able to play similar roles and complement each other functions (Ferbinteanu et al., 2003), making the dorsal-to-ventral functional specialization not so clearly defined as the anatomical segregation.

1.3 Where neural stem cells reside: The Dentate Gyrus

The dentate gyrus (DG), a V-shaped structure (Fig. 1c), is a subregion integrated into the curved Cornus Ammonis composed by CA3, CA2 and CA1 regions (Toni et al., 2015). The DG plays a role, in learning, episodic memory, in the recognition of the surrounded environment, in pattern separation processes (Gonçalves et al., 2016).

The DG can be divided mainly into three layers: the molecular layer (ML), granular cell layer (GCL) and the hilus (Toni et al., 2015) (Fig. 1c).

The molecular layer, the most external layer, is quite acellular, containing the dendrites of granule cells (Claiborne et al., 1990), axons from the entorhinal cortex and scarcely any somas of interneurons (Claiborne et al., 1990, Toni et al., 2015). Beyond that, this layer is composed by the fibers of the perforant path originated in the entorhinal cortex and from other extrinsic inputs that terminate in this region (Toni et al., 2015, Amaral et al., 2007).

The GCL, the middle layer, is densely composed by glutamatergic granule cells and it are five to eight cells deep (Shapiro et al., 2005, Toni et al., 2015, Bermudez-Hernandez et al., 2017). The cell body of the pyramidal basket cells is frequently located within the GCL (Amaral et al., 2007).

The hilus, a polymorphic layer, lies between the GCL and the area CA3, have larger neuronal somata, axons of granule cells known as mossy fibers, GABAergic interneurons and, mainly, mossy cells (Toni et al., 2015, Shapiro et al., 2005, Kesner et al., 2018). The mossy fibers (granule cell axons) are involving in the neural circuits. The fibers enter the hilus and then pass into area CA3 where they terminate on pyramidal cells and interneurons (Altman et al., 1996).

Between the granule cells layer and the hilus, prevails a region where new neurons are continuously produced, the subgranular zone (SGZ). It includes neural stem cells and progenitor cells as well as the bodies of GABAergic basket cells (Toni et al., 2015, Kempermann et al., 2015). Beyond the precursor cells, glial cells and endothelia cells, it is very likely to comprise immune cells, microglia and macrophages and an extracellular matrix (Kempermann et al., 2015).

After neural stem cells go through all development stages to form mature and functional cells in the DG, they can contribute to the neuronal plasticity and to the formation of new memories and learning (van Praag et al., 2002).

In latter subsections, I will briefly describe the main cell types composing the polymorphic hippocampal layer.

1.3.1 Granule cells

One of the main type of cells that are present in the dentate gyrus. Their cell body is elliptical and are firmly packed together. Their dendrites with a spiny apical morphology appear in cone-shapped tree extending throughout the molecular layer (Amaral et al., 2007).

They are the main excitatory neurons in the DG and the receiver of the majority of inputs into the DG (Kempermann et al., 2015, Cameron et al., 2015). They continue to be produced in large number throughout life (Cameron et al., 2015, Shapiro et al., 2005). They receive inputs from the entorhinal cortex and project through their axons along the mossy fiber to the CA3 area. They give to the pyramidal

cells residing in the CA3 excitatory inputs (Ge, et al., 2005). However, they rarely fire, and their excitatory activity is regulated by the interneurons in the DG and in the hilus (Ge, et al., 2005).

During their development, several hallmarks that can be distinguished as a discrete event of their maturation: establishment of neuronal polarity and migration; gaminobutyric GABAergic synapse formation onto apical dendrites; glutamertergic synaptogenesis; circuit integration followed by the final steps of maturation and refinement (Kesner et al., 2018). This process requires several weeks.

2. On the maturation of proliferating cells

2.1 Canonical maturation process of newborn granule cells in the dentate gyrus

Within the hippocampus, neural stem cells reside in the SGZ and the mature cells are mainly found in the GCL (Gonçalves et al., 2016). The subgranular region, a so-called neurogenic niche, provides the ideal environment propitious to the adult neural stem cells (NSCs) population to proliferate (Kempermann et al., 2015; Gonçalves et al., 2016). During the maturation process, these cells will commit to neuronal or non-neuronal cell lineages and differentiate passing through several development stages before integrating the hippocampal circuitry: proliferation, differentiation, survival, migration, maturation and integration (Gonçalves et al., 2016, Li et al.,2009) (Fig. 1a).

Adult neurogenesis may be divided into different phases. Firstly, the production of precursor cells, followed by an early survival phase and a maturation phase and, finally, the functional integration phase (Kempermann et al., 2015, Bordiuk et al., 2004).

At the beginning of the neurogenic process, the neural precursor cells proliferate broadening the pool of cells that might differentiate into neurons (Kempermann et al., 2015). However, early after cell-cycle exit, substantial changes in cell numbers occur because most newborn cells are eliminated within days after they are born (Kempermann et al., 2015) (Fig. 1a). Then, the neural precursors move to the SGZ where the axons and dendrites will grow, and subsequently, they will be incorporated into existing hippocampal circuits, where they will be differentiated into specific type of cells (Bordiuk et al., 2004, Kempermann et al., 2015). It has been described that the entire period of adult neurogenesis takes around 4/5 to 7 weeks (Kempermann et al., 2015), encompassing 1 month for neuronal maturation (Vivar et al., 2013).

Neurogenic processes in the adult hippocampus closely resemble the processes throughout embryonic development, since they follow a similar model of morphology and functional maturation (Esposito, 2005).

Adult neurogenesis starts with a population of precursor cells with glial properties (Kempermann et al., 2015) the type 1 radial glia-like (RGLs) (Fig 2b). These cells are thought to represent neural stem cells (NPCs) population (Gonçalves et al., 2016) and their cell body appears in the SGZ with a radial morphology, with apical processes extended through the GCL and branches into the molecular layer (Toni et al., 2015, Kempermann et al., 2015). Type 1 cells generate proliferating intermediate progenitor cells (IPCs, type 2 cells) (Gonçalves et al., 2016, Kempermann et al., 2015). They present a small cell body, irregular shaped dense nucleus and short, horizontal processes without long radial processes (Steiner, et al., 2006). These cells reside in the SGZ and have short processes (Toni et al., 2015) and they have the capacity to proliferate rapidly (Kempermann et al., 2015). IPCs include a subset of cells, the type 2a, that continue to express glial markers such as Glial Fibrillary Acidic Protein (GFAP) (Toni et al., 2015, Kempermann et al., 2015). However, at this point, they not present radial morphological characteristics. Type 2a cells express Nestin, similarly, to type 1 cells, and Sox2 as well (Kempermann et al., 2015). Other subset of IPCs, the type 2b cells, express additional transcription factors, namely NeuroD1 and Prox1 (Kempermann et al., 2015). Furthermore, both subsets express the transcription factor Tbr2 (Hodge et al., 2008). Of notice, Type 2 cells already start to receive synaptic GABAergic inputs which promotes their neuronal differentiation (Wang et al., 2005) (Fig. 2b).

Type 2 cells give rise to neuroblasts, the type 3 cells (Toni et al., 2015). These cells do not express Tbr2, and immature neural markers such as doublecortin (DCX) and Prox1 are expressed (Gonçalves et al., 2016, Toni et al., 2015, Kempermann et al., 2015) (it is important to highlight that DCX starts to be expressed a little earlier, already in the type 2b cells (Kempermann et al., 2015)). Polysialylated-neural cell adhesion molecule (PSA-NCAM) is another marker whose expression overlaps with DCX and it is recognized as an adult neurogenic marker. Type 3 cells have a short vertical process and have a little proliferative activity (Toni et al., 2015, Kempermann et al., 2015). Type 3 cells differentiate into mature dentate granule cells (Gonçalves et al., 2016, Toni et al., 2015). Their axons toward the CA3 region and the dendrites into the molecular layer (Toni et al., 2015).

After cell-cycle exit, the new neurons start to express NeuN (Kempermann et al., 2015) and the transient marker calretinin (Brandt et al., 2003). At this point, the number of cells decrease dramatically within a few days by apoptosis (Sun et al., 2013). For that reason, the number of cells that label for NeuN may decreased within few days. Usually, this elimination occurs before the cells have incorporate the neural circuits, meaning the functional connections in the CA3 are not establish yet or receive the correct dendritic inputs from the entorhinal cortex in the molecular layers (Kempermann et al., 2015). After this time point, very small changes happen in the number.

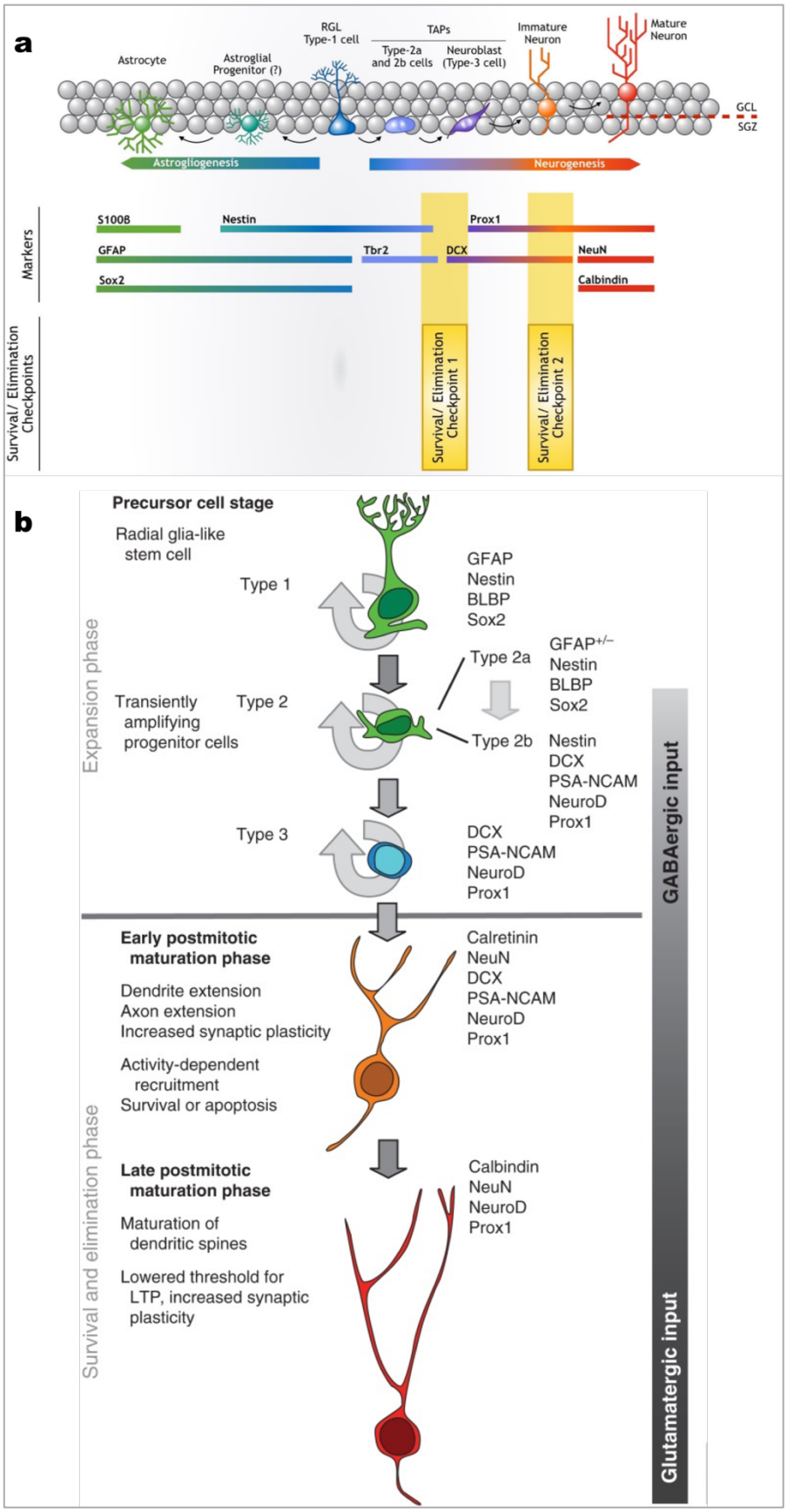


Figure 2 Adult hippocampal neurogenesis process. a) Histochemical and morphological characterization of newborn cells at different stages of development. The neural precursor cells (NPCs) can differentiate into mature astrocytes or neurons. During their maturation process different markers help to understand the development stage the newborn cell is in the moment of analysis. Adapted from Mateus Pinheiro, 2016. b) Maturation process of newborn cells committed with the neuronal path. Characterization of major morphological changes and the corresponding markers. Adapted from Kempermann et al., 2015.

At the same time, after the cell-cycle exit the dendrites starts to develop. However, the time when this process starts seems to be variable. On the other hand, dendrites development itself is actually well established (Kempermann et al., 2015). The new cells, 21 days after their born, send their axons from the dentate gyrus through the CA3 subfields exhibiting a regular laminar organization along the hippocampal septo-temporal axis where they form synapses (Sun et al., 2013). In the process the axons from new neurons have to compete with the existent synapses in the mossy fiber boutons which may be detected 10 days after their birth (Li et al.,2009). During this process, the dendritic network is also developing (Sun et al., 2013).

The kind of synaptic input and the extension of the dendritic tree are closely linked (Ambrogini et al., 2004). When cells are still in an initial differentiation stage and the dendritic tree is confined to the GCL, they present GABAergic input (Ambrogini et al., 2004). Throughout the maturation process, neurogenesis may be modulated by the neuronal network activity and by the GABA levels, since GABA levels might be an indicator of neuronal activity (Ge, et al., 2005). These cells sense the ambient before being sequentially innervated by GABA and glutamate synaptic inputs (Ge, et al., 2005).

As soon as cells elongate their dendrites, increase their complexity and they reach the molecular layer a switch happens in their phenotype (Ambrogini et al., 2004, Tozuka et al., 2005). A reduction of GABA depolarization on the new neurons which leads to alterations on morphology through maturation and also leads to the formation of glutamatergic synapses (Tozuka et al., 2005, Chancey et al., 2013).

After maturation phase, the cells suffer a few final adjustments. In this last period calretinin is expressed for 3 to 4 weeks which coexist with the dendritic maturation (Kempermann et al., 2015). After the integration of those cells into the neuronal network, the newborn cells switch their calcium-binding protein from calretinin to calbindin (Brandt et al., 2003, Toni et al., 2015). However, some more weeks are necessary in order these cells become electrophysiologically indistinguishable from the pre-existing cells (Ambrogini et al., 2004).

3. Functional integration of newborn cells in the dentate gyrus

3.1 Neural hippocampal circuits

The perforant pathway is the principal source of inputs from the entorhinal cortex to the DG (Vivar et al., 2013) (Fig. 3a). This is a unidirectional link that provides access to the region of the hippocampus formation forming the trisynaptic circuit [(Amaral et al., 2007). Therefore, the DG does not return the projection into the entorhinal cortex. The entorhinal cortex gives cortical sensory information to the hippocampus to execute its functions (Amaral et al., 2007).

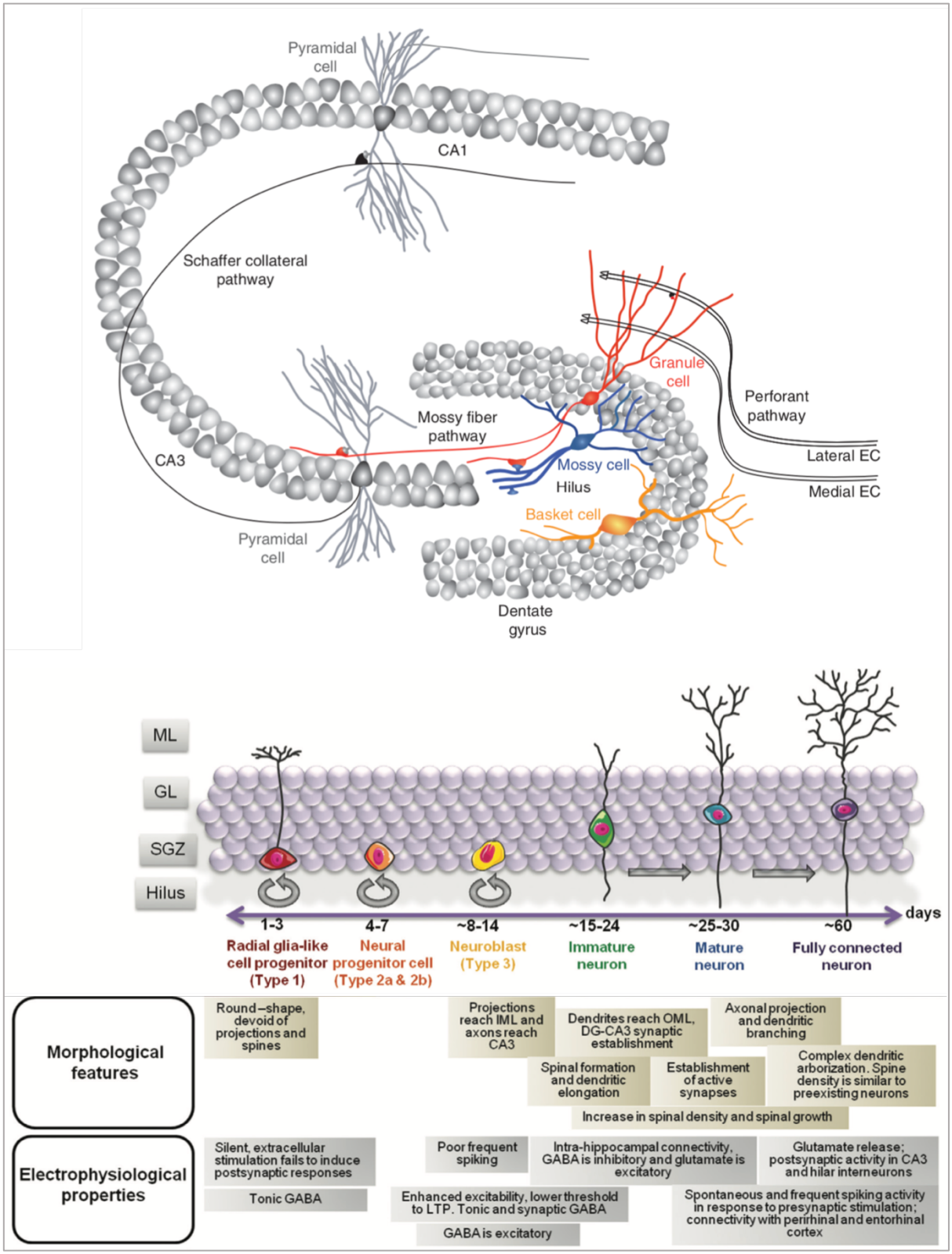


Figure 3 Functional integration of the newborn cells in the DG. a) Circuits of the hippocampus. The perforant pathway is the main source of inputs from the entorhinal cortex to the DG The unidirectional connection provides access to the region of the hippocampus formation forming the trisynaptic circuit. The DG does not return the projection into the entorhinal cortex. The entorhinal cortex gives cortical sensory information to the hippocampus to execute its functions. Adapted from Toni et al., 2015. Morphological and electrophysiological features during the maturation process which contribute to the neuron's integration in the preexisting neural circuits. Adapted from Aguilar-Arredondo et al., 2015

The starting point of the trisynaptic circuit lies in the entorhinal cortex. Neurons in the layer II of the entorhinal cortex project their axons to the DG and CA3 through the subiculum. However, the projection may come from layers V and VI, too (Steward et al., 1976) In rodents, the perforant pathway may be divided into two parts, the lateral and medial perforant paths takin into account if the projections are originated from the lateral or medial entorhinal cortex (Amaral et al., 2007). Thus, the projections terminate in the middle third of the molecular layers if the DG if they originate from the medial entorhinal cortex and terminate in the most superficial third of the molecular layer where they form asymmetric synapses if they came from lateral entorhinal cortex. These projections may also terminate in the CA3 and CA2 regions (Li et al.,2009).

The CA3 pyramidal cells project to other levels of the CA3 and to CA1, too (Li et al.,2009). The projection from CA3 to CA1 is called Schaffer collateral projection (Chronister, 1979, Vivar et al., 2013). The subiculum and the CA1 receive projections from the layer III of the entorhinal cortex and project to the deep layers of the entorhinal cortex through their pyramidal cells (Li et al.,2009).

The granule cells project to the CA3 through the mossy fibers (Vivar et al., 2013). The mossy fibers may also contact mossy cells and interneurons in the hilus (Li et al.,2009).

3.2 Integration of the newborn cells in the pre-existing neural circuits

Newborn cells in the adult brain have an important role in the hippocampal function. The addition of newborn neurons on existing neural circuitry contribute for cognitive processes under normal physiological and pathological conditions (Deng et al., 2010). They have been implicated in different brain functions, such as learning, memory, pattern separation and cognitive flexibility (Toda et al., 2018).

However, to attain functional maturation, new cells need to go through several steps until they integrate the complex network. As stated before, the environment and the activation of circuits during the development of newborn cells is a crucial factor for the time at which those cells reach their mature state and, also, for their functional integration (Aguilar-Arredondo et al., 2015).

Newborn neurons integrate gradually into existing circuits. Morphological analysis using molecular markers and electrophysiology are the commonly used techniques to determine the anatomical and physiological maturation of new neurons (Fig. 3b). Despite that fact, histological analysis is not the best method to study the maturation process. The maturation process is a continuous and not stage-defined process, and more than one marker could overlap the same or part of a particular stage of development (Aguilar-Arredondo et al., 2015). Another technique, retrograde tracing (Stanfield et al., 1988), is an effective method to analyze morphology and connectivity of the newborn cells. Retrograde tracing resorts to retroviral particles expressing fluorescent proteins such as GFP (van Praag et al., 2002), and has also been widely used.

Around 7 days after birth, new cells are round shaped cells without any projections and spines, but they could have short processes parallelly to the GCL without reaching the molecular layer (Esposito, 2005). Around this time point, the cells exhibit immature electrophysiological properties which are characterized by small amplitude, small numbers of spontaneous action potentials ((Aguilar-Arredondo et al., 2015, Esposito, 2005).

Around day 10, new cells reach the inner molecular layer, and 4 days after, they reach the middle of the molecular layer and start to show a more elaborate arborization (Aguilar-Arredondo et al., 2015). A few days later, new neurons begin to elongate dendrites and generate spines (Zhao, 2006). Within this period of time, new neurons also start to show electrophysiological properties which gives them more excitability, low input resistance and lower thresholds for long term potentiation (Aguilar-Arredondo et al., 2015). At maturation day 21, neurons finally reach the outer molecular layer and form axo-dendritic and axo-spinous synapses essentially with hilar interneurons, but also establish connections with other cells residing the hippocampus, including preexisting mature granule cells (Aguilar-Arredondo et al., 2015). At this moment, stimulation of glutamatergic receptors is crucial for the survival of newborn neurons and for increasing synaptic plasticity (Kempermann et al., 2015). On the other hand, GABA levels are extremely important for recognition of the neural activity (Ge, et al., 2005). The result of network activity on the new neurons is a tool to keep homeostatic mechanisms verifying that the integration of new cells will respond to the network's needs (Sahay et al., 2007).

Around day 28, new neurons already display a complex axonal and dendritic arborization and establish synaptic contacts (Zhao, 2006). At this moment, new neurons are able to respond to glutamatergic and GABAergic stimulation since at this stage, newborn neurons express both types of receptors (Esposito, 2005). Thus, from this moment new neurons resemble the preexisting mature neurons (Zhao, 2006).

Interestingly, around one month after birth, new neurons receive inputs from perirhinal cortex and lateral entorthinal cortex and have many glutamatergic afferents (glutamatergic synaptogenesis) (Vivar et al., 2012, Esposito, 2005). Electrophysiologically, at 4 weeks post-birth, new neurons exhibit excitatory and inhibitory inputs, generate spikes and release neurotransmitters onto postsynaptic neurons (Mongiat et al., 2011). Thus, the new cells are considered to be integrated, functionally, into the neural circuits at this stage. Then, by the second month of life, new neurons exhibit spontaneous spiking activity with higher frequency when it responds performant pathway stimulation, start to have an action potential and develop perisomatic GABAergic contacts (van Praag et al., 2002) and start to express glutamate receptors (Laplagne et al., 2006).

However, some authors clame the process of development and maturation is only given as fully completed approximately 6 months after birth (Toni et al., 2007). Interestingly, electrophysiology studies in mouse showed that maturation process may depend of the region that neurogenesis happens. In the dorsal DG, 21 days it is enough to exhibit properties of mature neurons, whereas in the ventral, the new cells need 28 days. In addition, the time required for cells maturation is greatly dynamic and modulators such as physical activity could increase the rate (Piatti et al., 2011). Despite the fact dorsal and ventral hippocampi have different behavioral roles, these regions also undergo different maturation processes based on histochemical (Bekiari et al., 2014) and electrophysiology studies (Piatti et al., 2011).

4. Classic modulators of hippocampal neurogenesis

4.1 Classic extrinsic modulators

The hippocampus is a highly plastic and adaptable brain region. For that reason, the number of new cells generated in the dentate gyrus may differ, taking into account the hippocampus reacts differently depending on the kind of stimuli that it receives. In this regard, neurogenesis is a process that contributes to process, adapt and respond to extrinsic stimuli (Duman et al., 2001). Previous studies have identified modulators that are either pro- or anti- neurogenic regulators in the SGZ of the dentate gyrus. Due to its particular relevance to work included in this thesis, I will focus on physical activity and treatment with antidepressants, as a pro-cytogenic modulators. In contrast, maladaptive stressful context is a well-known anti-cytogenic modulators.

Table 1 Stimulus that affected the hippocampus cytogenesis positively (pro-cytogenic) or negatively (anti-cytogenic).

Anti-cytogenic stimuli	Pro-cytogenic stimuli
Acute Stress Chronic Stress	Antidepressants Antidementials Physical activity

4.1.1 Stress as a neurogenic modulator

Stressful and threatening situations lead to a rapid response which will activate the sympathetic nervous system (SNS) and the hypothalamus-pituitary-adrenal (HPA) axis (Conrad, 2008). The SNS is responsible for stress response and the HPA axis operates a negative feedback response by releasing, shortly after stress exposure, glucocorticoid hormones from the adrenal gland (De Kloet et al., 1998) namely corticosteroids in the case of humans and corticosterone in most rodents (Conrad, 2008). The corticosteroids or corticosterone binds to glucocorticoids receptors located in hippocampus, more specifically in the DG (De Kloet et al., 1998) and the paraventricular nucleus of the hypothalamus (Joëls, et al., 2004) which, at this point, has increased level of c-fos in consequence of the enhanced local neuronal activity (Bain et al., 2004). This hormone has been shown to regulate neural progenitor cells' population in the adult DG (Kirby, et al., 2009).

Stress response stops when the perception of the *stress*-related *harmful* stimuli no longer exists. If exposure to stressful conditions persists, constant activation of stress response and the high levels of glucocorticoids may become harmful, as high levels of these hormones increase vulnerability of the hippocampus to damage (Conrad, 2008) reducing neurogenesis and impairing synaptic plasticity and the dendritic arborization (Sousa et al., 2000).

At basal conditions, corticosteroid hormone levels vary during day. Normally, levels in the morning and under rest are minimum. On the other hand, after stress exposure and during circadian peaks (end of the day), glucocorticoids receptors are considerably activated (Joëls, et al., 2004).

4.1.2 Acute exposure to stress

Situations of imminent danger will cause a fight or flight response, with an underlying neurologic substrate. Few hours of stress are sufficient to observe an increase in quantity of cells in the dorsal DG, but not on the ventral (Kirby, et al., 2009).

In addition, acute stress exposure may, in some circumstances, improve memory formation and cognitive flexibility (Kirby, et al., 2009) as a consequence of the newborn cells since the improvement is only observed 2 weeks after the stress exposure when the new neurons start play a role (Kirby, et al., 2009). Actually, this phenomenon is explained by the inverted U-shape function model. The model elucidates the consequences of stress for the healthy brain (Kirby et al., 2013). This model states that while severe and persistent stressors are harmful, moderated and short time stress may increase neural function (Kirby et al., 2013).

4.1.3 Chronic stress exposure

A model that has been frequently used by researchers to reproduce the etiologic characteristics of stress exposure in the development of stress-related behavior is the Unpredictable Chronic Mild Stress (uCMS) model. In consists in exposing, unpredictably, animals to mild stressors for several weeks. This model induces a depressive-like state characterized, essentially, by anhedonia and apathy (Nollet et al., 2013), along with anxious-like behavior and mild cognitive disfunction.

In contexts of chronic stress exposure, several impairments occur at the level of the hippocampus. Proliferation, survival and differentiation of newborn cells in the adult DG decrease, cognitive functions and emotion-related behavior are impaired and the levels of glucocorticoids increase (Mirescu et al., 2006).

Chronic stress and high levels of glucocorticoids lead to the impairment of hippocampal function which, consequently, will dysregulate the HPA axis (Conrad, 2008). High concentration of these hormone contributes for the susceptibility to hippocampal damage (Conrad, 2008). And, for such reason, glucocorticoids levels are often used as an outcome measure of the model (Nollet et al., 2013), that is, a proxy measure of stress levels, and, consequently the possible injury of hippocampal cells (Conrad, 2008).

4.2 Neuropsychopharmacologic effect

Recent studies demonstrate how neurogenesis can be affected by different pharmacological stimuli (Duman et al., 2001). Antidepressants have been shown to reverse the effect of stress on synaptic and dendritic structure of neural cells. The hippocampus is highly dynamic and plastic and the increased number of new cells observed when antidepressants are administered may be explained by the action of the antidepressants on the neural network (Sahay et al., 2007).

4.2.1 Fluoxetine

Fluoxetine is a selective serotonin reuptake inhibitors (SSRI) which belongs to second generation of antidepressants (commercially branded as Prozac®). This drug has a counteracting effect on stress and depressive-like status and when administrated chronically it has been showed to increase neurogenesis (Alboni et al., 2015).

Fluoxetine blocks the reuptake transporter protein placed in the presynaptic terminal of the serotonin neurons in order to prevent the reuptake of serotonin (Alboni et al., 2015. Serotonin seems to play a role in depression since low concentrations are found in the cerebrospinal fluid of animal models of depression, as well as patients diagnosed with depression (Patrício et al., 2014). SSRI antidepressants

are widely prescribed worldwide (Patrício et al., 2014); several studies show their effects on reverting depression-like behavior (Bessa et al., 2008), increasing neurogenesis (Malberg et al., 2000), reducing HPA axis activity and enhancing long-term potentiation within the trisynaptic circuit (Alboni et al., 2015).

Antidepressants allow to reverse emotional and cognitive impairments which are usually present in patients with depression symptoms (Mateus-Pinheiro et al., 2013). Interestingly, some of those ADs' effects were related to their ability to restore hippocampal neurogenesis and gliogenesis (Mateus-Pinheiro et al., 2013). However, different ADs have distinct impact.

4.2.2 Memantine

Recently, alternative drugs have been put forward as having a role in the treatment of anxiety and stress conditions. One of these, memantine, is an Ionotropic non-competitive glutamate receptor N-methyl-D-aspartate (NMDA) receptor antagonist commonly used for Alzheimer's disease, as and anti-demential drug (Sani ate al., 2012). Evidence shows that glutamatergic mechanisms could be involved in non-demential psychiatric disorders (Sani ate al., 2012). NMDA is a glutamate receptor and it has been implicated in anti-depressive responses (Skolnick et al., 2009). Interestingly, Ketamine, another NMDA receptor antagonist, has just recently put forward as short-term antidepressant drug, available for patients with depression (Quan et al., 2011). Memantine is currently used in moderate to severe Alzheimer's disease treatment (Kishi et al., 2017). This drug presents low-to-moderate affinity for noncompetitive NMDA receptor antagonist which binds to NMDA receptor (Kishi et al., 2017). Memantine counteracts the elevation of glutamate levels that otherwise would conduct to neuronal dysfunction (Danysz et al., 2003) and has been shown to regulate neuroplasticity, learning and memory (Deutschenbaur et al., 2016). Memantine and ketamine have similar pharmacological mechanisms, but memantine (Kishi et al., 2017) is less aversive and present less harmful side-effects than ketamine at therapeutic doses (Gideons et al., 2014).

Hence, glutamatergic neurotransmission seems to play an important role in depression treatment. Antidepressants such as ketamine cause a burst of glutamate which leads to synaptogenesis increase (Deutschenbaur et al., 2016). In the other hand, memantine blocks NMDA receptors located on inhibitory GABAergic neurons, and this will increase the levels of glutamate (Deutschenbaur et al., 2016).

4.3 Physical activity

Physical activity has known positive effects on several dimensions of heath in both humans and other mammals (Saur et al., 2013).

Contrary to external insults such stress exposure, physical activity has been shown to increase formation of new neurons in the adult brain, to improve cognitive functions, to counteract anxiety and depressive symptoms (Saur et al., 2013) and to play a neuroprotective effect in such scenarios.

In the case of rodents, environmental factors such as running activity is a favorable stimulus for synaptic plasticity (Cameron et al., 2015), cell survival (Chancey et al., 2013) and for adult neurogenesis (Cameron et al., 2015). Running increases, the quantity of newborn cells in the DG of adult rats (Stranahan et al., 2006).

Similarly, to stress response, running also involves changes in the HPA axis (Droste et al., 2003), with a hyper-activation only during physical activity (Stranahan et al., 2006). But, interestingly in this case, it is associated with a positive effect on brain and behavior (Stranahan et al., 2006). Studies in which animals that ran for 4 weeks, showed changes on HPA axis response. These finding seemed unexpected, considering the classic HPA axis response in aversive conditions; nevertheless, animals that practiced sustained exercise where shown to decrease anxiety-related behavior. However, is important to highlight that these changes on corticosterone levels are not observed in short-term exercise (Droste et al., 2009).

5. On Hilar Plasticity

5.1 Hippocampal hilus

At low magnification, hilar cells seem to present similar morphology between them. However, when they are carefully analyzed, one can distinctly notice how different cell subtypes populate the hippocampal hilus.

After embryonic development, large numbers of mitotic cells reside in the hilus. Knowledge about the hilar region is scarce in the adult mammalian brain. However, some information is documented. Until now, there are few insights on what cells are residing in this region. Some studies, based on Golgi analysis, show the presence of neurons with identical morphology to granule cells (Scharfman et al., 2006). These cells are called hilar ectopic granule cells (HEGCs) (Shapiro et al., 2005). Despite the fact that, at the beginning these cells were confused with interneurons. However, this theory was disregarded since it is very unusual for interneurons to present long spined multipolar morphology, in which the axonal plexus elongated along the septotemporal axis of the dentate gyrus (Amaral et al., 2007). This type of cells has a small cell body, with oval shape and could have a few somatic spines. On the other hand, these cells could also have a spherical cell body and numerous spiny dendrites (Shapiro et al., 2005).

Within the hilus several fusiform cells are typically present. The classic method to distinguish those cells is by the identification of the spines length spines (Andersen et al., 2007). Amaral (1978) described

a long-spined multipolar cells which are currently recognized as HIPP cell (hilar perforant path-associated cell) [58]. This type has two or three main dendrites which are localized parallel to the granule cell layer possibly extending for nearly the entire transverse length of one blade of the granule cell layer (Andersen et al., 2007).

The HICAP cells (hilar commissural-associational pathway-related cells) have a multipolar or triangular shape with thin, spiny dendrites that spread within the hilus and in the molecular layer (Andersen et al., 2007). Their axons extend throughout the granule cell layer and branch inside the molecular layer (Andersen et al., 2007).

The principal cells residing in the hilus are the mossy cells (Andersen et al., 2007). Their cell body is usually triangular or multipolar shape and large (25-35 μm). From the cell body, three or more thick principal dendrites come out and can reach long distances within the hilus region. Each of them bifurcates once or twice and, in general, generate few side branches within the hilus. The axonal plexus ramifies and extends throughout the hilus to penetrate the granule cell layer at noticeable distances from the soma (Andersen et al., 2007).

There is evidence showing that there are cells proliferating in the hilar region at the end of gestation period and also after birth. The pool persists during the granule cell neurogenesis and it has been put forward that a pool of hilar stem cells may also persist and form new neurons during adulthood (Andersen et al., 2007).

One of the hypotheses for the presence of these hilar cells lie on their migration from the hippocampal ventricular zone. A study showed that when a single X-ray irradiation protocol is applied to rats, neonatally, the generation of new cells in the hilus and ventricular zone was affected, on the contrary of the others neurogenic populations (Andersen et al., 2007).

Moreover, in humans, a similar process occurs. Cells proliferating in the hilus are present in the last phase of gestation, after birth and in adulthood (Andersen et al., 2007). Just like neurogenesis process in the subgranular layer, also in the hilus the percentage of newborn cells drops off considerably during the postnatal period (Andersen et al., 2007).

In light of these evidences, it seems reasonable to consider that the hilus may constitute a secondary germinal layer of the dentate gyrus, whose characterizations is still scarce.

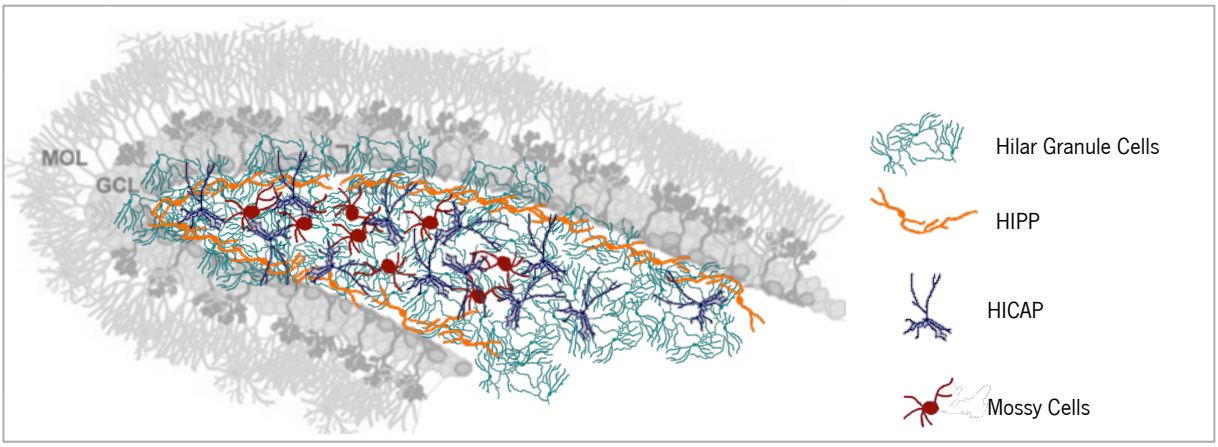


Figure 4 Hilar architecture characterization. Several studies have been increasing the knowledge of the hilar region. Most post-mitotic cells residing in the hilar region that have been described are hilar granule cells, HIPP cell (hilar perforant path-associated cell), HICAP cells (hilar commissural-associational pathway-related cells) and mossy cells.

RESEARCH OBJECTIVES

In light of the premises that I have previously discussed, in this thesis work we aim to bring to light key insights on adult neural plasticity of the hippocampal hilus, both in the healthy and *stressed* brain. As such, three main objectives were defined:

1. Characterization of hilar cytogenesis determining the phenotype and fate of the hilar cells in rodent model by immunofluorescence analysis;
2. Study of the modulation of hilar cytogenesis by extrinsic stimuli. Pro-cytogenic stimuli such as physical activity and psychotropic drugs were used as positive modulators, in opposition to acute- and chronic stress exposure.
3. Functional characterization of hilar dependent circuits. We have conducted a wide range of physiological and behavioral analysis in order to attain a first perspective on the role of newborn hilar cells. The identification of neural network of the new cells, using retroviral tracers, will allow for future elucidation of hilar cells function.

METHODS

1.1 Animals

Wistar Han rats, 8 weeks old males, from Charles River Laboratories, were used. Animal experiments were performed according to European Union Directive 2010/63/EU and were submitted to the local ethics committee. This project was approved by the Ethics Subcommittee for Life and Health Sciences (SECVS) of the University of Minho (SECVS 068.2017).

1.2 Acute Stress

Animals (control group, n=10; stressed group, n=9) were exposed to restrain stress for 3 hours in plastic restrainers. We administrated BrdU, *via* intraperitoneal injection (100mL/kg) twice in the interval of 1h during this procedure (Fig. 5a). The animals were sacrifice 24h after.

As a surrogate measure for acute stress exposure response, we collected blood, before sacrifice, by tail venous puncture to perform corticosterone analysis.

1.3 Unpredictable Chronic Mild Stress (uCMS)

To induce an anxiety- and anhedonic-like behavior in animals, a subgroup of animals was exposed to mild stressors during 6 weeks (Fig. 5c), (supplementary data). We divided the animals in four groups: CT, control group (n=12); uCMS, exposed to uCMS for 6 weeks (n=12); FLX, exposed to uCMS for 6 weeks and treated with fluoxetine (n=12); MEM, exposed to uCMS for 6 weeks and treated with memantine (n=11).

During the protocol, we weighted animals as a measure of the animals' well-being using as a human-endpoint 20% of the initial weight loss.

Drugs commonly prescribed in clinical settings, such as fluoxetine, as antidepressant, and memantine as an antidemential have a pro-cytogenic effect on the adult SGZ. Both drugs were administrated in last 2 weeks of stress protocol: fluoxetine -10 mg/kg and memantine - 20mg/kg. Untreated CT and uCMS groups were injected with saline solution during the last 2 weeks.

To label proliferative cells, we injected BrdU (50 mL/kg) in the last 5 days of stress protocol. Animals were sacrifice only 4 weeks after; with this timeframe, we provided time to newborn cells to migrate and differentiate into a mature phenotype.

At the end of uCMS exposure, as well as 4 weeks after its cessation, we determined the levels of circulating corticosterone in order to evaluate hormone imbalances caused by the stress exposure.

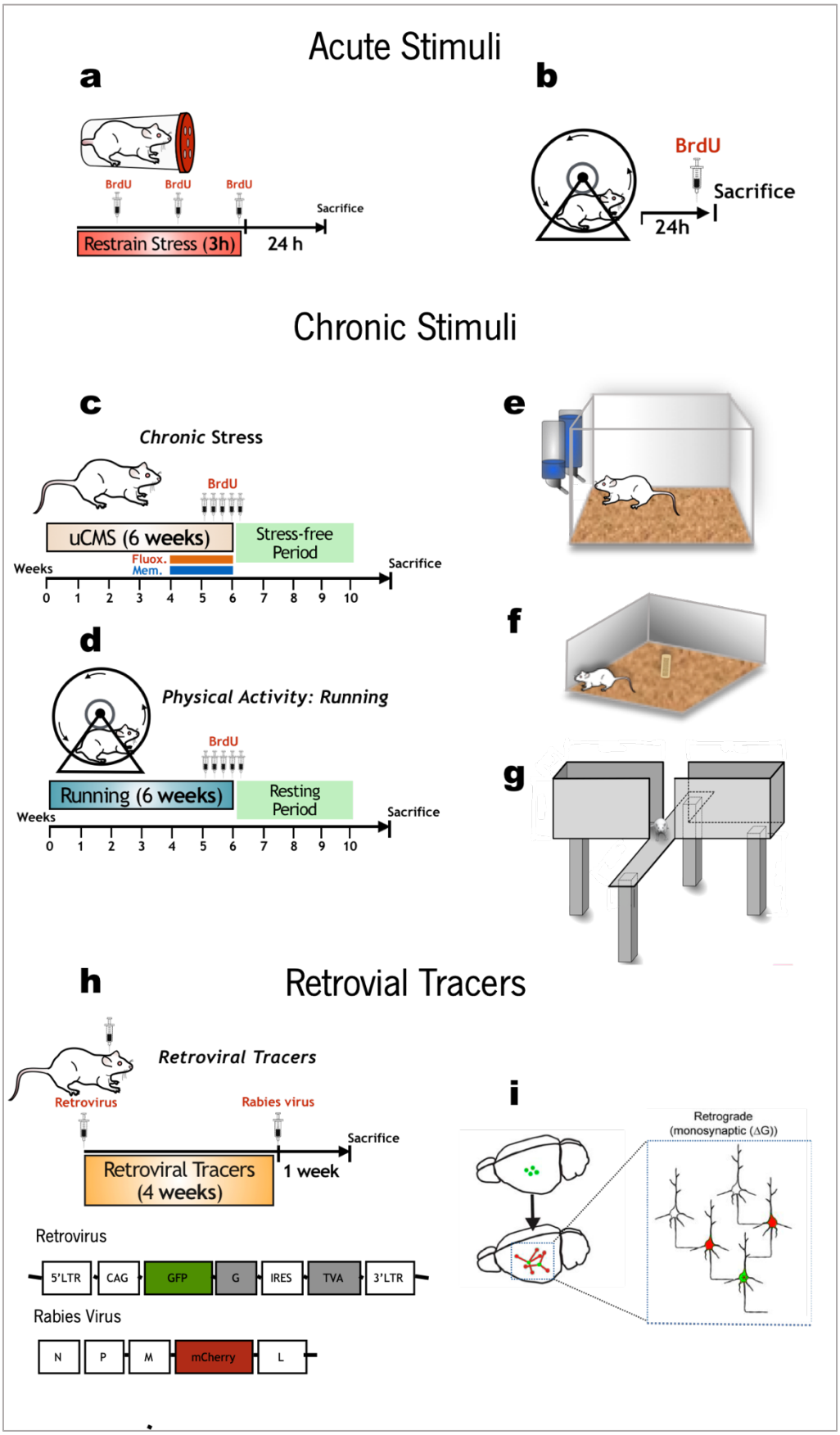


Figure 5 Methodology used to study the effect of extrinsic stimuli and neural circuits. a,b) Methodology for the acute stress and short-term physical activity experiments. c,d) Methodology for the chronic stress and sustained physical activity (6 weeks followed by 4 weeks of resting) experiments. e-g) Sucrose Preference Test (e), Novelty Suppressed Feeding test (f) and Elevated Plus-Maze test (g) were conducted in long-term experiments. h-i) Schematic depiction of the methodology used for neural circuits characterization. The first infection (retrovirus) is incorporated by the newborn cells only and is stained green (GFP). The second infection (rabies virus) stain the pre-synaptic neurons from the newborn cells stained in the first infection.

Before the beginning of treatment with fluoxetine/memantine, Sucrose Preference Test (SPT) was performed to evaluate whether animals present anhedonic behavior, as a pre-indicator of uCMS protocol efficacy. After drug treatment, behavioral tests were performed: SPT (Fig. 5e) and Novelty Suppressed Feeding (NSF) (Fig. 5f) to evaluate anhedonic and anxiety like-behavior, respectively. Furthermore, 4 weeks after stress cessation, we perform Elevated Plus Maze (EPM) (Fig. 5g) and SPT (methodology of behavioral tests can be found bellow).

1.4 Behavioral testing paradigms

1.4.1 Sucrose Preference Test

This test is performed was performed during the night-period (beginning at 9 pm) as to ensure animals were in their active phase during the trials. They had been food-deprived during the previous 12h. During the test, each animal was individually placed in cages with a pre-weighted bottle of water and second bottle with sucrose solution (2%) for 1 hour. At the end of the trial, bottles were re-weighted and preference for sucrose solution consumption was calculated (Mateus Pinheiro et al., 2013).

1.4.2 Novelty Suppressed Feeding

For this test, animals were food deprived 18 hours before the beginning of the trial. For the test trials, a food pellet is placed on the illuminated center of a dimly lighted open field arena, whose floor was covered with bedding. We evaluated latency to approach and take the food pellet as a measure of anxious-like behavior. After test completion, we assessed food consumption, by placing the animal on cage with pre-weighted food. Food consumption was used as a measure of appetite drive (Mateus Pinheiro et al., 2013).

1.4.3 Elevated Plus Maze (EPM)

The elevated plus maze consisted in an elevated, plus-shaped apparatus with two open and two closed arms (OA and CA, respectively). Animals were placed in the center of the plus-shaped apparatus and allowed to freely explore the structure for 5 minutes. For 5 minutes, animal movement was video recorded and subsequently automatically analyzed by Ethovion software. The percentage of time spent in the CA was used as a measure of anxious-like behavior (Mateus Pinheiro et al., 2013).

1.5 Short-term physical activity

To assess the impact of short-term physical activity (PA) on hilar cytogenesis animals, a small pilot assay was conducted (Fig. 5b). Animals were divided in two groups: CT (n=2), control animals not submitted to any experimental stimulus; RUN (n=2), group of animals that ran along one night.

Running wheels were adapted to the cages and the animals ran voluntary. Each animal had at their disposal one running wheel. The running wheel was connected to a rotations counter device. The session was also video recorded. In the next day, we administrated a single BrdU injection (100mg/kg) 2 hours before sacrifice.

1.6 Sustained long-term physical activity

To assess the impact of long-term physical activity on hilar cytogenesis, animals were divided in two groups: CT, control animals not submitted to any experimental stimulus (n=12); Run, animals that were allowed to run voluntarily for three days each week during 6 weeks (n=8) (supplementary data)(Fig. 5d).

Each night the number of wheel rotations was automatically registered. Since animals were group-housed (2 animals/cage), we divided that number of rotations by 2. To confirm that both animals ran equivalent distances, running sessions were recorded; when discrepancies between the two animals were observed, running distances were proportionally adjusted to each animal's video recorded running time.

In this running experimental layout, we performed the same behavior tests and at the same moment of the protocol as in the uCMS protocol. This means, SPT was performed in weeks 4, 6 and 10; NSF was performed at week 6 and EPM at week 10.

1.7 Neural Circuits

Viral tools (retrovirus and rabies virus) allow us to understand adult brain connectivity in which the newborn hilar cells are involved (Bergami et al., 2015, Deshpande et al., 2012).

A retroviral vector (CAG-G-TVA-GFP) encoding G (rabies virus (RABV) glycoprotein which is essential for retrograde transsynaptic virus monosynaptic transfer to presynaptic neurons (Bergami et al., 2015, Deshpande et al., 2012), TVA (avian receptor for the envelope protein EnvA used for pseudotyping RABV and for restricting the RABV infection to newborn neurons (Bergami et al., 2015) and the fluorescence reporter GFP (green colour) was injected intracranially in the hilar region. The vector integrates into the genome of dividing cells (Fig. 5 h and 5i).

After 4 weeks, the rabies virus was injected, intracranially, in the same region. The second injection contains the G deleted RABV, the envelope protein EnvA (which binds to the TVA receptor) and the fluorescence reporter mCherry. The fluorescence reporter stains the presynaptic neurons from those whose neurons incorporated the retrovirus (n=5) (Fig. 5h and 5i).

The stereotaxic surgery for both infections was performed under general anesthesia with a mixture of ketamine (75 mg/kg) and medetomidine (0.5 mg/kg) and a topic anesthetic, lidocaine. We delivery 2 µl of the AVV mix into the right dorsal dentate gyrus (DG) using the follow spatial coordinates relative to

Bregma: anterior–posterior = −4.36 mm; medial–lateral = 2.7 mm; dorsal-ventral = −3.4 mm (Paxinos et al., 2015). Analgesic (mixture of buprenorphine, 0.05 mg/Kg, and carprofen, 5 mg/Kg), and antisedative (Atipamezole, 1 mg/Kg) were administered subcutaneously at the end of the procedure. Post-surgery, the animals were monitored for four hours. In the following two days, every 12h the animals received regular analgesic treatment; animals were subsequently monitored daily for a week post-surgery. Sacrifice was performed one week after rabies virus infection.

1.8 ELISA

In order to assess corticosterone levels in plasma samples we used a Corticosterone Elisa Kit (SimpleStep Elisa, Abcam) (acute stress: CT, n=9; stress, n=8. Chronic stress: CT, n=8 ; CMS, n=8 ; FLX, n=8 ; MEM, n=8).

At the beginning, we prepared the standard solutions using as dilution factor 1:4 in 1X diluent M and, also, the plasma samples which are diluted at the concentration of 1:70 in 1X diluent M. Either for the standard solution or the samples we used two replicas of each. All the samples are placed in the plate (25µL), and then, 25 µL of biotinylated corticosterone protein is added to each well, cover it with sealing tape and left incubate for 2 hours. After, the plate is washed five times with 200 µL of 1X water buffer. Between washes, we tapped the place 4/5 times on absorbent paper towel to remove the liquid completely. Then, it is added 50 µL of 1X SP conjugate to each well, the plate is tapped, and it incubated for 30 minutes. Consecutively, we washed the plate as previously and added 50 µL of chromagen substrate for 20 minutes. Immediately before reading the plate in a microplate reader, we added 50 µL of stop solution.

1.9 Perfusion and tissue preparation

Animals were euthanized with pentobarbital (2ml/kg) and perfused transcardially with saline (0.9% NaCl) and 4% paraformaldehyde (PFA). Brains were removed and post fixed in 4% PFA for 24h at 4°C.

Brains were then transferred to a 30% sucrose solution at 4°C. Brains were subsequently frozen in liquid nitrogen and conserved in Optimal Cutting Temperature (OCT) compound at -20°C. Then, brains were sectioned on a cryostat, in 20 µm sections.

In addition, brains from animals infected with retroviruses were transferred to PBS 1X with azide after the post fixation with PFA and stored at 4°C. These were sectioned on vibratome, in 70 µm sections.

1.10 Immunofluorescence Analysis

Cryostat sections were incubated in PFA 4% for 30 minutes, washed (3 x 5 min) in 1X PBS (pH 7.4). Antigen retrieval was then performed for 20 minutes, using heated citrate buffer. Slides were then washed

with 0.5% Triton X-100 (3 x 5 minutes) following a permeabilization step for 10 minutes, and then, the sections were incubated in hydrochloric acid 2N for 30 minutes at room temperature (RT). Sections were then washed again with 0.5% Triton X-100 (3 x 5 min) and incubated in blocking serum (10% normal goat serum (NGS), abcam) for 30 min. Subsequently, sections were incubated with the primary antibody BrdU (1:200 Anti-BrdU rat, abcam); DCX (1:50 Anti-doublecortin mouse, Santa Cruz Biotechnology); GFAP (1:100 Anti-GFAP rabbit, DAKO); GFAP (1:100 Anti-GFAP mouse, ThermoFisher Scientific); S100β (1:200 Anti-S100b rabbit, DAKO) diluted in 0.5% Triton X-100 overnight at 4°C. In the next day, sections were washed with 1X PBS (3 x 10 minutes), and then incubated with secondary antibodies: 488 goat anti-mouse (1:1000, ThermoFisher Scientific), 594 goat anti-rat (1:1000, ThermoFisher Scientific); 488 goat anti-rabbit (1:1000, ThermoFisher Scientific); 647 goat anti-rabbit (1:1000, ThermoFisher Scientific) for 2 hours at RT. Then, it was washed 1X PBS (3 x 10 minutes). On double or triple stainins, a sequential overnight incubation protocol was followed. When primary antibody incubations were concluded, 2h incubation with the secondary antibodies was performed, followed by the incubation with DAPI (1:1000) for 10 minutes. Then, sections were washed again 1X PBS (3 x 5 minutes) and mounted with PermaFluor (ThermoFisher Scientific). Sections were stored at 4°C in the dark.

Vibratome sections were incubated in 0.5% Triton X-100 for 10 minutes followed by wash with 1X PBS for 10 minutes. Next, sections were incubated with the primary antibody GFP (1:1000 Anti-GFP rabbit, abcam) and mCherry (1:1000 Anti-mCherry chicken, Henbiotech) diluted in 0.5% Triton X-100 and 4% NGS overnight at 4°C. In the next day, they were washed with 1X PBS (3 x 10 minutes), and next incubated with secondary antibodies: 488 goat anti-rabbit and 594 goat anti-chicken for 2 hours at RT. Finally, sections were washed with 1X PBS (3 x 10 minutes), incubated with DAPI (1:1000) for 10 minutes, washed again as previously, and mounted. At the end, the sections were stored at 4°C.

1.11 Cell Countings

BrdU+ cells in the DG were counted in the subgranular zone and in the hilus, both in the dorsal and ventral dentate gyri. Cell densities were analyzed dividing the number of cells by the analyzed area.

Cells quantification or regions volumetry were performed in the fluorescence microscope, Olympus Widefield Upright Microscope BX61.

1.12 Statistics

Statistical analyses were performed using SPSS software. Normality test was used to test the normal distributions of the data. When two experimental group were compared, we used an unpaired two-sided Student's *t*-test and when the normal distribution was not achieved, we performed a nonparametric test,

Mann-Whitney *U* test. In the case of comparisons between more than one group, we performed a one-way ANOVA and a Krustal-Wallis when the normality was not assumed. For multiple-comparison post hoc analyses we used Tukey.

Graphs show mean values ± S.E.M, with 95% confidence interval.

RESULTS

1. Neuroproliferation in the adult hippocampal hilus

To address both young newborn cells and mature cells generated in the hilus, we adopted two experimental timelines (Fig. 6a): i) for a short-term proliferation/early cytogenesis analysis we injected BrdU on adult rats, and sacrificed them 24 hours post BrdU administration (n=4/group); ii) to characterize newborn hilar cells in terms of long-term survival and cell fate, we administered BrdU during 5 days, and sacrificed animals only 4 weeks later, to ensure newborn cells time to fully differentiate into their final cell fate (n=3/group). Within the hilus, as soon as 24 hours post BrdU injection, we were already able to observe hilar BrdU-positive cells, beyond those classically found in the SGZ (Fig. 6c). These cells were identified in both hippocampal poles (Fig. 6d). Due to the larger surface area of the hilar region, in comparison with thinner SGZ, hilar proliferation occurs at significant lower cell densities. Nevertheless, while BrdU-positive cells in the hilus represent approximately 41% of the DG proliferative cells pool (Fig. 6e), hilar proliferation is, proportionally, more expressive in the ventral hippocampal pole, where hilar proliferation accounts for 54% of all proliferation in the adult DG. Our immunostaining analysis allowed us to identify what we defined as *pseudo-DCX* cells within the hilus (Fig. 6g); these cells were specifically labelled with anti-DCX antibody but presented marked differences from their subgranular neuroblasts counterparts. Indeed, hilar BrdU/DCX double-positive cells lack the classical dendrito-axonal extensions of neuroblasts, presenting a immature-like cell shape. These *pseudo-DCX* cells are prevalent in the dorsal and ventral hippocampal pole ($\bar{x}_{dorsal}$=4.22, SEM=1.03; $\bar{x}_{ventral}$=2.94, SEM= .38; F=2.487, p= .129, t(22)=1.010, p= .323, d= .42)(Fig. 6h).

Within the hilus, after 4 weeks post BrdU injection, we still observed newborn hilar cells (BrdU+ cells) in the hilus (Fig 7a and b). However, It is possible to identify more BrdU+ cells in the SGZ than the hilus either in dorsal ($\bar{x}_{SGZ}$=28.77, SEM=3.78; $\bar{x}_{hilus}$=5.70, SEM= .90; F=15.953, p< .001, t(22)=-5.943, p< .001, d=1.93) or ventral (Mnd_{SGZ}=36.15, Mnd_{hilus}=16.08, U=77, p< .001).

2. Hilar newborn cell-fate

Four weeks after BrdU administration we analyzed cell survival and fate (n=3/group) (Fig. 7a); while in less extension compared with the SGZ, 4 to 5 weeks old newborn cells can be found in the hilus, in both hippocampal poles (Fig. 7b e 7c). While an extremely small proportion of newborn mature cells are likely to be astrocytes (GFAP+ and S100 β+ with similar proportion) (Fig. 7d and 7e), a small proportion are also likely to be NeuN+ neurons (Fig. 9n). Even so, the vast majority of BrdU+ cells at this time point, was negative for the tested cell markers, commonly expressed in newborn subgranular cells.

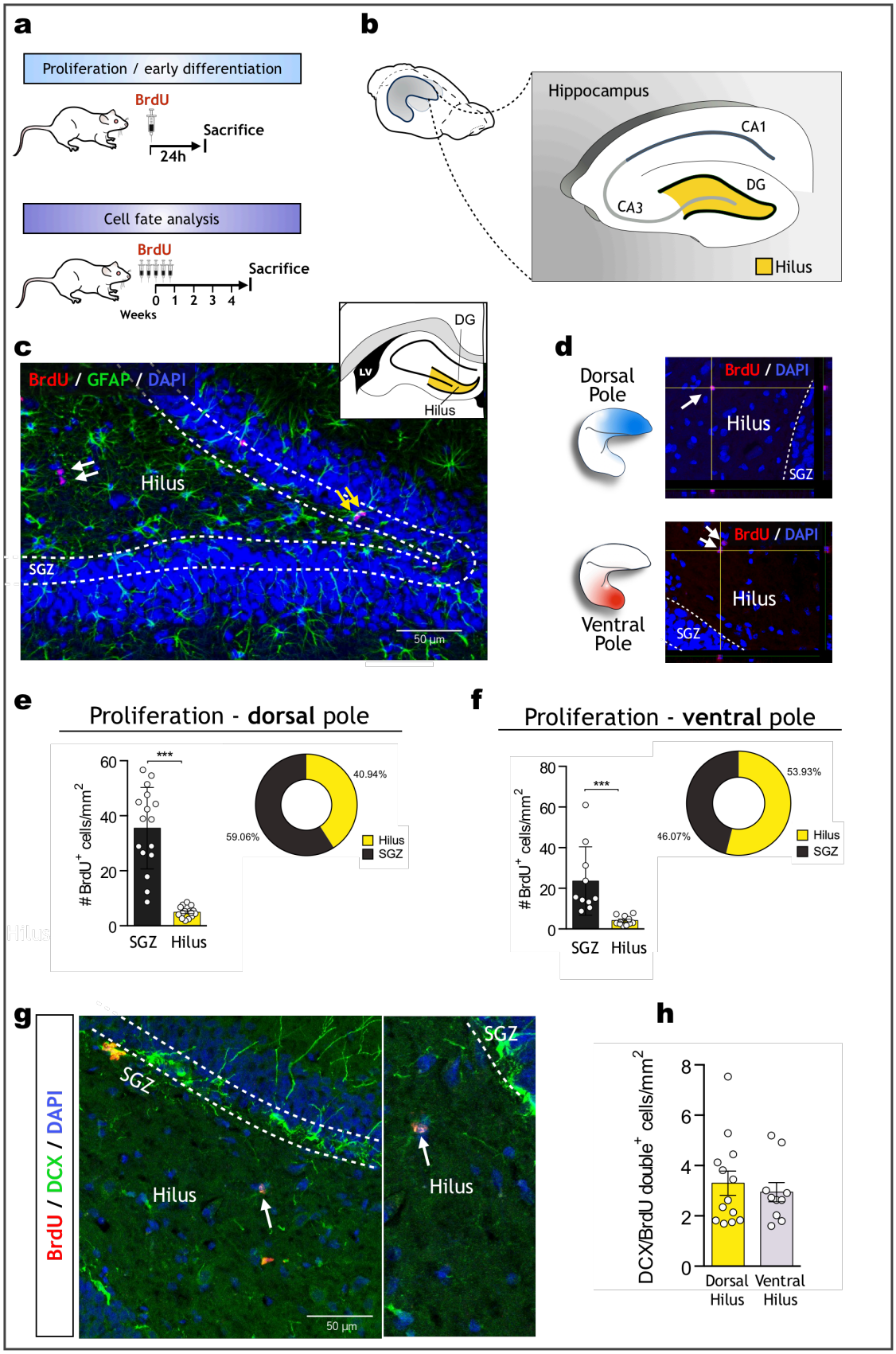


Figure 6 Neurogenesis in the subgranular zone and hilus in physiological conditions. a) Experimental timeline for the study of early proliferation and cell survival. b) Anatomical depiction of the hippocampal hilus. c) Hilar and subgranular proliferation (magnification 20x). d) Micrograph depiction of hilar proliferation in the dorsal and ventral poles (magnification 20x). e) Analysis of proliferation (BrdU-positive cells) in the dorsal pole (SGZ, n=17; hilus, n=15, ***, $p < .001$). f) Analysis of proliferation (BrdU-positive cells) in the ventral pole (SGZ, n=10; hilus, n=11, ***,$p < .001$). g) Proliferation analysis of neuroblasts in the dorsal hilus. Double-staining of BrdU (red) and doublecortin (green) in the hilar region. h) Cell quantification of hilar neuroblasts (dorsal, n=14; hilus, n=10). SGZ-Subgranular zone. Scale bar = 50µm

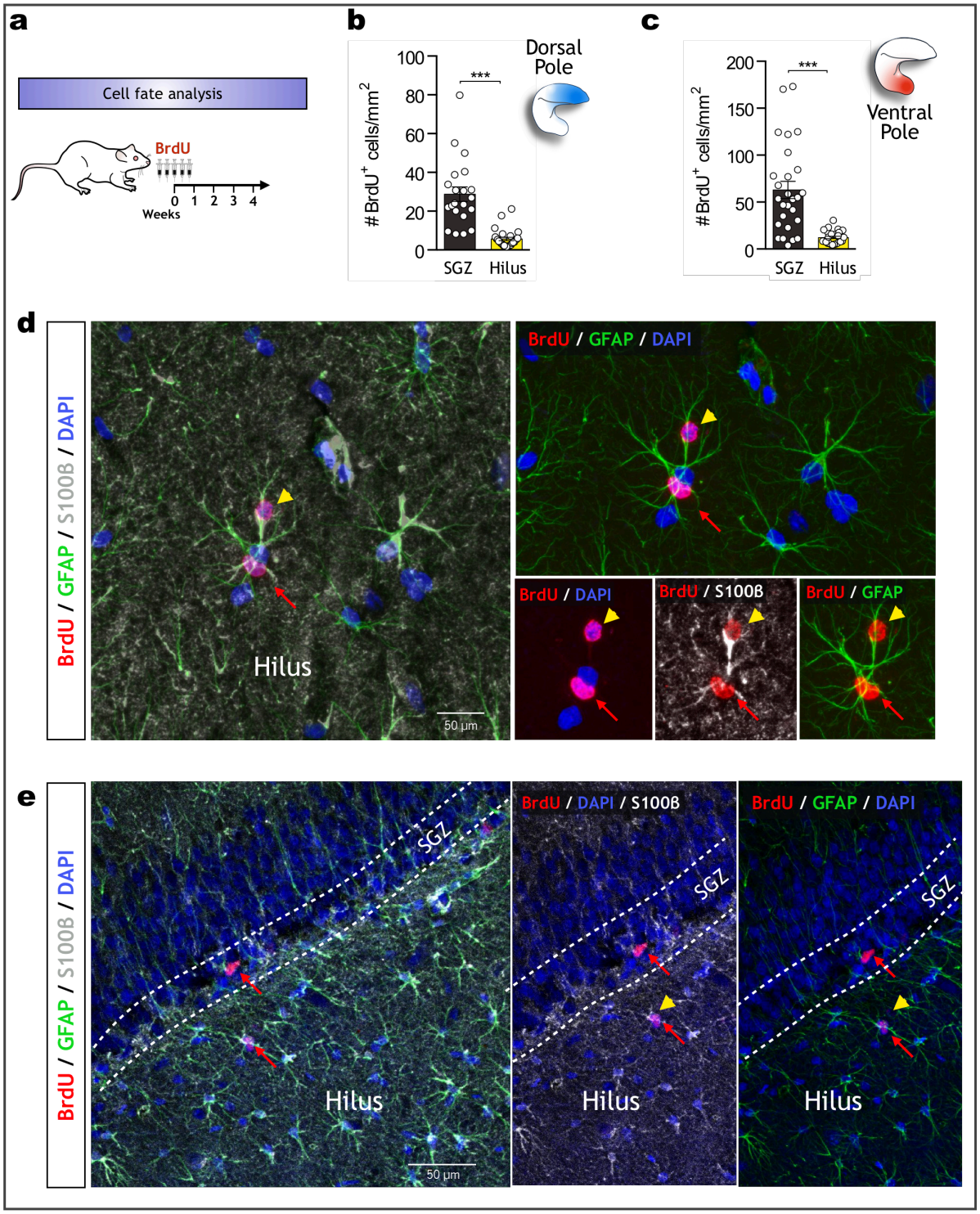


Figure 7 Phenotype of the newborn cells in the hilar region. a) Experimental timeline for long-term cell survival. a,c) Analysis of long-term cell survival in the dorsal pole (SGZ, n=21; hilus, n=27, ***, $p < .001$). (b) and in the ventral pole (SGZ, n=27; hilus, n=25, ***, $p < .001$). (c). d,e) Cell fate analysis using co-labelling of BrdU (red) and glial cell markers, GFAP (green) and S100β (grey). d) Analysis of one hilar cell labelled with BrdU (red) and S100β (grey) depicted by the yellow arrow and another BrdU-positive cell (red) not labelled with GFAP or S100β (grey) depicted by the red arrow. e) Analysis of hilar S100β (grey) and GFAP positive cell (green) co-labelled with BrdU (red) depicted by the yellow arrow. Red arrow depicted BrdU-positive cells. SGZ – subgranular zone. Scale bar = 50µm

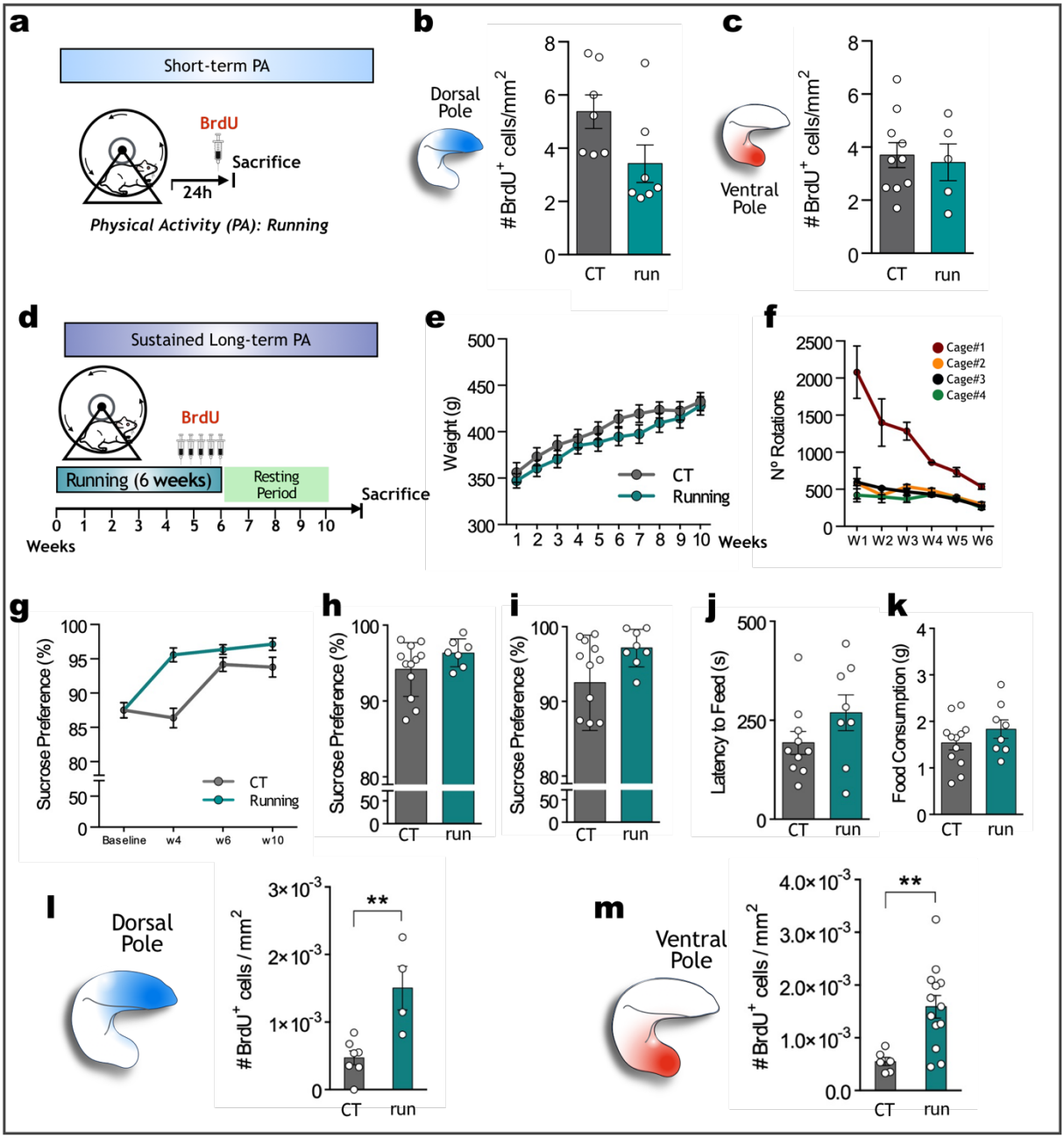


Figure 8 Effects of physical activity in cognitive functions and in neurogenesis. a) Experimental timeline of the protocol used for acute physical activity. b,c) Hilar proliferation in the dorsal pole (CT, n=7; run, n=7) (b) and ventral pole (CT, n=10; run, n=5) (c). d) Experimental timeline for chronic physical activity (6 weeks). e) Animal weight monitoring during the 6 weeks of running. f) Running activity monitoring; Number of rotations per cage for each week of exercise g) Analysis of anhedonic like-behavior by performing Sucrose preference test (SPT) along time. h,i) sucrose preference test of the control and running experimental groups at week 6 h) and week 10 i). j,k) Analysis of anxiety like-behavior by performing Novelty suppressed feeding (NSF) at the 6th week. j) Analysis of the time needed for the animal to reach the food. k) Analysis of the weight of food that the animal eats. l,m) Long-term hilar cell survival in control and running animals in the dorsal (CT, n=7; run, n=4; **, p= .001) (l) and in the ventral DG (CT, n=7; run, n=13; **, p= .001) (m). PA- Physical Activity; CT- Control group; run- animal's group running.

3. Chronic running elicits long-term hilar cytogenesis in both hippocampal poles

We next quantified the effects of a single running session on hilar cytogenesis (n=2/group). After the running session, animals were treated with BrdU and sacrificed 2h after (Fig. 8a). We successfully identified hilar BrdU-positive cells only 2h after BrdU administration (Fig. 8b and 8c). A single running

session did not produce any variation on the magnitude of hilar cytogenesis on the ventral pole or in the dorsal pole, but in the dorsal pole the proliferation on the SGZ is higher than in the hilus (Fig. 8b and 8c).

We next submitted adult rats to 3 voluntary running sessions per week, for 6 consecutive weeks, and analyzed the long-term effects on hilar cytogenesis (Fig. 8d), while weekly monitoring both weight (Fig. 8e) and running activity (n=8) (Fig. 8f). While animals who underwent running activity presented better hedonic status in the SPT (SPT week 6: $\bar{x}_{CT}$=94.17, SD=3.55; $\bar{x}_{RUN}$=96.15, SD=1.68; F=4.78, p= .042, t(16)=-1.70, p= .109, d= .68. SPT week 10: $\bar{x}_{CT}$=93.78, SD=4.80; $\bar{x}_{RUN}$=97.14, SD=2.51; F=7.45, p= .014, t(16)=1.98, p= .065, d= .84), no significant group effect was observed in comparison with control animals, most likely due to the already high values of sucrose preference this later group presented in this task (Fig. 8g-i). Moreover, we found no effects of chronic PA on anxiety-like behavior, in the novelty suppressed feeding task (Latency for feed: $\bar{x}_{CT}$=193.30, SD=91.80; $\bar{x}_{RUN}$=269.25, SD=127.89; F=1.162, p= .297, t(16)=-1.47, p= .161, d= .70. Food consumption: $\bar{x}_{CT}$=1.48, SD= .51; $\bar{x}_{RUN}$=1.84, SD= .55; F= .037, p= .850, t(16)=-1.47, p= .178, d= .67)(Fig. 3j and 3k).

Interestingly, chronic running elicited a marked pro-proliferative effect on the dentate hilus, as shown by the increase of BrdU-positive cells both in the dorsal (Fig.8l) and in the ventral hippocampal poles (Fig. 8m).

4. On the effects of maladaptive stress and psychotropic drugs over hilar cytogenesis

Our results show that animals subjected to an acute restrain stress presented the classical hyperactivation of the HPA axis, as shown by the acute peak on corticosterone secretion, markedly increased when compared to naive animals (Mnd_{CT}=5.83, Mdn_{CMS}=12.56, U=7.5, p= .004) (Fig. 9a and 9b). Nonetheless, acute stress exposure did not influence cell turnover on the dorsal dentate hilus (Dorsal pole: Mnd_{CT}=26.48, Mdn_{CMS}=24.14, U=2.75, p= .575) (Fig. 9c)but the number of new cells in the ventral decrease significantly (Ventral pole: $\bar{x}_{CT}$=4.96, SEM= .076; $\bar{x}_{CMS}$=3.00, SEM= .43; F=7.415, p= .012, t(24.23)=2.244, p= .034, d= .071 (Fig. 9d).

Next, we explored the effects of chronic stress exposure on the hilar cytogenic process (n=12/group) (Fig. 9e). As previously described, stress exposure promoted a significant weight loss on untreated animals (Fig. 9f); During the drug administration period, both fluoxetine and memantine aggravated weight loss ($X^2(2)$=17.23, p< .001, CMS vs MEM: p= .005, CMS vs FLX: p< .001), which was fully recovered in the weeks following stress cessation. Moreover, uCMS exposure disrupted the normal circadian corticosterone secretion pattern, observed in naive animals, abrogating nadir-to-zenith fluctuations (CMS: $\bar{x}_{nadir}$=66.04, SEM=8.07; $\bar{x}_{zenith}$=65.69, SEM=22.01; F= .056, p= .817, t(13)= .030, p< .977, d= .02) (Fig. 9g). Chronic stress exposure induced anhedonic behavior in untreated animals (Week

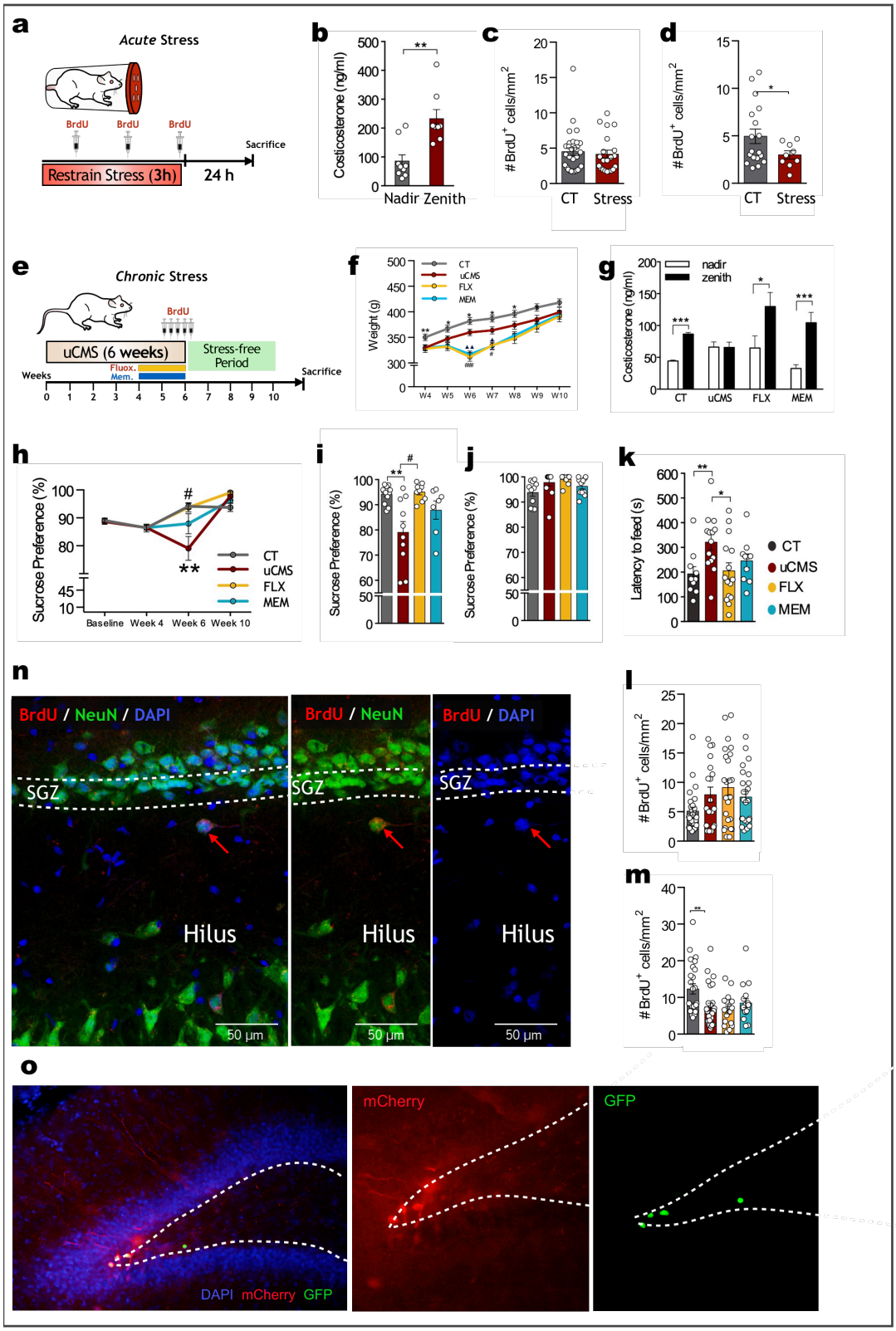


Figure 9 Effects of acute and chronic stress in cognitive functions and in neurogenesis. a) Experimental timeline. b) Corticosterone measurement in acutely stressed animals from the blood collected before the sacrifice (nadir: n=9, zenith): n=8). c,d) Hilar proliferation in the dorsal (CT, n=29; CMS, n=21) (c) and ventral poles of the DG (CT, n=18; CMS, n=9; *, p= .01) (d). e) Experimental timeline for the chronic stress. f) Weight monitoring in each week. g) Corticosterone measurement in control and chronically stressed experimental groups (with and without treatment) (n=8/group). h) Analysis of anhedonic like-behavior by performing Sucrose preference test (SPT) of the stress and pharmacological experiment at week 6, (**, p= .001; #, p= .01) and week 10 j). k) Analysis of anxiety like-bahavior by performing the Novelty suppressed feeding (NSF) test. Time needed for each animal to reach the food. (*, p= .01; **, p= .001). l,m) Long-term cell survival in stressed animals in the dorsal (CT, n=26; CMS, n=19; FLX, n=24; MEM, n=24) (l) and in the ventral DG (CT, n=25; CMS, n=26; FLX, n=17, MEM, n=17; **, p= .001) (m). n) Analysis of mature neurons - hilar cells with double staining for BrdU (red) and NeuN (green). o) Hilar retroviral tracing using the fluorescent marker GFP (green) (is incorporated by newborn cells) and mCherry (red) (stain the presynaptic neurons). uCMS- Unpredictable Chronic Mild Stress; CT- control group; FLX- Fluoxetine; MEM- Memantine. Scale bar = 50µm

6: $\bar{x}_{CT}$= 94.17, SD=13.47; $\bar{x}_{CMS}$=79.06, SD=13.47, F= 15.57, *p*= .001, t(10)=3.448, *p*= .006, *d*= 1.61. Week 10: Mnd_{CT}=8.09, Mdn_{CMS}=15.58, U=23, *p*= .007) (Fig. 9i and 9j), which were reverted by fluoxetine treatment ($X^2(2)$=19.02, *p*= .007, CMS vs FLX: *p*= .014) but not by memantine ($X^2(2)$=19.02, *p*= .007, CMS vs FLX: *p*= .051) (Fig. 9i). Normal hedonic status was reverted after the stress-free period (Fig. 9j). Animals submitted to uCMS presented additional emotional deficits as shown by an increase latency to feed in the novelty suppressed feeding task ($\bar{x}_{CT}$= 193.30, SD=91.80; $\bar{x}_{CMS}$=321.43, SD=112.87, F= .618, *p*= .440, t(22)=-2.95, *p*= .007, *d*= 1.22) (Fig. 9K); heightened anxiety, was reverted by fluoxetine treatment (F(2,36)=3.836, *p*= .031, n_p^2= .176, CMS vs FLX: *p*= .028), but not by memantine(Fig. 9K).

When analyzing the effects of uCMS exposure on hilar cytogenesis, our results showed that chronic stress exposure has no significant effects on the dorsal pole (Fig. 9l). Contrastingly, in the ventral pole, chronic stress exposure significantly decreased the number of hilar BrdU-positive cells (Fig. 9m).

Furthermore, none of the pharmacological treatments produced differential effects on hilar cytogenesis.

5. Neural circuits

Regarding the analysis of the neural circuits, during this thesis work we were able to perform intra-cerebral stereotaxic injections and to perform post-op staining analysis that confirmed the viral vectors were successfully injected in the interested region (n=5) (Fig.9o). This will allow for future work on the characterization of newborn hilar cells connectivity.

DISCUSSION

1. The dentate hilus as new neurogenic milieu

The SGZ is a neurogenic region canonically accepted. However, in this work, we were able to report newborn cells within the adult hippocampal dentate hilus. These newborn cells, that incorporated BrdU, are identifiable in the hilus as soon as 2h after BrdU administration. Moreover, 24h and 4 weeks post-BrdU injections, these cells persist in the hilus, showing long-term survival in this region. Therefore, newborn cells are likely generated in the hilus (rather than ectopically migrating to this region) where they are able to differentiate and survive. In this work, however we are not able to conclusively exclude that these cells can be generated in the SGZ, to only then ectopically migrate towards the hilus; even so, the early identification of newborn BrdU-positive cells 2h after BrdU administration in central hilar positions strengthens the possibility of an *in situ* cytoproliferative process.

Furthermore, our results show, that hilar cytogenesis occurs longitudinally through the entire septo-temporal axis of the hippocampal formation, as newborn cells were observed both in the dorsal and ventral poles. However, when comparing the magnitude of hilar cytogenesis with its SGZ counterpart, this process is proportionally higher in the ventral hippocampus, where the Hilus to SGZ newborn cells ratio is about 1:1. In the dorsal pole, this ratio was found to be reduced (with a Hilus-to-SGZ relation of 40% to 60%). In fact, the dorsal and ventral poles of the DG have been suggested to contribute to different functions in the brain. The dorsal DG has been more related to cognitive processes whereas the ventral DG has been shown to contribute mostly to emotional functions (Moser et al., 1995; Fanselow et al., 2010; Kheirbek et al., 2013). In the SGZ, a canonical neurogenic region, the increase of newborn cells number has been shown to be highly important for learning, memory, pattern separation, cognitive flexibility and emotional processes (Toda et al., 2018). In this work, we were unable to ascertain the functional relevance of the dorsal-to-ventral regional dichotomy; in light of the pivotal role of the temporal pole in emotional processing, one may speculate on the putative importance of hilar cytogenesis to these behavioral domains, as further discussed bellow.

2. On the cellular phenotype of newborn hippocampal hilar cells.

New cells have to pass through several phases to reach complete maturation and attain mature functional properties. The generation of new neurons in the SGZ has been already well-characterized. The phases of development of a new cell in the adult brain can be divided in proliferation, differentiation, survival, migration, maturation and integration (Gonçalves et al., 2016, Li et al.,2009). In the beginning of the cells development, NPCs proliferate in the SGZ, then cells differentiate into a specific type of cells

and during the maturation process the axons and dendrites develop and maturate increasing their complexity. Consequently, those cells become capable of receiving information and integrate the neural circuits. The cell's maturation process is a highly complex process that could take almost 1 month to be completed (Vivar et al., 2013). On the other hand, cell cytogenesis in the hilar region is not described at all. In this work, we show that proliferative cells reside in the hilus too, but the maturation method is not known yet.

Our early-differentiation analysis shows DCX-positive cells within the hippocampal hilar region; however, these *pseudo-DCX* hilar cells present what seems to be an incipient DCX labelling, with a poorer arborization. Since it is well known that synaptic input from surrounding mature cells is critical for a successful differentiation of newborn cells (Kempermann et al., 2015) it becomes likely that the decreased cell density of the hilar milieu, in comparison to the much denser granular region, provides only few synapses to the newborn cells, contributing to less development of their axo-dendritic structure. Hence, while these results may suggest early neuronal lineage commitment, the above stated particularities highlight the necessity to conduct further immunocytological analysis to confirm this claim. In this regard, future studies of 3D morphological reconstruction of these cells using techniques such immuno-golgi (Scharfman et al., 2006) or structural analyses using electron microscopy may contribute to the confirmation of the neuronal properties of newborn hilar cells.

Additionally, when analyzing long-term cell fate, most new hilar cells were negative for the mature neuronal cell marker, NeuN, typically expressed in mature newly generated granular neurons in the DG. Moreover, most of these cells we also negative for the glial cell marker, GFAP or S100β. However, we found very scarce hilar cells staining for both of these glial marker (Fig 7d, e). Our results show that this very small population or newborn astroglial cells are not significantly modulated by external cues, and most likely represent disperse points of astrogliogenesis, known to occur ubiquitously all over the adult mammalian brain. Two points, at opposite ends of the differentiation process, must still be addressed in future work. First, progenitor cell markers must be identified in newborn hilar cells, to ascertain whether progenitor cells are indeed resident in this region; secondly, terminal differentiation neuronal markers must be assessed, in light of the negative staining for NeuN. Classic markers such as the transient marker calbindin, or calretinin can prove to be useful in this analysis.

3. Physical activity as a modulator of hilar cytogenesis

In general, running activity is a favorable stimulus for brain functions. Contrary to the stress stimuli, the animals showed no signs of anxiety-like or anhedonic behavior. The voluntary physical activity was shown to increase hippocampal neurogenesis and to improve cognitive functions (van Praag et al, 1999).

Similar to what happens in the SGZ, physical activity (PA) was found to be a positive modulator of adult hilar cytogenesis. However, short-term PA was not sufficient to significantly bolster hilar cytogenesis. As animals were isolated during physical activity and isolation is known to be a mitigating factor which leads to decrease of neurogenesis in the SGZ (Stranahan et al, 2006), this might have also induced a decrease of the number of newborn cells in the hilus and mask the effects of PA. According to research in the field, 4 days of PA is enough to observe an increase in the number of NPCs in the SGZ. In fact, only when submitting adult rats to repeated running sessions for 6 weeks, we were able to find increased proliferation and cell survival, in both hilar poles. However, we did not observe any improvements on cognitive or emotion-related behavioral tasks, as control animals displayed already very high performances in the conducted tests.

Stress impairs hilar neurogenesis in a regional-specific manner: only ventral DG is susceptible to acute/chronic stress exposure. Cell cytogenesis in the SGZ, in both dorsal and ventral poles, was already shown to be reduced after stress exposure being correlated with cognitive and emotional deficits (Warner-Schmidt and Duman, 2006).

Animals exposure to acute restraining stress promoted impairments in the corticosterone secretion pattern; these animals presented blood hypercorticosteronemia, an expected and well described effect that results from HPA axis overactivation. This was a surrogate measure of acute-stress exposure efficacy. Consequently, brain cytogenesis levels also undergo alterations. Indeed, contrarily to the short-term insensibility of hilar proliferative cells to PA, a short-term exposure to stress was sufficient to significantly decrease hilar cytogenesis. Importantly, the observed effect was not seen throughout the entire septo-temporal axis of the hippocampus, but rather only in its ventral pole. Interestingly, the ventral (temporal) domain of the hippocampus (the “hot” hippocampus, as it has already been called) has been more directly linked to emotion-related behavioral dimensions, namely in what concerns for instance to hedonia, affective states, motivation or fear/anxiety (Moser et al., 1995, Fanselow et al., 2010, Kheirbek et al., 2013). This regionally specific effect of stress, was also observed after uCMS exposure, as decreased newborn cells’ long-term survival was only observed in the ventral pole. Although chronic stress exposure is known to affect particular cognitive functions (Mateus-Pinheiro et al., 2013), it is widely used to produce core symptoms of psychiatric disorders, such as anhedonic or anxious-like behaviors, as those we described in the results of this thesis. Hence, and despite the fact we cannot establish a definitive causal relation between variations in hilar cytogenesis and behavioral alterations, the specificity of this effect to ventral domains raises the possibility that newborn hilar cells might be actively involved in emotion-related behaviors control. It is important to highlight how connectivity analyses, that we have started in this thesis,

will allow for future identification of which neural substrates are these new hilar cells modulating, providing a consequential link to what behavioral domains are they controlling.

4. Regulation of hilar cytogenesis by fluoxetine and memantine

A final discussion note to address the observed effects of psychotropic drugs on hilar cytogenesis. SGZ neurogenesis can be affected by different pharmacological stimuli. Drugs such as fluoxetine have an opposing effect of the stress and depression and when administrated chronically in those cases, they display the ability to increase neurogenesis (Alboni et al., 2015, Mateus Pinheiro et al., 2013). In addition, this antidepressant has been shown to revert the emotional and cognitive deficits induced by stress exposure (Mateus Pinheiro et al., 2013). Recently, alternative drugs such as memantine have been put forward as having a role in the treatment of anxiety and stress conditions and, as fluoxetine, memantine treatment can revert anhedonia (Réus et al., 2012). Behavior tests we have performed show that while fluoxetine has a therapeutic effect on anxiety and anhedonic like-behavior, memantine was not able to revert any of these deficits.

Interestingly, neither of the two chronic pharmacologic treatments produced a reversion on the anti-cytogenic effects of uCMS in the hilar region. Again, this highlights how hilar cytogenesis displays particular properties in modulation by extrinsic factors, different from those observed in the SGZ. In fact, by integrating our data with available literature on SGZ cytogenesis, it is possible to put the participation of both niches in the pathogenesis of stress-related disorders into perspective: similarly to what is described in SGZ cytogenesis, the hilar region serves as pathogenic gateway of the stress response, as seen by an impaired proliferation, specific of the ventral pole; contrastingly, ADs do not seem to modulate hilar proliferation and, therefore, their effects on recovering neural plasticity seem to be conveyed only through the classical SGZ niche.

To study the degree of interdependency of these two cytogenic niches, and exact functional importance of both of them will likely unveil key aspects on how our environmental context reshapes and modulates mammalian brains function.

FINAL REMARKS

Taken all together, this work reinforces how fundamental textbook neuroscience knowledge is in constant mutation and needs to be revisited.

Indeed, we have identified a new cytogenic subregion in the adult brain, with specific responses to external stimuli, that differ from those of previously known canonical neurogenic niches. Although we are still not able to ascertain the full functional implications of this observation, our results put forward the possibility that cytogenesis may play different functional roles in the hilus, versus the SGZ, in processing and control of complex cognitive and emotion domains.

To pinpoint the exact mature phenotype of these cells and their circuitry will be fundamental to answer these questions and to better understand the importance of this newly identified and conserved neural plasticity niche.

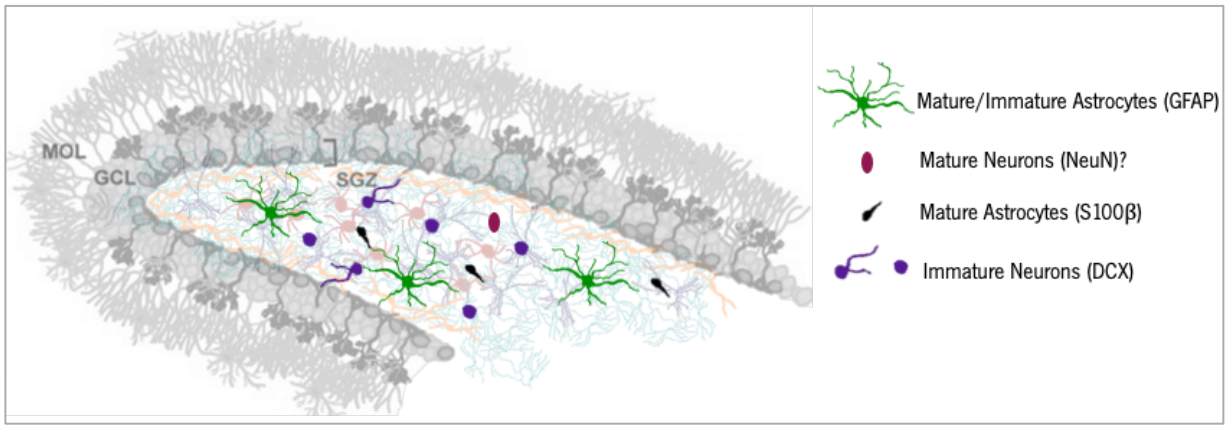


Figure 10 New hilar architecture characterization. This work improves the knowledge of the hilar region showing that post-mitotic cells residing in the hilar region seem maturate in glial cells but not in neurons. However, in initial stages of development the newborn cells seem to be immature neurons.

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Supplementary data

Annex 1: Unpredictable Chronic Mild Stress Protocol

Week 1	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Tilted Cage	Cage replacement	Strobe Lights	Inacc. Food	Confinement		
10h - 11h		Empty Bottle		Startle Noise			
11h - 12h		Startle Noise	Confinement		Tilted Cage		
12h - 13h							
13h - 14h	Confinement		Tilted Cage				
14h - 15h	Strobe Lights	Overcrowding		Overcrowding	Strobe Lights		
15h - 18h							
O/N	Water Dep. + ON Illumination	Tilted cage	food deprivation	Wet Bed	Inv. Light + Tilted Cage		

Week 2	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Tilted Cage	Inacc. Food	Empty Bottle	Overcrowding	Confinement		
10h - 11h		1h food intake	Tilted Cage (6h)		Strobe Lights		
11h - 12h		Startle Noise					
12h - 13h							
13h - 14h	Confinement			Cage Switch	Tilted Cage		
14h - 15h	Strobe Lights	Overcrowding					
15h - 18h			Confinement (2h)	Startle Noise			
O/N	Food Dep.+tilted cage	Water Dep. + ON Illumination	Strobe Lights	Wet Bed	Rev. Light + Tilted Cage		

Week 3	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Confinement	Inacc. Food	Empty Bottle	Cage Switch	Startle Noise		
10h - 11h		1h food intake	1h water intake				
11h - 12h	Startle Noise	Strobe Lights	Overcrowding		Confinement		
12h - 13h							
13h - 14h		Tilted Cage	Confinement				
14h - 15h	Overcrowding		Startle Noise	Tilted Cage	Overcrowding		
15h - 18h							
O/N	Food Dep. + wet bed	Water Dep. + ON illumination	Strobe Lights	Wet Bed	Rev. Light + Tilted Cage		

Week 4	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Confinement	Inacc. Food	food/water deprivation	Cage Switch	Overcrowding		
10h - 11h		1h food intake	Startle Noise				
11h - 12h	Strobe Lights	Overcrowding					
12h - 13h							
13h - 14h	Tilted cage		Strobe Lights	Startle Noise	Tilted Cage		
14h - 15h				Confinement			
15h - 18h		Confinement			Confinement		
O/N	Food Dep. + wet bed	ON illumination	SPT 4th week + wet bed	Strobe lights	Rev. Light + Tilted Cage		

Week 5	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Overcrowding	Inacc. Food	Empty Bottle	Tilted Cage	Confinement		
10h - 11h		1h food intake	1h water intake				
11h - 12h		Tilted Cage	Startle Noise	Cage Switch	Startle Noise		
12h - 13h	Startle Noise						
13h - 14h			Overcrowding	Strobe Lights	Overcrowding		
14h - 15h	Strobe Lights	Confinement					
15h - 18h							
O/N	Wet Bed + food deprivation	water Dep. + O/N Illum.	Strobe lights	Wet Bed	Rev. Light + Tilted Cage		

	BrdU (50mg/kg)						
Week 6	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h	Strobe Lights	Inacc. Food	Empty bottle	Food/water deprivation	Confinement		NSF
10h - 11h		1h food intake	1h water intake	Tilted Cage			
11h - 12h		Cage Switch	Confinement	Startle Noise	Tilted Cage		
12h - 13h							
13h - 14h	Confinement		Overcrowding	Cage Switch			
14h - 15h	Overcrowding	Tilted Cage			Strobe Lights		
15h - 18h							
O/N	Wet Bed + food deprivation	water Dep. + O/N Illum.	Strobe lights	SPT 4th week + wet bed	Rev. Light + Tilted Cage		

Week 7	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h		NSF	Blood collection				
10h - 11h							
11h - 12h							
12h - 13h							
13h - 14h							
14h - 15h							
15h - 18h							
O/N			Blood collection				

Week 10	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h						EPM	SPT
10h - 11h							
11h - 12h							
12h - 13h							
13h - 14h							
14h - 15h							
15h - 18h							
O/N							

Week 11	Mon	Tue	Wed	Thu	Fri	Sat	Sun
9h - 10h		Blood collection	Sacrifice				
10h - 11h							
11h - 12h							
12h - 13h							
13h - 14h							
14h - 15h							
15h - 18h							
O/N		Blood collection					

Annex 2: Chronical Physic Activity Protocol

Week 1	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h	Group A	Group B	Group A	Group B	Group A	Group B	

Week 2	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h	Group A	Group B	Group A video record	Group B video record	Group A	Group B	

Week 3	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h	Group A	Group B	Group A	Group B	Group A	Group B	

Week 4	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h	Group A	Group B	Group A	Group B	Group A	Group B	SPT

Week 5	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h	Group A	Group B	Group A	Group B	Group A	Group B	

Week 6	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h	Group A	Group B	Group A video record	Group B video record	Group A	Group B	SPT

Week 7	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h			NSF				

Week 11	Mon	Tue	Wed	Thu	Fri	Sat	Sun
20h - 9h	EPM	SPT		Sacrifice			