Universidade do Minho
Escola de Medicina

Ana Catarina Oliveira Ferreira

Dissecting Lipocalin-2 as a novel modulator of brain processes

março de 2017

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Dissecting Lipocalin-2 as a novel modulator of brain processes

Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação da
Prof. Doutora Fernanda Cristina Gomes de Sousa Marques
e do
Prof. Doutor João Carlos Sousa

março de 2017

STATEMENT OF INTEGRITY

I hereby declare to have conducted my thesis with integrity and I confirm that I have not used plagiarism or any form of falsification of results in the process of the thesis elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

University of Minho, 29 de março de 2017

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‘To me, that’s the beauty of science:
to know you will never see everything, but you never stop wanting to;
that when you learn something, for a second you feel crazy smart,
and then stupid all over again, as new questions come tumbling in.
It’s a urge that never dies, a game that never ends.’

Robert Krulwich

ABSTRACT

The continuous generation of new neurons in the adult mammalian brain comprises one of the most robust forms of neural plasticity occurring during adulthood. Throughout life, and specifically in the dentate gyrus (DG) of the adult mammalian hippocampus, resident neural stem cells (NSCs) give rise to new functional and mature neurons. It is a highly dynamic process, achieved through a stereotypic developmental sequence, controlled by the interplay between neurogenic niche-derived signals and intracellular pathways. New generated neurons will then integrate into the neuronal circuits and fulfill important functions in hippocampal plasticity and function. In fact, it is hypothesized that impaired neurogenesis contributes to the pathophysiology of several neurodegenerative diseases and neuropsychiatric pathologies. As so, the demonstration of an active neurogenic process in the adult brain opens perspectives for central nervous system repair strategies, based on cell replacement therapies. This, in turn, urges the need for the identification of the factors that control NSCs maintenance and neuronal integration for a proper brain functioning.

In recent years, the iron-trafficking protein Lipocalin-2 (LCN2) has emerged as an important regulator of brain homeostasis and behaviour, since it controls plasticity in the form of dendritic spine morphology, neuronal excitability and synaptic activity. Also, by binding to its specific cell-surface receptor (24p3R), and by regulating iron cell content, LCN2 serves diverse cellular functions that include cell proliferation, migration and survival, and death. However, it is still poorly recognized the existence of a fine-tuned mediation of intracellular iron by LCN2 for cellular homeostasis in the specific context of the brain. As so, in this thesis, we used a mouse model with the deletion of the *Lcn2* gene (LCN2-null mice) to explore its importance in the regulation of brain functioning and cell genesis during adulthood, and dissect the respective molecular underpinnings, both at physiological conditions and after stimulus. In our first study, we characterized the impact of LCN2 absence in the maintenance and survival of NSCs, and how this contributed for the integrity of brain plasticity and function under physiological states. Molecular and cellular analysis in the DG neurogenic niche revealed that LCN2 absence induced deficits in NSCs cell cycle progression, proliferation and commitment, culminating in impaired discriminative behaviour in the contextual fear discrimination task. Specifically, LCN2-null mice presented an accumulation of type-1 NSCs, as the consequence of a G0/G1 cell cycle arrest induced by an iron-mediated increase in endogenous oxidative stress. The additional treatment with the iron-chelating agent deferoxamine rescued NSCs oxidative stress, promoted the progression of NSCs in the cell cycle, and improved contextual discrimination. In this

sense, in our second work, we further dissected the impact of LCN2 absence when neurogenesis is externally manipulated. For that, we submitted LCN2-null mice to a chronic protocol of voluntary running, in order to increase hippocampal neurogenesis, while the chronic administration of corticosterone was applied to reduce the generation of new cells in the hippocampus. Analysis of cellular and behavioural outcomes revealed that exercise promoted NSCs proliferation and differentiation in LCN2-null mice, as it increased the proportion of progenitors in cycle and that survived and differentiated into new neurons, thus reducing anxiety and improving contextual discrimination. On the other hand, no effect on cell genesis and behaviour were observed in LCN2-null mice upon stress. The chronic treatment with corticosterone did not render LCN2-null mice a worsen phenotype, as the one observed under physiological conditions. Nevertheless, these results add an important piece of evidence in implicating LCN2 as a mediator of neural brain plasticity and functioning during adulthood, either at physiological conditions or upon stimulation. Moreover, and considering the little information that exists regarding the mechanisms that control the process of aging, along with the descriptions of LCN2' role in neurodegeneration, herein we characterized the contribution of LCN2 absence on the effects of aging in animal behaviour. Evaluation of anxiety, depressive-like and cognitive behaviours revealed that aging in LCN2-null mice reduced anxiety, while it sustained the depressive-like behaviour and impaired cognition observed at younger ages. These behavioural changes were further accompanied by a significant decrease in cell survival and neuronal differentiation, suggesting hippocampal neurogenesis as the basis for age-related changes in behaviour.

All together, our findings contributed to the identification of LCN2 as a novel molecular player in brain neural plasticity. Moreover, it unravels new and unique underlying mechanisms in the process of adult hippocampal neurogenesis, both in the context of the physiological brain, and after a stimulus or in pathology. Also, our results added new and important evidence on the way we understand and perceive the process of adult neurogenesis, shedding light on the basic principles of adult plasticity, with future implications in regenerative strategies.

RESUMO

A contínua formação de novos neurónios no cérebro adulto de mamíferos constitui uma das formas mais robustas de plasticidade neuronal que ocorre durante a idade adulta. Especificamente no giro denteado (GD) do hipocampo, as células estaminais neuronais residentes dão continuamente origem a novos neurónios maduros e funcionais, que integram os circuitos neuronais pré-existentes e contribuem para o funcionamento do cérebro. Este é um processo dinâmico, que requer um estreito controlo dos processos de proliferação e diferenciação das células dentro do nicho neurogénico. De fato, alterações neste processo estão associadas à manifestação de doenças neurodegenerativas e psiquiátricas. Assim, a existência de um processo de neurogénese ativo no cérebro adulto abre perspetivas para a regeneração do sistema nervoso central e, por seu lado, urge à necessidade em identificar fatores capazes de regular as células estaminais neuronais e a integração de novos neurónios.

Recentemente, a proteína Lipocalina-2 (LCN2), envolvida no tráfico de ferro, surgiu como sendo importante na manutenção da homeostasia do cérebro e do comportamento animal, uma vez que controla a plasticidade neuronal ao nível da morfologia dos neurónios, da excitabilidade e da atividade sináptica. De salientar, a LCN2, através da ligação específica ao seu receptor 24p3R presente à superfície das células, é capaz de regular os níveis intracelulares de ferro e, assim, influenciar processos celulares distintos que incluem a proliferação, migração e sobrevivência, e a morte celular. Contudo, ainda é pouco reconhecido o controlo de ferro pela LCN2 na manutenção da homeostasia celular no contexto do cérebro. Deste modo, no presente trabalho, e com o intuito de explorar a sua importância no funcionamento do cérebro adulto e na génese de novas células, usou-se como modelo animal um murganho que não expressa *Lcn2* (LCN2-null). Assim, no primeiro estudo, caraterizamos o impacto da ausência da LCN2 na manutenção e sobrevivência das células estaminais neuronais, assim como a sua contribuição para o funcionamento do cérebro em condições fisiológicas. Análises moleculares e celulares no GD revelaram que a deleção de LCN2 induz défices na progressão do ciclo celular das células estaminais, da sua proliferação e diferenciação, culminando em défices cognitivos avaliados na tarefa de discriminação de contextos. Especificamente, animais LCN2-null apresentaram um aumento da população de células estaminais, como consequência da incapacidade das células progredirem na fase G0/G1 do ciclo celular, devido ao aumento do stress oxidativo induzido pela acumulação de ferro. O tratamento dos animais com o quelante de ferro deferoxamina permitiu o resgate do stress

oxidativo, a progressão no ciclo celular, e melhorou o índice de discriminação de contextos, o que sugere que a capacidade de tráfico de ferro pela LCN2 tem um papel central na regulação da neurogénese. Neste sentido, no segundo trabalho, dissecamos o impacto da ausência da LCN2 na regulação externa da atividade neurogénica. Para isso, os animais LCN2-null foram submetidos a um protocolo de exercício, de modo a promover o aumento da neurogénese, enquanto que a administração crónica de corticosterona serviu para reduzir a formação de novas células no hipocampo. De notar, o exercício melhorou significativamente os défices apresentados pelos animais LCN2-null, promovendo o aumento da proporção de progenitores no ciclo celular e da sua diferenciação em novos neurónios, resultando numa redução da ansiedade e num melhor índice de discriminação. Por outro lado, nenhum efeito foi observado após o stress: o tratamento crónico dos animais LCN2-null com corticosterona não agravou o fenótipo observado em condições fisiológicas, no que diz respeito à formação de novos neurónios e ao comportamento. Mesmo assim, estes resultados elucidam e implicam a LCN2 como um importante mediador da plasticidade neuronal e do funcionamento do cérebro adulto, quer em condições fisiológicas, quer após estimulação. Além disso, e considerando a quase inexistente informação relativa aos mecanismos que controlam o processo de envelhecimento, juntamente com as descrições na literatura do papel da LCN2 na neurodegeneração, neste trabalho caraterizamos o impacto da ausência de LCN2 no comportamento durante o processo de envelhecimento. Análise de níveis de ansiedade, depressão e cognição revelaram que o envelhecimento induz uma redução da ansiedade nos animais LCN2-null, mantendo o comportamento depressivo e os défices cognitivos observados em idades mais jovens. Estas alterações foram acompanhadas por uma significativa redução na sobrevivência celular e diferenciação neuronal no hipocampo, o que sugere a génese de novos neurónios como a base para as alterações observadas no comportamento com o envelhecimento.

Em suma, os resultados aqui apresentados contribuíram para a identificação da LCN2 como um novo fator capaz de regular a plasticidade neuronal. Além disso, revelamos novos e únicos mecanismos subjacentes ao processo neurogénico no cérebro adulto, em condições fisiológicas e patológicas. Igualmente, os nossos resultados adicionam novas e importantes evidências sobre a forma como entendemos o processo neurogénico, abrindo caminho para o nosso conhecimento sobre os princípios básicos da plasticidade no cérebro adulto, com importantes e futuras implicações em estratégias regenerativas.

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LIST OF ABBREVIATIONS

A

ACTH – adrenocorticotropic hormone
AD – Alzheimer's disease
Ascl1 – achaete-scute complex homolog 1
ANP – amplifying neural progenitor

B

BDNF – brain derived neurotrophic factor
BIM – Bcl-2-interacting mediator
BrdU – bromodeoxyuridine
BMP – bone morphogenetic protein

C

CA – cornus ammonis
Calb – calbindin
Casp3 – caspase 3
CFC – contextual fear conditioning
CORT – corticosterone
CNS – central nervous system
CRF – corticotropin releasing factor
CREB – cAMP response element-binding protein

D

DAPI – 4,6-diamidino-2-phenylindole
DCX – doublecortin
DFO – deferoxamine
DG – dentate gyrus
Dlx2 – distal-less homeobox-2
DMEM – dulbecco's modified Eagle's medium
DNA – Deoxyribonucleic acid

E

EC – entorhinal cortex
EDTA – ethylenediamine tetraacetic acid
EGF – epidermal growth factor
EFGR – epidermal growth factor receptor
EPM – elevated plus maze

F

FAC – ferric ammonium citrate
FBS – foetal bovine serum
FGF-2 – fibroblast growth factor
FST – forced swimming test

G

GABA – γ-aminobutyric acid
GCL – granule cell layer
GFAP – glial fibrillary acidic protein
Gpx4 – glutathione peroxidase 4
GR – glucocorticoid receptor

H

HCl – hydrochloric acid
HPA axis – hypothalamus-pituitary-adrenal axis
H_2O_2 – hydrogen peroxide

I

IGF – insulin growth factor
IHC – immunohistochemistry
i.p. – intraperitoneally

L

LCN2 – Lipocalin-2
LTP – long term potentiation
LTD – long term depression
LV – lateral ventricles

M

ML – molecular layer
mGluR – metabotropic glutamate receptor
MR – mineralocorticoid receptor
MS – multiple sclerosis
MWM – Morris water maze

N

NAC – *N*-acetylcysteine
NaCl – sodium chloride
NeuN – neuronal nuclei
NeuroD1 – neuronal differentiation 1
NMDA – N-Methyl-D-aspartate
NGAL – neutrophil gelatinase-associated lipocalin
NGF – nerve growth factor
NPC – neural progenitor cell
NSC – neural stem cell
NSF – novelty suppressed feeding

O

OB – olfactory bulb
Oligo2 – oligodendrocyte transcription factor 2

P

Pax6 – paired box protein 6
PBS – phosphate-buffered saline
PCR – polymerase chain reaction
PFA – paraformaldehyde
PFC – prefrontal cortex
Prox1 – prospero homeobox 1
PSA-NCAM – polysialylated-neural cell adhesion molecule
PVN – paraventricular nucleus

Q

QNP – quiescent neural progenitor

R

RGL – radial glia-like
RMS – rostral migratory stream
ROS – reactive oxygen species
RT – room temperature

S

SEM – standard error of mean
SGZ – subgranular zone
Shh – sonic hedgehog
Sox2 – sex-determining region Y-box 2
SOD – superoxide dismutase
SVZ – subventricular zone

T

TAP – transient amplifying progenitor
Tbr2 – T-box brain protein 2
TGF – transforming growth factor

V

VEGF – vascular endothelial growth factor

W

Wt – Wild-type
WAT – White adipose tissue

THESIS LAYOUT

CHAPTER I presents a general introduction into the research theme and a state of the art covering fundamental aspects of neural plasticity in the adult brain, focusing particularly in the hippocampus, along with the description of its regulatory mechanisms and major functional roles. Also, at section 5 of this chapter, it is provided an adaptation of the review article published in *Progress in Neurobiology* (2015), which is presented at the end of the chapter as an Appendix A.

CHAPTER II comprises the research work that focused on the study of the role of Lipocalin-2 (LCN2) in the regulation of adult neurogenesis and its impact on brain function. We show that, through iron regulation, LCN2 controls neural stem cells cell cycle progression and death, self-renewal and commitment, with ultimate impact on behaviour. This chapter is presented as an original paper accepted for publication in *Molecular Psychiatry*.

CHAPTER III addresses the effects of voluntary running in hippocampal neurogenesis in the absence of LCN2, as well as the relevance of LCN2-regulated hippocampal neurogenesis in response to glucocorticoids. Particularly, it is shown that exercise-mediated increase in neurogenesis is sufficient to overcome LCN2-null mice cellular and behavioural impairments, while in the absence of LCN2, neurogenesis and behaviour are not affected by the exposure to chronic corticosterone.

CHAPTER IV presents the address on the impact of aging in behaviour and the role of LCN2 in age-related behavioural and plasticity changes. In this chapter, we aged the knockout mice model for LCN2 (LCN2-null) until 12- and 18-months of age, and addressed several dimensions of behaviour that included mood, anxiety and cognition, and estimated the generation of newborn neurons, comparative to young-adult mice.

CHAPTER V encompasses the thesis general discussion, in which an integrative overview of the major findings is debated. Also, it unveils future perspectives and delineates possible complementary experiments to further understand our current results.

CHAPTER I

Introduction

1. Neural plasticity in the adult brain

In the last decades, our view of the mature vertebrate brain has changed significantly. Previously seen as structurally stable and unchangeable soon after birth, the adult brain is now generally recognized as capable of structural and functional changes, in response to intrinsic and extrinsic signals, and throughout life (Fuchs and Flugge, 2014). This so-called neural plasticity, as the ability to "change itself", encompasses a series of physiological changes that occur in response to environmental factors, and that include re-wiring of neural circuits, synaptic-dependent activity, remodelling of dendrites and synapses, and the generation of new cells. Also, two forms of functional neural plasticity include long-term potentiation (LTP) and depression (LTD), as the enhancement and reduction, respectively, of signal transmission between two neurons after synchronous stimulation (Bliss and Cooke, 2011). For instance, synaptic plasticity, the activity-dependent modification of the strength or effectiveness of synaptic transmission at pre-existing synapses, plays a central role in the capacity of the brain, and particularly the hippocampus, to incorporate transient experiences into persistent memory traces (Citri and Malenka, 2008).

Several regions in the adult brain are described to undergo different forms of plasticity, among which the hippocampus. The overall changes that this and other brain regions undergo allows the adult brain to rapidly respond and adapt, to maintain functional homeostasis, to cope with environmental challenges and, ultimately, achieve adaptation (Tavosanis, 2012). Among the different forms of neural plasticity described to occur in the adult brain, the generation of new neurons, i.e. the process of adult neurogenesis, is the most fascinating phenomenon. Given its significant functions and relevance on brain physiology and pathogenesis, the identification of the underlying molecular modulators in this form of plasticity, and their contribution for neuronal integration into hippocampal circuits, is of the utmost relevance. This becomes further relevant considering the potential of neurogenesis in regenerative medicine to modulate neural circuits and brain function.

As so, for the purpose of this thesis, we will next review the literature related to the neurogenic process, its regulatory mechanisms and functional implications in the adult brain.

2. Neurogenesis

2.1 Historical perspective

Neurogenesis is the process by which new neurons are generated from neural precursor cells.

Traditionally, and for a long time, this phenomenon was believed to be limited to the embryonic and early postnatal stages, and that the adult brain could not form new neurons. Such "non-neurogenic view" of the adult brain derived mainly from the work of Ramón y Cajal, that claimed that neurons are generated exclusively in the developing prenatal brain (Ramon y Cajal, 1913). This view was widely accepted and, in fact, there was no clear evidence for the existence of a stem cell pool in the adult brain. However, this central dogma in neuroscience started to change when, in the 1960's, Altman and colleagues, using tritiated (^{3}H)-thymidine cell labelling, were able to radiographically trace cells born at the time of injection, therefore providing the first anatomical evidence for the presence of newly generated granule cells in the adult rat brain (Altman, 1963). New cells were described to be present in the hippocampus (Altman and Das, 1965), neocortex (Altman and Das, 1966) and olfactory bulb (Altman, 1969), in both physiological conditions and in response to injury (Altman, 1962). Several works followed, by Kaplan and Nottebohm, which corroborated the idea that new neurons are being generated and integrated in the adult brain and in several species, from birds to primates (Kaplan and Hinds, 1977; Kaplan, 1981; Kaplan and Bell, 1984; Paton and Nottebohm, 1984; Burd and Nottebohm, 1985; Gould et al., 1999a). Still, the fact that young neurons were continuously incorporated into the adult brain was not widely accepted until the mid 1990s. The development of novel techniques precipitated this shift, including immunohistologial techniques for labelling dividing cells with another exogenous thymidine analogue, bromodeoxyuridine (BrdU). This was vital for such immunolabelling assessments, which coupled with confocal imaging allowed the identification of the fate of dividing cells through the co-labelling of BrdU with other cellular markers, such as the neuronal marker neuronal nuclei (NeuN) or the astrocytic marker glial fibrillary acidic protein (GFAP) (Corotto et al., 1993). Interestingly, BrdU labelling remains widely used nowadays, along with other similar molecules, IdU and CldU (iodide and chloride equivalents, respectively). In addition, the development of tracing techniques (Luskin, 1993) and others, allowed to disclose that the incorporation of young neurons does not only occur at developmental stages, and that is a process highly regulated by external factors, such as stress, age and exercise (Cameron and Gould, 1994; Kempermann et al., 1997; Kempermann et al., 1998a). Finally, the major breakthrough and interest in adult neurogenesis came in the late 90's, when Eriksson and colleagues (1998) reported, for the first time, that new neurons were produced in the adult hippocampus of humans and also in aged individuals, evidencing the translational potential of this phenomena (Eriksson et al., 1998). Nevertheless, and although human neurogenesis in the hippocampus is nowadays generally accepted, it has been only characterized in limited cases

(Spalding et al., 2013).

Currently, the challenge in this field of research is focused on understanding the heterogeneity and modulation of the brain regions where neurogenesis occurs in adulthood, and the exact molecular mechanisms underlying the formation and functional integration of the newly formed cells. The ultimate goal is to gain insights for the manipulation of these niches for regenerative purposes.

2.2 Adult neurogenesis – where in the brain?

Adult neurogenesis is defined as the process comprising the generation, differentiation and integration of new neurons from resident neural stem cells (NSCs) in the adult brain (Gage, 2000). This process requires four main consecutive developmental steps: (i) NSCs proliferation and commitment, (ii) progenitors migration, (iii) differentiation and maturation and (iv) integration of the neuronal progeny into pre-existent neuronal circuitries (Ming and Song, 2005). Despite most of the research in the context of adult brain has focused on the generation of new neurons, it is important to highlight that also new glial cells (astrocytes and oligodendrocytes) can be generated from adult NSCs. In fact, gliogenesis, compared to neurogenesis, is more prevalent in the adult mammalian brain and in a more wide spread manner (Cameron et al., 1993; Frisen, 2016; Rusznak et al., 2016). Still debatable is how these two cell lineages (neurogenesis and gliogenesis) are related but is known that, under certain circumstances, both cell genesis can interact and facilitate cell repair.

In the adult mammalian brain, the generation of new neurons is spatially restricted to two specific brain regions: the adult subventricular zone (SVZ) lining the lateral ventricles (also known as subependymal zone), and the subgranular zone (SGZ) of the dentate gyrus (DG) at the hippocampus (Figure 1). Nevertheless, a recent growing number of studies has been showing that neurogenesis can also occur in other brain regions [Figure 1; (Gould, 2007)], including the neocortex (Altman and Das, 1966; Gould et al., 2001; Dayer et al., 2005), striatum (Gould et al., 1999b; Bernier et al., 2002; Dayer et al., 2005; Ernst et al., 2014), hypothalamus (Huang et al., 1998; Fowler et al., 2002; Kokoeva et al., 2005) and amygdala (Gould et al., 1999b; Bernier et al., 2002; Fowler et al., 2005), among others (Figure 1). However, conflicting data exist, since several studies failed to confirm such findings (Kornack and Rakic, 2001; Koketsu et al., 2003; Bhardwaj et al., 2006), or have described neurogenesis in such regions only after damage or pharmacological manipulations (Van Kampen et al., 2004; Chen et al., 2005; Collin et al., 2005). Still, it remains debatable if in these brain areas new functional adult neurons are formed from NSCs (Gould, 2007).

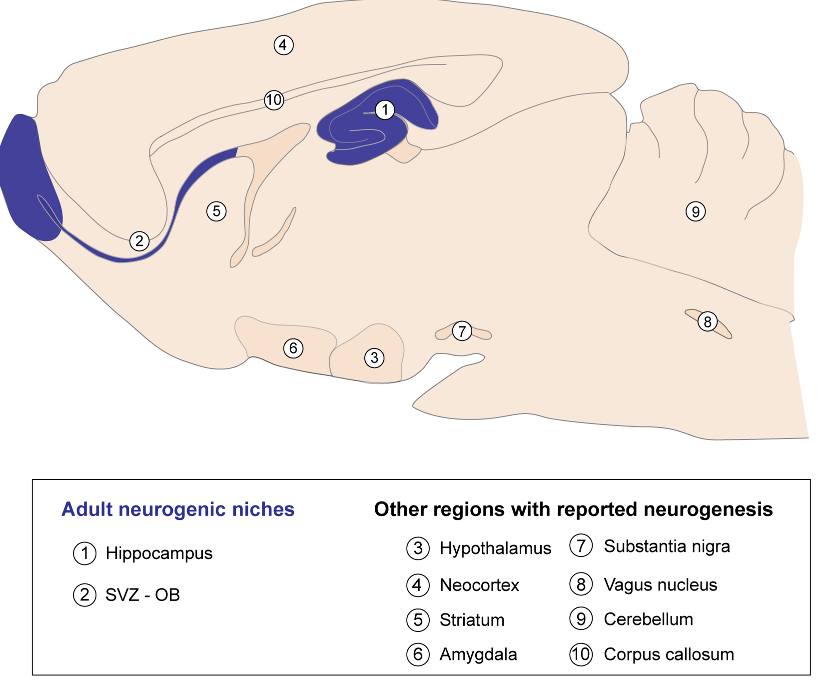


Figure 1. Neurogenic niches in the adult mammalian brain. Adult neurogenesis is widely accepted to occur in the adult mammalian brain in two major brain regions: the subventricular zone (SVZ) of the lateral ventricles that generates neuroblasts that migrate through the rostral migratory stream (RMS) towards the olfactory bulb (OB), and the hippocampal dentate gyrus (DG) (both highlighted in blue). Currently, other different regions in the adult brain are controversially reported to also present neurogenesis.

The progenitor cells that are continuously generated at the SVZ migrate anteriorly along the rostral migratory stream (RMS) to the olfactory bulb (OB) to become interneurons (Figure 1). In the DG of the hippocampus, new granule neurons are continuously born in the SGZ from neural progenitors, and migrate a short distance into the granule zone of the DG and integrate locally into the existing circuitry (Ming and Song, 2005). Importantly, both neurogenic regions are unique in its cellular composition. Various cellular types and tissue components are an integrated part of the niche and include the stem/progenitor cells, their immediate progeny and immature neurons, glia (astrocytes, oligodendrocytes and microglia) and vascular cells (Mercier et al., 2002), immune cells and macrophages, and an extracellular matrix (Kempermann et al., 2015). Together, they form a microenvironment that is permissive for neuronal development to occur, providing structural framework and a transduction of signals that is crucial for NSCs activation and dynamic regulation.

2.3 Neural stem cells in the adult brain

Adult NSCs in the neurogenic niches are defined based on two fundamental properties: the capacity to self-renew, and to give rise to differentiated progeny (Gage, 2000). As in the developing brain, adult NSCs stemness is characterized by the capacity of cells to undergo unlimited self-renewal by cell division, in order to maintain the number of stem cells in a given compartment at a steady level, or to increase at particular conditions (Kempermann, 2011). As multipotent cells, a single adult self-renewing NSC is able to generate all neural cell lineages: neurons, astrocytes and oligodendrocytes. These particular features of adult NSCs were originally suggested by the *in vitro* long-term expansion of neurospheres (non-adherent, spherical cultures of clonally derived precursors), and subsequent differentiation into the neural lineages (Reynolds and Weiss, 1992). Of interest, this has been shown for both adult rodent (Palmer et al., 1999) and human brains (Roy et al., 2000; Nunes et al., 2003). Current *in vivo* studies using nucleotide analogue labelling, retroviral lineage-tracing, and genetic fate-mapping strategies, confirmed and revealed NSCs dynamics, differentiation capacities, regulatory mechanisms, and heterogeneity (Bond et al., 2015).

In opposition to embryonic stem cells, characterized by high rates of proliferation, adult NSCs present two distinct characteristics that define their unique identities: the acquisition of a quiescent state, and the confinement to a complex and stable cellular niche (Urban and Guillemot, 2014). Adult NSCs are largely quiescent *in vivo*, remaining for long periods at G0 phase (Doetsch et al., 1999). This quiescence state is thought to allow NSCs to resist metabolic stress, and to be crucial for tissue homeostasis maintenance and to avoid stem cell exhaustion over a lifetime (Orford and Scadden, 2008; Simons and Clevers, 2011). On the other hand, the confinement of NSCs to the described adult neurogenic niches renders them to an enriched microenvironment that favours such quiescence and the maintenance at undifferentiated states (Morrison and Spradling, 2008). Within the niches, a great variety of signals are provided by the composing cell types and structures, which modulate the behaviour of adult NSCs and adjust the production of new cells according to the needs of the tissue (Fuchs et al., 2004; Blank et al., 2008; Faigle and Song, 2013).

The identity of the NSCs that give rise to new neurons in the adult central nervous system (CNS) has been a subject of intense debate for the past years. Several cell types have been proposed as the resident adult NSCs, including astrocytes (Doetsch et al., 1999), multiciliated ependymal cells (Johansson et al., 1999) and subependymal cells (Morshead et al., 1994). The current most well accepted hypothesis is that, similarly to the embryo, resident adult NSCs are quiescent radial glia-like cells (Doetsch et al., 1999) with structural and molecular characteristics of astrocytes (Alvarez-

Buylla and Lim, 2004). Evidence supporting this arise from studies using anti-mitotic treatment recovery (Seri et al., 2001), genetic ablation (Garcia et al., 2004), and transgenic fate-mapping (Dranovsky et al., 2011). In the adult SVZ, multipotent NSCs, present along the lateral ventricle walls, are GFAP-, Nestin and Prominin1-expressing cells (Lim and Alvarez-Buylla, 2016), whereas in the SGZ, resident NSCs express GFAP, Nestin and sex-determining region Y-box 2 (Sox2) cellular markers, and possess a defining radial branch extending through the granule layer (von Bohlen und Halbach, 2011). Also in the SGZ, nonradial stem cells can generate new neurons (Suh et al., 2007). These cells express Sox2, but not GFAP, and are characterized by the lack of any radial process, with some containing parallel extensions to the dentate granule layer (Suh et al., 2007). While radial NSCs are typically quiescent and slow dividing cell, nonradial horizontal stem cells are faster dividing cells (Seri et al., 2004).

In the prevailing model of adult hippocampal neurogenesis, and during the process of lineage fate choice, quiescent radial glia-like NSCs can undergo different model of identities for self-renewal and differentiation to produce new cells (Figure 2). In accordance with the model suggested by Bonaguidi and colleagues (2011), radial NSCs can either remain in a quiescent state or become activated and enter the cell cycle (Bonaguidi et al., 2011). Based on *in vivo* clonal analysis, it was proposed that the activated stem cell proceeds to one of the three modes of self-renewal: (i) symmetric self-renewal to expand the pool, (ii) neurogenic or (iii) astrogliogenic asymmetric self-renewal to generate a differentiated progeny, while maintaining the pool (Bonaguidi et al., 2011). Also, radial NSCs can move to quiescence and maintain stemness, or differentiate into an astrocyte via transition astroglia (Bonaguidi et al., 2011). It is also possible that a quiescent radial NSC can directly differentiate into an astrocyte without cell division [Figure 2a; (Bonaguidi et al., 2011)]. This model of “repeated self-renewal” was further corroborated in the work of Dranovsky and co-workers (Dranovsky et al., 2011). Contrastingly, others presented a model in which the potential of the precursor cells is fixed and the precursor cell population becomes exhausted with age (Encinas et al., 2011). Accordingly to this “disposable stem cell” model, a radial glia-like stem cell is quiescent for the entirety of its postnatal life and, when activated, it undergoes several rapid asymmetric divisions to generate only the neuronal lineage, without returning to quiescence [Figure 2b; (Encinas et al., 2011)]. This suggests that decrease of hippocampal stem cells is directly linked to the production of new neurons and new astrocytes (Encinas et al., 2011). Also, of interest, a “Nonradial precursor” model exists, where proliferative cells lacking a radial process are capable of generating neurons, astrocytes, and even radial-glia cells (Suh et al., 2007).

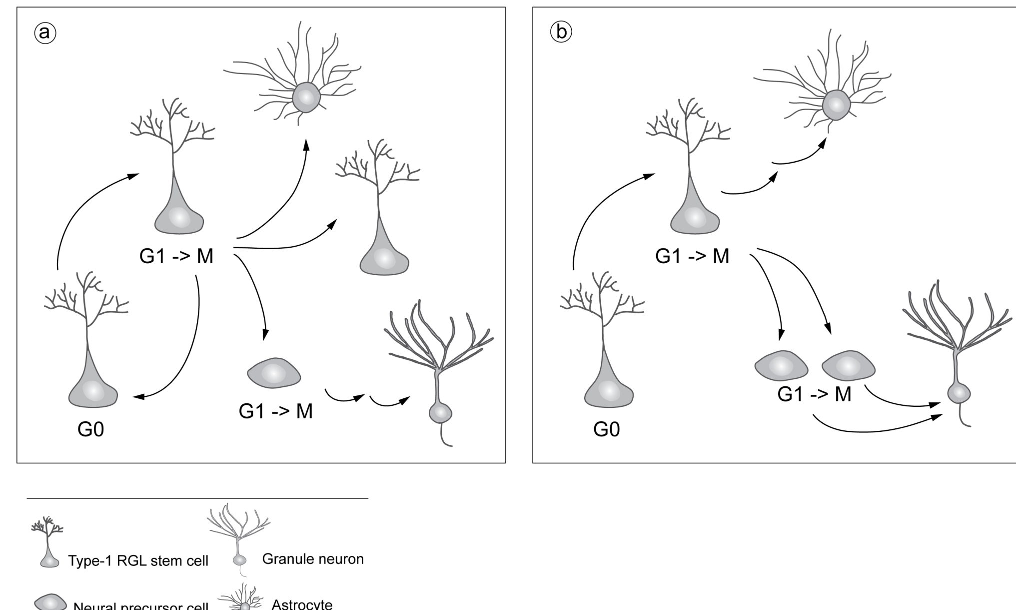


Figure 2. Models for neural stem cells (NSCs) behaviour in the adult hippocampus. (**a**) Adult NSCs can transit between quiescent and mitotic states by exiting and entering the cell cycle, respectively. Once activated, NSCs can divide symmetrically to generate additional NSCs or asymmetrically to produce neuronal or astroglial lineages. (**b**) Alternatively, once activated, NSCs can repeatedly divide to generate a neuronal lineage without returning to quiescence or terminally differentiate into astrocytes. M, mitosis; G0, gap 0 phase; G1, gap 1 phase; RGL, radial glia-like. Adapted from (Bonaguidi et al., 2012).

Together, these described findings demonstrate that radial glia-like cells, although presenting apparent similar morphology and molecular identity, exhibit distinct levels of self-renewal and differentiation capacity. Whether and how this suggestive heterogeneity that exists among radial glia-like NSCs (Gebara et al., 2016) reflects differences in intrinsic properties or extrinsic regulation remains unclear. A recent study revealed that only few genes were specific to the quiescent type of NSCs (Shin et al., 2015), while others demonstrated that a single NSC is not long-term self-renewing (Barbosa et al., 2015; Calzolari et al., 2015), and appear to be already committed to the generation of specific neural cell types (Taverna et al., 2014). The most obvious example of such heterogeneity among adult NSCs is the different progeny originated in the two neurogenic niches. In the adult SVZ, NSCs generate interneurons at the OB and oligodendrocytes at the corpus callosum, whereas SGZ NSCs generate dentate granule neurons and astrocytes (Ming and Song, 2011). Notably, NSCs derived from both niches generate all three neural lineages once propagated in culture with high

concentrations of growth factors (Reynolds and Weiss, 1992; Palmer et al., 1997). This may suggest that, *in vivo*, the niche may limit adult NSC potential. In fact, Rolando and colleagues (2016) have recently reported that adult hippocampal NSCs inherently possess multilineage potential and that oligodendrogenesis in the hippocampus is repressed by the action of Drosha, a ribonuclease enzyme involved in the execution of the initial step of microRNA processing (Rolando et al., 2016).

Within the niche, quiescent NSCs coexist with active forms of stem cells (Li and Clevers, 2010), which are more responsive to damage/injury. This, in fact, opens perspectives for endogenous NSC-based CNS regeneration. Moreover, the recent advances in adult NSC biology have raised great expectations for the usage of these cells as potential resources for neuronal replacement therapy after injury or CNS degeneration. However, before the full potential of adult NSCs can be applied for regenerative medicine, we need to identify the sources of stem cells, the mechanisms regulating their proliferation and fate and, most importantly, in the case of neuronal lineages, to characterize their functional properties.

In the following sections, we will review some of these aspects for the SVZ, and particularly for the SGZ hippocampal niche, since it is the main object of study in this thesis.

3. Neurogenesis in the subventricular zone and the olfactory bulb

The SVZ neurogenic niche constitutes the major source of new neurons generated in the adult brain. Composed by a thin layer of cells, the rodent SVZ niche is located below the ependyma along the wall of the lateral brain ventricles [Figure 3; (Alvarez-Buylla and Garcia-Verdugo, 2002)]. In the literature, the SVZ is also commonly designated as subependymal zone, mostly due to its location and to be distinguished from the embryonic SVZ. In fact, in light of the recent findings showing that adult NSCs from this niche can also present their cell bodies in direct contact with the ventricle, both designations are accepted (Mirzadeh et al., 2008).

3.1 Adult subventricular zone cellular composition, regulation and functional relevance

Along the lateral ventricle walls, radial glia-like NSCs are type-B1 cells that, once activated, give rise to fast proliferating cells, the transient amplifying progenitors (TAPs) or type-C cells, which generate neuroblasts (type-A cells) that migrate anteriorly in the RMS to the OB [Figure 3; (Doetsch et al.,

1997)]. Structurally, type-B1 cells are organized in pinwheel arrangements with a primary cilium that is in direct contact with the ventricular lumen (Mirzadeh et al., 2008), that enables them to sense signals present in the cerebrospinal fluid, while extending their basal processes into the perivascular niche, which also represents an important source of signals (Figure 3). Upon asymmetric division, type-B1 cells give rise to type-C cells (Ortega et al., 2013), which represent the largest population of proliferating SVZ progenitor cells, and are identified by the transcription factors distal-less homeobox-2 (Dlx2), achaete-scute complex homolog 1 (Ascl1/Mash1), and epidermal growth factor receptor (EGFR) (Ciccolini et al., 2005). Type-C cells then divide symmetrically three times before becoming type-A cells, which represent the neuronal precursors expressing the polysialylated form of the neural cell adhesion molecule (PSA-NCAM) and doublecortin (DCX) (Figure 3). Type-A cells then move along one another within elongated cellular aggregates that are unsheathed by GFAP-positive cells (Lois and Alvarez-Buylla, 1994), forming the RMS (Doetsch and Alvarez-Buylla, 1996). This neuroblast chain migration converges into the OB (Figure 3), where immature neurons migrate radially outward, differentiate into different subtypes of inhibitory interneurons, and integrate in distinct layers of the OB (Altman, 1969; Doetsch and Alvarez-Buylla, 1996; Ming and Song, 2011). The majority of these newborn cells become GABAergic granule neurons expressing NeuN and Calbindin (Lim and Alvarez-Buylla, 2016).

Other cell types also reside along the lateral wall and compose the SVZ neurogenic niche, conferring physical and signalling support for the ongoing neurogenesis. Type-B2 astrocytes, or niche astrocytes, distinguishable from type-B1 cells according to their ultrastructural differences, present a highly branched morphology and are frequently found in the interface between the SVZ and the striatum (Doetsch et al., 1997). Also, and characteristic of the adult SVZ, ependymal cells form a monolayer of cells that outlines the ventricular walls (Mirzadeh et al., 2008). Organized into pinwheel formations, ependymal cells are covered in cilia, at their apical surface, that beat the cerebrospinal fluid inducing its movement throughout the ventricle (Figure 3). Ependymal cells also secrete signalling factors that allow the activation of NSCs.

Tanycytes (Doetsch et al., 1997; Chojnacki et al., 2009), microglia (Thored et al., 2009) and endothelial cells of the blood vessels (Tavazoie et al., 2008) are also cellular components of the SVZ niche.

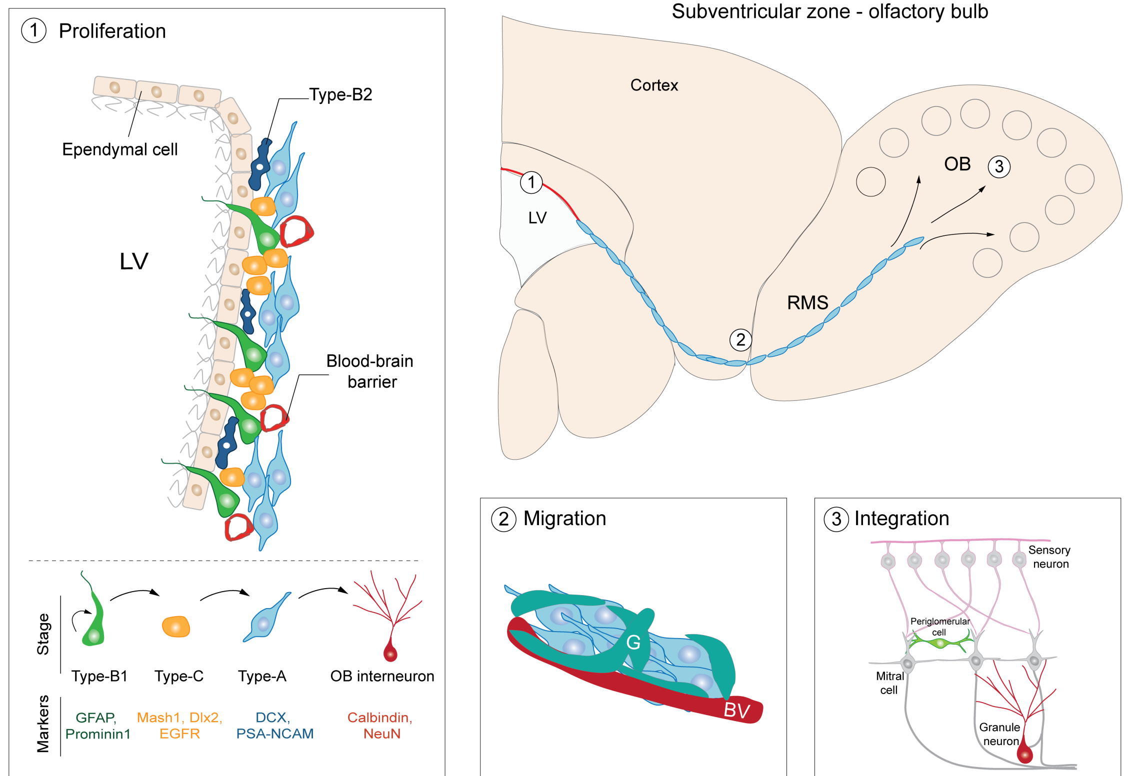


Figure 3. Adult neurogenesis at the subventricular zone (SVZ). (**1**) At the lateral ventricles, type-B1 radial glia-like stem cells give rise to fast proliferating type-C cells that in turn originate type-A cells (neuroblasts). Type-A cells at the SVZ then migrate through the RMS towards the OB (**2**), where they differentiate into granule neurons and integrate into the local circuitries (**3**). Specific cellular markers identify each stage-specific step of proliferation in the ventricular walls. BV, blood vessel; DCX, doublecortin; Dlx2, distal-less homeobox-2; EGFR, epidermal growth factor receptor; G, glial sheath; GFAP, glial fibrillary acidic protein; LV, lateral ventricle; Mash1, achaete-scute complex homolog 1; NeuN, neuronal nuclei; OB, olfactory bulb; PSA-NCAM, polysialylated neural cell adhesion molecule; RMS, rostral migratory stream.

Subventricular zone regulation

The SVZ adult neural stem and precursor cells population is not considerably changed throughout life and is maintained at a steady-state level due to the tight balance between extrinsic and intrinsic factors. In fact, many soluble molecules/proteins/factors and signalling pathways have been described to be critical in the adult SVZ and OB, in the control of NSCs proliferation, self-renewal and differentiation. The intrinsic factors responsible for homeostasis maintenance include a set of signals that are produced by the progenitors itself, which act together with exterior microenvironment cues to instruct distinct neurogenic phases. As an example, the transcription factors expressed by NSCs,

and all the intracellular receptors, are involved in progenitor's proliferation and neuroblasts migration. Among them, Sox2 and Paired related homeobox 1 are examples of transcription factors responsible for SVZ NSCs self-renewal (Lim and Alvarez-Buylla, 2016). Also, in type-C cells, Mash1, Neurogenin-2 and Oligodendrocyte transcription factor 2 (Oligo2) are responsible for the neuronal and glial lineage commitment, respectively (Lim and Alvarez-Buylla, 2016), while PSA-NCAM (Gascon et al., 2007) and DCX (Ocbina et al., 2006) are implicated in progenitors migration, and β1 integrin (Belvindrah et al., 2007) and Slit proteins (Sawamoto et al., 2006) in neuroblasts migration and attraction to the OB, respectively. In addition, and recently, regulation of SVZ by epigenetic and microRNAs was described to be fundamental in the control of NSCs multipotency and differentiation (Lim and Alvarez-Buylla, 2016).

Extrinsic regulation of SVZ neurogenesis comprises several trophic and growth factors [fibroblast growth factor-2 (FGF-2), epidermal growth factor (EGF), transforming growth factor (TGF)-α, ciliary neurotrophic factor (CNTF) and vascular endothelial growth factor (VEGF)], neurotransmitters [γ-aminobutyric acid (GABA), serotonin, histamine], morphogens (e.g. Sonic hedgehog, Shh; bone morphogenetic proteins, BMP), neurotrophins, hormones and cytokines (Lim and Alvarez-Buylla, 2016). Also, other signalling molecules, such as the Wnt family and the Notch signalling pathway, are described to participate in the modulation of neurogenesis (Lim and Alvarez-Buylla, 2016). These regulatory signals have origin in diverse sources, mostly from neurons, NSCs, ependymal cells, endothelial cells of the blood-brain barrier (Ramirez-Castillejo et al., 2006), microglia cells (Thored et al., 2009), and the choroid plexus (Sawamoto et al., 2006). For instance, adult SVZ NSCs have direct access to cerebrospinal fluid (produced by the choroid plexus at the brain ventricles), which represents a complex reservoir of diverse signals capable of modulating neurogenesis in the SVZ (Falcao et al., 2012).

Functional relevance of subventricular zone neurogenesis

Under physiological conditions, the major function of the SVZ neurogenic niche represents the continuous production of new neurons to the OB to promote plasticity of the existing circuitry (Ming and Song, 2011). Moreover, and in accordance with its described functions in odour, OB neurogenesis has been shown to play important roles in short-term memory and flexible olfactory associative learning, including in mating and social recognition (Feierstein et al., 2010), odour discrimination (Gheusi et al., 2000) and long-term olfactory memory (Lazarini et al., 2009). Also under pathological or challenging conditions, including neurodegenerative diseases such as

Alzheimer's disease (AD) and multiple sclerosis (MS) (Haughey et al., 2002; Nait-Oumesmar et al., 2007), SVZ is highly responsive and plays diverse roles depending on the condition. For instance, after demyelinating injuries, oligodendrocyte precursors from adult SVZ have been shown to migrate to the corpus callosum to myelinate axons (Xing et al., 2014). This responsiveness provides a source of potential endogenous therapies for some CNS disorders, which has also opened important perspectives for regeneration.

4. Hippocampal neurogenesis

The adult mammalian hippocampus is endowed with considerable adaptive and regenerative capacity, since it harbours one of the two neurogenic niches of the adult brain. This adds an extra level of complexity to both structural and functional aspects of this region, accounting for its functions in memory processing and emotional behaviour, as we will discuss later. But firstly, we will briefly specify key notions of hippocampal activity and intra-regional organization.

4.1 Hippocampal organization and structure

The hippocampus is a highly plastic region located at the medial temporal lobe of the brain. With a heterogeneous topographical organization and function, the hippocampal formation is part of a brain system that includes the *cornus ammonis* (CA) fields (subdivided in CA1, 2 and 3), the DG, the subiculum and the entorhinal cortex (EC) (Amaral et al., 2007). Here, pathways of unidirectional signals flow to form the intrinsic hippocampal circuit, the trisynaptic loop, from the EC, propagated by excitatory synaptic relays in the CA and in the DG, and then redirected back into the EC (Figure 4). Most external inputs are received from the EC, via the perforant path that arise from layer II of the EC, and terminate in the CA3 and DG [Figure 4; (Deng et al., 2010)]. Within the DG, granule cells project their axons (mossy fibers) to the proximal apical dendrites of CA3 pyramidal neurons that, in turn, convey signal input towards the ipsilateral CA1 pyramidal cells through Schaffer collaterals (Figure 4). To close the loop, CA1 pyramidal cells then project their axons to the subiculum, or directly to deep layers of the EC (Amaral et al., 2007).
Structurally, the hippocampus is a long curved structure that extends along a dorsal (septal) to ventral (temporal) axis in rodents, corresponding to an anterior-posterior orientation in humans (Strange et al., 2014). This disposition allows the hippocampal formation to establish distinct

extrinsic connectivity with multiple cortical and subcortical structures, such as the prefrontal cortex (PFC) or the amygdala, and that are the basis of hippocampal functions in cognition and emotion. Still, and despite that the basic intrinsic structure and circuitry is maintained along the septo-temporal axis of the hippocampus, a clear functional dissociation between dorsal and ventral hippocampal regions is observed. Accounting for such differences are the distinct connections with cortical and subcortical areas in the dorsal *versus* ventral parts. Specifically, the most dorsal part of the hippocampus establishes connections with, for example, retrosplenial and anterior cingulate cortices, thus mediating more cognitive-related processes such as spatial navigation and learning and memory (Moser et al., 1995; Fanselow and Dong, 2010). On the other hand, the ventral part of the hippocampus connects with brain regions related to emotional processing and general affective states regulation, including the medial PFC, amygdala, nucleus accumbens and the hypothalamic endocrine and autonomic nuclei (Fanselow and Dong, 2010), thus participating in anxiety and fear control, motivation and sexual behaviour, among other functional correlates.

Notably, connectivity and gene expression data further sustain the hypothesis of a functional gradient rather than a simplistic dorsal-ventral dichotomy (Strange et al., 2014).

Within the hippocampal structure, the DG is the only subregion where neurogenesis occurs (Kempermann et al., 2015). Of notice, neurogenesis in the hippocampus generates only one type of neurons, the glutamatergic granule cells. These cells are the main excitatory neurons of the DG, representing the major input region to the hippocampus and, therefore, the principal contributors for the roles associated with that structure. These neurons receive primary inputs from perforant path fibers, originated in the EC (Figure 4). In addition, they receive commissural inputs from the contralateral hippocampus, diverse neuromodulatory afferents, most notably cholinergic inputs from the septum, dopaminergic inputs from the midbrain, feedback inputs from CA3, glutamatergic inputs from mossy cells, and inhibitory inputs from interneurons in the hilus, as well as granule and molecular layers (Goncalves et al., 2016). In turn, granule cells provide excitatory input to the CA3 pyramidal cells by sending their axonal projection along the mossy fiber tract [Figure 4; (Kempermann et al., 2015)].

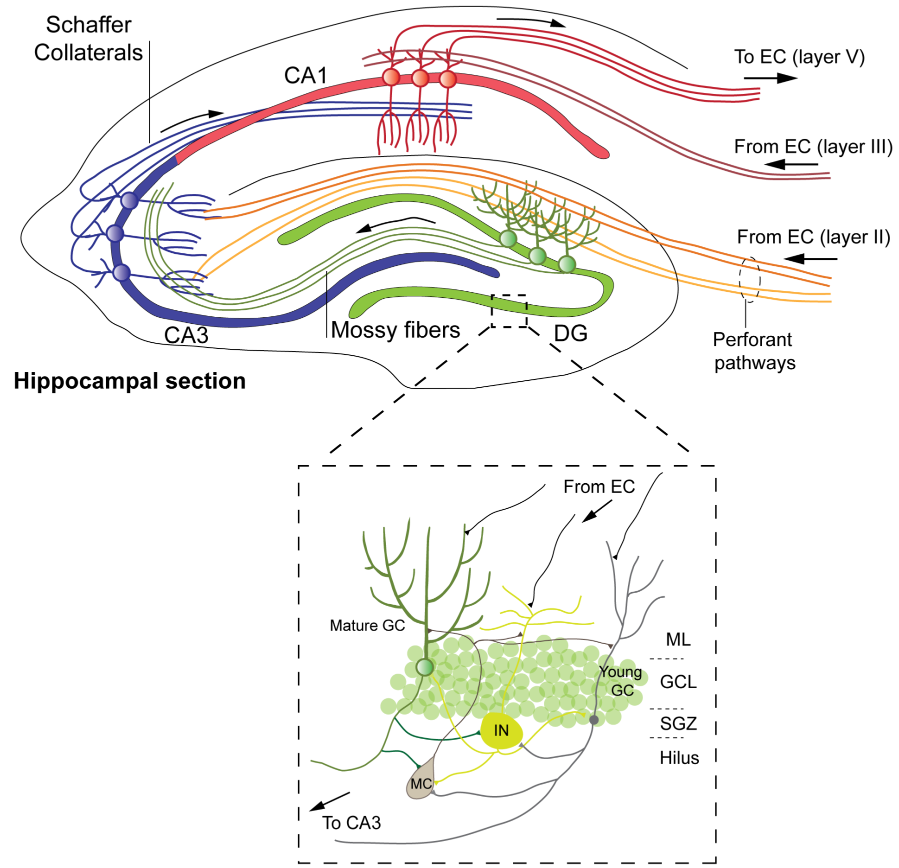


Figure 4. Hippocampal anatomy and intrinsic organization and circuitry. The basic functional hippocampal unit corresponds to the classical trisynaptic circuit, in which perforant path inputs from the entorhinal cortex (EC), carrying processed sensory information, innervate DG granule cells and pyramidal cells of both CA1 and CA3. The unidirectional and intrinsic processing of this sensory information is crucial for hippocampal function in learning, memory and cognition, emotion, and stress response. CA1, CA3, *cornus ammonis* 1 and 3; DG, dentate gyrus; EC, entorhinal cortex; GC, granule cell; GCL; granule cell layer; IN, inhibitory interneuron; ML, molecular layer; MC, mossy cell; SGZ, subgranular zone.

Moreover, granule cells are characterized to fire very sparsely, with their activity being modulated by a large number of interneurons in the DG and the hilus (Figure 4), and only a small population is activated by the described inputs at any given time. This, in fact, is crucial for creating non-overlapping responses to different experiences, thereby keeping memories distinct (Li et al., 2013).

Anatomically, the DG area contains significantly more principal neurons than its input or output regions, and consists of three layers: the molecular layer (ML), the granule cell layer (GCL) and the hilus (Figure 4). In the border between the GCL and the hilus is located the SGZ, where NSCs are

located and from which new granule neurons are generated (Kempermann et al., 2015). This remarkable and unique ability of the hippocampus to generate new neurons throughout life, highly contributes to its neuronal plasticity, both synaptic and morphological, being considered as an important underlying mechanism involved in hippocampal functions.

In the following sections, we will review the current knowledge on adult hippocampal neurogenesis with respect to cellular composition, regulatory factors and its functional relevance.

4.2 Neurogenesis in the adult hippocampus

In the adult SGZ neurogenic niche, type-1 radial and nonradial NSCs, placed at the border between the inner granule cells layer and the hilus, represent the first step of the cell genesis process (Figure 5). Upon division, and horizontally to the SGZ, type-1 cells give rise to intermediate progenitor type-2 cells, TAPs or NPCs (Figure 5), that divide actively (approximately 2.5 times) before exiting the cell cycle (Encinas et al., 2011), are Ki67-positive cells, and are greatly responsible for the expansion of a pool of progenitor cells (Ehninger and Kempermann, 2008). Within this cell population, two subsets have been identified: type-2a cells, that no longer display radial morphology, but still present radial glial markers (GFAP and Nestin), and type-2b cells, that start to present the first marks of neuronal lineage choice, namely the T-box brain protein 2 (Tbr2) marker. In turn, type-2 cells give rise to neuroblasts, or type-3 cells (Figure 5), which are slowly proliferating cells that exit the cell cycle and initiate the post-mitotic development of immature cells (Ehninger and Kempermann, 2008). The highly variable morphology of type-3 cells reflects this developmental transition, with cells presenting processes of various lengths and complexities that gradually change from horizontal to vertical orientations (Ehninger and Kempermann, 2008). These immature neurons expressing DCX and PSA-NCAM markers, migrate into the inner GCL and start to receive GABAergic inputs (Tozuka et al., 2005). Approximately two thirds of these immature cells, if not recruited into function, are eliminated by apoptosis (Biebl et al., 2000; Sierra et al., 2010), once the cells have exited the cell cycle. These apoptotic corpses of new generated cells are rapidly phagocytized from the niche by inactivated microglia in the adult SGZ (Sierra et al., 2010). The remaining cells will then fully differentiate into mature newborn calbindin/NeuN-expressing granule neurons.

Although the great majority of the new cells generated in the adult hippocampus from NSCs are neurons, there is also a considerable proportion of new glial cells being generated. While the regulation of their differentiation process is largely undetermined, some authors suggest the

existence of distinct subsets of progenitors that may be committed to a glial lineage (Encinas et al., 2011).

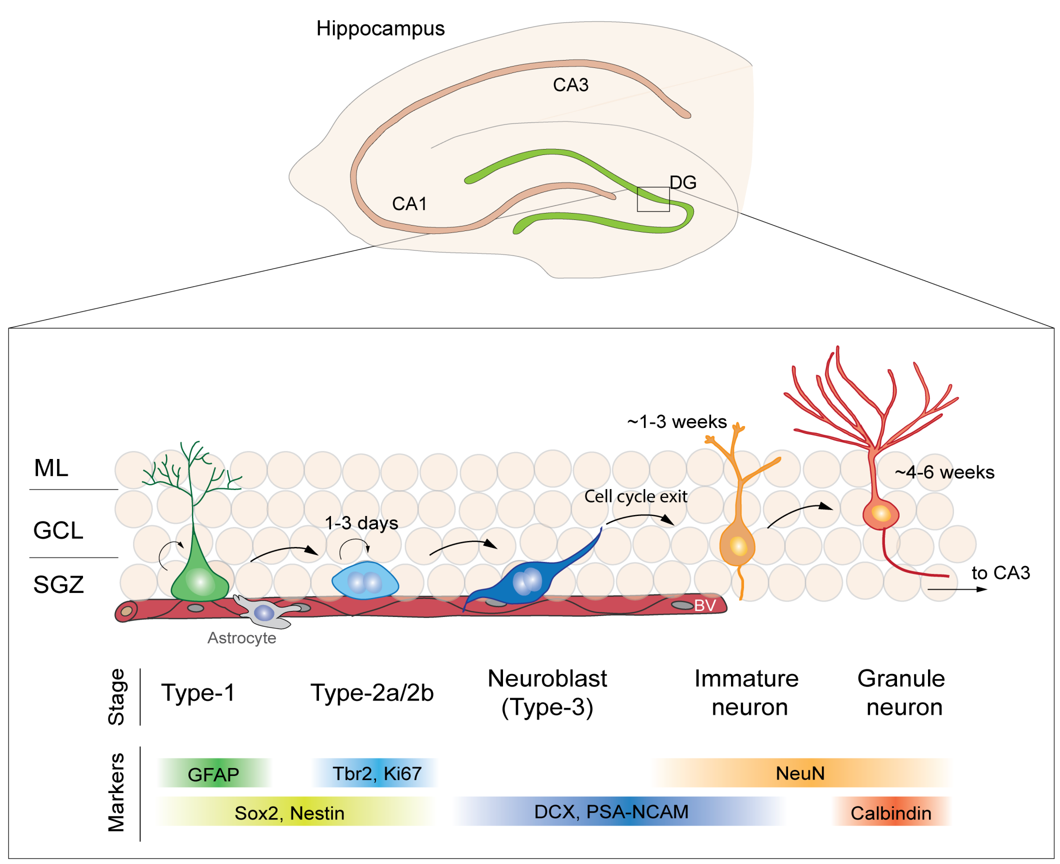


Figure 5. The adult hippocampal neurogenic niche. Within the hippocampal dentate gyrus (DG), resident neural stem cells (type-1 radial glia-like cells) in the subgranular zone (SGZ) give rise to progenitor cells (type-2 and type-3 cells) that migrate towards the granular cell layer (GCL) where they fully differentiate into mature granule neurons. Each sequential step can be identified using specific immunohistochemical markers, as depicted. BV, blood vessel; DG, dentate gyrus; DCX, doublecortin; GCL, granule cell layer; GFAP, glial fibrillary acidic protein; ML, molecular layer; NeuN, neuronal nuclei; NPC, neural progenitor cell; PSA-NCAM, polysialylated neural cell adhesion molecule; SGZ, subgranular zone; Sox2, sex-determining region Y-box 2; Tbr2, T-box brain protein 2.

Newborn generated neurons in the DG will then, rather than replacing pre-existing granule cells, contribute to the ongoing turnover (addition and subtraction) of newborn neurons in a restricted part of the DG (Ehninger and Kempermann, 2008). During the course of synaptic integration into the existing circuitry, these new neurons follow a stereotypic process. Within days post-mitosis, newborn neurons extend dendrites towards the ML, and project axons to the hilus, as early as 7 days,

reaching the CA3 region of the hippocampus at 10-11 days (Zhao et al., 2006). The first synaptic inputs are of GABA excitatory and tonic currents, released from local interneurons at the SGZ and the hilus (Esposito et al., 2005), in a 'priming' process where pre-existent mature granule cells activate parvalbumin GABA-releasing interneurons to feed back onto the developing cells (Alvarez et al., 2016). As early as 10 days after birth, phasic GABA and glutamate post-synaptic input currents start to originate from cells in the ML and hilar mossy cells (Deshpande et al., 2013), respectively, and by the third week, GABA currents are inhibitory (Ge et al., 2006). By around 16 days of age, dendritic spines are formed, in a process that appears to be controlled by local astrocytes (Zhao et al., 2006). During the first month, other synaptic inputs are essential for the correct development and integration of adult-born granule cells (Ge et al., 2006), which include a back-projection from CA3 and a transient input from mature granule cells in the DG (Vivar et al., 2012), as well as inputs from the subiculum and the EC. By the end of the first month, adult-born neurons are fully mature, integrated in the circuitry of the hippocampus and their morphological growth is mostly complete (Goncalves et al., 2016). Cells receive incoming synaptic inputs and actively inhibit local circuitry as they establish large mossy fiber connections with CA3 pyramidal neurons and innervate hilar basket interneurons (Freund and Buzsaki, 1996). However, at this stage, their electrophysiological features are distinct from those of mature granule cells, giving them unique properties that are crucial for their functional role. Newborn neurons are more excitable, present reduced LTP induction and increased LTP amplitude, and have more depolarized resting membrane potentials, which allows for the cells to respond to a broad range of stimuli (Ming and Song, 2011; Gu et al., 2012). This corresponds to a period of unusual activity and plasticity, essential for the function of adult-born neurons and their contribution to enhance local neural and structural plasticity which, in turn, influences global brain function. As newborn neurons mature further, they come under stronger inhibitory control, and the range of stimuli that elicit firing becomes finer, resulting in a sparser activity, typical of mature granule cells (Danielson et al., 2016).

The functional contribution of newly formed granule neurons will next be described in detail.

4.3 Functional role of adult-born neurons in the hippocampus

The growing understanding of the mechanisms underlying the neurogenic process has contributed to our knowledge on how new cells can reshape hippocampal intrinsic circuits and affect its behavioural functions. Adult newborn neurons are considered to influence behaviour only after

integration in the DG networks; still, their presence is thought to have impact around the fourth and sixth weeks post-mitosis, due to its increased excitability and plasticity (as described above). In fact, optogenetic silencing of newborn neurons at 4-weeks, but not at 2- or 8-weeks, impairs hippocampal memory retrieval (Gu et al., 2012).

Early studies on the function of neurogenesis have some discrepancies and contradictory findings, which reflect the complexity that emerges from the difficulty to precisely segregate the functions of newborn cells in the hippocampus. This entropy can also result from the diversity of models and approaches that have been tackling this issue. Adult-born neurons display distinct functions depending on the environmental inputs and cognitive demands present during their maturation (Goncalves et al., 2016). Experience shapes the timing of neuronal integration into hippocampal networks and their connectivity, being conceivable that distinct demands can result in different functions by newborn neurons. Also, there is supportive evidence that functional roles of adult hippocampal neurogenesis vary according to the animal models studied and the time-window of analysis.

Immediately after the initial discovery of neurogenesis in the postnatal rat hippocampus, Altman suggested that the new neurons formed were critical for learning and memory processes (Altman, 1963). Many following studies have, since then, shown that adult neurogenesis and specific behaviours are affected in a reciprocal fashion manner. In general, the functions of newborn neurons have been inferred from experiments in which adult neurogenesis was ablated or enhanced, and found to result in specific cognitive alterations. The first experimental evidence came when the blockage of neurogenesis in the adult mouse, using the cytostatic agent methylazoxymethanol, disrupted trace eye-blink conditioning and trace fear conditioning (Shors et al., 2001). Since then, many other experimental manipulative approaches, including irradiation (Clelland et al., 2009), transgenic models (thymidine kinase-based approaches) (Deng et al., 2009; Snyder et al., 2011) and, more recently, optogenetic (Kheirbek et al., 2013; Danielson et al., 2016), have also contributed to our understanding on the functions of hippocampal neurogenesis in cognition and memory. Spatial memory was affected in many instances, particularly in long-term memory retention in the Morris Water maze (MWM) task (Snyder et al., 2005). Context-dependent memory at the contextual fear conditioning (CFC) test was also found to rely on neurogenesis (Saxe et al., 2006). Additional evidence proposes that newborn neurons are required to reduce interference between memories that occur at different times (Rangel et al., 2014), in forming distinctive memories for individual episodes (Snyder et al., 2005), to regulate forgetting in old

memories (Akers et al., 2014), and even for the ability to adopt new strategies to complete a previously learned task, i.e. cognitive flexibility (Ming and Song, 2011). Yet, not every task that requires the hippocampus also requires new neurons (Becker and Wojtowicz, 2007). For instance, spatial learning in the MWM is disrupted by hippocampal lesions in rats (Gilbert et al., 2001), but not by irradiation of new cells (Snyder et al., 2005).

Additionally, a growing body of evidence associate adult neurogenesis in the DG with improved performance in pattern separation behavioural tasks (Nakashiba et al., 2012; Danielson et al., 2016). Pattern separation is defined as a process that produces differentiated outputs from similar inputs, in the case of memories, by reducing the overlap in their representations (Deng et al., 2010). Even though several brain regions contribute to pattern separation, this process has for long been associated with the DG (as described above). The permanent ablation of adult neurogenesis by X-ray irradiation impaired the ability of mice to perform in spatial pattern separation tests (Clelland et al., 2009). Other authors had concurring findings, by showing that the genetic ablation (Nakashiba et al., 2012) or the transient optogenetic inhibition (Danielson et al., 2016) of adult-born neurons impairs pattern separation.

Conversely, increasing neurogenesis, either by behavioural interventions (such as exercise and enriched environment) or by genetic enhancement of new neurons survival, renders better performances in CFC tasks that required distinguishing between similar environments. Sahay and colleagues (2011) showed that increasing the number of newborn neurons, by deleting the proapoptotic gene encoding for BCL2-associated X (Bax) protein from adult neural precursors and their progeny, enhances the animals' ability to distinguish between overlapping contextual representations (Sahay et al., 2011). Also, exercise and environmental enrichment were associated with enhanced spatial learning performance in the MWM task (van Praag et al., 1999). On the other hand, it is known that the generation of new cells decreases with age, stress and degeneration, both in humans and rodent models, with impact in learning tasks performances (Lledo et al., 2006; Aimone et al., 2010).

Adult hippocampal neurogenesis and diseases of the CNS

When considering the contribution of adult-born neurons in brain functioning at physiological states, it becomes increasing clear that our understanding on which role adult neurogenesis plays at a disease state, or which consequences may arise from its involvement, may also contribute to disclose some basic principles of newborn neurons physiological functions. Until now, there is no

clinical evidence of an isolated impairment of adult hippocampal neurogenesis in the absence of other anomalies, but several studies have reported an association between alterations in the generation of new neurons and several neurological and psychiatric disorders, thus providing a link between adult neurogenesis and brain pathogenesis. Even in some cases, alterations in neurogenesis are considered to contribute to disease symptoms and progression. This is explained by the fact that newborn neurons account for a disproportionally large fraction of DG activity and, even though DG responses are sparse, even small changes in the activity can have a significant impact.

One of the first hints on the potential association between adult neurogenesis and, for instance, mood disorders, were provided by the demonstration that corticosterone (CORT) suppressed the generation of new neuronal and glial cells in the adult DG (Gould et al., 1992). Moreover, anhedonic behaviour has been reported after suppression of cell proliferation in rodents (Snyder et al., 2011), and after bilateral hippocampal irradiation in non-human primates (Perera et al., 2011), while others found no association (Bessa et al., 2009). The same was true for increased immobility in the forced swimming test (FST). Another critical aspect is the question of whether hippocampal neurogenesis is required for the action of pharmacological treatments, such as antidepressants. In fact, according to the neurogenic hypothesis of depression, a reduced production of new neurons in the hippocampus is related to the pathogenesis of depression, while a successful antidepressant treatment requires the enhancement of hippocampal neurogenesis (Duman et al., 2001; Santarelli et al., 2003). Mice with ablated hippocampal neurogenesis blocked the ability of antidepressants to produce a successful behavioural response (Santarelli et al., 2003), and increasing hippocampal neurogenesis *per se* is sufficient to counteract depression and anxiety (Miller and Hen, 2015). However, the participation of hippocampal newborn neurons in the control of anxiety is quite controversial. Anxiety-like behaviour after ablation of neurogenesis has been reported in several test paradigms, namely in the novelty supressed feeding (NSF) (Bessa et al., 2009), elevated plus maze (EPM) (Revest et al., 2009) and light/dark test (Revest et al., 2009; Fuss et al., 2010). Yet, several other studies that followed with different ablation approaches and experimental timeframes presented apparently discordant results (Santarelli et al., 2003; Saxe et al., 2006).

Alterations in adult neurogenesis, and reduction in the size of the hippocampus, have also been reported in schizophrenia (Duan et al., 2007). For example, seizure-induced SGZ neurogenesis contributes to epileptogenesis and long-term cognitive impairment (Jessberger et al., 2007). Many candidate genes involved in the pathology of schizophrenia have been suggested to also play critical

roles in adult neurogenesis (Duan et al., 2007). Also, several neurodegenerative diseases, including AD, Parkinson's and Huntington's diseases, have also been associated with alterations in adult neurogenesis [reviewed in (Winner and Winkler, 2015)].

Our understanding of the physiological function of adult neurogenesis, and the mechanisms underlying its regulation, not only provides new prospective on the plasticity of the mature nervous system, but also sheds light on the etiology and pathophysiology of some brain disorders, and even for future regenerative approaches.

4.4 Modulation of adult hippocampal neurogenesis

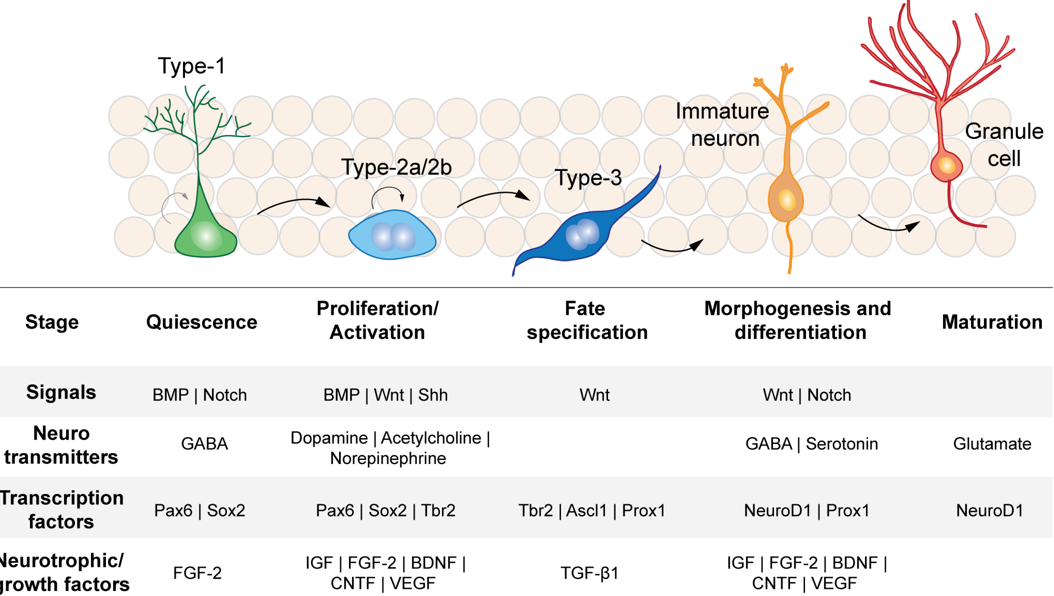
The ability of NSCs to self-renew, proliferate and differentiate into all neural cell types makes critical the understanding of these cells and of the factors that regulate this process. Numerous studies over the past years have identified several key factors and signalling mechanisms (intrinsic and extrinsic) that regulate adult neurogenesis within the hippocampal niche. Since NSCs pass through a series of genetically and morphologically stages to generate new cells, every step of their development is meticulously regulated and critical to the overall net of neurogenesis. For instance, NPCs proliferation can also occur in other regions of the adult brain, but the cells do not differentiate into neurons, becoming glia instead (Aimone et al., 2014).

Converging signalling mechanisms for the regulation of adult hippocampal neurogenesis occurs at several levels (Figure 6), both within and in close proximity of stem cells. As previously mentioned, the cellular composition of the niche provides the required microenvironment for signalling gradients of growth factors, neurotransmitters and transcription factors, which allows the anatomical and functional control of each neurogenic step (Ehninger and Kempermann, 2008; Ming and Song, 2011). In fact, the regulatory process of neurogenesis is a complex bidirectional interaction between intrinsic signalling and extrinsic cues, produced by the surrounding cells of the neurogenic niche. For instance, adult NSCs release factors that contribute to an autocrine and paracrine signalling. Immediate progeny of NSCs also provide diffusible or contact-mediated signals to regulate stem cells quiescence as a part of the feedback mechanism for homeostasis. Astrocytes secrete cytokines, such as interleukins -1β and -6 (Barkho et al., 2006) to promote neuronal differentiation (Song et al., 2002). In addition, NSCs can be found in dense clusters in close proximity to blood vessels (Palmer et al., 2000), presenting cell-to-cell communication, vascular endfeets and connexin-mediated gap junctions with the vasculature, which allows quiescent NSCs and progenitors to communicate and

receive local regulatory cues and factors (Balu and Lucki, 2009). A complex relationship exists between endothelial cells and hippocampal precursor cells, with the vasculature representing an abundant source of extrinsic factors that modulate adult neurogenesis due to such physical location to progenitors (Figure 5). Emerging evidence indicates that VEGF participates in this crosstalk, being secreted by endothelial cells to promote NSCs self-renewal, and also neurotrophin-3, which favours quiescence and long-term maintenance (Delgado et al., 2014). In addition, the blood-brain barrier is leaky near clusters of proliferating NSCs, which allow these cells to more easily access factors in the blood (Tavazoie et al., 2008).

Growth factors and neurotrophins also play an important role in regulating late progenitors (Figure 6), but their impact on NSCs is less well described (Faigle and Song, 2013). Among the neurotrophic factors described to control neurogenesis, brain derived neutrophic factor (BDNF) has been the most extensively studied one and shown to promote progenitors proliferation and survival (Scharfman et al., 2005), and to improve memory acquisition and consolidation (Bekinschtein et al., 2007). Various other growth factors have also been shown to regulate adult hippocampal neurogenesis, including FGF-2 (Kang and Hebert, 2015) and insulin-growth factor (IGF) (Aberg et al., 2000), among others (Table 1), described to promote NPCs proliferation and differentiation, and the survival of new neurons. Contrastingly, evidence suggests that EGF does not play an important role in the regulation of adult hippocampal neurogenesis (Kuhn et al., 1997).

Regulation of gene expression by transcription factors represents one fundamental mechanism that regulates adult NSCs. Over the years, many proteins were identified to be expressed at specific stages of the neurogenic process in the adult hippocampus, and have since been used as specific cellular markers (Figure 5). For example, the transcription factor Sox2, the most extensively studied transcription factor in NSCs behaviour and function, is expressed in type-1 and type-2a cells (Figure 5) and controls their multipotency and proliferative capacities (Favaro et al., 2009). Other transcription factors predominantly active in NSCs are the proneuronal transcription factor Ascl1/Mash1, expressed in dividing type-2a cells to promote proliferation and dictate cell fate (Jessberger et al., 2008), and paired box protein 6 (Pax6), shown to be essential in the production and maintenance of GFAP-positive early progenitor cells [Figure 6; (Maekawa et al., 2005)]. Many other transcription factors that are stage-specific (Figure 6; Table 1) exert predominant functions.



Stage	Quiescence	Proliferation/ Activation	Fate specification	Morphogenesis and differentiation	Maturation
Signals	BMP | Notch	BMP | Wnt | Shh	Wnt	Wnt | Notch	
Neuro transmitters	GABA	Dopamine | Acetylcholine | Norepinephrine		GABA | Serotonin	Glutamate
Transcription factors	Pax6 | Sox2	Pax6 | Sox2 | Tbr2	Tbr2 | Ascl1 | Prox1	NeuroD1 | Prox1	NeuroD1
Neurotrophic/ growth factors	FGF-2	IGF | FGF-2 | BDNF | CNTF | VEGF	TGF-β1	IGF | FGF-2 | BDNF | CNTF | VEGF	

Figure 6. Signals, neurotransmitters and transcription/growth factors regulators during adult hippocampal neurogenesis. Stage- and cell-specific effects of different intrinsic signalling pathways and external factors during lineage progression. Ascl1, achaete-scute complex homolog 1; BNDF, brain neurotrophic derived factor; BMP, bone morphogenetic protein; CNTF, ciliary neurotrophic factor; FGF-2, fibroblast growth factor-2; GABA, γ-aminobutyric acid; IGF, insulin growth factor; NeuroD1, neuronal differentiation 1; Prox1, prospero homeobox 1; Sox2, sex-determining region Y-box 2; Shh, Sonic hedgehog; Tbr2, T-box brain protein 2; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

Morphogens such as Notch (Ables et al., 2010) and Shh (Lai et al., 2003) have been found to be required for NSCs self-renewal and hippocampal progenitor cells proliferation, respectively. Other signal molecules also known to control stage- and cell-specific steps during neurogenesis (Table 1) include BMP signalling, described to regulate the *equilibrium* between proliferation and quiescence (Bond et al., 2014), and Wnt signalling pathways, which induce progenitors differentiation toward the neuronal lineage (Lie et al., 2005). Additionally, the DG is enriched with inputs from many other brain regions that release different neurotransmitters. Among the classic ones, glutamate and GABA directly regulate proliferation and maturation of NSCs, and the integration and survival of newborn neurons (Lledo et al., 2006; Balu and Lucki, 2009; Kempermann et al., 2015). Specifically, glutamate regulates the survival of newborn neurons in the adult SGZ (Tashiro et al., 2006), while reduction of GABA signalling results in NSCs activation and symmetrical division [Figure 6; (Song et

al., 2012)].

Moreover, regulators of the cell cycle are also important in dictating NSCs proliferation. This control occurs mainly during the G1 phase, where factors such as cyclins and cyclin-dependent kinases are crucial in the control of cell cycle entry by NSCs, its progression or arrest, and cell cycle length and exit, thus determining the net result of each cell cycle round (Lledo et al., 2006). For example, cyclin E is associated with G1/S phase transition, and is highly expressed by Nestin- and GFAP-positive NSCs, being consequently involved in mediating cell cycle entry from quiescence (Ikeda and Ikeda, 2015).

Other additional control includes, for instance, hormonal regulation by estrogen, cellular metabolic states (Shin et al., 2015), and epigenetics (Goncalves et al., 2016).

Adult neurogenesis is also strongly modulated by microglia and inflammation. While some reports show that the release of inflammatory cytokines, including interleukin-6, from microglia cells sharply inhibits neurogenesis (Ekdahl et al., 2003), others demonstrated that if the balance of secreted molecules in the neurogenic niche is anti-inflammatory this can, in fact, promote neurogenesis (Battista et al., 2006). For example, in response to exercise, microglia stimulates neurogenesis through the chemokine ligand 1 signalling pathway (Vukovic et al., 2012).

An interesting hallmark of adult hippocampal neurogenesis concerns its sensitivity to physiological and pathological stimuli. In the last decade, a large number of evidence has demonstrated that different external and environmental stimuli can impact both positively and negatively in cell proliferation and neuronal survival. For instance, physical exercise, such as running, strongly increases proliferation (van Praag et al., 1999), while enriched environment promotes new neurons survival (Kempermann et al., 1998a). Also, learning modulates adult neurogenesis in a complex, yet specific manner. For example, adult SGZ neurogenesis is only influenced by learning tasks that depend on the hippocampus, since subjecting animals to specific learning paradigms mostly regulates the survival of new neurons, but the effects largely depend on the timing of cell birth and learning phases (Ming and Song, 2011). Contrastingly, factors such as aging (Kempermann et al., 1998b), and stress and its effectors (Lucassen et al., 2010), have shown to exponentially reduce neurogenesis in the DG (Table 1).

In the next following sections, due to the scope of this thesis, a more detailed regulation of hippocampal neurogenesis by exercise, aging and stress will be provided. Also a special attention to oxidative stress and related processes will be given.

Table 1: Summary of the reports describing the roles of intrinsic and extrinsic regulators of adult hippocampal neurogenesis.

Regulators	Model	Stage- and cell-specific effects	References
Transcription factors			
Pax6	*Pax6*-deficient rat	Reduces GFAP+ cells, but increases PSA-NCAM progenitors	(Maekawa et al., 2005)
Sox2	Nestin-Cre *Sox2*-deleted mutant	Complete loss of NSCs, but not of cell genesis	(Favaro et al., 2009)
Mash1/Ascl1	Retroviral-mediated overexpression	Redirects the fate of proliferating progenitors	(Jessberger et al., 2008)
Tbr2	Nestin-Cre conditional ablation	Loss of intermediate progenitors and neuronal differentiation	(Hodge et al., 2012)
NeuroD1		Decreases survival and maturation of newborn neurons	(Gao et al., 2009)
Prox1		Premature NSCs differentiation	(Lavado et al., 2010)
Signalling			
BMP	BMP4 lentivirus in the DG	Increases NPCs cell cycle exit, reduces neurogenesis	(Bond et al., 2014)
Wnt	Mutant Wnt1 lentivirus in the DG	Decreases BrdU+ and DCX+ cells	(Lie et al., 2005)
Notch	Notch1 deletion in Nestin-expressing NSCs	Fewer type-1 NSCs and neuroblasts	(Ables et al., 2010)
Shh	Shh-adenoviral vector overexpression in the DG	Increases cell proliferation and differentiated NeuN+ cells	(Lai et al., 2003)
	Pharmacological inhibition in the hippocampus	Reduced proliferative BrdU+ cells	
Neurotrophic/growth factors			
EGF	EGF i.c.v. infusion	No alterations in newborn cells number	(Kuhn et al., 1997)
FGF-2	FGF receptors conditional deletion	Decreases NSCs, progenitors and immature neurons	(Kang and Hebert, 2015)
BDNF	CamKII-conditional mutant	Increases cell proliferation; no effect on cell survival or fate	(Chan et al., 2008)
		Decreases newborn cells survival	(Choi et al., 2009)
CNTF	Forebrain CNTF injection	Improves cell proliferation and neuronal differentiation	(Emsley and Hagg, 2003)
IGF	Peripheral infusion of IGF	Increases progenitors proliferation and differentiation	(Aberg et al., 2000)
TGF-β1	GFAP-TGF-β1 overexpression transgenic mice	Reduces proliferating neuroblasts	(Buckwalter et al., 2006)
VEGF	VEGF i.c.v. administration	Augments proliferation and differentiation	(Jin et al., 2002)
Neurotransmitters			
Glutamate	Retrovirus-mediated KO of NMDA glutamate receptor	Reduces the survival rate of newborn neurons	(Tashiro et al., 2006)
GABA	GABA receptor agonist	Decreases type-2 cells; promotes neuronal differentiation	(Tozuka et al., 2005)
Dopamine	Dopamine D2 receptor antagonism (by haloperidol)	Increases NSCs proliferation	(Kippin et al., 2005)
Serotonin	Serotonin neurotoxin injection into the raphe nuclei	Reduces the number of PSA-NCAM neurons	(Brezun and Daszuta, 1999)
Acetylcholine	Cholinergic agonist systemic administration	Reduced proliferation and short-term survival	(Mohapel et al., 2005)
Norepinephrine	Pharmacological depletion	Decreases proliferation, but not survival or differentiation	(Kulkarni et al., 2002)
Hormones			
Estrogen	Ovariectomized female rats	Diminishes the number of BrdU-labelled cells	(Tanapat et al., 1999)
CORT	Acute CORT treatment	Decreases cell proliferation	(Cameron and Gould, 1994)
Cell cycle			
E2F1	E2F1-KO mice	Reduces cell proliferation and newborn neuronal cells and progenitors division	(Cooper-Kuhn et al., 2002)
Cyclin D2	Cyclin D2-mutant mice	Neuronal precursors proliferation decreased	(Kowalczyk et al., 2004)
ROS	NOX-generated ROS inhibition	Reduces NSCs proliferation	(Le Belle et al., 2011)
Aging	12- to 27-months old rats	Decreased cell proliferation and differentiation	(Kuhn et al., 1996)
Stress	Physical and psychosocial stress in monkeys	Reduces precursor cells proliferation	(Gould et al., 1998)
Antidepressants	Chronic treatment (different classes)	Improves neurogenesis	(Malberg et al., 2000)
Behaviour			
Running	Mice housed with running wheels	Increases cell proliferation and survival	(van Praag et al., 1999)
Enrichment	Mice living in an enriched environment	Promotes neuronal precursor cells survival	(Kempermann et al., 1997)
Learning	Learning in the water-maze test	Increases the number of newborn cells	(Dobrossy et al., 2003)
Pathological conditions			
Ischemia	Transient global ischemia	Increases neuronal differentiation	(Liu et al., 1998)
Inflammation	MS mice model	Enhances NPCs proliferation	(Giannakopoulou et al., 2013)
AD	Human patients	Increased neuroblasts population	(Jin et al., 2004)

The table is based on results from publications on adult neurogenesis (Ming and Song, 2005; Lledo et al., 2006; Balu and Lucki, 2009; Goncalves et al., 2016). It is intended to summarize some examples of adult hippocampal neurogenesis regulators, as well as the models used to disclose its roles.

Abbreviations: BDNF, brain derived neurotrophic factor; BMP, bone morphogenetic protein; CORT, corticosterone; CNTF, ciliary neurotrophic factor; DG, dentate gyrus; EGF, epidermal growth factor; FGF-2, fibroblast growth factor; GABA, γ-aminobutyric acid; HPA, hypothalamic-pituitary-adrenal; i.c.v., intracerebroventricular; IGF, insulin growth factor; KO, knockout; MAPK, mitogen-activated protein kinase; MS, multiple sclerosis; NeuroD1, neuronal differentiation 1; NPCs, neural progenitor cells; NSCs, neural stem cells; Pax6, paired box protein-6; Prox1, prospero homeobox-1; ROS, reactive oxygen species; Shh, Sonic hedgehog; Tbr2, T-box brain protein 2; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

4.4.1 Exercise in the modulation of adult hippocampal neurogenesis

It is widely accepted that exercise produces long-lasting beneficial effects in the health of individuals. With positive outcomes that extend beyond the periphery to the CNS, exercise facilitates the recovery from injury, improves cognition and counteracts age-related memory decline (van Praag, 2008). In this sense, many studies are currently focused on understanding the effects of exercise on hippocampal neurogenesis, its kinetics, as well as its putative therapeutic significance.

In the current literature, rodent models of aerobic activity include the voluntary running, where animals are housed under standard conditions with free access to a running wheel, and the forced running, where animals are placed on a treadmill and forced to run. In both models, exercise promotes hippocampal neurogenesis and benefits memory (Hamilton and Rhodes, 2015); however, there are still some inconsistencies and variability across the different studies. Notably, not only the type of running model needs to be taken into account, but also variables such as the genetic background of the animal model, the housing environment, and the duration and intensity of the exercise, will all determine the beneficial effects of running on neurogenesis (Patten et al., 2015). For instance, voluntary running increases the number of proliferating cells in the hippocampus only 3 days after a short-term running, returning to basal levels after 35 days of running, while an effect on cell differentiation and survival has been shown to require at least 10 days of running (Kronenberg et al., 2006).

In the late 90's, van Praag and colleagues (1999) were pioneers in describing the pro-proliferative effects of exercise (van Praag et al., 1999). Upon housing animals under standard conditions with running wheels, voluntary exercise was observed to increase hippocampal cell proliferation and to enhance neurogenesis (van Praag et al., 1999). This first description has been countlessly replicated along the years, with others additionally reporting the regional effects of running (Ehninger and Kempermann, 2003), and the consequences of exercise in the biology of each cell type that composes the hippocampal neurogenic niche. Moreover, many have also repeatedly demonstrated the positive correlation between running distances and the levels of hippocampal neurogenesis (Rhodes et al., 2003). It is clear that running influences adult neurogenesis by promoting proliferation, but the effects on cell differentiation and survival are also for long well established. Voluntary wheel running has been reported to induce the proliferation of type-2b Nestin-positive cells (Kronenberg et al., 2003) and type-2 Tbr2-positive cells (Hodge et al., 2008), as well as the increase of the number of DCX-positive type-3 neuroblasts (Brandt et al., 2010) and NeuN/BrdU-positive newborn neurons (Bednarczyk et al., 2011). Importantly, these immediate pro-proliferative cellular

effects of running have been shown to rely on cell cycle dynamics. For instance, some have shown that running induces quiescent NSCs to enter into the cell cycle (Lugert et al., 2010), while promoting cell cycle exit of fast progenitor cells (Brandt et al., 2010). Moreover, Farioli-Vecchioli and colleagues (2014) showed that 12 days of running expands type-2b and -3 progenitor cells by shortening the length of the S phase of the cell cycle (Farioli-Vecchioli et al., 2014). Conversely, others described increased cell proliferation following 5 days of running but not caused by a shortening of the cell cycle length (Fischer et al., 2014). For sure, the fact that the duration of the running protocol and the type of cell population analysed were distinct between studies, highly contributed to these contradictory results.

Also, although quite intriguing, exercise activates the proliferation of quiescent radial glia-like type-1 stem cells (Lugert et al., 2010). Even though these cells seem to not highly contribute to hippocampal neurogenesis, due to their quiescence status, the fact that they respond to running supports the view that radial NSCs are a reserve pool of cells that can be "called" to increase the neurogenic process in response to changes under certain conditions (Lugert et al., 2010).

Exercise does not only promote cell proliferation and the generation of new cells, but it also influences their individual morphology. Of interest, wheel running enhances the maturation of newborn neurons by increasing spine motility and the proportion of mushroom spines, as well as it improves spine growth during early maturational stages, thereby promoting cells recruitment into neuronal activation (Zhao et al., 2006). In fact, it is strikingly that newborn cells generated by running heightens LTP synaptic plasticity (van Praag et al., 1999) and improves learning (van Praag et al., 2005). In fact, several have extensively reported the beneficial effects of exercise on learning and memory, both in humans (Pereira et al., 2007) and rodents (van Praag et al., 1999). Particularly, both voluntary and forced running were shown to enhance spatial learning, using different types of mazes (such as water, radial and y-maze) and training paradigms (Patten et al., 2015). Running also improves performance in other hippocampal-dependent tasks, including spatial pattern separation (Creer et al., 2010), CFC (Baruch et al., 2004) and novel object recognition (O'Callaghan et al., 2007). The particular importance of adult neurogenesis for exercise-promoted cognition was clearly illustrated when Clark and colleagues (2008) used focal gamma irradiation to ablate neurogenesis and showed its requirement to enhance spatial learning in response to running (Clark et al., 2008).

The boost of hippocampal neurogenesis by running is a robust phenomenon, since, for instance, it has no effect on neurogenesis in the adult SVZ and OB (Brown et al., 2003). A multitude of cellular

and molecular factors triggered by exercise are instrumental for its beneficial effects on hippocampal neurogenesis. There is evidence that exercise significantly boosts the generation of glia cells (astrocytes and oligodendrocytes) in the hippocampus, and also in other brain regions (Mandyam et al., 2007), but the functional significance of this is still undetermined. Moreover, running increases cerebral blood flow, blood-brain barrier permeability and glucose metabolism, which combined raises the levels of circulating hormones and growth factors (Aimone et al., 2014). Particularly, BDNF is considered one of the most important factors up-regulated by physical exercise (Vivar et al., 2013). The increase of other factors, such as nerve growth factor (NGF) (Neeper et al., 1996) and FGF-2 (Gomez-Pinilla et al., 1997) is considered transient and less pronounced. Also, vasculature-associated trophic factors, including IGF and VEGF, have been implicated as mediators of running-induced neurogenesis, since both are increased in running animals (both serum and hippocampal levels) (Carro et al., 2000; Fabel et al., 2003) and, conversely, their blockage abolishes running-mediated increased neurogenesis (Trejo et al., 2001; Fabel et al., 2003). A number of additional signalling pathways have been shown also to be involved and that includes changes in the neurotransmitter system, such as GABA and glutamate, and in hippocampal genes expression related to synaptic plasticity (van Praag, 2008).

Exercise-induced hippocampal neurogenesis is currently explored as a strategy to overcome and rescue decreased neurogenesis and memory associated with pathological states. In conditions as diverse as irradiation, aging, AD, or even stroke, exercise has proven to be effective in counteracting the negative effects linked with pathology [reviewed in (Patten et al., 2015)]. For example, exercise was described as a potent strategy to reduce age-related cognitive decline and neurogenesis (van Praag et al., 2005), and to delay hippocampal neurodegeneration and rescue spatial memory deficits in a mouse model of AD (Huttenrauch et al., 2016). For sure, the beneficial effects in disease do not imply that exercise can alter the pathology itself, but rather stimulates compensation by promoting neural plasticity.

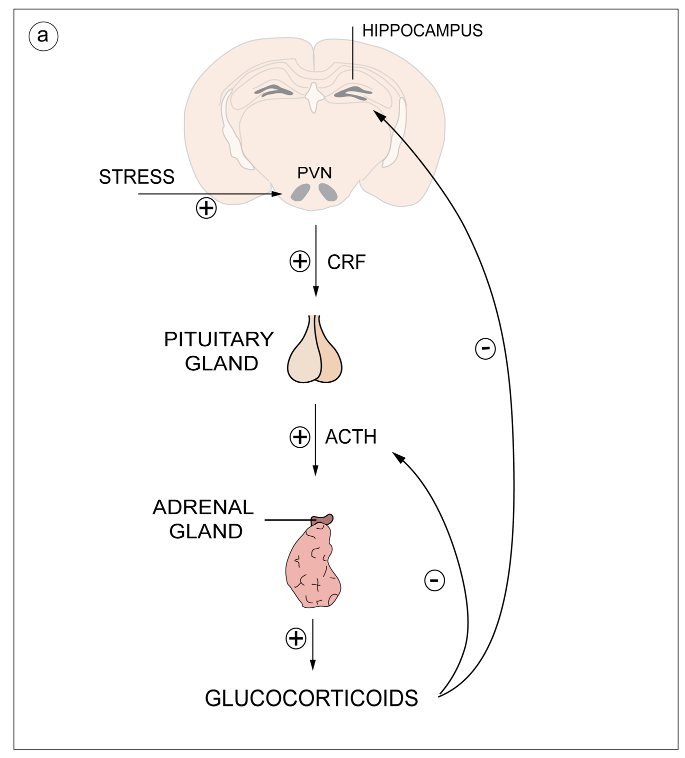
4.4.2 Regulation of adult hippocampal neurogenesis by stress and glucocorticoids

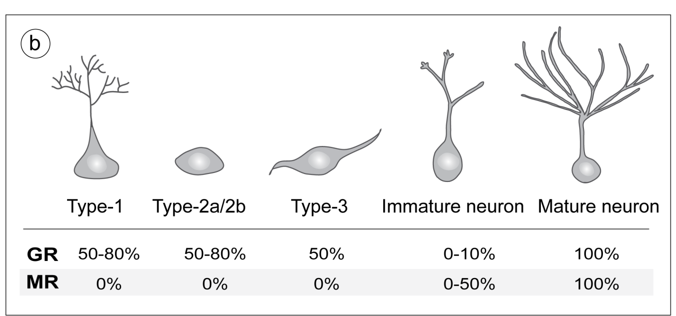
Stress is defined as any event interpreted as a threat to the physiological/psychological integrity of an individual that produces biological and/or behavioural responses (McEwen, 2000). These responses allow the individual to cope with the situation, and have important adaptative functions

(McEwen, 2000). However, if sustained and prolonged over time, an imbalance can occur resulting in the development of an array of adverse effects.

In the mediation of the stress response, the CNS integrates the information sensed by peripheral organs and adjusts the body activity through the activation of systemic stress responses. These are driven by the activation of the hypothalamus-pituitary-adrenal (HPA) axis (please see Figure 7a for a more detailed regulation of the axis) and the catecholominergic system. As end-effectors of the HPA axis, glucocorticoid hormones (cortisol in humans and CORT in rodents) are released from the adrenal cortex into the bloodstream to exert a negative feedback in the brain (Smith and Vale, 2006). In here, the hippocampus is central in the termination of the axis response and in the articulation of neuroendocrine, physical and behavioural responses to ensure homeostasis reinstatement, by perceiving sensory inputs from the external environment and internal inputs from the body and providing a negative feedback control (Fulford and Harbuz, 2005).

Glucocorticoids promote the regulation of both brain and peripheral functions primarily via two specific receptors: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) (Lucassen et al., 2010). The hippocampus is highly enriched in both GR and MR, rendering it a higher sensitivity to the effects of glucocorticoids (Lucassen et al., 2010). In fact, the HPA axis, through its effectors, has been shown to strongly impact on hippocampal functions, including cognition, behaviour and mood. Actually, the hippocampus and its associated DG is one of the forebrain limbic structures that, along with the PFC and amygdala, is involved in the regulation of the autonomic and HPA axis stress response, also serving as a link between stress response and the development of neuropsychiatric disorders (e.g. depression) (Smith and Vale, 2006). In this sense, it is not surprisingly that hippocampal neurogenesis can also be profoundly affected by stress exposure. The pioneer studies in the early 1990's by Gould and colleagues (1992) suggested an inhibitory role for stress-induced glucocorticoids in hippocampal neurogenesis, since the administration of adrenal hormones in rats negatively affected the incorporation of H^3-thymidine, while the removal of adrenal hormones, by adrenalectomy, boosted proliferation (Gould et al., 1992; Cameron et al., 1993). The general view is that the effects of stress on neurogenesis occur by primarily inhibiting cell proliferation, being the effect generalized across species, stressors and duration (Lucassen et al., 2010).





	Type-1	Type-2a/2b	Type-3	Immature neuron	Mature neuron
GR	50-80%	50-80%	50%	0-10%	100%
MR	0%	0%	0%	0-50%	100%

Figure 7. Hypothalamus-pituitary-adrenal (HPA) axis regulation during stress. (**a**) As part of the stress response, activation of the paraventricular nucleus (PVN) in the hypothalamus results in the secretion of corticotropin releasing factor (CRF), which, in turn, promotes the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland into the blood stream. This hormone stimulates glucocorticoids (cortisol in humans and CORT in rodents) release from the adrenal cortex. Glucocorticoids will then promote the regulation of both brain and peripheral function by interacting with their receptors in several target tissues. In the brain, the hippocampus is central in the regulation of the negative feedback of the axis. (**b**) Glucocorticoid (GR) and mineralocorticoid receptors (MR) expression varies during hippocampal neurogenesis. ACTH, adrenocorticotropic hormone; CRF, corticotropin releasing factor; GCL, granular cell layer; GR, glucocorticoid receptor; ML, molecular layer; MR, mineralocorticoid receptor; PVN, paraventricular nucleus; SGZ, subgranular zone.

Both psychosocial and physical stressors, as well as acute and chronic stress exposures, have a potent suppressive effect on proliferation, but are also described to inhibit one or more phases of the neurogenic process, including neuronal differentiation (Lucassen et al., 2010). Particularly, acute social defeat and acute predator odour exposure was observed to decrease neuronal differentiation (Egeland et al., 2015). In addition, the exposure to unpredictable chronic mild stress, an experimental paradigm commonly used to model depression in rodents, causes a reduction in the number of proliferating hippocampal progenitor cells and of newborn neurons (Santarelli et al., 2003). This, in addition, causes despair- and anxiety-like behaviour (Santarelli et al., 2003). Also, injection of glucocorticoids in animals exerts, overall, similar effects as those induced by stress exposure. The exogenous chronic systemic administration of CORT, which has been proved to be a reliable animal model to study stress-triggered responses (Sousa et al., 2000), reduces cell proliferation and the survival of hippocampal progenitors (Murray et al., 2008; Brummelte and Galea, 2010), with reports also describing increased apoptosis of both neural progenitors and immature granule neurons (Yu et al., 2010). Noticeable, and from a translational point of view, the *in vitro* treatment of human hippocampal-derived stem cells with cortisol was also observed to induce a reduction on both cell proliferation and differentiation (Anacker et al., 2011). Nevertheless, the relationship between glucocorticoid levels and adult neurogenesis is far more complex than a simple linear inhibitory role. In fact, some have demonstrated persistent inhibition of neurogenesis despite restoration of normal levels of glucocorticoids (Mirescu and Gould, 2006) and, at certain conditions, glucocorticoids increase by stress revealed no change, or even an increase in cell proliferation and neurogenesis (Kirby et al., 2013). Certain stress paradigms, such as restraint stress and active avoidance shock training, have been shown to elevate CORT levels but no measurable effect on cell proliferation. Also, footshock and exposure to a novel environment were shown to increase proliferation and even improve memory (Kirby et al., 2013). Notably, other manipulations that increase stress hormone secretion, such as exercise, similarly stimulate adult neurogenesis (Kronenberg et al., 2006). It is clear, then, that the relationship between hormones and neurogenesis also depends on other factors, still to be clearly elucidated.

The described effects of glucocorticoids on hippocampal neurogenesis are achieved, in part, indirectly by the orchestration of several different signalling mechanisms, or by direct effects through GR and MR receptors. Both receptors are expressed during the different stages of neuronal development (Egeland et al., 2015). Particularly, stress can directly target the precursor cell pool, since the large majority of quiescent type-1 NSCs and type-2a amplifying progenitors express GR

(Figure 7b). Interestingly, such expression ceases in the intermediate progenitors (type-2b), starts to resume in type-3 neuroblasts, and is abundantly expressed once the cells become fully mature (Figure 7b). The MR, on the other hand, is only expressed at the mature phase of the cells (Egeland et al., 2015). Additional regulation proposed to underlie the stress effects on neurogenesis includes pro-inflammatory cytokines, neurotrophic factors and neurotransmitters (Balu and Lucki, 2009; Egeland et al., 2015). Also, chronic stress was shown to, not only reduce the number of proliferating cells, but concomitantly to elevate the levels of p27Kip1, an endogenous cell cycle inhibitor (Heine et al., 2004). Moreover, the close contact of hippocampal DG NSCs with the blood vessels in the vicinity ideally places stem cells as easy targets for the increased levels of glucocorticoids in circulation in a situation of stress.

To notice, hippocampal neurogenesis can also reversely facilitate the normalization of glucocorticoid levels after stress (Snyder et al., 2011). In fact, many have proposed a bi-directional relationship between adult hippocampal neurogenesis and HPA axis regulation. Specifically, neurogenesis-deficient mice showed more pronounced glucocorticoid levels after acute stress (Schloesser et al., 2009; Snyder et al., 2011), highlighting the role of hippocampal neurogenesis in enhancing glucocorticoid-mediated negative feedback on the HPA axis. This, in turn, has been shown to be important in buffering the behavioural response to stress, but also in physiological conditions. Particularly, the total ablation of neurogenesis precipitates behavioural despair and anhedonia, even under baseline conditions when mice were not exposed to any stress (Snyder et al., 2011). Together, stress/glucocorticoid-mediated regulation of hippocampal neurogenesis has implication to mood disorders. Chronic stress, for instance, is a precipitating factor for major depression and significantly influences the course of the disease by altering hippocampal neurogenesis (Anacker and Pariante, 2012). Indeed, counteracting the impairments in adult neurogenesis is considered to be a promising strategy for future neuropsychiatric treatments.

4.4.3 Aging in the regulation of hippocampal neurogenesis

Adult neurogenesis is widely accepted as a lifelong process and, although it is described to decline along with age, the generation of new neurons still persists in older individuals at very low levels. This assumption exists since the first description of adult neurogenesis by Altman (Altman and Das, 1965), and it has been reported since then. From the 90's, several have demonstrated that the levels of hippocampal neurogenesis in aging and in aged animals are only a fraction of that seen in

younger adults. The decreased production of new neurons is significant, in the order of 80% or more (Kuhn et al., 1996), and starts relatively early in the aging process, at 2-months of age (Ben Abdallah et al., 2010). Only a modest, if any, additional decline occurs during senescence (at 9-months of age) (Ben Abdallah et al., 2010). Interestingly, the observations that the number of precursor cells reduces across aging mostly denotes the decline of the number of TAPs (Kempermann, 2015). Consistently, the proportion of NSCs that can survive and differentiate into newborn neurons is reduced to half of that of young animals (Kuhn et al., 1996), which was also observed for neurogenesis in the OB (Molofsky et al., 2006).

In the current literature, several theories exist to explain age-associated decline in adult neurogenesis. One possibility it that, as animal ages, less progenitor cells maintain their neurogenic potential as they differentiate, meaning that, with aging, more progenitors become astrocytes and less differentiate into neurons (Encinas et al., 2011). This, in fact, explains why neurogenesis decreases with aging, while gliogenesis not. On the other hand, it is suggested that age-related reduction in neurogenesis is due to the progressive loss of stem cells, particularly of the actively dividing progenitor cells (Encinas et al., 2011). Indeed, this reduction occurs due to the drastic decrease of cells in division (Lugert et al., 2010), in part suggested by the progressive cell exhaustion that occurs as consequence of the increased cell death or differentiation into a specialized cell type. Still, others have shown that the overall number of Sox2-positive cells, and of the number of neurosphere-forming cells, remains constant throughout aging (Ahlenius et al., 2009); yet, the percentage of these cells expressing proliferation markers (BrdU and Ki67) is drastically reduced with age (Hattiangady and Shetty, 2008). This, in turn, suggests that, rather than a decrease in the total number of precursors, aging results in an increased quiescence of NSCs and, therefore, in the decline of the proportion of active precursors.

Aging is quite often designated as a strong (or even the strongest) negative regulator of adult hippocampal neurogenesis. Nevertheless, the decline is attributed as the by-product of age-related changes in other cellular systems. These include alterations in the cellular immune response, production of inflammatory mediators and hormonal levels. Also in the brain, both astrocytes and microglia show increased activation with aging (DeCarolis et al., 2015; Rodriguez-Arellano et al., 2016), possibly as a part of the increased immune activation, which undoubtedly changes their secretory profile. For example, secretion of neurogenic factors derived from glia, such as FGF-2 and VEGF, decreases prominently with age (Shetty et al., 2005). There is correlative evidence that intracerebroventricular infusion of FGF-2 into old mice partially ameliorates the aging-related decline

in the number of BrdU-positive cells (Riddle and Lichtenwalner, 2007). Other factors, including BDNF and EGF, have been shown as important mediators of aging-related neural changes in the brain (Riddle and Lichtenwalner, 2007). Also, altered levels of neurotransmitters release (Riddle and Lichtenwalner, 2007), or accumulation of inflammatory molecules (Huber et al., 2011), constitute only some of the examples of additional factors that can inhibit neurogenesis and impair hippocampal functions during aging (Villeda et al., 2011). Furthermore, the vasculature also deteriorates with age, thus providing less blood flow and regulatory factors to the niche (DeCarolis et al., 2015). In fact, using the heterochronic parabiosis model, Villeda and colleagues (2011, 2014) showed that the proliferative capacity of resident stem cells is modulated by the "age" of the systemic circulation (Villeda et al., 2011; Villeda et al., 2014). Heterochronic young mice had significantly reduced numbers of new DCX-labelled neurons in the hippocampus, whereas their older counterpart showed a partial restoration of neuroblasts (Villeda et al., 2011). Factors, including the circulating C-C motif chemokine 11, were associated with age-related decline in hippocampal neurogenesis (Villeda et al., 2011).

Additionally, the age-related increase in serum glucocorticoids levels has been proposed as the main cause for the age-dependent decline of new neurons (Montaron et al., 2006). There is an interesting correlation, during aging, between chronically elevated CORT levels and the reduction of neurogenesis. In fact, adrenalectomy in senescent rats dramatically reduces CORT levels (Montaron et al., 1999) and increases cell proliferation and neurogenesis (Cameron and McKay, 1999; Montaron et al., 1999), evidencing age-related decrease in neurogenesis as a consequence of an imbalance in environmental signals.

Consistent with the idea supported by several lines of evidence that neurogenesis is involved in memory processing in the young adult brain, similarly many reports linked age-associated changes in neurogenesis with deficits in memory and learning (Rapp and Amaral, 1992). Although merely correlative, studies have predicted the performance of aged rats on hippocampal-dependent tasks according to their levels of neurogenesis (Driscoll et al., 2006). Individuals with lower rates of neurogenesis might be more vulnerable to neurogenesis-related memory loss, whereas increased neurogenesis at younger ages might be preventive (Seib and Martin-Villalba, 2015). In fact, 20-months old rats reported to individually exhibit better spatial memory performance in the MWM presented more new neurons generated (Drapeau et al., 2003), and the decreased survival of newborn cells in the DG of 28-months old rats is associated with deficits in CFC test (Wati et al., 2006). More important, it has been proposed that the age-related decline in neurogenesis may even

contribute to the precipitation of pathological conditions, such as AD. In this sense, modulation of neurogenesis in the aged brain has been considered as useful in preventing or treating the development of memory disorders during the course of normal aging. In line with this, a growing body of evidence suggests that exercise interventions hold the potential to counteract age-associated declines (Intlekofer and Cotman, 2013), by restoring cell survival (van Praag et al., 2005).

4.4.4 Reactive oxygen species in neural stem cell regulation

Until very recently, the importance of cellular metabolism has been largely neglected for the role it may play in determining the functional properties of NSCs and in the generation of new neurons. However, in recent years, it is becoming increasingly clear that the metabolic state of adult NSCs sets the cell's energy status and, therefore, its behaviour (Knobloch and Jessberger, 2016). Recent single-cell RNA-seq analysis of the adult NSCs provided the first evidence that lineage progression in adult hippocampal neurogenesis is functionally coupled to the activity of a specific metabolic program (Shin et al., 2015). Quiescent radial glia-like NSCs have a predominant glycolytic profile, and high levels of oxidative phosphorylation are associated with termination of proliferation and neuronal differentiation. This shift in cells metabolic profile is required due to the high-energy demands that the differentiation process has (Hall et al., 2012; Steib et al., 2014; Bond et al., 2015). In here, a controlled delivery and utilization of oxygen is then essential for the balance between energy generation and the avoidance of unwarranted oxidative reactions (Vieira et al., 2011).

In the adult brain, the oxygen tension is estimated to range between 0.1–5.3%, and low rates of oxygen are commonly observed in regions of high cellular density, such as the hippocampal DG (Prozorovski et al., 2015). This creates a hypoxic microenvironment that contributes to the maintenance of a low oxidative phosphorylation in quiescent stem cells, and has important physiological roles in protecting cells from DNA damage and in promoting NSCs self-renewal (Simon and Keith, 2008). On the other hand, although still controversial, evidence suggests that hypoxia also contributes to the generation of free radicals, including reactive oxygen species (ROS) in particular subcellular compartments (Vieira et al., 2011). Also, oxidative phosphorylation that takes place in mitochondria during cell differentiation generates ROS as a by-product (Madhavan, 2015).

Mainly known to cause oxidative stress upon cellular accumulation, and to contribute to disease and cell death, ROS at lower nontoxic levels can, actually, promote cell proliferation and survival

(Blanchetot and Boonstra, 2008). Intracellular ROS exists primarily in the forms of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO), nitric oxide radical (NO), and peroxynitrite ($ONOO^-$) and, under normal physiological conditions, the negative effects of ROS are antagonized by antioxidant mechanisms. These include, among others, the action of the antioxidant enzymes superoxide dismutase (SOD) and glutathione peroxidase (GPX), with this last one being the most abundant antioxidant synthesized by the cells (Bigarella et al., 2014). However, when ROS production outpaces its scavenging, this leads to the excessive accumulation of ROS, which shifts the intracellular redox environment to a more oxidized state, and promotes oxidative reactions that produce adverse effects on multiple cellular components. In fact, oxidative stress imposed by the cellular accumulation of ROS is a major contributor to disease and cell death (Dixon and Stockwell, 2014). Nevertheless, in recent years, it is becoming evident that ROS, at low non-toxic levels, is important for signalling and can partly regulate the appropriate balance between self-renewal and differentiation for stem cell function (Blanchetot and Boonstra, 2008; Bigarella et al., 2014). For example, in the hematopoietic system, a low endogenous cellular ROS status contributes to the maintenance of the quiescent state of the hematopoietic stem cells, whereas a higher ROS state is associated with a greater proliferation leading to a premature exhaustion of self-renewal (Jang and Sharkis, 2007). This has led to the hypothesis that keeping ROS levels low within the stem cell niche is an important feature of stemness, which is directly related to the quiescent state of stem cells. This also holds true for the NPCs, where ROS was shown to promote cell proliferation (Yoneyama et al., 2010; Le Belle et al., 2011).

Among the forms of ROS that exist, the anion superoxide (O_2^-) is the principal one that was described to control NSCs function, being mainly produced by the mitochondrial respiratory chain complexes, and by several oxidases, such as the plasma membrane NADPH oxidase (Prozorovski et al., 2015). While NADPH oxidase complex shifts redox homeostasis during the activation of NSCs to trigger self-renewal, mitochondria-derived ROS promotes NSCs differentiation (Prozorovski et al., 2015). Early steps of neuronal differentiation are associated with progressively increase mitochondrial biogenesis, ATP production and concomitant rise in ROS formation (Yoneyama et al., 2010; Le Belle et al., 2011). The redox potential also changes as cells progress through the cell cycle. Upon stimulation for cell division, quiescent NSCs that normally present low levels of ROS, progressively increase endogenous ROS to guarantee proliferation (Yoneyama et al., 2010; Le Belle et al., 2011). A flux of ROS induces cell cycle entry and progression, while the accumulation of reducing factors in the nucleus at the S phase of the cycle allows successful DNA replication for cell division. The levels of

superoxide radicals increase as cells transit from G1 to M phase, and the balance between hydrogen peroxide and superoxide concentration influences the decision to enter or exit the cycle. In fact, overproduction of ROS and their accumulation in the nucleus during the G1 phase, which may also affect the flux of reducing factors into the nucleus, forces the cell into cycle arrest or to exit for differentiation (Ostrakhovitch and Semenikhin, 2013). In addition to the external environment, intracellular ROS production also increases as cells proceed through the differentiation process. Within the hippocampal redox environment that favours differentiation, a more oxidized environment favours differentiation toward the astroglial lineage, whereas a more reduced environment favours neuronal differentiation [Figure 8; (Le Belle et al., 2011; Prozorovski et al., 2015)].

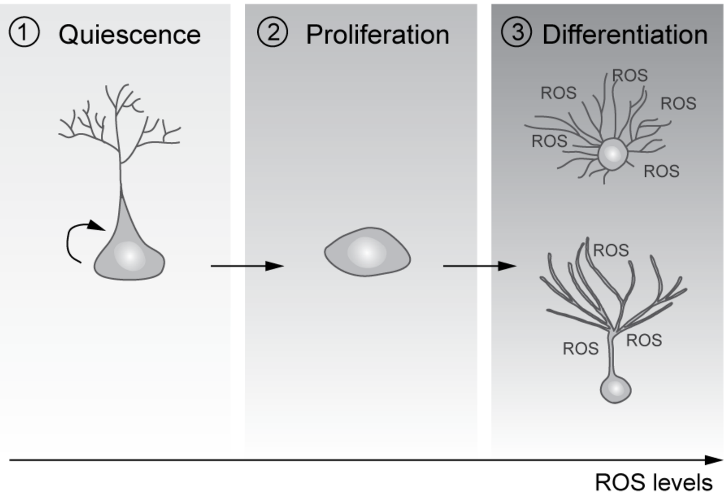


Figure 8. Redox potential and cell fate decision. (**1**) Neural stem cells reside in a relatively hypoxic environment that favours quiescence and self-renewal. As the environment becomes more oxidized, cell proliferation and differentiation is activated (**2**). The differentiation process, itself, also leads to increased production of intracellular oxygen free radicals. Higher numbers of astroglial cells are generated in an environment where the redox potential is more oxidized, while a more reduced environment potentiates neuronal differentiation (**3**). ROS, reactive oxygen species.

Interestingly, the neurogenic process itself generates ROS (Walton et al., 2012). As a high-energy consuming process, adult neurogenesis was observed to transiently generate localized oxidative stress, thus contributing to ROS-mediated effects within the niche (Walton et al., 2012). An increased expression of oxidized markers were observed in the SGZ, and further *in vitro* approaches revealed that induction of NSCs differentiation results in an immediate increase in overall ROS

production (Walton et al., 2012). Moreover, neurogenesis ablation reduced oxidative stress within the SGZ, therefore highlighting that oxidative stress is generated in the normal course of neurogenesis (Walton et al., 2012).

Accumulating evidence suggests that ROS influences cell proliferation by functioning as second messenger in intracellular signal transduction pathways. Specifically, ROS modifies the actions of proteins through the reversible oxidation of essential cysteine residues. Particularly in the neural precursors, ROS may contribute to regulation through intracellular signalling of EGF receptors (Yoneyama et al., 2010). Nevertheless, other mechanisms have been proposed, including targeting cell cycle regulators such as cyclin D1 (Burch and Heintz, 2005).

Even though essential for NSCs function, ROS-mediated signalling can also negatively influence stem cell biology and contribute to impaired neurogenesis. Perturbations in the redox balance within the neurogenic microenvironment by, for instance, impaired efficacy of the antioxidant enzyme SOD, can lead to changes in the production, functional integration, and long-term survival of new neurons. Studies of SGZ neurogenesis in mouse models deficient in each of the SODs isoforms (Huang et al., 2012) revealed a significant reduction in the production of new neurons (Rola et al., 2007; Fishman et al., 2009), reduced long-term survival of newborn cells (Rola et al., 2007), and/or the shift towards gliogenesis (Fishman et al., 2009). This advises for the importance in maintaining a normal redox environment in all subcellular compartments for a proper neurogenic process and further hippocampal integrity. In fact, cellular changes caused by oxidative stress at the hippocampal neurogenic niche were also associated with important functional and behavioural defects, since assessment of cognitive function of SODs-deficient mice, in the MWM and in the novel object recognition task, revealed significant impairments (Huang et al., 2015). In line with this, oxidative stress imposed by the overproduction of ROS has also been implicated with the precipitation of neuropsychiatric and neurodegenerative disorders, such as AD, and in normal aging (Huang et al., 2015), putatively through the intense activation of apoptosis in neuronal cells. Also, blood levels of specific antioxidants were shown to be decreased in anxiety and major depression, which were normalized after therapeutical treatment (Atmaca et al., 2004; Hovatta et al., 2010). Of interest, also moderate exercise prevents oxidative stress-induced anxiety-like behaviour, putatively through the pro-neurogenic effects of running (Salim et al., 2010).

As so, and given the fundamental role of redox homoeostasis in NSC function, it is becoming clear that understanding the molecular dynamics underlying intracellular redox regulation will reveal key mechanisms controlling NSCs survival and regeneration.

4.4.4.1 Iron contribution to reactive oxygen species production and cellular function

Iron is one of the most abundant elements in the living organisms and considered to be essential for life. Physiological processes such as DNA synthesis and cell division, oxygen transport and storage, require enzymes with iron as a cofactor (Mladenka et al., 2006), and, in the CNS, iron is involved in neurotransmission and myelination (Weinreb et al., 2013). On the other hand, free or loosely bound iron is also potentially toxic, since it participates in redox reactions (e.g. Haber-Weiss and Fenton reactions) that lead to the formation of ROS. As a result, iron-mediated oxidative stress leads to cell death and a number of chronic degenerative conditions, including hepatocellular carcinoma, chronic kidney tubular injury and diabetes, among others (Dixon and Stockwell, 2014).

The initial reactive oxygen intermediate produced in almost all cases related to oxidative stress is O_2^-, which is rapidly converted to H_2O_2 by the action of SODs (Figure 9). Noticeably, H_2O_2 and other peroxides are relatively non-reactive molecules against most cellular constituents, but rather need the presence of transient metals, such as iron, in order to catalyse the formation of reactive radicals (Galaris and Pantopoulos, 2008). In fact, there are indications that some of the H_2O_2-induced signalling effects are dependent on iron availability (Galaris and Pantopoulos, 2008). Specifically, iron and iron derivatives (e.g. heme or iron-sulfur [Fe-S] clusters) are incorporated into and are essential for the function of ROS-producing enzymes, including NADPH oxidases (Figure 9), and are also present at the active sites of the H_2O_2-destroying enzyme catalase (Dixon and Stockwell, 2014). However, available pools of labile, redox-active iron ions in the cytosol, the mitochondrial matrix and the lysosomes (Dixon and Stockwell, 2014), are capable of directly catalysing free radicals via Fenton reaction (Figure 9). By specifically reacting with H_2O_2, iron-catalysed ROS produces extremely reactive hydroxyl radicals (Figure 9) that, along with the depletion of cellular antioxidants and of glutathione peroxidase activity, results in cellular oxidative stress, increased membrane lipid peroxidation, DNA damage and protein oxidation and misfolding (Zecca et al., 2004). Also, O_2^- can reduce Fe^{3+} back to Fe^{2+}, thus allowing iron to act as a catalyst of the reaction (Figure 9).

Of interest, an important line of defence against oxidative stress-mediated toxicity includes the chelation of catalytically active iron by agents such as deferoxamine (DFO) or deferasirox (Dixon and Stockwell, 2014). Specifically, DFO is effective in preventing cellular damages caused by elevated ROS production since, upon endocytosis by the cell, it chelates iron in the lysosomes, therefore modulating overall iron homeostasis in different cell compartments (Galaris and Pantopoulos, 2008). Moreover, chelators such as DFO have been implemented or proposed as treatments for diverse

pathologies linked to iron overload syndromes and/or ROS accumulation (Dixon and Stockwell, 2014).

Figure 9. The role of iron in reactive oxygen species (ROS) metabolism. The reaction of peroxides with ferrous (Fe^{2+}) ions to yield soluble hydroxyl radicals is referred as the Fenton reaction. Examples of enzymes that contribute to ROS formation or detoxification are represented in blue. Fe^{2+}, ferrous ion; Fe^{3+}, ferric ion; SOD, superoxide dismutase.

Of notice, a form of iron-dependent, oxidative cell death designated ferroptosis was recently described in mammalian cells (Dixon et al., 2012). Ferroptosis is morphologically, biochemically and genetically distinct from apoptosis, autophagy and reported forms of necrosis, and is characterized by the overwhelming iron-dependent accumulation of lethal lipid ROS (Dixon et al., 2012). Moreover, glutathione peroxidase 4 is an essential regulator of ferroptotic cell death (Yang et al., 2014), and it can be prevented by lipophilic antioxidants, such as vitamin E, and by the iron chelator DFO (Dixon et al., 2012), but not by apoptosis or necrosis inhibitors. Of interest, ferroptosis and lipid peroxidation processes were recently identified as metabolic checkpoints required for direct neuronal reprogramming (Gascon et al., 2016). Ferroptosis inhibitors improved the generation of inducible neurons from a range of somatic cells, and *in vivo* after brain injury (Gascon et al., 2016).

Iron homeostasis

A fine balance exists at the cellular and systemic levels in order to maintain iron concentration meticulously controlled. Interestingly, there is no biological mechanism to excrete iron from the body, and the balance of iron levels is tightly controlled at the absorption level at the enterocytes. For this process, hepcidin, produced in the liver, when in circulation binds to ferroportin, the transporter responsible for the iron efflux from the cells, and targets it to lysosomal degradation

(Anderson and Frazer, 2005; Galaris and Pantopoulos, 2008). Consequently, iron is retained inside the cells, namely the enterocytes, avoiding its absorption to the blood. Whenever iron levels increases in the body, there is also an increase in hepcidin levels that blocks the iron uptake at the enterocytes. The accepted model is that low hepcidin levels trigger increased iron absorption from the duodenum and iron release from reticuloendothelial macrophages. By contrast, high hepcidin levels result in decreased iron absorption and iron retention in macrophages. Once in the blood, iron is bound to transferrin that delivers its cargo to the cells upon binding to transferrin receptors that are expressed in most cell types, and provides the major route for iron acquisition (Gomme et al., 2005). Upon binding, transferrin enters an endocytic pathway and, within endosomes, iron dissociates from the receptor and is transported into the cell cytoplasm, where it can be stored bound to ferritin (Harrison and Arosio, 1996).

Specifically in the CNS, the brain is unique among the organs with regard to iron metabolism. The existence of the blood-brain barrier limits the entrance of plasma iron but holds transport mechanisms that allow for iron to move from the luminal surface of the endothelial cells to the brain (Zecca et al., 2004). Precisely, iron transportation in the barrier occurs due to the presence of transferrin receptors on the luminal surface of the capillary endothelial cells, allowing for the complex uptake by endocytosis and consequent expel from the abluminal surface of the endothelia into the brain interstitial fluid (Li and Reichmann, 2016). Moreover, the concentration of iron in several brain regions varies significantly. For instance, brain regions more associated with motor functions tend to have more iron than non-motor-related ones, which can, at least in part, explain why movement disorders are commonly associated with iron imbalance (Zecca et al., 2004). Specifically, oligodendrocytes are commonly enriched in iron, even though also neurons and microglia present ferritin, thus indicating that all neural cell types have the capacity to store iron (Zecca et al., 2004). And, in fact, iron is required for many physiological brain functions, including neural respiration, myelin synthesis, neurotransmission and synaptic plasticity (Gozzelino and Arosio, 2016). This importance of iron in brain homeostasis is also observed by the retardation and impaired cognitive abilities caused by its deficiency during early development, while axonal degeneration and neuronal death is triggered by its overload (Gozzelino and Arosio, 2016). The physiological accumulation of iron in the brain occurs during the aging process, where the reduction in the expression of antioxidant proteins and repair mechanisms contribute to senescence processes and iron-mediated oxidative stress (Gozzelino and Arosio, 2016). In fact, it is well known that the levels of iron increase disproportionally in many neurodegenerative diseases, including Parkinson's

disease and AD (Gozzelino and Arosio, 2016). Still, the precise mechanisms of abnormal iron metabolism in those neurodegenerative diseases need to be further elucidated.

In recent years, alternative pathways for iron cell delivery have been proposed in the literature. With important roles in the mediation of cell proliferation, apoptotic cell death and development (Yang et al., 2002), lipocalin-2 (LCN2) has been shown to transport and delivery iron to cells through its specific cellular membrane receptor (Devireddy et al., 2005). To notice, the described iron-delivery pathway mediated by LCN2 has been suggested to be present in cells and to constitute an alternative mechanism in transferrin-iron trafficking (Yang et al., 2002). This has been shown to be critical in, for instance, the innate immune response to infection (Flo et al., 2004), in kidney development (Yang et al., 2002), in the attenuation of iron-related oxidative stress (Yamada et al., 2016), and even in the regulation of hippocampal neuronal dendritic spine density and morphology (Mucha et al., 2011). Moreover, LCN2 has been attributed important roles in the context of neurodegeneration, including MS (Marques et al., 2012), AD (Naude et al., 2012; Mesquita et al., 2014) and, more recently, in Parkinson's disease (Kim et al., 2016).
The precise mechanisms and roles of LCN2 in cell physiology and brain homeostasis, along with other important functions in disease initiation and progression, will be next described in more detail at section 5 of this chapter and are present at Appendix A.

5. Lipocalin-2

Over the last decades, the need and interest in the identification of molecules that can serve both a physiological role in the organism and allow for the detection of disease onset and progression has emerged. In this sense, LCN2 has become increasingly relevant in recent years, due to its crucial role in physiological development (Yang et al., 2002) and in the innate immune response (Flo et al., 2004), but also in the pathophysiology of many diseases, being even suggested by many as an important biomarker in kidney injury (Schmidt-Ott et al., 2007) and neurodegeneration (Choi et al., 2011; Marques et al., 2012; Naude et al., 2013). The existence of the knockout mouse for the coding sequence of LCN2 [the LCN2-null mice; (Flo et al., 2004)] has allowed, throughout the years, to clearly define many of the currently known and accepted functions of LCN2, both in the periphery and in the CNS.

As part of the lipocalin protein family of secreted proteins (Flower, 1994, 1996), and similarly to other lipocalins, LCN2 acts as a carrier/transporter and binds to specific cell-surface receptors to modulate its cellular functions. Specifically, LCN2-mediated cell homeostasis is highly dependent on its interaction with the specific cell-surface receptors 24p3R (also known as solute carrier family 22 member 17) (Devireddy et al., 2005), and megalin (Hvidberg et al., 2005). Moreover, LCN2 has high affinity to bind and transport iron, but only when iron is complexed with low molecular mass iron-binding ligands, the siderophores (Goetz et al., 2002; Yang et al., 2002; Devireddy et al., 2005). These ligands are small, high-affinity iron chelators secreted by microorganisms for iron acquisition during invasion, but have also been recently described to be endogenously produced by mammals (Bao et al., 2010; Devireddy et al., 2010). This ability of LCN2 to bind to iron, through siderophores, is, indeed, intimately related to its roles in the innate immune response (Flo et al., 2004), and to the modulation of cell physiological processes, including cell proliferation, differentiation and apoptosis (Goetz et al., 2002; Yang et al., 2002; Devireddy et al., 2005). This, in turn, provides an important link between physiology and pathology. Nevertheless, the literature is still contradictory with respect to some of the functions of LCN2, especially in the pathophysiology of diseases (Ferreira et al., 2015). Moreover, the broad expression of LCN2 and its regulation by several factors [as reviewed in (Ferreira et al., 2015)] is intimately related to its functions. For instance, the acute increased concentration of LCN2 in the serum following intraperitoneal injection of lipopolysaccharide is intimately related to its function as an acute-phase protein in the innate immune response to bacterial infection (Flo et al., 2004).
In the following sections, these and other functions will be further reviewed, but for more detailed descriptions, please see Appendix A.

5.1 Lipocalin-2 functions

The replication of many invading pathogenic microorganisms largely depends on the availability of iron from the invaded host (Schaible and Kaufmann, 2004). In mammals, the fact that iron is mainly intracellular and in circulation bound to transferrin (Gkouvatsos et al., 2012), creates an iron-poor environment for the invading pathogens. To overcome this and to acquire iron, small iron-binding siderophores are secreted by pathogens, which have higher affinity for iron than the host iron carriers (Miethke and Marahiel, 2007). In turn, and as part of the innate immune response, mammalian cells produce and secrete LCN2 to tightly chelate iron-loaded bacterial catecholate-type

siderophores, and limit iron availability to pathogens (Flo et al., 2004; Berger et al., 2006). The importance of this response is evident from the lethality that bacterial infection causes in LCN2-null mice (Flo et al., 2004). The posterior discovery of the existence of a mammalian endogenous siderophore, to which LCN2 can bind (Bao et al., 2010; Devireddy et al., 2010), has opened novel perspectives on additional functions for LCN2, particularly in iron trafficking under physiological conditions. To add, the fact that LCN2 binding to the mammalian siderophore significantly improves in the presence of ferric iron (Bao et al., 2010), revealed a feature of intracellular iron homeostasis regulation by LCN2 that is conserved from bacteria to mammals.

The acquisition of iron by cells is critical for proliferation, survival and differentiation (Andrews, 2008). In mammals, cells acquire iron via receptor-mediated endocytosis of iron-loaded transferrin (Garrick and Garrick, 2009). However, LCN2-mediated iron trafficking has also been shown to occur, and independently of transferrin (Yang et al., 2002). It has been proposed that, through the specific interaction to its cellular receptor 24p3R (Devireddy et al., 2005), LCN2 regulates a fine-tuning mediation of iron cell content that is irrespectively of the cell type and based upon the state of the ligand (Devireddy et al., 2005). Specifically, LCN2-cointaining iron (holo-LCN2) binds to 24p3R, is internalized, traffics to endosomes and releases iron from the complex, thereby increasing intracellular iron concentration [Figure 10; (Devireddy et al., 2005)]. On the other hand, upon apo-LCN2 (LCN2 iron-free) internalization through 24p3R, iron is chelated and transferred to the extracellular medium, thus reducing intracellular iron concentration (Devireddy et al., 2005). Importantly, this LCN2-mediated modulation of iron cell content has been shown to impact on cell proliferation and apoptosis, respectively, the later occurring through the pro-apoptotic Bcl-2-interacting mediator of cell death (BIM) (Devireddy et al., 2005).

Many reports have been supporting the role of LCN2 in physiological intracellular iron homeostasis. For instance, LCN2-containg iron was identified in the developing mammalian kidney and shown to deliver iron into cells to induce epithelial cell differentiation (Yang et al., 2002). Also, the delivery of iron to spermatozoa, at the caudal epididymis, was demonstrated to occur through the internalization of holo-LCN2 (Elangovan et al., 2004), while it was shown to induce apoptosis of hematopoietic cells, with importance during acute anaemia (Miharada et al., 2005). In addition, LCN2 expression was demonstrated to be increased in situations of increased iron utilization (such as anaemia and hypoxia), as a physiological response to cope with the reduction of iron and/or to promote iron utilization (Jiang et al., 2008).

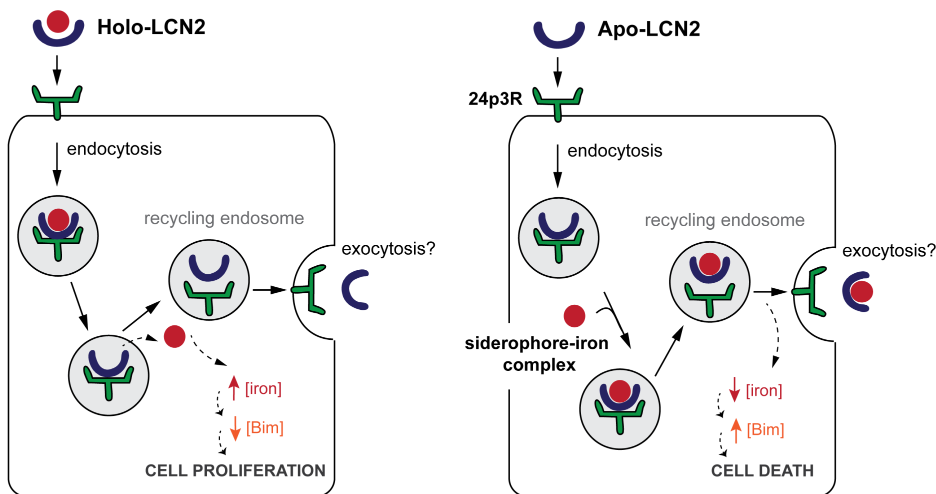


Figure 10. Iron metabolism and apoptosis regulation by LCN2. The proposed model for iron trafficking by LCN2 includes the donation of iron to cell via the 24p3R upon holo-LCN2 binding. This internalization leads to the release and intracellular increase of iron that potentiates cell proliferation. On the other hand, LCN2 binding to 24p3R in its apo-form is internalized and becomes complexed with the mammalian siderophore-iron complex and releases iron to the extracellular medium. This iron depletion induces the expression of the pro-apoptotic molecule *Bim*, which induces apoptosis. Bim, Bcl-2-interacting mediator. Adapted from (Devireddy et al., 2005; Richardson, 2005).

In accordance with its central role in iron transport and, although still limited, it is speculated that circulating LCN2 levels may reflect the body's iron status, at least in haemodialysis patients (Bolignano et al., 2009). Moreover, LCN2-null mice were described to exhibit increased intracellular labile iron and lower circulating iron levels (Srinivasan et al., 2012), which rendered the animals a higher sensitivity to lipopolysaccharide-induced sepsis, associated with an increased oxidative stress (Srinivasan et al., 2012). In fact, and as previously mentioned, despite being crucial for DNA replication and the control of cell cycle progression or arrest and, therefore, of cell growth and survival, iron can also be highly reactive and contribute to the formation of a ROS-mediated oxidative environment and cell death. Noticeably, in this context, LCN2 was shown to induce oxidative damage through intracellular iron overload (Kagoya et al., 2014). Specifically, hematopoietic cells when treated with LCN2 protein showed increased levels of ROS, due to high intracellular levels of iron, which in turn led to increased apoptosis and, consequently, decreased cellular proliferation (Kagoya et al., 2014). This phenotype was consistent with other reports showing that LCN2

treatment induces cardiomyocyte caspase-3-mediated apoptosis by increasing intracellular iron accumulation (Xu et al., 2012). Others have similarly shown that up-regulation of LCN2 in an ovarian clear cell carcinoma cell line increases intracellular iron concentrations (Yamada et al., 2016). However, in this case, LCN2 contrastingly attenuated iron-related intracellular ROS levels, DNA damage and apoptosis by increasing the intracellular concentration of the antioxidant regulator glutathione; this, ultimately, enhanced cell survival (Yamada et al., 2016). In this sense, LCN2 was even suggested to have antioxidant properties (Yamada et al., 2016), in line to what has been also described by others, and even to be a putative useful biomarker to identify oxidative stress (Roudkenar et al., 2007). For instance, LCN2 expression was shown to be induced upon cellular oxidative stress, specifically after H_2O_2 treatment (Roudkenar et al., 2007), and by metabolic stress (Zhang et al., 2014), to confer antioxidant and anti-apoptotic protection (Roudkenar et al., 2008; Roudkenar et al., 2009; Roudkenar et al., 2011) by inducing the expression of SOD-1 and -2 (Bahmani et al., 2010) and of glutathione (Yamada et al., 2016). On the other hand, down-regulation of LCN2 expression in lung adenocarcinoma cells increased ROS levels and promoted apoptotic cell death via nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 signalling, which, in turn, was effectively prevented by the treatment with the antioxidant *N*-acetylcysteine (NAC) (Song et al., 2015). To add, the regulation of oxidative environments by LCN2 has even been suggested to be relevant in disease context, including cancer (Yamada et al., 2016), cardiomyopathy (Chan et al., 2015) and sepsis (Srinivasan et al., 2012), among others.
Altogether, LCN2 functions pertain to its capacity to traffic iron within cells and the impact of such modulation, whether under physiological conditions or in pathology, are crucial for cellular equilibrium and homeostasis.

5.2 Lipocalin-2 in the brain

In the CNS, as in the periphery, LCN2 functions are debatable [reviewed in (Ferreira et al., 2015)], and the precise impact of LCN2 in the CNS is considered to be multifaceted but far from being completely unravelled. In fact, the contradiction already begins in the identification of the neural cell sources of LCN2 and in what context. While most of the reports describe LCN2 expression in response to brain injury and inflammation (Thouvenot et al., 2006; Marques et al., 2008; Ip et al., 2011), studies showing the physiological expression of LCN2 in the adult brain are scarcer. Nevertheless, in recent years, LCN2 expression was described, only in some reports, to occur in

specific brain regions of the physiological adult rat brain (Chia et al., 2011). Specifically, LCN2 (both mRNA and protein) was detected, at lower levels, in most of the brain regions of the normal adult brain, but with higher expression in the OB, brainstem and cerebellum (Chia et al., 2011). Further single and double immunostaining identified astrocytes as the major producers of LCN2 in the physiological brain (Chia et al., 2011). Nevertheless, LCN2 expression by neurons in the hippocampus (Mucha et al., 2011) and the amygdala (Skrzypiec et al., 2013) has also been reported. Still, astrocytes are considered the major producers of LCN2 in the brain, not only in physiology (Chia et al., 2011), but also in pathogenesis (Marques et al., 2012; Bi et al., 2013).

In a variety of brain injury models, the strong induction of LCN2 in reactive astrocytes, endothelial cells (Marques et al., 2008) and microglia (Jin et al., 2014), occurred for LCN2 to act as a pro-inflammatory signal to amplify neuroinflammation and, thereby, exert neurotoxic effects [reviewed in (Ferreira et al., 2015)]. However, others have considered LCN2 expression as a rescue signal to help preventing further damages and restore brain homeostasis [reviewed in (Suk, 2016)]. For instance, many have reported increased expression of LCN2 (mRNA and protein) in the context of several brain pathologies, including MS (Marques et al., 2012) and AD (Naude et al., 2012), to mediate neuronal toxicity with relevance for disease progression. For instance, and in the particular case of MS, the presence of LCN2 and its increased expression has been reported by many (Berard et al., 2012; Marques et al., 2012; Nam et al., 2014), but while some studies point to its neurodeleterious effects (Nam et al., 2014), others suggest that it may be neuroprotective (Berard et al., 2012). Moreover, the strong up-regulation of LCN2 in cortical brain regions of an AD mouse model suggests for its involvement in the establishment of the initial inflammatory response and in the sensitization of nerve cells to amyloid-β toxicity (Wu et al., 2006). In fact, LCN2 was shown to be required for amyloid-β toxicity to astrocytes, since the survival of wild-type astrocytes was decreased upon amyloid-β stimulation, while astrocytes from LCN2-null mice were not affected (Mesquita et al., 2014). Additional recent reports demonstrated that LCN2 expression is up-regulated in the lesioned dopaminergic system, being suggested as pathogenic factor in the course of Parkinson's disease (Kim et al., 2016).

Even though in some cases controversial, it is well recognized that LCN2 represents a key mediator in the course of some neurodegenerative diseases. The disclosure of the precise contribution of LCN2 to CNS pathogenesis, in fact, have been discussed to be crucial for our understanding in disease progression, and might even have implications for the development of neuroprotective therapeutic strategies. Nevertheless, and even before the knowledge on LCN2 contribution to

pathology, we believe it is critical to firstly recognize and understand the roles of LCN2 in brain physiology (Figure 11).

Interestingly, the secretion of LCN2 by astrocytes is considered to be one of the mechanisms through which astrocytes interact with neurons to regulate neuronal homeostasis. In fact, and at physiological conditions, neurons constitutively express the 24p3R specific receptor for LCN2 (Ip et al., 2011; Rathore et al., 2011), with a particular and relevant expression described to occur in the hippocampus (Chia et al., 2015). Specifically, 24p3R, in cultured hippocampal neurons, binds and internalizes LCN2, when both apo- and holo-forms are applied (Chia et al., 2015). Consistently with the mechanism of receptor-mediated endocytosis described for other cells (Yang et al., 2002; Devireddy et al., 2005), also in the brain, treatment of hippocampal neurons with holo-LCN2 increased *Bim* expression and decreased cell survival, suggesting that holo-LCN2 is pro-apoptotic in primary hippocampal neurons (Chia et al., 2015).

In addition, the iron-binding capability of LCN2 was shown to be a key element in the regulation of dendritic spine density and morphology in hippocampal neurons (Mucha et al., 2011). Specifically, dendritic spine retraction and morphology was shown to be regulated in an iron-dependent manner, since the *in vitro* treatment of hippocampal neurons with holo-LCN2 caused a decrease in spine density, further potentiated by the treatment with apo-LCN2 (Mucha et al., 2011). Moreover, spines shape was also affected by apo- and holo-LCN2, with the proportion of thin spines increased by holo-LCN2, and the percentage of mushroom decreased, further pronounced upon apo-LCN2 treatment (Mucha et al., 2011). The observed effects on spine plasticity by LCN2 were due to a decrease in actin's mobile fraction, which is a component of the spine cytoskeleton and that regulates its shape (Mucha et al., 2011). Of notice, mushroom spines, considered the mature spines in the CNS, are responsible for the maintenance of established neuronal networks and in long-term memory. Even though LCN2 is certainly not the only modulator of dendritic spine shape, the peculiar modulation of actin mobility by LCN2 and iron can have an impact on structural plasticity and, therefore, behaviour. In fact, *Lcn2* ablation in mice induces alterations in the emotional status of the animal, by specifically promoting anxiety and learned helplessness, and also mild spatial reference memory impairment (Ferreira et al., 2013). Moreover, and adding to these behavioural alterations, significant changes in the morphology and spine density of hippocampal neurons were also observed in LCN2-null mice (Ferreira et al., 2013).

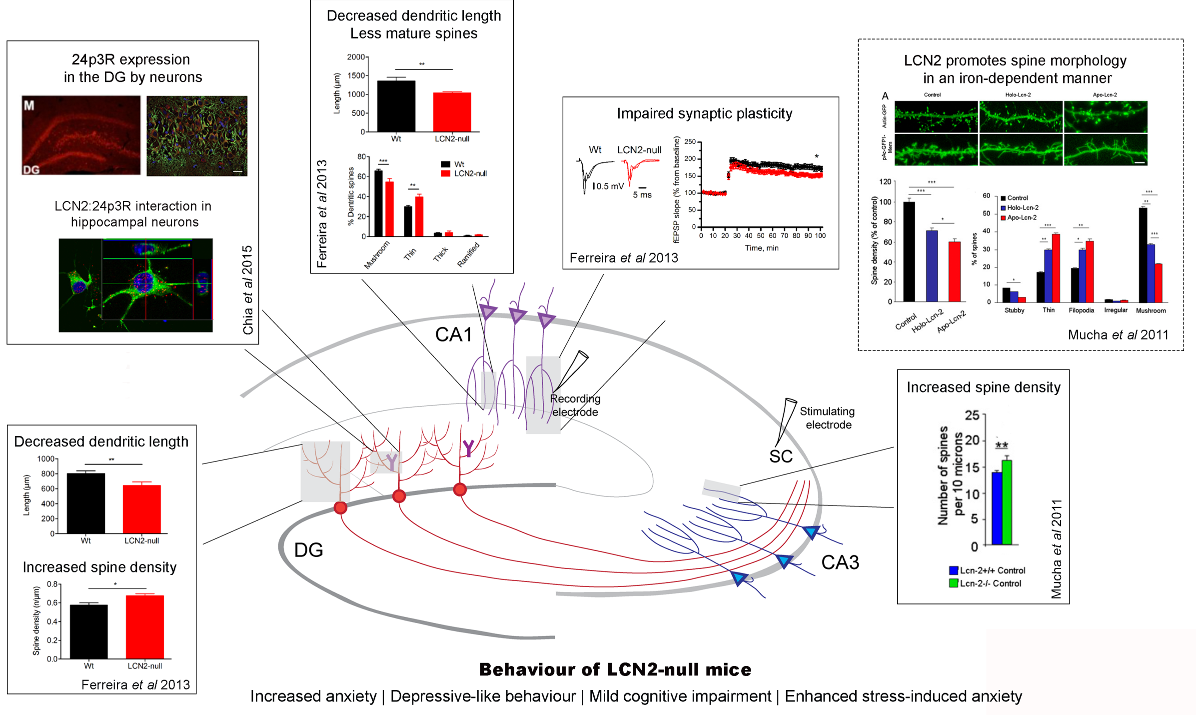


Figure 11. Summary view on the physiological roles of LCN2 in the adult hippocampus. Depicted are the main reports that have described the importance of LCN2 in brain physiology, particularly in neuronal morphology and plasticity, and synaptic function, with ultimate impact in animal behaviour. In such regulation, iron has an important role, through the specific interaction of LCN2 to its cellular receptor 24p3R.

The absence of LCN2 promoted neuronal hypertrophy at the ventral hippocampus, with a significant atrophy in neurons at the dorsal division, and a decrease in the proportion of mushroom type spines (Ferreira et al., 2013) (summary at Figure 11). This misregulation was accompanied by a sustained decrease in synaptic plasticity, as measured by LTP at the CA1 region of the dorsal hippocampus (Ferreira et al., 2013) (Figure 11). For sure, all these alterations at the cellular level in LCN2-null mice contribute to the described impaired behaviour (Ferreira et al., 2013). Nevertheless, how this can impact in, for instance, the normal course of aging and, therefore, on the regulation of neurodegenerative diseases, is still unclear.

The precise underlying mechanisms that may account for the effects seen in the absence of LCN2 are debatable. For sure, 24p3R is assumed as crucial for the mediation of LCN2 functions in the physiological brain. Further possible explanations on the contribution of LCN2 for hippocampal

plasticity and function have been commented based on the gliotransmission and the tripartite synapse (von Bohlen Und Halbach and Soreq, 2013). The deletion of LCN2 is suggested to affect the population of astrocytes, thus leading to changes in the activity-dependent synaptic plasticity of neurons (von Bohlen Und Halbach and Soreq, 2013). Therefore, LCN2 regulation connects between cell types, behavioural paradigms and even possibly developmental ones (von Bohlen Und Halbach and Soreq, 2013). Nevertheless, and considering the roles of LCN2 in brain physiology and in physiological iron trafficking, the putative modulation of iron cell content by LCN2 in the brain cannot be excluded. It is not known, whether similarly to what occurs in kidney development and haematopoiesis, if LCN2 modulates neural cells proliferation and apoptosis, by iron trafficking. This can be of relevance if we take into consideration the described roles of hippocampal cell genesis in the regulation of brain function, behaviour, and neuropsychiatry diseases, while to LCN2 are attributed roles in mood (Ferreira et al., 2013), depression (Naude et al., 2013) and synaptic plasticity (Mucha et al., 2011). In this sense, a thorough analysis on the contribution of LCN2 to cell genesis-mediated behaviour becomes necessary, not only to clarify LCN2 as a putative endogenous regulator of brain cell genesis, but also to identify the potential regulation of brain cell physiology and function based on iron trafficking. With this, it will be possible to establish novel critical mediators underlying physiological processes of cell proliferation and survival, and in response to different conditions.

6. Thesis aims

Despite the major breakthroughs in recent years, the precise regulation by LCN2 in the maintenance of CNS homeostasis is still far from being completely understood. Considering its promising diagnostic and prognostic value as described before, we believe that the precise disclosure of LCN2 roles in brain physiology is important. As so, and taking into account the reported modulatory roles of cell proliferation and apoptosis by LCN2 in other systems, based on its iron-traffic capabilities, in this thesis we aimed at addressing the role of LCN2 on cell proliferation and differentiation, focusing on the two well-described brain regions where cell genesis is described to occur, but given particular attention to the hippocampus. Moreover, we focused on disclosing the importance of adult neurogenesis modulation by LCN2 in response to environmental/external factors, including stress, exercise and aging.

In this context, and using the LCN2-null mouse model, the present thesis aim to:

- Dissect the role of LCN2 in the regulation of cell genesis in the adult brain, as well as the respective underlying mechanisms, and correlate it with the behavioural and neuroplasticity status of the brain (Chapter II).

- Clarify the relevance of LCN2-regulated hippocampal neurogenesis in the context of external modulation by exercise and stress, and its implications in mood and cognitive behaviours (Chapter III).

- Assess the contribution of LCN2 to age-related changes in behaviour and in hippocampal neurogenesis (Chapter IV).

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APPENDIX A

From the periphery to the brain: Lipocalin-2, a friend or foe?

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From the periphery to the brain: Lipocalin-2, a friend or foe?

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ABSTRACT

Lipocalin-2 (LCN2) is an acute-phase protein that, by binding to iron-loaded siderophores, acts as a potent bacteriostatic agent in the iron-depletion strategy of the immune system to control pathogens. The recent identification of a mammalian siderophore also suggests a physiological role for LCN2 in iron homeostasis, specifically in iron delivery to cells *via* a transferrin-independent mechanism. LCN2 participates, as well, in a variety of cellular processes, including cell proliferation, cell differentiation and apoptosis, and has been mostly found up-regulated in various tissues and under inflammatory states, being its expression regulated by several inducers.

In the central nervous system less is known about the processes involving LCN2, namely by which cells it is produced/secreted, and its impact on cell proliferation and death, or in neuronal plasticity and behaviour. Importantly, LCN2 recently emerged as a potential clinical biomarker in multiple sclerosis and in ageing-related cognitive decline. Still, there are conflicting views on the role of LCN2 in pathophysiological processes, with some studies pointing to its neurodeleterious effects, while others indicate neuroprotection. Herein, these various perspectives are reviewed and a comprehensive and cohesive view of the general function of LCN2, particularly in the brain, is provided.

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Abbreviations: LCN2, lipocalin-2; 24p3R, solute carrier family 22 member 17; NGAL, neutrophil gelatinase-associated lipocalin; BIM, pro-apoptotic Bcl-2-interacting mediator of cell death; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; AD, Alzheimer's disease; CP, choroid plexus; CSF, cerebrospinal fluid; LPS, lipopolysaccharide.

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1. An overview of lipocalin-2

The need and interest in the identification of molecules that can serve both a physiological role in the organism and allow for the detection of disease onset and progression has emerged over the last decades. Among the proteins identified as key players in health and disease, lipocalin-2 (LCN2) has became increasingly relevant in recent years as a biomarker in several diseases, including acute kidney injury (Kumpers et al., 2010), Alzheimer's disease (AD) (Choi et al., 2011), multiple sclerosis (MS) (Marques et al., 2012) and depression (Naude et al., 2013).

The existence of the knockout mouse for the coding sequence of LCN2 (LCN2-null mice) (Flo et al., 2004) has largely contributed to such findings allowing for the determination of many of the currently known functions of this 25-kDa secreted protein. LCN2-null mice are viable and reproduce normally, but display compromised functions at the postnatal level (Flo et al., 2004), albeit LCN2 expression begins already *in utero* (Mallbris et al., 2002). At postnatal stages, the diverse functions described for LCN2, in physiological and in pathological conditions in different mammalian organ systems, including the central nervous system (CNS) (Fig. 1), called the attention for its considerable interest within the scientific community. Among LCN2's functions, its antimicrobial activity is, undoubtedly, the best described. Specifically, LCN2 acts in the very first steps of antimicrobial defence, as part of the acute-phase response, to sequester bacterial siderophores (bacterial molecules with higher affinity for iron than the host iron-binding proteins) (Flo et al., 2004). Additionally, emerging evidence in the literature associates LCN2 to the modulation of cell physiological processes (proliferation, differentiation, apoptosis, cell activation and migration), providing a potential important link between physiology and pathology. The strongest evidence for its role in physiological conditions comes from the described involvement in kidney differentiation and in the regulation of epithelial morphogenesis (Yang et al., 2002). Concerning its role in disease, different conditions have been correlated with increased levels of LCN2. Particularly, studies indicate its altered expression in certain types of cancers (Rodvold et al., 2012), coronary diseases (Iqbal et al., 2013), metabolic syndrome (Jang et al., 2012; Yoo et al., 2014), insulin resistance and type 2 diabetes (Yan et al., 2007), chronic kidney disease (Hashikata et al., 2014), autoimmune diseases (Kamata et al., 2012) and in wound repair (Miao et al., 2014). In the brain, the involvement of LCN2 in autoimmune diseases such as MS (Berard et al., 2012; Marques et al., 2012; Nam et al., 2014) and other neurodegenerative disorders such as AD (Choi et al., 2011; Mesquita et al., 2014; Naude et al., 2012) is also suggested.

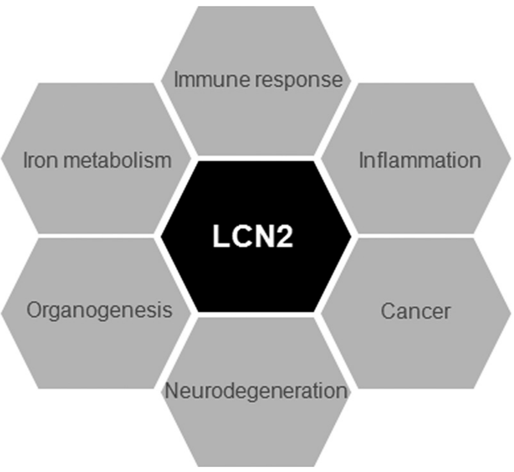

Fig. 1. Representative diagram for the broad and distinct dimensions where LCN2 has described functions. Lipocalin-2 is a secreted protein participating in several biological systems, by mediating processes that are crucial for cellular homeostasis.

Altogether, available data indicates a role for LCN2 in several organs in physiological conditions as well as in response to stress or injury. Furthermore, and of notice, LCN2 levels in biological fluids are generally low, being up-regulated and detectable in different stages of several diseases, which strongly indicates the potential of its use as a biomarker of disease onset and progression.

Although there is consensus on the relevance of LCN2 in the acute-phase response, the literature is still contradictory with respect to its role in the pathophysiology of disease. The present review aims to provide a state of the art on the roles of LCN2 in different biological contexts and to explore the current existing conflicting observations. We will first describe, in a general perspective, the well-known functions of LCN2 in the innate immune response associated to infection, iron trafficking and in the modulation of the inflammatory response and, later, focus in more detail on the possible functions of LCN2 in the CNS.

2. Lipocalin-2 as a member of the lipocalin protein family

Lipocalin-2 belongs to the lipocalin family, a family of diverse secreted proteins involved in health maintenance and effective in disease prevention (Flower, 1994, 1996). Lipocalins are small (160–180 amino acids in length), soluble and secreted proteins that act as carriers and transporters predominantly of lipophilic/hydrophobic molecules such as steroids, bilins, retinoids and lipids (Flower, 1994, 1996). For example, apolipoprotein D, α1-microglobulin and purpurin are lipocalins that bind to cholesterol, heme and retinol, respectively (Chakraborty et al., 2012; Flower, 1994). Consequently, several functions have been described for these members of the lipocalin family, including cell division regulation (*e.g.* α1-microglobulin), cell differentiation, cell-to-cell adhesion and survival (*e.g.* purpurin) (Flower, 1994). The known ability of lipocalins to form complexes with soluble macromolecules and to bind specific cell-surface receptors is considered to be the basis through which lipocalins modulate their functions (Flower, 1996). Of notice, and unlike most other protein families, members of the lipocalin family share low primary sequence identity, in some case as low as 20%, and membership to this family has been largely determined based on structural similarities (Flower et al., 2000). All members share a common secondary and tertiary structural feature – the so-called "lipocalin fold", which is a cup-shaped cavity where ligands bind (Flower et al., 2000). It is the difference in specific amino acids within this "lipocalin fold" that accounts for the diversity of ligands that lipocalins carry.

Similarly to other lipocalins, LCN2 acts as a carrier/transporter and binds to specific cell-surface receptors, therefore modulating several cellular functions. However, the LCN2 binding cavity is distinct from that of most of other lipocalins, since it is atypically polar and large enough to accommodate macromolecular ligands

(Goetz et al., 2002). Nevertheless, LCN2 can also weakly bind to some common ligands of lipocalins, including leukotriene B4 and platelet-activating factor (Bratt et al., 1999). Moreover, LCN2 is reported to have high affinity to bind and transport iron but only when iron is complexed with low molecular mass iron-binding ligands, the siderophores (Devireddy et al., 2005; Goetz et al., 2002; Yang et al., 2002). These ligands are small, high-affinity iron chelators, secreted by microorganisms for iron acquisition, but have been recently described to be endogenously produced by mammals (Bao et al., 2010; Devireddy et al., 2010). The capacity of LCN2 to bind iron through siderophores is responsible for its role in the immune response (Flo et al., 2004) and in the regulation of cell proliferation and cell death (Devireddy et al., 2005; Yang et al., 2002). The modulation of cell homeostasis is highly dependent on the interaction of LCN2 with specific cell-surface receptors, namely 24p3R (also known as solute carrier family 22 member 17) (Devireddy et al., 2005) and megalin (Hvidberg et al., 2005), through the regulation of iron cell content (Devireddy et al., 2005). Finally, LCN2 was just recently shown to bind to membrane phosphatidylethanolamine and to induce lipid raft movement in a protein kinase A-dependent manner, with impact in the modulation of sperm maturation (Watanabe et al., 2014). Still unexplored is whether this feature influences specifically sperm maturation or may also be related with other cellular processes such as migration and cell-to-cell interaction.

The specific interaction of LCN2 with iron and its surface cellular receptors, as well as its regulatory expression and clinical significance, will be described and discussed in detail in the following sections.

3. General considerations on lipocalin-2

Lipocalin-2 was first identified as the product of an over-expressed gene of murine kidney cells infected with simian virus (Hraba-Renevey et al., 1989). The human protein analogue, on the other hand, was found stored in human neutrophils in close association with matrix metalloproteinase-9 [(MMP-9), a gelatinase secreted by neutrophils for extracellular matrix degradation and remodelling] and showed a high degree of similarity with the deduced sequence of the rat α2-microglobulin related protein (Kjeldsen et al., 1993). At that time, no specific function was attributed to this protein and its covalent association to MMP-9 led to its initial designation as neutrophil gelatinase-associated lipocalin (NGAL) (Kjeldsen et al., 1994; Kjeldsen et al., 1993). Nowadays, while LCN2 is the formal designation (HUGO Gene Nomenclature Committee), it is also known as 24p3, NGAL, oncogene 24p3, human neutrophil lipocalin, α2-microglobulin related protein, siderocalin and uterocalin. This diversity of designations truly reflects the diverse mechanisms and pathways in which LCN2 has been implicated, and as such, of its potential. Lipocalin-2 is broadly expressed in several tissues and in diverse conditions, with a modulatory induction by specific factors.

3.1. Lipocalin-2 expression regulation

In the mouse, LCN2 expression begins *in utero* during the foetal stage at the level of the proximal tibia and metatarsal (Owen et al., 2008). In 10-days-old foetal mice, LCN2 is strongly expressed in proliferating and prehypertrophic chondrocytes, and remains as such in prehypertrophic and hypertrophic chondrocytes in 17-days-old foetal mice (Owen et al., 2008). In humans, the expression of LCN2 during embryonic development, specifically in the skin, has been described to occur in a spatio-temporal manner at 20–24 weeks of gestation in the interfollicular epidermis, with a posterior progression towards the hair follicles (Mallbris et al., 2002). In postnatal stages, evidence derived from mouse tissues indicates that LCN2 is strongly expressed in normal healthy tissues such as the bone marrow, liver, spleen, heart, lungs, kidney and thymus (Aigner et al., 2007). However, as age advances, LCN2's expression progressively declines, particularly in the liver, kidney and spleen (Garay-Rojas et al., 1996). Also, as a component of neutrophils cytoplasmic granules, LCN2 is synthesized during the early stages of neutrophil maturation (Kjeldsen et al., 1993).

Many factors and conditions have been described to trigger the induction of LCN2 in various tissues and cell types. Of interest, the expression of LCN2 is up-regulated in tissues that are more prone to infection such as the mucosal (Kjeldsen et al., 2000) and epithelial (Cowland and Borregaard, 1997; Friedl et al., 1999) barriers, where it behaves as an acute-phase protein. It is also secreted by neutrophils at sites of infection, by adipocytes (Kjeldsen et al., 1993), activated leukocytes and peritoneal cells (Flo et al., 2004), macrophages (Meheus et al., 1993), endothelial (Liu and Nilsen-Hamilton, 1995; Marques et al., 2008) and choroid plexus (CP) epithelial cells (Marques et al., 2008). The regulatory and core elements described to be present in the *Lcn2* gene explain, at least partially, how its expression can be regulated by specific molecules (summarized in Table 1). In the mouse, the LCN2 gene has several *cis* regulatory elements in its 5′-upstream region, including a TATA box-like element (between nucleotides 28 to 23 relative to the transcription start site) and *cis* binding elements for several transcription factors (Garay-Rojas et al., 1996). Inducers of LCN2 expression include cytokines, growth factors, retinoic acid, glucocorticoids, phorbol esters and lipopolysaccharide (LPS). Among these, LPS has been shown to be the major inducer of LCN2 expression (summarized in Table 1). Specifically, LCN2 is highly expressed in cultured rat peritoneal macrophages (Sunil et al., 2007) and macrophage cell lines (Meheus et al., 1993) after *in vitro* LPS stimulation. In *in vivo* experiments, intraperitoneal administration of LPS has been shown to induce LCN2 expression in the CP (Marques et al., 2008), liver and lungs (Sunil et al., 2007). Importantly, the induction of LCN2 expression mediated by LPS stimulation occurs through the activation of the Toll-like receptor (TLR)-4 (Flo et al., 2004). Also *in vivo*, LCN2 has been shown to act as an acute-phase protein in the liver as a response to the injection of turpentine (Liu and Nilsen-Hamilton, 1995).

As indicated above, LCN2 expression can also be induced by several cytokines and growth factors, which include interleukin (IL)-1β (Cowland et al., 2003), IL-6 (Hamzic et al., 2013), IL-10 (Vazquez et al., 2014), IL-17 (Chiricozzi et al., 2014), IL-22 (Le et al., 2014; Raffatellu et al., 2009), tumour necrosis factor (TNF) (Bu et al., 2006; Landro et al., 2008), insulin-like growth factor-1 (IGF-1) and transforming growth factor-α (TGF-α) (Sorensen et al., 2003). The induction of LCN2 expression by such inducers is known to be dependent on the activation of NF-κB transcriptional activity, with NF-κB being suggested as a positive regulator of LCN2 expression itself (Iannetti et al., 2008). In fact, the *Lcn2* gene promoter has an NF-κB response element and four signal transducer and activator of transcription-1 sites (Zhao and Stephens, 2013). Studies using IL-17 and IL-1β indicate that the induction of LCN2 may also require multiple other transcription factors including AP-1 and a CCAAT/enhancer-binding protein site (Cowland et al., 2003; Shen et al., 2006). Similarly, *in vitro* stimulation of mouse liver cells (Liu and Nilsen-Hamilton, 1995), L-cells (Garay-Rojas et al., 1996) and primary thymocytes (Devireddy et al., 2001) with dexamethasone induces an increase in LCN2 mRNA levels. This specific expression regulation by dexamethasone is mainly explained by the existence of two glucocorticoid responsive core elements in the *Lcn2* gene promoter (Garay-Rojas et al., 1996).

Of interest, the expression of LCN2 is also regulated by hormones, including progesterone [suggested to be a negative regulator of LCN2 expression, at least in the uterus (Huang et al.,

Table 1
Summary of the molecules described to modulate the expression of LCN2 and the pathways through which such modulation is known to occur.

Inductor of LCN2 expression		LCN2 expression	Cells/Tissue	Pathways demonstrated to be involved	References
LPS		↑	Macrophages	–	Meheus et al. (1993)
		↑	Immortalized epithelial cells	–	Bu et al. (2006)
		↑	Macrophages	TLR4; NF-κB	Sunil et al. (2007)
Dexamethasone		↑	L cells	GRE	Garay-Rojas et al. (1996)
		↑	Liver cells		Liu and Nilsen-Hamilton (1995)
		↑	Primary thymocytes		Devireddy et al. (2001)
Oestrogen	Estradiol	↓	Breast cancer cell lines	ERE	Seth et al. (2002)
	17-β estradiol	↓	Aortic segments		Gao et al. (2006)
		↑	Mammary glands		Seth et al. (2002)
Cytokines	IL-6	↑	Brain vascular cells	–	Hamzic et al. (2013)
	IL-1β	↑	Immortalized epithelial cells; embryonic fibroblasts	NF-κB; C/EBPδ(CCAAT/enhancer-binding protein)-binding site	Bu et al. (2006); Cowland et al. (2003); Shen et al. (2006)
	IL-22	↑	Primary urothelial cells; intestinal epithelial cells		Le et al. (2014); Raffatellu et al. (2009)
	IL-17	↑	Epidermis; intestinal epithelial cells; embryonic fibroblasts		Chiricozzi et al. (2014); Raffatellu et al. (2009); Shen et al. (2006)
	IL-10	↑	Primary and J774A.1 macrophages	–	Vazquez et al. (2014)
	TNF	↑	Whole blood culture; immortalized normal epithelial cells	NF-κB	Bu et al. (2006); Landro et al. (2008)
Retinoic acid		↑	L cells	–	Garay-Rojas et al. (1996)
Insulin		↑	Adipose tissue explants	MEK/PI-3K pathways	Tan et al. (2009)
Growth factors	IGF-1, TGF- α	↑	Primary keratinocytes	–	Sorensen et al. (2003)

GRE, glucocorticoid response element; ERE, oestrogen response element.

1999)], and particularly oestrogen, for which *Lcn2* contains a response element in the promoter region (Gao et al., 2006; Seth et al., 2002). In *in vitro* studies, while the stimulation of aortic segments from ovariectomized mice with 17-β estradiol induces the down-regulation of *Lcn2* mRNA levels (Gao et al., 2006), the treatment with estradiol of breast cancer cell lines selected for positive oestrogen receptor leads to sustainable increased expression of LCN2 mRNA (Seth et al., 2002). Also, among other described modulations [reviewed in (Chakraborty et al., 2012)], LCN2 levels were found decreased in mammary glands of ovariectomized mice, which were restored by supplementation with oestrogen (Seth et al., 2002). Finally, other inductors described to promote increased expression of LCN2 include retinoic acid (vitamin A) (Garay-Rojas et al., 1996), which can be of importance for therapeutic proposes, and even insulin, as *in vitro* studies with adipose tissue explants stimulated with insulin induced the expression of LCN2 (Tan et al., 2009). This ultimate modulation has been associated to the described role of LCN2 in insulin resistance.

The broad expression of LCN2 and its regulation by several factors intimately relates to its functions. For instance, the acute increased concentration of LCN2 in the serum following intraperitoneal injection of LPS is related to its function as an acute-phase protein in the innate immune response to bacterial infection by sequestrating iron (Flo et al., 2004). Moreover, the inducible expression of LCN2 by pro-inflammatory molecules suggests that LCN2 plays an important role in inflammation and inflammatory-related diseases. These and other functions will be next further discussed (summarized on Table 2).

4. Lipocalin-2 functions

4.1. In the innate immune response

The metabolism and division of many pathogenic microorganisms depend on iron availability (Schaible and Kaufmann, 2004). In mammals, iron is predominantly intracellular, tightly bound to proteins such as ferritin; in the circulation, iron is mostly bound to transferrin (Gkouvatsos et al., 2012). To acquire iron, invading pathogens have evolved strategies to survive within such severe iron-poor environments by secreting small iron-binding siderophores (Miethke and Marahiel, 2007). These molecules remove iron from the host's protein-iron complexes, as they have a much higher affinity for iron than the host iron carriers (Miethke and Marahiel, 2007). On the other hand, in order to prevent bacterial iron acquisition, mammals have developed defence mechanisms to "withhold" iron (Cassat and Skaar, 2013; Ganz, 2009). One of such strategies, and as part of the innate immune response, is the production of LCN2 that functions as a chelator of bacterial siderophores (Flo et al., 2004; Goetz et al., 2002). In response to acute bacterial infection, LCN2 is released from the liver and spleen to sequester iron-loaded bacterial siderophores and exert a bacteriostatic effect (Berger et al., 2006). The importance of such response became evident from the lethality that bacterial infection causes in LCN2-null mice (Flo et al., 2004). When exposed to a sublethal dose of a clinical strain of *Escherichia coli*, H9049, LCN2-null animals develop bacteraemia, sepsis and showed increased lethality, with 80% of the LCN2-null mice dying within the first 42 h post-infection (Berger et al., 2006; Flo et al., 2004). When present, LCN2 expression is markedly induced during innate immunity through the activation of TLR4 (Flo et al., 2004) and/or through several pro-inflammatory cytokines (Saiga et al., 2008). Being secreted by neutrophils at sites of infection (Kjeldsen et al., 1994), LCN2 is essential in opposing the scavenge of host iron by bacterial siderophores.

Interestingly, different pathogenic bacteria secrete different types of siderophores (Henderson et al., 2009), but the capacity of LCN2 to bind bacterial siderophores is limited to the catecholate-type (Holmes et al., 2005). Among those are siderophores secreted by *E. coli*, *Salmonella* spp. and *Klebsiella pneumonia*. In infections triggered by these agents, LCN2 has been shown to be protective for the host (Chan et al., 2009; Nairz et al., 2009; Wu et al., 2010). For instance, LCN2 is up-regulated in macrophages in response to *Salmonella* spp. to confer resistance to the host (Nairz et al., 2009) and LCN2-null animals have impaired lung bacterial clearance upon infection with *K. pneumonia* (Chan et al., 2009).

Although LCN2 is described to only bind a certain type of siderophores to modulate its bacteriostatic effects, this does not preclude a major role for LCN2 in bacterial infectious that do not produce catecholate-type siderophores. In fact, a role for LCN2 in

Table 2
Concise description of the functions attributed to LCN2.

LCN2 functions			
Innate immune response (Flo et al., 2004)			
Role	Strain	Outcome	References
Chelator of bacterial siderophores	*E. coli*	LCN2-null animals develop bacteraemia, sepsis and showed increased lethality (80% of the null mice die within the first 42 h)	Berger et al. (2006); Flo et al. (2004)
	Salmonella spp.	LCN2 up-regulated in macrophages to confer resistance to the host	Nairz et al. (2009)
	K. pneumonia	LCN2-null animals present impaired lung bacterial clearance	Chan et al. (2009)
Bacterial control by a siderophore -independent mechanism	*S. pneumoniae*	LCN2 limits proliferation of strains that require enterobactin-like siderophores	Nelson et al. (2005)
	H. influenzae		
	C. albicans	LCN2 is induced in an IL-17-dependent manner, but not essential for host defence	Ferreira et al. (2014)
Antiplasmodial regulator	*Plasmodium*	LCN2 secretion for the control of iron redistribution during blood-stage malaria	Zhao et al. (2012)
Iron homeostasis (Devireddy et al., 2005; Yang et al., 2002)			
	Model	Outcome	
Inductor of cell proliferation/ cell differentiation	Renal tubular cells	*Lcn2* silencing inhibits proliferation	Viau et al. (2010)
	Metanephric mesenchymes	Cellular uptake of the LCN2-iron complex induces epithelial cell differentiation	Yang et al. (2002)
	Mouse spermatozoa	Holo-LCN2 is internalized for ferric ion exchange	Elangovan et al. (2004)
Inductor of cell death	Haematopoietic/erythroid cells	LCN2 promotes apoptosis[a] and inhibits cell survival and differentiation	Liu et al. (2011); Miharada et al. (2005)
	Neonatal myocytes	LCN2 regulates cardiomyocyte apoptosis by intracellular iron accumulation[b]	Xu et al. (2012)
Others iron metabolism-related	Anaemia/hypoxia	LCN2 expression is increased to promote iron utilization/ mobilization from stores	Jiang et al. (2008)
	Thalassemia intermedia	Increased expression of LCN2 for survival facilitation of the less damaged thalassemic erythroid precursors	Patsaoura et al. (2014)
	Mouse uterus	Oestrogen-induced LCN2 expression for iron utilization during uterine cell growth	Stuckey et al. (2006)
	Breast, liver and pancreatic cancers	LCN2 expression for cellular iron trafficking	Li and Chan (2011); Torti and Torti (2013)
Modulation of the inflammatory response			
	Model	Outcome	
Anti-inflammatory modulator	Obesity-related inflammation	Antagonist of the effects of inflammatory molecules (TNF and IL-6)	Zhang et al. (2008)
		Anti-inflammatory regulator in macrophage activation	Guo et al. (2014)
	Nephrotoxic nephritis	Expression in innate immune cells confers protection by inducing apoptosis	Eller et al. (2013)
Pro-inflammatory modulator[c]	Psoriasis	Sustained increased levels of LCN2 during pathogenesis, decreased once the lesions are healed	Ataseven et al. (2014)
	Autoimmune myocarditis	LCN2 levels increment according with disease progression, decreasing upon recovery	Ding et al. (2010)
	HIV-infected patients	Decreased serum LCN2 levels, increased upon anti-retroviral therapy due to decreased neutrophil function	Landro et al. (2008)
	Polymorphonuclear neutrophils	Paracrine chemoattractant indispensable for neutrophil function during inflammation	Schroll et al. (2012)
	Heart transplantation	LCN2 required for the initiation of the inflammatory response	Aigner et al. (2007)
	Mycobacterial pulmonary infection	Promoter of neutrophil recruitment and regulator of chemokine production	Guglani et al. (2012)
	Lupus/arthritis	Increased LCN2 to boost immune cells recruitment for the inflammatory process initiation	Shashidharamurthy et al. (2013)
	Osteoblasts	IL-17-induction of LCN2 to amplify inflammation	Shen et al. (2005)
Metabolism regulator[d]	Obesity	Increased LCN2 expression correlates with expression profile of pro-inflammatory cytokines	Auguet et al. (2011)
		LCN2 increased expression in relation to body mass index and increased activities of MMP-2/9	Catalan et al. (2009); Yoo et al. (2014)

[a] Not directly connected to iron delivery, but shown to be important during acute anaemia.
[b] As a consequence of the generation of reactive oxygen species.
[c] In some cases intimately connected to its bacteriostatic role.
[d] By the control of inflammatory responses.

determining the establishment or maintenance of mucosal colonization by *Streptococcus pneumoniae* and *Haemophilus influenzae* has been described (Nelson et al., 2005). An outstanding feature of these bacteria that contrasts with enteric organisms is their independence from siderophore-mediated iron acquisition (Nelson et al., 2005). As so, the increased expression of LCN2 upon such colonization is suggested to occur to limit the proliferation of competitor species that can colonize the host and that require enterobactin-like siderophores (Nelson et al., 2005). Similarly, LCN2 mediates immunity to fungi such as *Candida albicans*, as it is

strongly induced in an IL-17-dependent manner, but surprisingly does not seems to be required for the host overall defence (Ferreira et al., 2014).

The recent discovery of the existence of a mammalian endogenous siderophore, to which LCN2 binds (Bao et al., 2010; Devireddy et al., 2010), was a major breakthrough, providing novel cues on possible functions for LCN2, particularly as an iron-trafficking protein in physiological conditions. The described mammalian siderophore is a small metabolic product called catechol (Bao et al., 2010) and, while alone it binds poorly to LCN2, the addition of ferric iron significantly improves its affinity to LCN2 (Bao et al., 2010). In addition, the iron-binding moiety of catechol was described to be 2,5-dihydroxybenzoic acid, which is similar to the bacterial component (2,3-dihydroxybenzoic acid) and even the enzyme responsible for its synthesis, the 3-OH butyrate dehydrogenase-2, is homologue of the bacterial EntA (Devireddy et al., 2010). This finding revealed a feature of intracellular iron homeostasis that is conserved from bacteria to humans, in which LCN2 is involved. Of notice, supplementation with the mammalian siderophore was shown to enhance bacterial growth *in vitro*, and mice lacking the mammalian siderophore resist to *E. coli* infection (Liu et al., 2014), bringing into light the concept that bacteria are also able to use the mammalian siderophore to scavenge iron from the host (Liu et al., 2014). Nevertheless, and to counteract this use by bacteria, the mammalian host is capable of suppressing 3-OH butyrate dehydrogenase-2 expression (decreasing the production of the mammalian siderophore), while up-regulating LCN2 expression *via* TLR4 signalling (Liu et al., 2014). This reciprocal regulation of both LCN2 and the mammalian siderophore is considered to be a protective mechanism to limit microbial access to iron.

In addition to antibacterial and anti-fungal roles, LCN2 was also shown to act as an antiplasmodial defence molecule (Zhao et al., 2012). Described to be up-regulated during malaria infection in mice (Coban et al., 2007), LCN2 was reported to have a pivotal role in controlling parasite levels as well as host innate and adaptive responses during *Plasmodium* blood-stage malaria infections (Zhao et al., 2012). Importantly, such modulation seems to involve the host iron status, with LCN2 controlling iron redistribution during the infection (Zhao et al., 2012).

Moreover, the existence of a NF-κB-dependent expression of LCN2 (Bu et al., 2006; Matsuo et al., 2007), in line with its involvement in innate and adaptive immune systems, points to an immunomodulatory role of LCN2. In accordance, La Manna and colleagues (2014) observed that the *in vitro* stimulation of peripheral blood mononuclear cells with LCN2 (both iron-loaded and iron-free forms) potentiated the expression of the human leucocyte antigen G, known to be a tolerogenic molecule (La Manna et al., 2014). The treatment also affected the stimulation of regulatory T-cells, suggesting the putative involvement of LCN2 in the regulation of specific immune pathways (La Manna et al., 2014).

Altogether, new and varied evidence have emerged that point to other roles for LCN2 than those directly related with the acute-phase response and control of bacterial growth. For instance, the evidence that LCN2-null mice also present multiple apoptotic defects in haematopoiesis (Liu et al., 2011), impairments in neuronal excitability (Mucha et al., 2011) and in emotional behaviours (Ferreira et al., 2013) opens perspectives for additional roles of LCN2.

4.2. In iron-homeostasis: impact on cell proliferation, differentiation and death

The acquisition of iron by cells is critical for cell survival, proliferation and differentiation (Andrews, 2008). Almost all mammalian cells are described to acquire iron by receptor-mediated endocytosis of iron-loaded transferrin (Garrick and Garrick, 2009). However, the capacity of LCN2 to interact with iron, through a catechol complex, in physiological conditions (Bao et al., 2010; Yang et al., 2002), has paved the way to propose the existence of an LCN2-mediated iron-delivery pathway, with potential impact on cell homeostasis. Interestingly, some observations reinforce LCN2 as an alternative to transferrin in iron trafficking. For instance, hypotransferrinemic mice (Trenor et al., 2000) and atransferrinemic humans (Hamill et al., 1991; Hayashi et al., 1993) have severe defects in haematopoiesis and in CNS development, but display normal development of most epithelial organs, and even mice lacking the transferrin receptor-1 initiate organogenesis (Levy et al., 1999). In addition, transferrin expression only begins at embryonic day 12, when organogenesis is already advanced (Gustine and Zimmerman, 1973). This suggests that other proteins, such as LCN2, may provide iron to the cells.

Moreover, the participation of LCN2 in iron-delivery related processes were strengthen upon its identification as an inducer of rat metanephric mesenchyma conversion into epithelia (Yang et al., 2002). Specifically, LCN2 containg iron was identified in the developing mammalian kidney and shown to deliver iron into cells, thus inducing epithelial cell differentiation (Yang et al., 2002). Of interest, LCN2-mediated iron delivery occurred preferentially in the epithelial progenitors at an early stage, while transferrin-mediated iron delivery occurs in late epithelial progenitors (Yang et al., 2002). In fact, down-regulation of *Lcn2* in mouse renal tubular cells lead to a significant decrease in epidermal growth factor-induced cell proliferation *in vitro*, and the silencing of LCN2 gene in mice inhibited tubular proliferation (Viau et al., 2010).

The role of LCN2 in iron delivery to cells, *via* the cellular uptake of the LCN2-iron complex (Yang et al., 2002), requires the existence of cellular receptors that mediate LCN2 uptake with affinity. Hvidberg and colleagues (2005) were the first to demonstrate megalin as one of the cellular receptors for LCN2, since it mediated the endocytosis of LCN2 in polarized epithelia (Hvidberg et al., 2005). In the same year, the isolation and identification of 24p3R revealed the existence of a fine-tuning mediation of iron content in the cell by LCN2 (Devireddy et al., 2005). Such mediation, irrespectively from the cell type, is largely dependent upon the state of the ligand: iron-containing (holo-) and iron-free (apo-) LCN2 (Devireddy et al., 2005). Specifically, holo-LCN2 binds to 24p3R, is internalized, traffics to endosomes and releases iron from the complex, thereby increasing intracellular iron concentration (Devireddy et al., 2005). By contrast, when apo-LCN2 is internalized upon binding to 24p3R, it chelates iron and transfers it to the extracellular medium, reducing intracellular iron concentration (Devireddy et al., 2005). Importantly, this LCN2-mediated modulation of cell iron content has been shown to impact on cell proliferation and apoptosis, respectively, the later occurring through the pro-apoptotic Bcl-2-interacting mediator of cell death (BIM) (Devireddy et al., 2005; Richardson, 2005).

With the exception of a report questioning the role of LCN2 in the modulation of iron metabolism (Correnti et al., 2012), which suggested that LCN2 does not induce cellular iron efflux, many others studies support the participation of LCN2 in cellular iron homeostasis. For instance, the delivery of iron to spermatozoa, at the caudal epididymis, was demonstrated to occur through the internalization of holo-LCN2, disclosing a physiological role for LCN2 in spermatozoa in the context of protein-ligand complex internalization (Elangovan et al., 2004). Also, LCN2 has been shown to induce apoptosis of haematopoietic cells (Devireddy et al., 2001; Lin et al., 2005; Liu et al., 2011; Miharada et al., 2005) and to inhibit the survival and differentiation of erythroid cells *in vitro* (Miharada et al., 2005). Although a direct relation to iron levels was not described, this regulation was demonstrated to be important

during acute anaemia (Miharada et al., 2005). In addition, the observation that LCN2-null mice develop a progressive accumulation of lymphoid, myeloid and erythroid cells strengths the notion that LCN2 is an important regulator of the haematopoietic compartment (Liu et al., 2011).

Similarly, recombinant LCN2 induces cardiomyocyte apoptosis (Xu et al., 2012), an important mechanism of cardiac remodelling leading to heart failure, by promoting intracellular iron accumulation (Xu et al., 2012). Interestingly, this induction of apoptosis triggered by increased intracellular iron content by LCN2 is the opposite of what has been described to occur in other organs (Richardson, 2005). However, in this case, this is considered to occur as a consequence of the generation of reactive oxygen species. In fact, the chemical chelation of free intracellular iron attenuated LCN2-induced caspase-3 activity (Xu et al., 2012). On the other hand, the sustained expression of LCN2 in situations of increased iron utilization, as is the case of anaemia and hypoxia, is suggested to occur as a physiological response to cope with the reduction of iron and to promote iron utilization and mobilization from stores (Jiang et al., 2008). Also, the sustained expression of LCN2, in both mouse models and patients with thalassemia intermedia (an inherited blood disorder), is considered to represent a survival response mechanism in the sense that it can facilitate the maintenance of the few but still functional erythroid precursors, or as a consequence of the abnormal iron regulation in the disease. However, no correlation has been found between LCN2 levels and either the parameters of erythropoiesis (haemoglobin, foetal haemoglobin, reticulocytes and soluble transferrin receptor), or of iron storage (ferritin and non-transferrin-bound iron) (Patsaoura et al., 2014). Likewise, the temporally coordinated induction of LCN2 expression, among other iron homeostasis genes, was observed to occur by oestrogen in the mouse uterus, evidencing an important role of iron metabolism during sex steroid hormone-induced uterine cell growth and differentiation (Stuckey et al., 2006).

The additional reported expression of LCN2 in a number of cancers (Li and Chan, 2011), including breast, liver and pancreatic cancer, has been considered to occur as a source of iron for cells to proliferate (Torti and Torti, 2013). With descriptions ranging from a pro- to an anti-cancer role, LCN2 is engaged in key events known to contribute to malignancy (apoptosis, proliferation and invasion). For instance, transfection of MCF7 human breast cancer cells with human LCN2 expression constructs increases cell proliferation (Fernandez et al., 2005) and potentiates angiogenesis (Yang et al., 2013). In accordance, inhibition of LCN2 reduces breast tumorigenesis (Sun et al., 2011), whereas in the case of the colorectal cancer, LCN2 expression has been either correlated with a decreased overall survival of patients (Sun et al., 2011) but also with reduced metastasis (Lee et al., 2006). Nevertheless, it is important to highlight that in the majority of the above-described reports, it is not clear whether the observed effects for LCN2 are directly related with alterations in the intracellular iron levels.

4.3. As a modulator of the inflammatory response

Several studies highlight the increased production of LCN2 upon diverse pro-inflammatory stimuli. In fact, the acute up-regulation of LCN2 suggests its involvement in inflammatory diseases, either by contributing to the progression of the inflammatory state or, on the contrary, to the development of an anti-inflammatory state.

In addition, in some cases, the increased expression of LCN2 closely accompanies the disease process, which lead to the suggestion of its potential usefulness to monitor disease progression (further discussed in section 6). For instance, the described increased levels of LCN2 in the skin of patients with psoriasis, where chronic inflammation plays a role in disease pathogenesis, is followed by a decrease in LCN2 levels, once the lesions are healed (Ataseven et al., 2014). In addition, serum LCN2 levels are increased in animal models of autoimmune myocarditis at an early stage of the disease, and remain high during the inflammatory stage, but decreased upon recovery (Ding et al., 2010). In the case of lymphopenic HIV-infected patients, serum LCN2 levels are low and increase upon anti-retroviral therapy (Landro et al., 2008). In the case of HIV, the low levels of serum LCN2 are considered to reflect a decrease in neutrophil number and function, since a positive correlation between neutrophil counts and LCN2 levels was observed (Landro et al., 2008).

Consistent with its role in anti-inflammatory responses, LCN2 has been shown to antagonize the effects of TNF on adipocytes and macrophages, and to attenuate the stimulatory effects of LPS on the gene expression of IL-6 and monocyte chemoattractant protein-1 in adipocytes (Zhang et al., 2008). Moreover, LCN2-null mice were described to display an up-regulated expression of the M1 macrophage marker (Cd11c) and a down-regulation of the M2 marker (arginase 1) in adipose tissue and liver after a high-fat diet feeding (Guo et al., 2014). Also, LCN2-null bone marrow-derived macrophages were more sensitive to LPS stimulation and presented an up-regulation of pro-inflammatory markers expression, which was attenuated upon treatment with recombinant LCN2 (Guo et al., 2014). Altogether, this suggests that LCN2 plays a role as an anti-inflammatory regulator in macrophage activation. In addition, in murine nephrotoxic serum nephritis, an inflammatory kidney disease characterized by increased infiltration of leukocytes, LCN2 is considered to be an endogenous inhibitor of inflammation, since its expression in innate immune cells is protective by inducing apoptosis of macrophages and by limiting cytokine production *via* TLR2 signalling (Eller et al., 2013).

On the contrary, the role of LCN2 as a pro-inflammatory modulator has also been described and is mainly related to its already described role as a bacteriostatic protein. The inflammatory status that underlies a defence response against pathogens is considered protective and necessary to remove, control and/or neutralize injurious microorganisms. Indeed, the acute inflammation triggered initially represents the first line of defence in response to invading pathogens or to tissue damage. Under this circumstance, and at low bacterial density, it is well established that LCN2 exerts its iron-depletion strategies for bacterial proliferation. As bacterial density increases, LCN2 becomes a pro-inflammatory molecule, serving as a signal of uncontrolled bacterial replication to induce chemokines' expression that, in turn, potentiates the influx of neutrophils into the site of infection (Bachman et al., 2009). As such, LCN2 was considered a paracrine chemoattractant indispensable for neutrophil function in inflammation (Schroll et al., 2012). The addition of recombinant LCN2 to primary cultures of human and murine polymorphonuclear neutrophils significantly stimulated neutrophil migration, while a significantly reduced neutrophil chemotactic activity and impaired cellular adhesion is observed in cells obtained from LCN2-null mice (Schroll et al., 2012). In the same line of evidence, the number of infiltrating granulocytes, after reperfusion, in murine heart transplantation, was described to be reduced in LCN2-null grafts, suggesting a role for LCN2 in the initiation of the inflammatory response (Aigner et al., 2007). Similarly, during mycobacterial pulmonary infection, LCN2 was shown to restrain inflammation by inhibiting inflammatory chemokines production but at the same time to promote neutrophil recruitment (Guglani et al., 2012). In addition, the increased levels of LCN2 in autoimmune disorders, such as the case of lupus and arthritis, boost the recruitment of immune cells to the site of inflammation, a process essential for the initiation, perpetuation, and resolution of the inflammatory processes (Shashidharamurthy et al., 2013).

Additionally, it is hypothesized that LCN2 is released by granulocytes at sites of inflammation and, therefore, mediates local tissue injury (Li and Chan, 2011). This specific contribution derives from the description of LCN2 secretion in tissue damage also following ischaemia, namely to recruit and chemoattract other immune cells that participate in the inflammatory response (Aigner et al., 2007). Also the description that LCN2 produced at the sites of inflammation can act as an inducer of apoptosis may also be contributing for local tissue injury. In cultured osteoblasts, LCN2 induction by IL-17 was observed to promote cell apoptosis and to modulate subsequent inflammatory events (Shen et al., 2005). Both are considered to work synergistically to amplify inflammation.

In parallel, more modulatory effects have been described for LCN2 under inflammatory conditions, some through mechanisms that are still to be disclosed. Among these, exercise-induced inflammation modulates bone marrow homeostasis leading to an increase in leucocyte turnover and a decrease in erythroid compartment (Spiropoulos et al., 2010). Of interest, it seems that LCN2 is the main factor that regulates the production and mobilization of erythroid progenitor cells (Spiropoulos et al., 2010). In a different context, the sustained expression of LCN2 upon liver damage is considered to be a reliable indicator of damage and has significant hepatoprotective effects (Borkham-Kamphorst et al., 2013). Despite the absence of correlation between human LCN2 serum levels and the degree of liver fibrosis, a positive correlation with the inflammatory status was observed (Borkham-Kamphorst et al., 2013). The proteomic profiling of LCN2-null mice under normal conditions and after exposure to inflammatory stimuli (LPS) reveals several altered proteins that are described to have functions in detoxification, metabolism of nutrients and in cell adhesion, thus suggesting an essential function for LCN2 in liver homeostasis and in the onset of inflammatory responses (Labbus et al., 2013).

Remarkably, LCN2 is also involved in the regulation of metabolism, through the control of the inflammatory response. The increased production of LCN2 in the adipose tissue and in the liver as a response to metabolic stress and to cytokines, points for the involvement of LCN2 in adipocyte metabolism and inflammation (Zhang et al., 2014). In fact, a function for LCN2 in insulin resistance and glucose metabolism has already been described. Suggested by some authors as a potential adipokine (Yoo et al., 2014; Zhang et al., 2008), LCN2 was found increased in serum of obese patients and in adipose tissue and liver of obese mice (Zhang et al., 2008) as well as in a metabolic syndrome rat model (Yoo et al., 2014). Importantly, such levels are strongly correlated with the expression profile of pro-inflammatory cytokines (Auguet et al., 2011) and of other circulating markers of inflammation (Yoo et al., 2014). Also, the increased adipose tissue expression of LCN2 in obesity was correlated to body mass index (Yoo et al., 2014) and to increased activities of both MMP-2 and MMP-9, reinforcing its potential participation in the low-grade chronic inflammation associated with obesity (Catalan et al., 2009). Moreover, LCN2 was also shown to impact in the energy metabolism, even in the absence of increased inflammation, since exogenous recombinant LCN2 is able to increase glucose production in hepatocytes (Yan et al., 2007). The precise function and relevance of LCN2 in obesity and in energy metabolism is, nonetheless, quite controversial since studies with LCN2-null mice were inconclusive. In one study, LCN2-null mice were shown to be protected from diet-induced obesity, insulin resistance and inflammation by modulating 12-lipoxygenase and TNF levels in adipose tissue (Law et al., 2010). In another one, LCN2 absence significantly potentiated diet-induced obesity, dyslipidemia, fatty liver disease, and insulin resistance (Guo et al., 2010). Also, LCN2 expression is differentially regulated in diabetes, since its levels were found to be increased in human subjects in response to insulin infusion (Tan et al., 2009); still, whether this increase reflects a protective response or contributes to disease progression is not yet known.

In summary, there is still some inconsistency regarding the effects of LCN2 in the inflammatory response. Such contradictions are even more marked with respect to brain diseases, which will be next discussed in detail.

5. Lipocalin-2 in the central nervous system

In the CNS, as in the immune/inflammatory response, the functions of LCN2 are similarly controversial. A first report described its up-regulation in whole brain homogenates in response to peripheral turpentine-induced inflammation (Liu and Nilsen-Hamilton, 1995). Later descriptions demonstrated LCN2 up-regulation in the brain upon a peripheral inflammatory stimulus (with LPS) (Marques et al., 2008) and in animal models of CNS diseases such as experimental autoimmune encephalomyelitis (EAE) (Marques et al., 2012), AD (Naude et al., 2012) and in spinal cord injury (Poh et al., 2012; Rathore et al., 2011). In this context, LCN2 is likely to be engaged in identical mechanisms as those described in the periphery such as in the modulation of the innate immune response (through siderophore binding and iron homeostasis), in the balance between pro- and anti-inflammatory responses, in cellular activation and cellular migration, and, eventually, also in mechanisms still undetermined or not clearly identified (Lee et al., 2011; Lee et al., 2009).

5.1. Where in the brain?

In the brain, LCN2 expression is described to occur mainly in response to an injury or inflammatory status (Ip et al., 2011; Marques et al., 2008; Thouvenot et al., 2006), but the basal and physiological expression of LCN2 was also described to occur in specific regions of the adult rat brain (Chia et al., 2011). Particularly, Chia and colleagues (2011) showed that low levels of LCN2 mRNA and protein expression are detected in most regions of the normal adult brain, except for the olfactory bulb, brainstem and cerebellum, where the expression levels are higher (Chia et al., 2011). Single and double immunostaining revealed the presence of LCN2 in astrocytes in these regions in the normal brain (Chia et al., 2011). However, the presence of LCN2 in the brain, under physiological conditions, is quite controversial since other authors have reported the absence of labelling or expression (Ip et al., 2011; Marques et al., 2012; Mesquita et al., 2014). Still, LCN2 sustained expression is accepted to occur when an injury is imposed to the CNS or when a CNS disease is installed. In such cases, LCN2 expression is restricted to the affected brain region, although controversy exists on the type of cells producing it (Table 3). In initial reports, LCN2 was shown to be induced after an acute peripheral injection of LPS at the barriers of the brain, both in the CP epithelial cells and in the endothelial cells of the capillaries that irrigate the brain parenchyma (Marques et al., 2008). Of interest, not all CP epithelial cells produce LCN2 simultaneously and this secretion is not observed when the peripheral stimulus is repeated (Marques and Sousa, 2015) or become chronic, such as in the EAE mouse model of MS (Marques et al., 2012). In the later cases, LCN2 expression in the CP derives from infiltrating neutrophils (Marques et al., 2012) (Fig. 2). Yet, it is now know that LCN2 expression in the brain at inflammatory conditions is not restricted to the barriers, but extends to the brain parenchyma. For instance, in the EAE mice model, LCN2 was found in activated astrocytes particularly in brain regions typically affected in MS patients (Marques et al., 2012) (Fig. 2). Notably, the production of LCN2 by astrocytes and infiltrating neutrophils in the EAE mice likely contributes to its presence in the cerebrospinal fluid (CSF) during the active phases

Table 3
Summarized description of the diverse reports demonstrating the differential cellular expression of LCN2 in the brain, in various disease models, and the discrepancy in the outcomes when LCN2-null mice are used.

	Model	LCN2 expression		24p3R expression	LCN2-null mice phenotype	Proposed role for LCN2	References
		Brain region	Brain cells	Brain cells			
Ischaemia	Transient focal cerebral ischaemia	Peri-infarct cortex	Neurons[a]	–	–	Help-me signal for glial activation	Xing et al. (2014)
	Transient middle cerebral artery occlusion; Hypoxic-ischaemic brain injury	Ipsilateral hemispheres	Astrocytes, microglia	Neurons, astrocytes, endothelial cells	Decreased inflammatory phenotype	Glial activation	Jin et al. (2014b)
Neuroinflammation	LPS-induced neuroinflammation	Whole brain	Endothelial cells, astrocytes, microglia	–	Reduced glial activation and neurotoxicity	Promoter of neurotoxic glial activation, neuroinflammation	Jin et al. (2014a)
		Brain barriers, brain parenchyma	CP epithelial cells, endothelial cells	–	–	Part of the innate immune response	Marques et al. (2008)
	In vitro CP epithelial cells stimulated with LPS	–	CP epithelial cells	–	–	Neuronal growth, differentiation and function	Thouvenot et al. (2006)
	Transgenic rats expressing mutant human TDP-43	Forebrain, spinal cord	Astrocytes	Reactive astrocytes, neurons	–	Neuronal death	Bi et al. (2013)
	Intracerebral haemorrhage	Ipsilateral basal ganglia, cortex	Astrocytes, some neurons	–	–	Modulation of iron homeostasis	Dong et al. (2013)
	LPS-induced endotoxemia	Whole brain, parenchyma, brain barriers	Endothelium, microglia, CP epithelial cells	Neurons, CP	Similar inflammatory response to controls	Not an essential modulator of gliosis in response to LPS *in vivo*	Ip et al. (2011)
	Kainate-induced excitotoxic model	Hippocampus	Astrocytes	–	–	Iron transport	Chia et al. (2011)
	Spinal contusion injury	Spinal cord, brain barriers	Astrocytes, neurons, endothelial cells	Astrocytes, neurons	Improved recovery from contusion	Inflammatory regulator	Rathore et al. (2011)
Infection	West Nile virus encephalitis	Whole brain	Ependymal cells, astrocytes, CP epithelial cells, neurons, parenchymal cells	–	Tissue viral load and survival similar to controls	Dispensable as an immunoregulatory factor	Nocon et al. (2014)
Pain	Complete Freund's adjuvant injection	Hindpaw	Microglia	Microglia	Mechanical allodynia diminished	Central sensitization and nociceptive behaviour	Jha et al. (2014)
	Formalin injection; peripheral nerve injury	–	–	–	Nociceptive behaviour attenuated		Jha et al. (2013)
	Spared nerve injury	Spinal cord	Neurons	Neurons, microglia	Less pain sensitivity	Mediator of neuropathic pain	Jeon et al. (2013)
MS	Chronic model	Spinal cord	Astrocytes	Astrocytes, microglia	Disease phenotype attenuated	Modulator of autoimmune inflammation	Nam et al. (2014)
	Relapsing-remitting EAE model	Brain barriers[b], cerebellum, brain stem	CP stromal cells, astrocytes	–	–	Putative molecule for MS diagnosis	Marques et al. (2012)
	Relapsing-remitting EAE model	Spinal cord[a,c]	Astrocytes	Astrocytes, microglia	Disease severity increased	Modulator of EAE pathogenesis	Berard et al. (2012)
AD	*In vitro* TNF stimulation	[a,b]	Primary culture neurons, astrocytes, and microglia	–	–	Sensitization of nerve cells to β-amyloid toxicity	Naude et al. (2012)
	In vitro Aβ cellular stimulation		CP epithelial cells, astrocytes	CP epithelial cells, astrocytes, neurons	Increased cellular viability	Required to mediate Aβ toxicity	Mesquita et al. (2014)
Behaviour	Behavioural characterization of LCN2-null mice		–	–	Emotionally altered, cognitive impaired; decreased LTP	Modulator of synaptic activity and behaviour	Ferreira et al. (2013)
	Acute restraint stress		Neurons, astrocytes	–	Stress-induced anxiety	Regulator of neuronal excitability	Mucha et al. (2011); Skrzypiec et al. (2013)

CP, choroid plexus; CSF, cerebrospinal fluid; LPS, lipopolysaccharide; MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; LTP, long-term potentiation; AD, Alzheimer's disease.

[a] Also in post-mortem human brain tissue.
[b] Also detected in CSF of patients.
[c] In serum and CSF of patients.

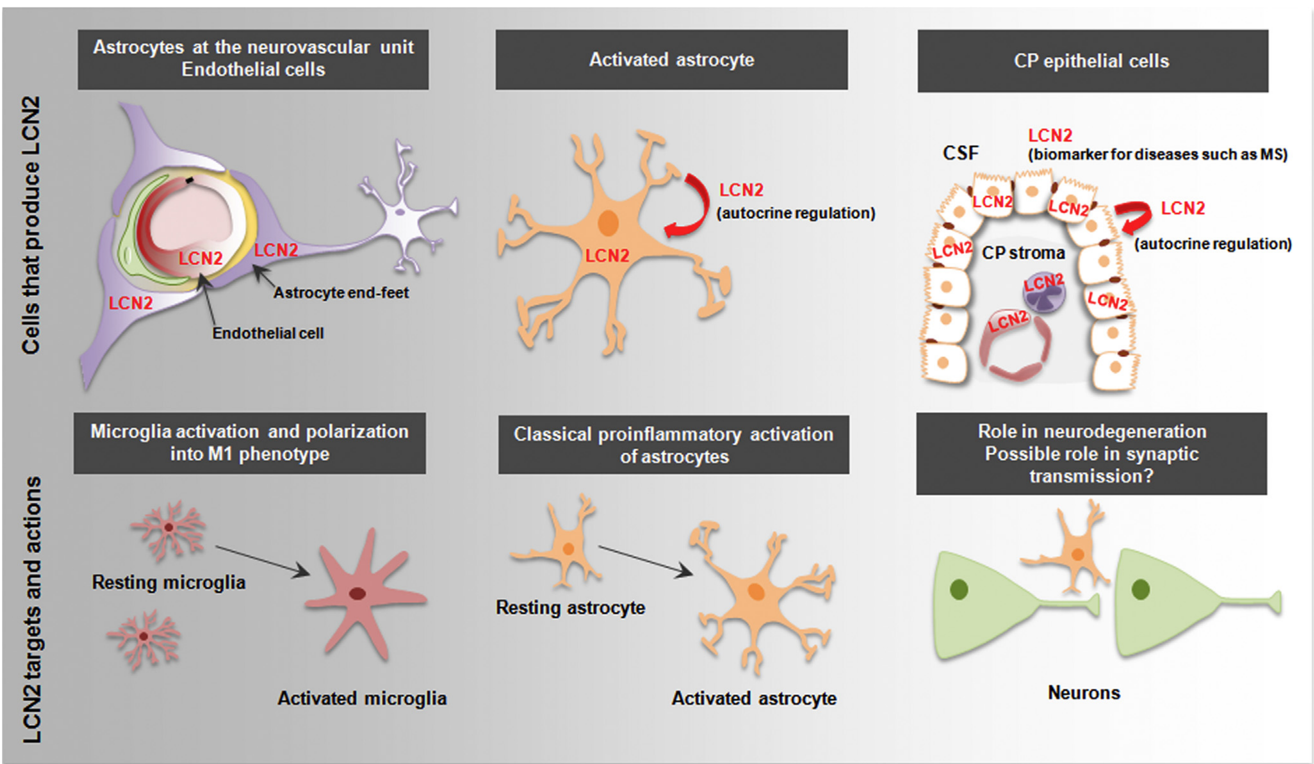

Fig. 2. Schematic overview of which CNS cells produce LCN2 and in which cells/processes LCN2 acts. Although controversy exists on which cells produce LCN2, it is largely described that its expression occurs when an injury is imposed or a disease state is installed. Lipocalin-2 is produced by the endothelial cells and the astrocytes at the neurovascular unit, and by activated astrocytes, where it is considered an autocrine regulator of reactive astrocytosis. Lipocalin-2 is also expressed by some (but not all) CP epithelial cells but only in response to acute stimulus. In the CSF, LCN2 presence has been postulated as a potential biomarker of disease, such as in the active phases of MS. When produced, LCN2 is described to act on microglia to amplify M1-polarized phenotypes over M2 and on astrocytes through the promotion of the classical pro-inflammatory activation. By acting in neurons, LCN2 is considered to have a role in neurodegeneration and even possibly in synaptic transmission. CSF, cerebrospinal fluid; CP, choroid plexus; MS, multiple sclerosis.

of disease (Marques et al., 2012). In addition, other authors have shown that in the ischaemic brain and after transient middle cerebral artery occlusion, LCN2 is predominantly produced by the brain endothelium and by astrocytes, whereas its receptor (24p3R) is expressed in neurons, astrocytes and endothelial cells (Jin et al., 2014b) (Table 3). Similarly, increased levels of LCN2 mRNA and protein were found in the CP, vascular endothelium, macrophage/microglia and astrocytes in an animal model of West Nile virus encephalitis (Nocon et al., 2014). Interestingly, in this study, the authors also evidenced that some neuronal subsets contained LCN2 protein, but no detectable mRNA expression (Nocon et al., 2014). Again, in a rat animal model of brain injury after intracerebral haemorrhage, LCN2 expression was found markedly increased in the ipsilateral basal ganglia and cortex, with most LCN2 positive cells being astrocytes (Dong et al., 2013) (Table 3). The precise identification of the brain cells that express, secrete and take up LCN2 are yet to be clearly defined, since contradictory reports exist. Most authors, as mentioned until now and including our group, were unable to detect LCN2 expression by neurons or microglial cells in the brain parenchyma (Marques et al., 2009); however, few studies suggest that indeed both cell types are able to produce it. Specifically, when assessing the role of LCN2 in the pathogenesis of neuropathic pain using a mouse model of spared nerve injury, LCN2 was detected in neurons of the spinal cord, simultaneously with the expression of the 24p3R in spinal neurons and microglia (Jeon et al., 2013). Additionally, LCN2 was described as one of the most highly up-regulated transcripts detected by microarray analysis in the mouse hippocampus (Mucha et al., 2011) and in the amygdala (Skrzypiec et al., 2013), after an acute restraint protocol of stress. In these studies, LCN2 expression was found in the pyramidal cell layers of CA1–CA3 and in the granule cell layers of the dentate gyrus of the hippocampus, co-localizing with the neuronal marker NeuN (Mucha et al., 2011). At the nucleus of the basolateral amygdala, LCN2 was shown to co-localize mostly with neurons and, to a lesser extent, with the glial fibrillary acidic protein astrocyte marker (Skrzypiec et al., 2013). Furthermore, the presence of LCN2, and its release by neurons, was described in post-mortem human brain samples of stroke patients, compared to the contralateral cortex, and in the rat animal model of focal cerebral ischaemia (Xing et al., 2014).

Of relevance, the controversy of whether LCN2 is produced by neuronal cells also persists even in *in vitro* studies. Experiments have demonstrated the expression of LCN2 by neurons in response to TNF (Naude et al., 2012) and after oxygen–glucose deprivation (Xing et al., 2014), while others showed no expression by these cells in response to amyloid beta (Aβ) or LPS (Mesquita et al., 2014). This inconsistency may be related to the different stimuli or type of insult applied or possibly due to neuron-enriched culture contamination by astrocytes that can easily occur (Mesquita et al., 2014). Interestingly, *in vitro* studies showed that LCN2 expression by astrocytes occurs only when they are reactive (Bi et al., 2013), corroborating the described *in vivo* expression of LCN2 by astrocytes only in diseased brain regions, as we will further discuss.

5.2. How? From the innate immune response to iron trafficking

When the levels of LCN2 were reported to be increased at the brain barriers in response to peripheral administration of LPS, a role in protecting the brain from invading microorganisms,

through sequestration of iron, was suggested (Marques et al., 2008). This argued in favour of a LCN2 bacteriostatic action in the CNS, similarly to that described for the periphery (Flo et al., 2004). Still, the relevance of LCN2 production by the brain barriers will depend on the pathogen that is invading the CNS since, for instance, the function of LCN2 in the host response to West Nile virus encephalitis infection was shown to be dispensable as the infected brains of LCN2-null and WT mice contained similar numbers of infiltrating cells and no differences in tissue viral loads or survival was observed (Nocon et al., 2014).

As mentioned before, in addition to CP epithelial cells and to endothelial cells (Marques et al., 2008), some have described the production of LCN2, at the brain parenchyma, by neurons (Mucha et al., 2011; Naude et al., 2012) and by microglia (Ip et al., 2011; Naude et al., 2012) although this is controversial. Nevertheless, consensus exists on the synthesis of LCN2 by astrocytes, which are considered the major producers of LCN2 in the brain (Bi et al., 2013; Chia et al., 2011; Marques et al., 2012). As such, its secretion is assumed to be one of the mechanisms through which astrocytes interact with neurons and regulate neuronal homeostasis. This possible role in neuronal homeostasis pertains to iron transport by LCN2. This is conceivable because neurons constitutively express 24p3R even in physiological conditions (Ip et al., 2011; Rathore et al., 2011). Indeed, the iron-binding capability of LCN2 has been shown to be a key element in the regulation of dendritic spine density and morphology (Mucha et al., 2011). Specifically, this type of regulation was demonstrated to occur in an iron-dependent manner, since *in vitro* treatment of hippocampal neurons with holo-LCN2 caused a decrease in spine density but treatments with apo-LCN2 further potentiate those processes (Mucha et al., 2011). Moreover, both apo- and holo-LCN2 affected spines shape, by increasing the proportion of the thin type and decreasing the density of mushroom spines, being again the effects with apo-LCN2 more pronounced (Mucha et al., 2011). In this work, Mucha et al. (2011) also showed that this effect on spine plasticity is due to a decrease in actin's mobile fraction, which is a component of the spine cytoskeleton and that regulates its shape (Mucha et al., 2011). It is generally accepted that mushroom spines are mature spines and that are implicated in the maintenance of established neuronal networks and long-term memory ("memory spines") and, although LCN2 is certainly not the only modulator of dendritic spine shape, this peculiar modulation of actin mobility may have an impact on structural plasticity and behaviour. In fact, disruption of *Lcn2* expression in mice promotes increased anxiety, depressive-like behaviour and mild spatial reference memory impairments (Ferreira et al., 2013). Importantly, these behavioural alterations are concomitant with significant alterations in the morphology of hippocampal neurons and spine density, along the dorsal-ventral axis (Ferreira et al., 2013). Regarding this, the absence of LCN2 promotes neuronal hypertrophy at the ventral hippocampus, with a significant atrophy in neurons at the dorsal division and a decrease in the proportion of mushroom type spines (Ferreira et al., 2013). Similarly, in response to stress, the ablation of LCN2 revealed to be deleterious by promoting stress-induced increase in spine density, which correlated with a higher excitability of CA1 neurons and stress-induced anxiety (Mucha et al., 2011). Nonetheless, since the brain responds differently depending on the presence of an acute or chronic stressful stimulus, the strategy of LCN2-null mice to cope with different stress paradigms requires further detailed studies.

Still to be unravelled is whether, similar to what occurs in kidney development (Yang et al., 2002), LCN2 is able to modulate cell proliferation in the brain through iron trafficking. This possible modulation can be crucial for brain homeostasis maintenance, with further impact in the neurogenic niches in response to injury. Interestingly, the involvement of LCN2 in neurodegenerative disorders, such as AD, where iron is a key player, has been shown (Mesquita et al., 2014; Naude et al., 2012). Indeed, iron homeostasis deregulation may lead to iron accumulation, which is a common feature of such neurodegenerative diseases (Crichton et al., 2011). In fact, the presence of LCN2 in the brain regions associated with AD pathology in human post-mortem brain tissue (Naude et al., 2012) and in the mediation of Aβ cytotoxicity (Mesquita et al., 2014) may relate to the modulation of iron homeostasis. The function of LCN2 as an iron-chelator in the brain has also been suggested after intracerebral haemorrhage, as part of the response to clear iron released from the haematoma during clot resolution (Dong et al., 2013).

5.3. In neuroinflammation: neuroprotective or neurodetrimental?

Inflammation in the CNS (termed neuroinflammation) is common to all neurodegenerative conditions and whenever an injury is imposed in the CNS. The cellular expression of LCN2 under such conditions is known to occur so it can engage critical roles in inflammatory progression. For instance, the secretion of LCN2 by activated astrocytes mediates neuronal toxicity, which can have implications in disease progression (Bi et al., 2013).

Upon stimulation, LCN2 is secreted by astrocytes to act as an autocrine mediator and to stimulate surrounding astrocytes and microglia to become reactive, thus amplifying the inflammatory response (Lee et al., 2007; Lee et al., 2009) and impacting on neurons. Whether this modulation by LCN2 can be considered deleterious or protective is largely controversial presently. Several reports point to LCN2 as a deleterious modulator since, for instance, in the injured brain after ischaemia, LCN2 was shown to contribute to neuronal cell death by potentiating, among others, ischaemic-induced glial activation, neutrophil infiltration and pro-inflammatory cytokine/chemokine levels (Jin et al., 2014b). Similarly, *Lcn2*-expression ablation attenuates LPS-induced glial activation in the brain (Jin et al., 2014a) and promotes better locomotor recovery after spinal cord injury (Rathore et al., 2011), reinforcing the view that, when present, LCN2 might contribute to the uncontrolled neurotoxic glial activation under conditions of excessive and chronic inflammation. These results are in accordance with the findings that LCN2 secreted by glial cells in inflammatory conditions up-regulates the expression of C-X-C motif chemokine-10 (Lee et al., 2011), participating in the recruitment of leukocytes to the CNS. This role as an immune cell attractant, through the modulation of chemokines, has implications, for instance, in central sensitization at the site of inflammation and in nociceptive behaviour in chronic inflammatory pain (Jha et al., 2014). Mechanical allodynia (Jha et al., 2014), nociceptive behaviour (Jha et al., 2013) and pain sensitivity (Jeon et al., 2013) are all features of pain processing that were decreased or attenuated in LCN2-null mice (Table 3). However, the observation that LCN2-null mice present a similar inflammatory response to LPS, as controls, suggests a non-essential modulation of gliosis in response to LPS *in vivo* (Ip et al., 2011).

Opposing to these neurodetrimental effects under inflammation, LCN2 has also been attributed a neuroprotective role. At least in one report concerning MS, LCN2-null mice presented an increased severity of disease manifestation and progression (Berard et al., 2012), accompanied by an increased pro-inflammatory response. It is, therefore, plausible to assume that LCN2 mediates differential effects depending on the type of CNS inflammatory response.

5.3.1. Lipocalin-2 in multiple sclerosis

The presence and relevance of LCN2 in different brain diseases is well accepted, but its precise role in disease onset and progression is still debateable. In the particular case of MS, the

presence of LCN2 and its increased expression has been reported by many (Berard et al., 2012; Marques et al., 2012; Nam et al., 2014), but while some studies point to its neurodeleterious effects (Nam et al., 2014), others suggest that it may be neuroprotective (Berard et al., 2012). Berard and colleagues (2012) demonstrated that the induction of EAE in LCN2-null mice leads to increased disease severity and pro-inflammatory responses, indicating that LCN2 is protective (Berard et al., 2012). Specifically, such increased disease severity was associated with increased lesion burden and IFN-γ and TNF expression, both pro-inflammatory cytokines with well-known implication in EAE pathogenesis (Berard et al., 2012). The absence of alterations in *IL-4* mRNA levels in LCN2-null mice suggests that the protective function of LCN2 in EAE involves the control of the T_h1 immune response, rather than the promotion of an T_h2 immune response (Berard et al., 2012). The described protective effects are considered to occur towards macrophages/microglia and reactive astrocytes, but not neurons, as those are the cell populations shown to express 24p3R during the course of the disease (Berard et al., 2012; Nam et al., 2014). On the other hand, Nam and colleagues (2014) showed that LCN2-null mice present a less severe disease phenotype, demonstrated by the lower mean of EAE scores and demyelination, reduced inflammatory infiltration, glial activation and inflammatory cytokine/chemokine expression, when compared to wild-type animals (Nam et al., 2014). Of interest, these authors also showed that upon the adoptive transfer of T cells from EAE-induced wild-type mice to a LCN2-null mice, the animals developed a normal EAE disease profile, while the transfer of T cells from EAE-induced LCN2-null mice to wild-type mice reduced disease severity, reinforcing the view that LCN2 is detrimental in EAE (Nam et al., 2014). In accordance with this deleterious effect is the observation that increased levels of LCN2 in the CSF coincide with the active phases of the disease and are reverted by natalizumab treatment (an antibody that blocks leucocyte entry into the CNS, currently used as therapeutics) (Marques et al., 2012). The specific engagement of LCN2 in the MS context can, indeed, be related to its previously described involvement in the amplification of M1-polarized phenotypes of microglia, over M2, which is curiously associated with the initiation and perpetuation of inflammation rather than with its resolution (Jang et al., 2013b) (Fig. 2). In addition, astrocyte-derived LCN2 promotes classical activation of astrocytes (Jang et al., 2013a), which also supports that rather than being involved in the resolution of the inflammatory response, LCN2 contributes to the initiation and installation of the disease. Of interest, LCN2 was also shown to have detrimental effects in a spinal cord injury model (Rathore et al., 2011). Particularly, LCN2-null mice showed better locomotor recovery after spinal cord contusion injury than wild-type mice, and this was accompanied by decreased neutrophil and monocyte influx to the injured spinal cords, as well as by a reduction in the expression of several pro-inflammatory chemokines, cytokines and inducible nitric oxide synthase (Rathore et al., 2011).

The attempt of the described studies to disclose if LCN2 is either protective or deleterious relies on the use of the LCN2-null mouse model, which as a full knockout system has its limitations. Additional studies are, therefore, necessary to further understand whether LCN2 acts in a context-depend manner, and which are the up- and/or down-stream mediators of such responses.

5.3.2. Lipocalin-2 in Alzheimer's disease

The engagement of LCN2 in the initiation of an inflammatory response is suggested to promote secondary tissue damage. Indeed, in *in vitro* studies LCN2 was shown to modulate cell death after inflammatory stimulation of cultured astrocytes: LCN2 increased expression and secretion induced cell death sensitization, stimulation of cell migration and morphological changes of reactive astrocytes (Lee et al., 2009). These observations point to LCN2 as an autocrine mediator of reactive astrocytosis (Lee et al., 2009). Of relevance, this LCN2-induced cytotoxic sensitization was demonstrated to involve iron metabolism and the pro-apoptotic BIM protein, not only in astrocytes but also in activated microglia and neurons (Lee et al., 2007; Lee et al., 2012). Furthermore, it was proposed that degenerating neurons activate quiescent astrocytes and these, in turn, secrete LCN2 to induce neuronal migration, morphological changes and neuronal cell death (Bi et al., 2013; Lee et al., 2012). The mediation of neuronal toxicity by LCN2 is of particular relevance for disease progression. For instance, the strong up-regulation of LCN2 in cortical brain regions of an AD mouse model which overexpresses mutated, but not wild-type forms, of the human amyloid precursor protein and presenilin-1, is suggestive of LCN2 involvement in the establishment of the initial inflammatory response and sensitization of nerve cells to Aβ toxicity (Wu et al., 2006). More recently, the stimulation of neuronal and glial (astrocytes and microglia) cultures with TNF was shown to lead to an increase in LCN2 levels (Naude et al., 2012). In addition, in RNA samples extracted from cortical neurons of mice lacking TNF type 1 receptor (TNFR1) or type 2 (TNFR2) and treated with TNF, LCN2 was specifically induced by the activation of TNFR1 (Naude et al., 2012). Remarkably, binding to this receptor generally leads to pro-inflammatory and pro-apoptotic responses, whereas binding to TNFR2 has been seen associated with neuroprotection. These observations are relevant in the context of AD, since it is known that TNF, through a TNFR2 signalling pathway, can be protective against Aβ-induced neuronal cell death (Patel and Brewer, 2008). However, when present, LCN2 can silence neuroprotection against the AD-associated excitotoxic factors *via* TNFR1-specific signalling (Naude et al., 2012). Of interest, in this same study, LCN2 levels were found to be decreased in CSF of patients with mild cognitive impairment and AD, but increased in brain regions associated with AD pathology in human post-mortem brain tissue, with no changes in the serum levels (Naude et al., 2012). However, other authors described, in a distinct cohort of patients, significantly higher LCN2 serum levels in mild cognitive impaired patients, when compared to healthy controls and AD patients (Choi et al., 2011).

In addition to these findings, LCN2 was recently showed to modulate the cellular response to Aβ (Mesquita et al., 2014). In this work, LCN2 production was up-regulated in both CP epithelial cells and astrocytes, but not in microglia or neurons, in response to Aβ (Mesquita et al., 2014). Moreover, LCN2 was shown to be required for Aβ toxicity to astrocytes, as the survival of wild-type astrocytes was decreased upon Aβ stimulation, while astrocytes from LCN2-null mice were not affected (Mesquita et al., 2014). This protection was found to result from both a lower expression of the pro-apoptotic gene *Bim* and a decreased pro-inflammatory response (Mesquita et al., 2014), which is in accordance with a role for LCN2 in promoting pro-inflammatory response and degeneration.

In all of the described cases, LCN2 production by reactive astrocytes seems a critical step for the course of the disease, whether by potentiating or attenuating the inflammatory response. Further understanding on whether LCN2 is a marker of activated astrocytes by itself, or represents a key mediator in the involvement of astrocytes on CNS pathogenesis, might have important implications for the development of neuroprotective therapeutic strategies.

6. The promising diagnostic and prognostic value of lipocalin-2

In the recent years, the increased number of reports demonstrating the nature of secreted LCN2, in several diseases conditions, has suggested its potential value as a biomarker in clinical monitoring. In fact, it is becoming increasingly clear the

pathophysiologic role of LCN2 in diverse diseases, as described in the present review. In line with this, LCN2 has been proposed as a biomarker for various conditions and has also emerged as an attractive molecular tool with distinct clinical applications. Indeed, its small size, associated with the existence of commercially available immunoassays that quantify LCN2 levels, reinforces such emerging view. However, and although showing high sensitivity, the available immunoassays have low specificity, which is something that has to be improved in the future (Haase et al., 2009).

In the particular case of the kidney, the sustained expression of LCN2 in response to either acute or chronic kidney damage, has gained considerable importance and has led several authors to propose LCN2 as a serum and urinary biomarker for functional, toxic and ischaemic renal damage (An et al., 2013). In fact, several interesting findings have emerged from pre-clinical and clinical studies. For instance, urinary LCN2 levels have become a standard biomarker for both the early diagnosis and the prediction of prognosis of patients with renal injury (Schmidt-Ott et al., 2007). In acute kidney injury, a significant increase in the expression of LCN2 in the kidney is observed (Hirsch et al., 2007). Aligned with a poor sensitivity of creatinine in the early diagnosis of this disease (Kumpers et al., 2010), the positive correlation of LCN2 levels with disease severity (Kumpers et al., 2010) has strengthen the suggestion of LCN2 as an early diagnostic and prognostic marker for these patients. Similarly, in chronic kidney disease, the increased levels of LCN2 in tissue, blood and urine (Viau et al., 2010) correlate with the extent of renal damage. Importantly, in polycystic kidney disease, the levels of LCN2 in patients with rapid disease progression are significantly higher than those with slower progression (Viau et al., 2010). Also, in the cases of patients with stable angina, undergoing percutaneous coronary intervention, the increased level of urinary LCN2 is a strong predictor of mortality (Borkham-Kamphorst et al., 2013).

In addition, several authors are currently pursuing studies on the effectiveness of LCN2 as a marker and predictor of other diseases. The diversity of reports describing LCN2 expression, in animal models of disease and in humans, and under several conditions, strongly suggests for its predictive value. Examples of this are the proposals of LCN2 as a marker of disease progression in post-liver resection (Kienzl-Wagner et al., 2014), asphyxiated newborns (Surmiak et al., 2014), breast cancer (Wang and Zeng, 2013), chronic obstructive pulmonary disease (Wang et al., 2014), systemic lupus erythematosus (Li et al., 2014), heart failure (van Deursen et al., 2014) and nephrolithiasis (Zhu et al., 2014). Nevertheless, in most reports, the suggestion for the need of additional and further studies to dissect the precise reliability of LCN2 as a biomarker is noted. Still, the proposed role of LCN2 in such diseases paves the way for possible therapeutic targeting.

Whether detrimental or protective, also in the CNS context, LCN2 has been profoundly associated to alterations in brain homeostasis and in specific regions that are affected in neurodegenerative disorders, including AD and MS. Due to its peculiar mechanisms of induction and involvement in accelerating neuronal cell death, many authors are also proposing the use of LCN2 as a marker for the diagnosis and prognosis of several neurological diseases. The particular description of increased LCN2 plasma and CSF levels in patients with mild cognitive impairment (Choi et al., 2011), of plasma levels in depression (Naude et al., 2013), but also in the CSF of MS patients (Marques et al., 2012), is suggestive of LCN2's involvement in these pathologies. Specifically, the presence of high LCN2 levels in patients with mild cognitive impairment, considered to be a transitional state between normal and mild dementia, was considered not only to reflect overall CNS inflammation, but also an important marker of progression from one form of dementia to the other (Choi et al., 2011). In this sense, as much as it is risky to say, it is plausible to speculate that higher levels of LCN2 could be associated with a higher risk to develop AD (Choi et al., 2011). However, some cautious should be taken into consideration since in the study by Naude et al. (2012), no changes in the plasma levels for LCN2 in AD and in mild cognitive impairment was observed (Naude et al., 2012). In parallel, the increased plasma levels of LCN2 in subjects with a recurrent depression episode, compared to those in the first episode, points for its potential use as a marker of disease (Naude et al., 2013). This finding further suggests that LCN2 may be an indicator for symptoms of depression, which should be further investigated in detail.

Furthermore, the identification of increased LCN2 CSF levels in MS patients, which also occurs in the EAE animal model, suggests for its putative relevance and possible inclusion in the already existent panel of inflammatory MS markers (Marques et al., 2012). The major drawback at this point concerns the period in the course of the disease that the analyses are performed and, currently, it is not known whether LCN2 levels emerge earlier than current diagnostic markers of the disease (Marques et al., 2012). This can be of clinical relevance so to identify patients that would benefit from early treatment.

Irrespective of the great value of LCN2 from the clinical point of view, we believe that a single marker will be insufficient to track disease progression, particularly when its altered expression is common to such myriad of diseases. For instance, the levels of LCN2 overlap between depressed and non-depressed patients, which certainly limits its use as a single diagnostic marker, in this particular case. Ultimately, the best approach will always be the use of LCN2 in combination with other validated biomarkers. This is certainly a great challenge for the near future and for personalized medicine.

7. Final remarks

We performed here a comprehensive revision of the multifaceted LCN2, ranging from its known and postulated functions in the periphery to the latest findings of its involvement in diseases of the CNS. Lipocalin-2 has gained great interest in multidisciplinary fields since its discovery. With a denotable broad expression and rapidly induction upon stimulation, particularly in tissues more prone to infection, the relevance of LCN2 in the acute inflammation and innate immune response is nowadays well accepted. Lipocalin-2 is part of an efficient mechanism of sensing microbial metabolism to modulate the proper host immune response, with iron assuming an important role in such regulation. The existence of specific cell surface receptors for LCN2, and of an endogenous mammalian siderophore, strengthens the role of LCN2 in iron trafficking within cells and as a player on iron homeostasis. The impact of such modulation, whether under physiological or pathological conditions, has been demonstrated to be crucial for cellular equilibrium and homeostasis. Indeed, although iron is the best-known ligand for LCN2, which may explain the impact of LCN2 in innate immunity and in cellular processes such as cell proliferation and death, the role of LCN2 in the modulation of pro- and anti-inflammatory responses still warrants further research. Also unclear is the mechanism through which LCN2 modulates cellular processes such as cell migration. Is it possible that LCN2 can also bind to other ligands? Has the demonstrated LCN2 binding to phosphatidylethanolamine a role in cellular processes such as dendritic arborisation and cell-to-cell connections, known to be affected in LCN2-null mice?

The precise impact of LCN2 in the CNS is far from being completely unravelled and is considered to be multifaceted. The production of LCN2 by reactive astrocytes is likely to play significant roles in neurodegeneration. From a clinical perspective,

LCN2 could be targeted therapeutically to dampen pro-inflammatory astrocytic activation. Nonetheless, at this point, the absence of a specific antagonist for LCN2, aligned with a less elucidated mediation of astrocytic neurotoxicity by LCN2 through the binding to specific receptors, makes the task of counteracting LCN2 effects in the progression of CNS diseases very challenging. Filling these and other gaps of knowledge is crucial to assert the already described roles for LCN2 and, possibly, to uncover new ones.

Conflict of interests

The authors have no conflicting financial interests to declare.

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CHAPTER II

Lipocalin-2 regulates adult neurogenesis and contextual discriminative behaviours

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Lipocalin-2 regulates adult neurogenesis and contextual discriminative behaviours

Running title: Adult neurogenesis orchestration by lipocalin-2

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Abstract

In the adult mammalian brain, newborn granule cells are continuously integrated into hippocampal circuits, and the fine-tuning of this process is important for hippocampal function. Thus, the identification of factors that control adult neural stem cells (NSCs) maintenance, differentiation and integration is essential. Here we show that the deletion of the iron trafficking protein lipocalin-2 (LCN2) induces deficits in NSCs proliferation and commitment, with impact on the hippocampal-dependent contextual fear discriminative task. Mice deficient in LCN2 present an increase in the NSCs population, as a consequence of a G0/G1 cell cycle arrest induced by increased endogenous oxidative stress. Of notice, supplementation with the iron-chelating agent deferoxamine rescues NSCs oxidative stress, promotes cell cycle progression and improves contextual fear conditioning. LCN2 is, therefore, a novel key modulator of neurogenesis that, through iron, controls NSCs cell cycle progression and death, self-renewal, proliferation and differentiation and, ultimately, hippocampal function.

Keywords

Lipocalin-2; iron; oxidative stress; neural stem cells; adult neurogenesis; contextual discrimination

Introduction

In the adult mammalian brain, at the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus [1], resident neural progenitor cells give rise to new functional neurons that integrate into the pre-existing neuronal circuitry, and modulate local neural plasticity and network [1, 2]. This integration is important for hippocampal integrity and function, such as the regulation of learning, memory and pattern separation [3-5], emotion [6] and neurodegeneration [7]. As so, understanding the factors that control adult neural stem cells (NSCs) maintenance, differentiation and integration is critical.

Of interest, iron is necessary for cell growth and differentiation [8, 9] and its acquisition by cells is essential for cell survival and maturation. Accordingly, several studies showed that iron controls cell cycle progression [10] or arrest [11] and cell death-related genes [12]. On the other hand, iron is also potentially toxic due to its participation in redox reactions that lead to the formation of reactive oxygen species (ROS), thus creating an oxidative stress environment [13]. Noticeably, increasing evidence implicates ROS in the regulation of long-term potentiation (LTP) in hippocampal neurons [14], and in neuropsychiatric pathologies and cognitive performances [15-17]. Most mammalian cells acquire iron *via* receptor-mediated endocytosis of iron-loaded transferrin [18]. However, in recent years, lipocalin-2 (LCN2) has emerged as an alternative mechanism of physiological iron delivery and uptake [19-21]. As a member of the lipocalin family, the best described function for LCN2 concerns its secretion in the innate immune response to infection, to limit iron-availability for bacterial growth [22]. Additionally, LCN2 has the ability to interact with iron through a mammalian catechol complex [19, 23] and modulate iron cell content [21]. The existence of a fine-tuned mediation of intracellular iron by LCN2 is still poorly recognized, but in the context of the brain, the iron-binding capability of LCN2 was shown to be important in the regulation of hippocampal neuronal dendritic spine density and morphology [24], with impact on structural plasticity and function. In fact, *Lcn2* deletion is associated with increased anxiety, depressive-like behaviour, spatial reference memory impairments and decreased LTP [25]. Nevertheless, the contribution of LCN2 in the regulation of cell proliferation, putatively through iron trafficking and iron-mediated oxidative stress, has never been explored in the brain.

In this study, we addressed the role of LCN2 in the adult mammalian brain cell genesis, and its relevance for NSCs homeostasis maintenance, as well as for the integrity of hippocampal plasticity and function.

Material and Methods

Animal Experiments

Experiments were performed in 2-months old male mice lacking *Lcn2* expression (LCN2-null) and the respective wild-type (Wt) littermate controls, in a C57BL/6J background. All animal procedures were conducted in accordance with EU Directive 2010/63/EU and were approved by the Portuguese national authority for animal experimentation (ID: DGAV9457). Mice were intraperitoneally (i.p.) injected with 50 mg/kg of BrdU (Sigma Aldrich, St. Louis, MO, USA) 24 h before sacrifice (for fast proliferation protocol), or twice a day for 5 consecutive days, followed by a chase period of 28 days (for slow dividing cells analysis). To test the effect of iron chelation in cell cycle regulation, mice were i.p. injected with 200 μM of deferoxamine (DFO; Sigma), along with BrdU, and sacrificed 24 h later. To assess the capacity of iron chelation in contextual discrimination, mice were chronically treated with DFO (200 μM) by i.p. injection every other day for 28 days, and in the end tested for contextual fear conditioning (CFC). Animals were also pharmacologically treated with the antioxidant agent N-acetylcysteine (NAC; Sigma) by i.p. injection with 300 mg/kg, and sacrificed 24 h later. The respective control groups were injected with saline.

Immunohistochemistry and imaging

Brains were fixed by transcardial perfusion with 4% paraformaldehyde, sectioned in a cryostat and incubated overnight with primary antibodies, followed by the respective fluorescent secondary antibodies. Fluorescence images were acquired with the Olympus Fluoview FV1000 confocal microscope (Olympus, Hamburg, Germany) and the number of double-positive cells calculated using Olympus Fluoview FV1000 software.

Contextual fear conditioning

Contextual fear conditioning was conducted for 2 days and each test session was scored. On the conditioning day, mice were placed in a chamber with a stainless grid floor and pairings between light and shock (1 sec, 0.5 mA) was delivered, spared from each other with an interval of 20 sec (Figure 3a). On day 2, mice were re-exposed to the conditioning chamber for 3 min for contextual fear memory, and 2 h later presented to a novel context, where the grid was removed, black plastic inserts covered the floor and the walls of the chamber, that was also scented with a paper towel dabbed with vanilla extract. The session consisted on a 3-min trial and animal freezing was scored for the entire session and defined as the complete absence of motion for a minimum of 1 sec.

Neural stem cells culture

Stem cells were cultured from 1–3 days-old Wt and LCN2-null mice SGZ, as an adaptation of the protocol described elsewhere [26]. Parameters analysed included cell proliferation, self-renewal, cell cycle, cellular viability and death and ROS levels. Cell treatments included the exposure of LCN2-null-derived neurospheres to recombinant mouse LCN2 protein (R&D Systems, Inc., Minneapolis, MN, USA), at a concentration of 100 ng/ml for 24 or 72 h, and to 2 μM of DFO for 24 h. Also, Wt cultures were treated with 500 μM of ferric ammonium citrate (FAC; Sigma) for 24 h.

For a more detailed description of the experimental procedures, please see Supplementary information.

Statistical analyses

All experiments were performed and analysed by the same experimenter, blind to the animals' genotype or group treatment under assessment. Animals were assigned to groups according to their genotypes, and no method of randomization was applied. Sample sizes were determined by power analyses based on previously published studies [25]. Variables followed a Gaussian distribution as revealed by the D'Agostino & Pearson normality test. Data are reported as mean ± standard error (S.E.M.). Statistical significant differences between groups were determined using two-tailed Student's *t*-test for two comparisons, and two-way ANOVA, followed by Bonferroni's multiple comparison test for multiple comparisons. Values were considered to be statistically significant for $P \leq 0.05$ (* or #), $P \leq 0.01$ (** or ##), $P \leq 0.001$ (*** or ###) and $P \leq 0.0001$ (**** or ####). For the *in vitro* analysis, the number of independent experiments is specified in the legend of each Figure.

Results

The absence of LCN2 leads to decreased rates of cell proliferation

In order to understand the role of LCN2 in the process of adult neurogenesis, we first analysed the rates of proliferating cells in the SGZ of the DG in LCN2-null mice. For that, the number of cells entering the S-phase was analysed by injecting animals once with BrdU (Figure 1a) and 24 h later, quantification of the total number of BrdU$^+$ cells showed a significant reduction (P=0.004) in LCN2-null mice hippocampus when compared to Wt littermate controls (Figure 1b, c).

Interestingly, when we analysed the levels of LCN2 mRNA, by RT-PCR (not shown), and protein expression by western blot and immunohistochemistry in the DG of adult mice, we were not able to detect expression of LCN2 (Supplementary Figure S1a, b), which is in accordance with other reports [27, 28]. Nevertheless, analysis of protein expression revealed that LCN2 is highly present in the serum (Supplementary Figure S1a), suggesting that a possible regulation of adult neurogenesis by LCN2 may occur extrinsically to the brain, through its delivery by the blood vessels that surrounds the neurogenic niches, which deserves further clarification.

The subsequent analysis of the endogenous Ki67 marker of proliferation revealed that LCN2-null mice also presented a significant reduction (P=0.012) in the number of Ki67$^+$ cells (Figure 1b, c). Also, the effect of LCN2 absence on cell proliferation at the adult subventricular zone (SVZ) was studied and, similarly, LCN2 absence impaired cell proliferation (Supplementary Figure S2a-c).

LCN2-null mice present deficits in adult progenitors differentiation

The impact of LCN2 in the generation of newborn mature cells in the neurogenic niches was next investigated. Twenty-four hours upon BrdU injection, quantification of the number of proliferating neuroblasts (DCX$^+$ BrdU$^+$ cells; Figure 1d) revealed a significant reduction (P=0.01) in the SGZ of LCN2-null mice (Figure 1e, h). Concomitantly, analysis of newborn neurons (Calb$^+$ BrdU$^+$ cells; P=0.05) and of new mature astrocytes (S100β$^+$ BrdU$^+$ cells; P=0.04; Figure 1f-h) showed a significant decrease in LCN2-null mice. Similarly, the analysis of the DCX$^+$ BrdU$^+$ neuronal precursor cells, in LCN2-null mice SVZ, revealed a significant decrease (Supplementary Figure S3a-c). Nevertheless, the proportion of 28-days old newborn neurons that migrated into the olfactory bulb was similar between animals (Supplementary Figure S3d-f).

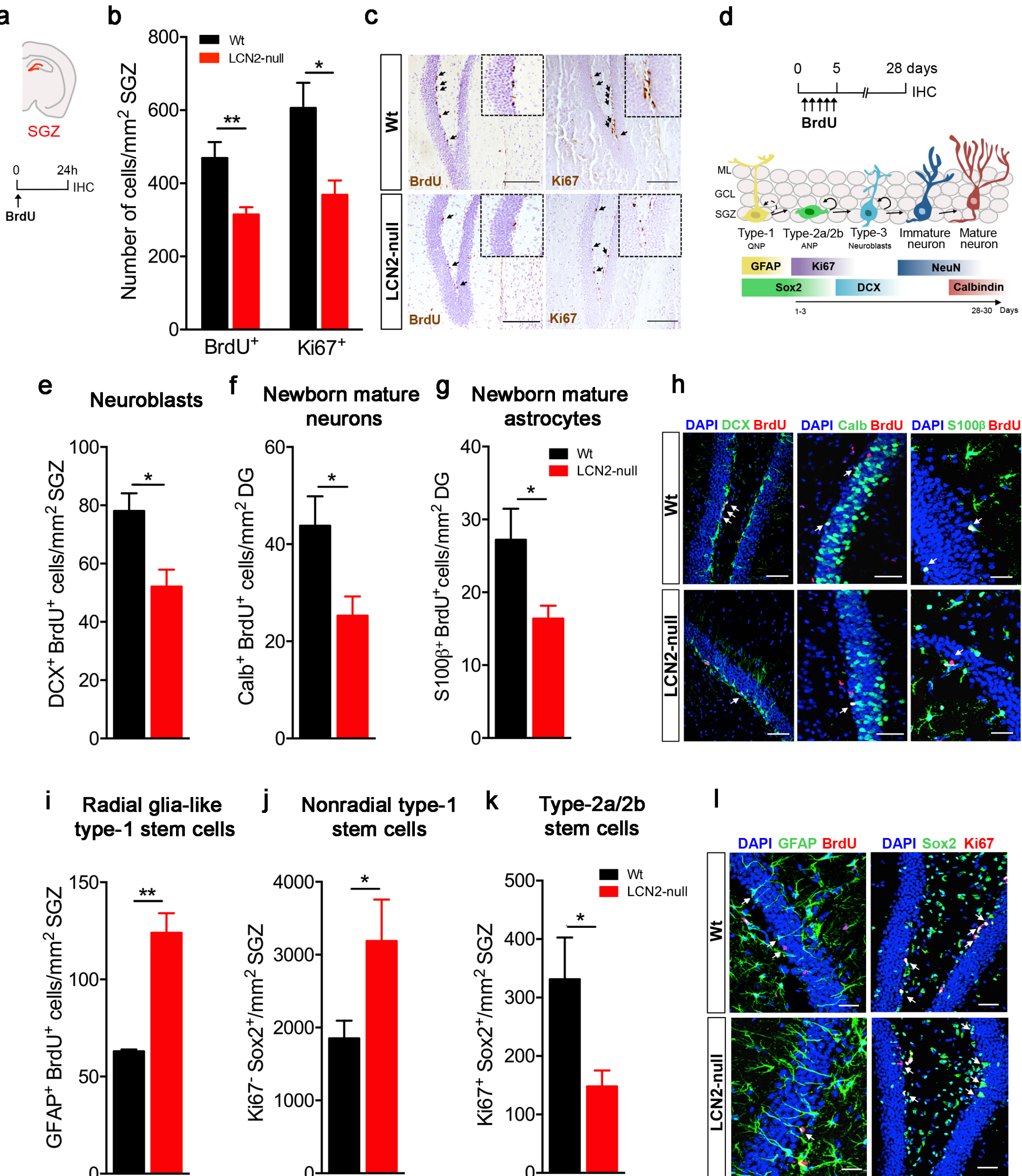

Figure 1. LCN2-null mice present reduced rates of cell proliferation and impaired NSCs proliferation, survival and maturation in the adult hippocampus. (**a**) Schematic diagram of the SGZ of the DG at the hippocampus and of the BrdU protocol used. (**b**) Quantification analysis of $BrdU^{+}$ and $Ki67^{+}$ cells in the SGZ (*n*=5 per group). (**c**) Representative images of BrdU and Ki67 immunostaining at SGZ (indicated by black arrows; scale bar, 50 μm). (**d**) Schematic diagram of the experimental protocol of BrdU used and illustration of the hippocampal neurogenesis process, including cellular types and their specific markers. (**e-g**) Reduction of DCX^{+} $BrdU^{+}$ neuronal precursors, of newborn mature neurons ($Calb^{+}$ $BrdU^{+}$) and astrocytes ($S100\beta^{+}$ $BrdU^{+}$) in LCN2-null SGZ, compared to Wt mice (*n*=6 per group). (**h**) Confocal images of DCX/BrdU, Calb/BrdU and S100β/BrdU co-labelling (indicated by black arrows; scale bar, 50 μm). (**i, j**) Increased number of radial glia-like $GFAP^{+}$ $BrdU^{+}$ type-1 stem cells and of nonradial $Ki67^{-}$ $Sox2^{+}$ type-1 stem cells in LCN2-

null mice (*n*=5 per group). (**k**) Decreased number of Ki67$^+$Sox2$^+$ type-2 progenitor cells in LCN2-null SGZ (*n*=5 per group). (**l**) Representative co-immunostaining of GFAP/BrdU and Ki67/Sox2 at DG (indicated by black arrows; scale bar, 50 µm). Data are presented as mean ± SEM analysed by two-tailed Student's *t*-test and are representative of two independent experiments. *$P \leq 0.05$, **$P \leq 0.01$. ANP, amplifying neural progenitor; Calb, calbindin; DCX, doublecortin; GCL, granular cell layer; IHC, immunohistochemistry; ML, molecular layer; QNP, quiescent neural progenitor; SGZ, subgranular zone.
See also Supplementary Figures S2 and S3.

The absence of LCN2 impairs neural stem cells proliferation and survival

Since LCN2 absence affected progenitors proliferation and differentiation, we next analysed if such impairments were dependent on the misregulation at the level of the stem cells pool. Firstly, we identified radial glia-like type-1 stem cells, as GFAP$^+$ BrdU$^+$ cells (Figure 1d), and observed a marked increase (*P*=0.009) in LCN2-null mice SGZ (Figure 1i, l). Consistently, for the SVZ, an increase in type-B1 stem cells was observed (Supplementary Figure S4a, b). Moreover, the transcription factor Sox2 was used to identify nonradial type-1 stem cells (Sox2$^+$ Ki67$^-$; Figure 1d) and type-2 amplifying progenitor cells (Sox2$^+$ Ki67$^+$). Even though the total number of Sox2$^+$ cells in the SGZ of LCN2-null mice was similar to the number observed in the Wt littermate controls (Supplementary Figure S5e), analysis of the nonradial type-1 stem cells confirmed that LCN2' absence leads to a significant (*P*=0.04) increase in this population (Figure 1j, l). On the other hand, quantification of type-2 stem cells (Sox2$^+$ Ki67$^+$) revealed a prominent decrease (*P*=0.04) in LCN2-null mice SGZ (Figure 1k, l).
Overall, and despite the total number of cells being similar, a consistent increase in quiescent type-1 stem cells (radial and nonradial types) and a prominent depletion of type-2 amplifying progenitor cells was evident in the SGZ of LCN2-null mice, which suggests the existence of a misregulation in cell transition from type-1 to type-2 progenitors when LCN2 is absent, with subsequent impact on neuronal and glial lineages.

LCN2 deletion affects progenitors cycling, promoting cell cycle exit and death

Data presented suggested the requirement of LCN2 for the specific regulation of the NSCs pool maintenance and differentiation, which next lead us to analyse the cell cycle regulation in LCN2-null mice SGZ. The number of cells exiting and re-entering the cell cycle at the SGZ within 24 h after the BrdU pulse were indexed (Figure 2a) and revealed that the proportion of cell cycle exit was

significantly increased (P=0.003) in LCN2-null mice; in contrast, a significant lower proportion of cells were observed to re-entry the cycle (P=0.03; Figure 2b, c, e). Interestingly, a similar regulation of the cell cycle at the SVZ was observed (Supplementary Figure S4a-c).

This increased cell cycle exit in LCN2-null DG could, at least in part, explain the observed decrease in the number of dividing progenitors and, consequently, of newborn mature cells; however, it did not clarify which cells exit the cycle. Quantification of the percentage of $Sox2^+$ cells in cycle showed a clear decrease (P=0.002; Figure 2d, e) when LCN2 was absent, further supporting the idea that LCN2 regulates cell cycle in type-1 stem cells and the transition to type-2 in the DG. Also, LCN2-null mice displayed increased hippocampal cell death, as quantified by the number of positive cells for activated caspase-3 ($Casp3^+$) (P=0.048), and further co-immunostaining analysis revealed that the number of radial type-1 stem cells expressing $Casp3^+$ was significantly increased in LCN2-null mice (P=0.03), when compared to Wt (Supplementary Figure S5a-d). As so, the data presented suggest that the impaired cell genesis in the adult hippocampus in LCN2-null mice is the consequence of a deficient cell cycle regulation and of substantial apoptosis of type-1 NSCs. More importantly, this seems to be due to a lack of an antioxidant regulation at the level of stem cells resultant from the LCN2 absence, since a significant (P=0.009) decrease of glutathione peroxidase-4 (Gpx4) expression by $Sox2^+$ stem cells was observed in LCN2-null mice (Supplementary Figure S5d, e).

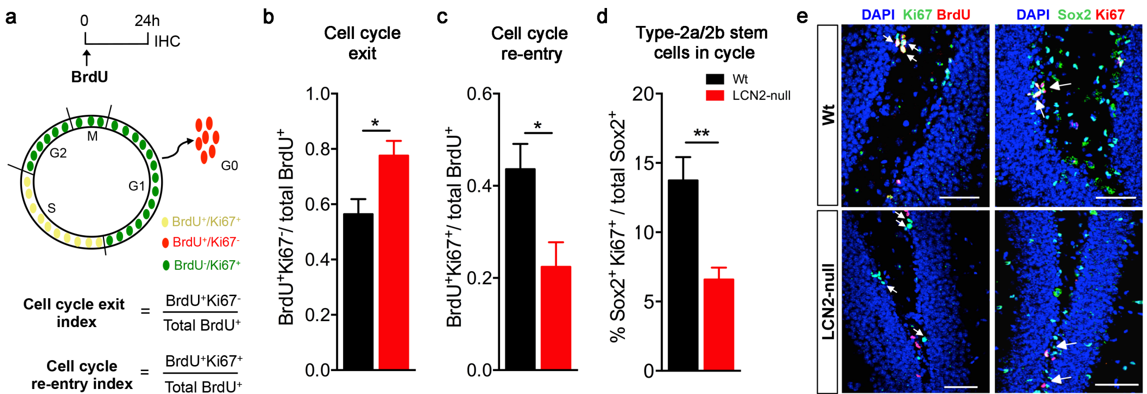

Figure 2. LCN2 deletion impairs the number of cycling DG progenitors by favouring cell cycle exit. (**a**) Schematic representations of the BrdU protocol, of the cell cycle phases that BrdU and Ki67 co-labelling correspond to, and of the indexes used for cell cycle exit and re-entry. (**b**) Increased proportion of cells exiting the cell cycle at the SGZ in the absence of LCN2 (n=5 per group). (**c**) Reduced number of cells re-entering the cell cycle in the SGZ of LCN2-null mice (n=5 per group). (**d**) Decreased percentage of type-2 progenitor cells in cycle at the SGZ (n=5 per group) in the absence of LCN2. (**e**) Representative confocal immunostaining images of BrdU/Ki67 and Sox2/Ki67 co-labelling (indicated by white arrows; scale bar, 100 μm). Data presented as mean ± SEM analysed

by two-tailed Student's *t*-test and are representative of two independent experiments. *$P\leq0.05$, **$P\leq0.01$. See also Supplementary Figure S4.

LCN2-null mice impaired hippocampal neurogenesis affects contextual discrimination behaviour

Consistent with the current knowledge that adult hippocampal neurogenesis contributes to enhance the extent of information encoded by the DG and improves pattern separation and contextual discrimination, the ability of LCN2-null mice to discriminate between overlapping contextual representations in a CFC task was next investigated. Assessment of animals' behaviour during training revealed that animals presented similar percentage of freezing (Block I-III; Figure 3b). Upon re-exposure to the conditioning context, 24 h after the training and with no cue presentation, LCN2-null mice showed a significant ($P=0.004$) decreased percentage of freezing (Figure 3c). Subsequent analysis of contextual discrimination, by exposing the animals to a novel context 2 h later, revealed that LCN2-null mice presented similar levels of freezing behaviour when compared to the training context, while Wt mice presented substantially less freezing behaviour upon exposure to the novel context (Figure 3c). This altered behaviour translated into a lack of context discrimination capacity by LCN2-null mice (discrimination index: $P=0.002$; Figure 3c, d). Overall, this reveals the importance of the observed impaired adult hippocampal neurogenesis in LCN2-null mice, since it translated into deficits in hippocampal function.

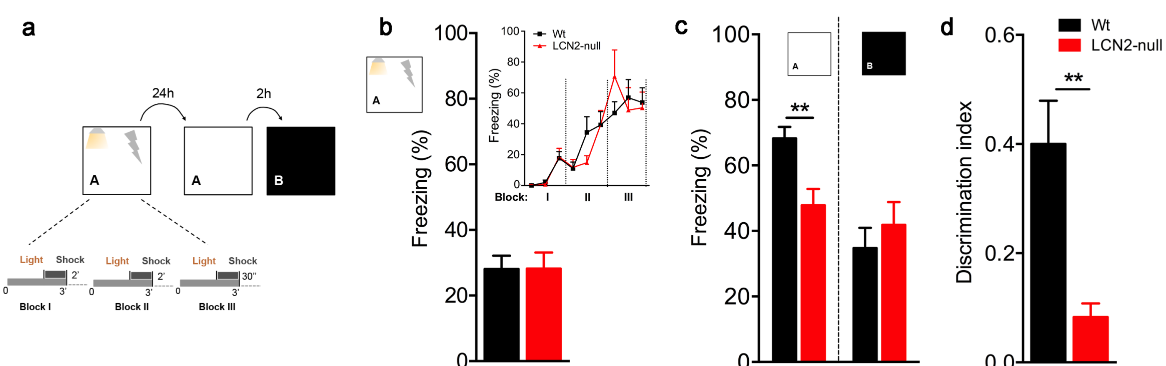

Figure 3. Impaired contextual fear conditioning in LCN2-null mice. (**a**) Experimental scheme of the used paradigm for contextual fear condition test. (**b**) Freezing behaviour during the training session, as total percentage of freezing and of freezing acquired along the trials of shock exposure (Block I-III). (**c**) Impaired contextual retrieval by LCN2-null mice at context A, but similar freezing between genotypes at context B ($n=10$ per group). (**d**) Discrimination index is impaired in LCN2-null mice.

Data are presented as mean ± SEM analysed by two-tailed Student's *t*-test and are representative of two independent experiments. ** $P \leq 0.01$.

The absence of LCN2 impairs in vitro neural stem cell proliferation and self-expansion

In order to mechanistically understand how the absence of LCN2 affects hippocampal precursors' proliferation and survival, with important consequences at the behavioural level, a further detailed characterization was performed using *in vitro* culture assays.

Analysis of BrdU^{+} cells [over total positive nuclei (DAPI^{+})] revealed that LCN2-null-derived neurospheres presented a significant (P=0.03) decrease in the percentage of proliferative cells (Figure 4a, b). Moreover, the clonal neurosphere assay showed that the total number of primary and secondary neurospheres generated from LCN2-null NSCs were significantly lower when compared to Wt (Figure 4c, e). Similarly, analysis of the mean size of the neurospheres revealed that both primary (P=0.03) and secondary neurospheres (P=0.03) obtained from LCN2-null SGZ were smaller (Figure 4d, e).

The effect of LCN2 absence on the self-renewal of SGZ cells was subsequently tested by adhering neurospheres for 48 h under proliferative conditions, and further labelled for Sox2 and BrdU. Quantification of the percentage of Sox2^{+} BrdU^{+} cells revealed a significant decrease in LCN2-null-derived NSCs (P=0.03), thus confirming a significant effect on self-renewal capabilities in NSCs from LCN2-null SGZ (Figure 4f, g). We anticipated that LCN2 exerted such particular modulation through its specific cellular receptor 24p3R[21]. In fact, *in vivo* analysis of 24p3R expression by immunofluorescence at the DG, in Wt animals, revealed that the receptor was present throughout the gyrus and in the hilus (Supplementary Figure S6a, a'). Specifically, additional co-labelling assessments showed that NSCs positive for Sox2 and Nestin (Supplementary Figure S6b, c) co-expressed 24p3R, as well as labelled-retaining BrdU^{+} cells at the SGZ (Supplementary Figure S6d). In addition, analysis of 24p3R expression in cultured neurospheres revealed that NSCs under proliferative conditions express the LCN2 receptor (Supplementary Figure S6e, f).

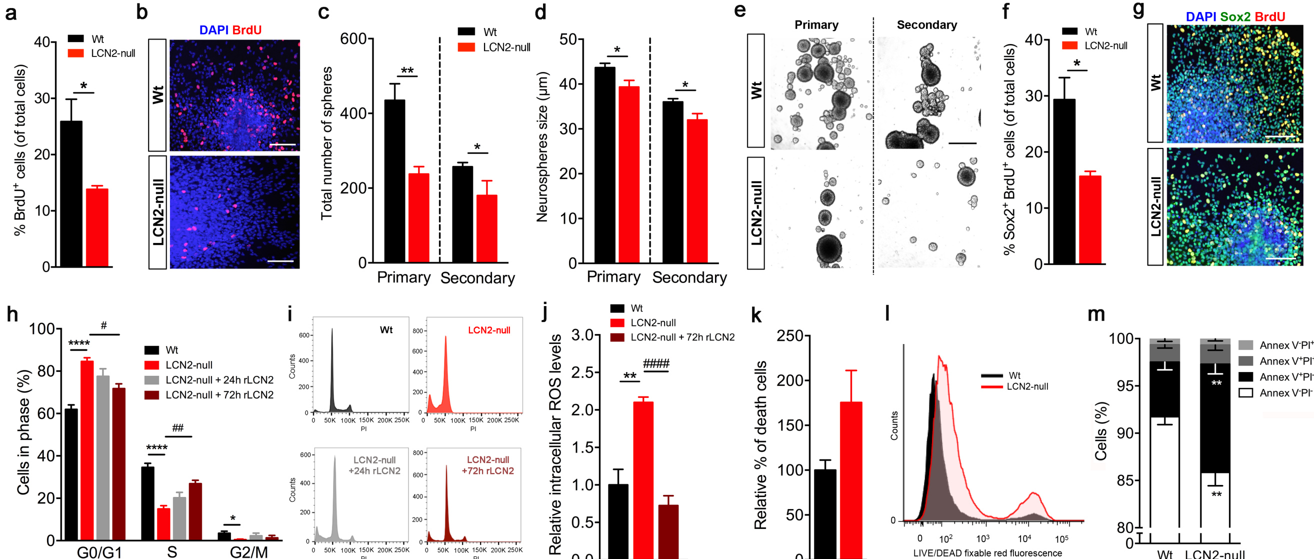

Figure 4. LCN2 is required for neural stem cells G0/G1 cell cycle transition, through the control of endogenous redox cellular status, and its absence impairs proliferation and self-renewal and promotes cell apoptosis. (**a, b**) Reduction of proliferating neurospheres represented by $BrdU^+$ cells/total cells in LCN2-null derived cells. (**c**) Reduction of the number of primary and secondary neurospheres in the absence of LCN2. (**d**) Average diameter of primary and secondary neurospheres. (**e**) Cultured primary and secondary spheres from Wt and LCN2-null SGZ. (**f, g**) NSCs sphere-renewal impaired in LCN2-null as quantified by $Sox2^+$ $BrdU^+$ cells. Scale bars, 100 μm. (**h, i**) Increased percentage of NSCs at G0/G1 phase of the cell cycle in LCN2-null mice, rescued upon delivery of exogenous rLCN2 protein. (**j**) Two-fold increased levels of intracellular ROS in LCN2-null derived neurospheres, restored upon rLCN2 treatment. (**k, l**) Live and dead assay by flow cytometry. (**m**) Increased NSCs apoptosis in LCN2-null mice. Data are presented as mean ± SEM, analysed by two-tailed Student's *t*-test and are representative of three independent experiments. **P*≤0.05, ***P*≤0.01, *****P*≤0.0001 for Wt *versus* LCN2-null; #*P*≤0.05, ##*P*≤0.01, ####*P*≤0.0001 for treatment *versus* LCN2-null alone. rLCN2, recombinant LCN2.

LCN2 regulates NSCs endogenous levels of ROS, which is required for a proper cell cycle progression

To assess the influence of LCN2 in neurospheres cell cycle progression, the proportion of NSCs at the different cell cycle phases were analysed by flow cytometry. After labelling 7 days-old cultured neurospheres with propidium iodide, the analysis of the DNA content in each genotype revealed that the percentage of cells at G0/G1 phase was significantly ($P<0.0001$) higher in LCN2-null cells (Figure 4h, i). In accordance, a significant ($P<0.0001$) decrease in the proportion of cells at S phase was observed, as well as for the G2/M phase ($P=0.01$; Figure 4h, i).

Interestingly, when LCN2-null neurospheres were treated with exogenous recombinant LCN2

(rLCN2) for 24 and 72 h, we found that 72 h of treatment significantly (P=0.02) decreased the percentage of cells at G0/G1, when compared to LCN2-null neurospheres alone, and that it allowed NSCs cell cycle progression, since it significantly (P=0.008) increased the percentage of cells at S phase (Figure 4h, i). These observations did not, however, provide information on how LCN2 determines the control of NSCs cell cycle progression. In recent years, the modulation of cellular redox states has been suggested to be important for the balance between self-renewal and differentiation in progenitor cells. Of notice, and consistent with the idea of LCN2' role in iron trafficking [19, 20], we further considered the putative contribution of LCN2' absence to a ROS-dependent cell cycle arrest and death. In fact, analysis of intracellular ROS levels in NSCs revealed a significant (P=0.03) increase in LCN2-null derived cells, which was successfully reverted by the addition of rLCN2 for 72 h (Figure 4j). Moreover, we observed that the overproduction of ROS in the absence of LCN2 sensitized NSCs to cell death, which was demonstrated by the increase in the percentage of non-viable cells in LCN2-null cells (P=0.06; Figure 4k, l) and of the percentage of apoptosis (Annexin V^+PI^-; P=0.002) (Figure 4m). Moreover, *in vivo* treatment of LCN2-null mice for 24 h with the antioxidant agent NAC (300 mg/kg; [29]) significantly reduced (P=0.02) the levels of $Casp3^+$ in the SGZ, when compared to the saline control group (Supplementary Figure S5h), which suggests that ROS accumulation in the absence of LCN2 is the likely inducer of the observed increased cell death.

Oxidative damage induced by LCN2 absence is iron-mediated

Recognizing that excessive intracellular iron promotes oxidative stress, the connection between iron regulation and ROS, by LCN2 and specifically in NSCs, was next studied. For that, NSCs intracellular iron levels were depleted by adding the membrane-permeable iron chelator DFO to LCN2-null neurospheres for 24 h [2 μM; [30]]. Noticeably, relative to LCN2-null cultures alone, DFO treatment significantly (P<0.0001) reduced the intracellular levels of ROS in neurospheres, to levels similar to those occurring in Wt animals (Figure 5a). Additionally, cell cycle analysis in LCN2-null neurospheres treated with DFO and labelled with propidium iodide, revealed that DFO significantly decreased the percentage of cells arrested at G0/G1, when compared to LCN2-null neurospheres alone (P=0.004; Figure 5b, c), and allowed the progression of NSCs in the cycle, since it increased the percentage of cells at S (P=0.09) and G2 (P=0.02) phases.

These results suggest that intracellular iron accumulation in NSCs, due to the absence of LCN2, is the key underlying mechanism promoting oxidative stress and cell cycle arrest. In fact, and interestingly, treatment of cultured Wt NSCs with an iron source (FAC) for 24 h [500 μM; [31]] similarly

induced a significant (*P*=0.02) increase in the production of ROS, when compared to control non-treated cultures (Supplementary Figure S7a). Importantly, this iron-mediated imposed oxidative stress also induced cell cycle arrest, as FAC significantly increased the proportion of cells at G0/G1 phase (*P*=0.007), not allowing for cells to progress in cycle (S phase: *P*=0.01; Supplementary Figure S7b, c), which largely resembles the phenotype observed to occur in the absence of LCN2.

Moreover, *in vivo* analysis of cell cycle indexes of exit and re-entry, after DFO treatment for 24 h [200 μM; [32]], showed that iron chelation was sufficient to improve progenitors cell cycle regulation in the SGZ of LCN2-null mice (treatment effect: $F_{1,13} = 19.3$, $P<0.0001$). Specifically, DFO significantly normalized cell cycle exit in LCN2-null SGZ (*P*=0.0005), promoting progenitors cell cycle re-entry (*P*=0.0007), to indexes similar to those occurring in Wt animals (Figure 5d-f). In fact, a significant increase in cell proliferation was observed in LCN2-null mice DG after treatment with DFO, of both $BrdU^+$ (treatment effect: $F_{1,14} = 20.5$, $P=0.0005$) and $Ki67^+$ cells (treatment effect: $F_{1,15} = 10.9$, $P=0.005$; Supplementary Figure S7a, b), with an additional prominent decrease (*P*=0.03) in the number of $Casp3^+$ cells in the SGZ of LCN2-null mice (Supplementary Figure S8c). Importantly, the additional chronic administration of DFO for 28 days significantly improved the performance of LCN2-null mice in the CFC. The observed decreased percentage of freezing presented by LCN2-null mice was significantly improved after DFO treatment (*P*=0.02), when compared to saline treated LCN2-null animals (Figure 5g). Moreover, context discriminatory indexes were also recovered in LCN2-null mice after DFO treatment (*P*=0.08; Figure 5h).

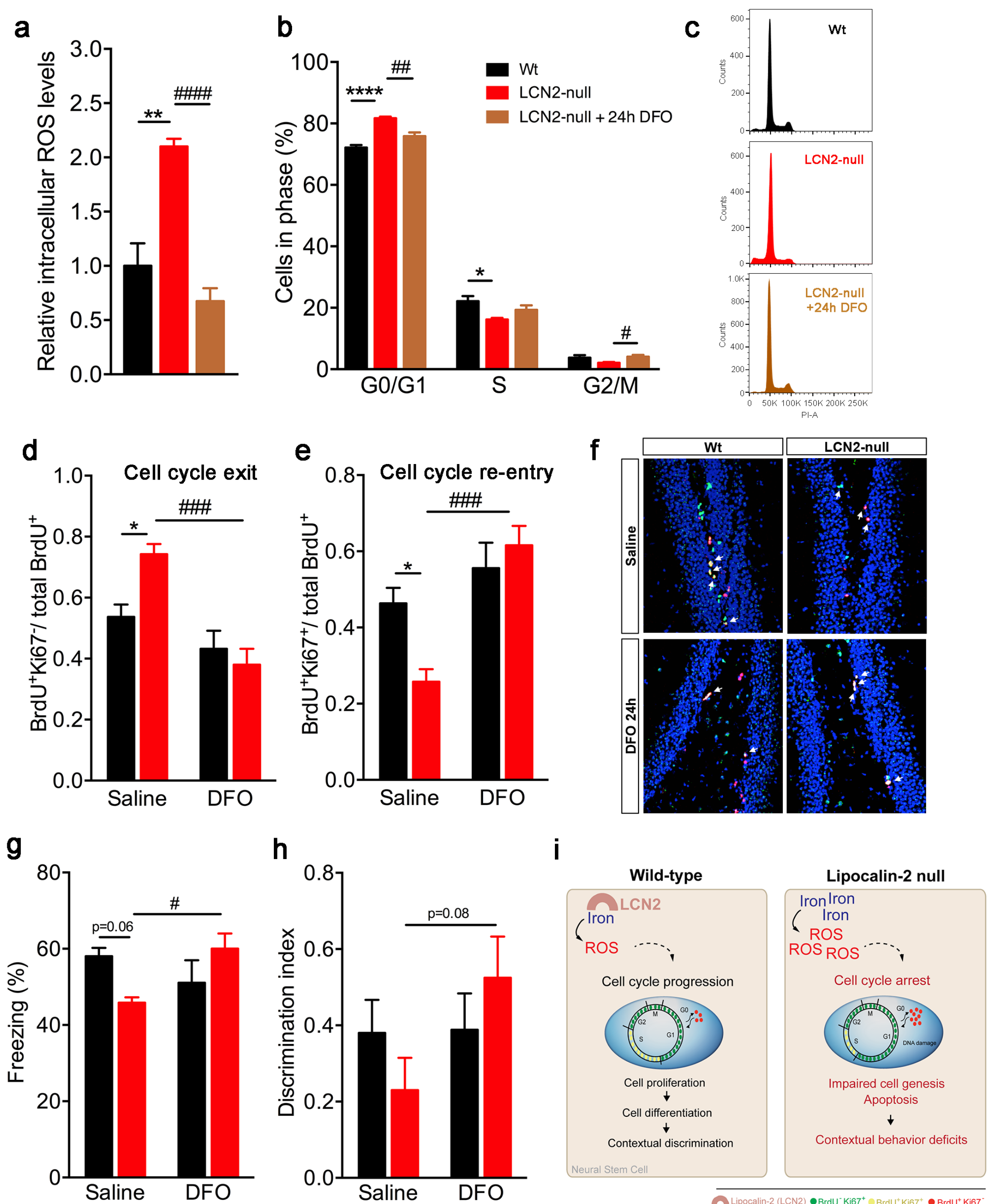

Figure 5. LCN2 absence imposed oxidative damage and cell cycle arrest is dependent on intracellular iron regulation. (**a**) Increased oxidative damage of LCN2-null NSCs is rescued by DFO. (**b, c**) Cell cycle arrest in LCN2-null mice derived neurospheres is restored after DFO treatment. (**d, e**) *In vivo* impaired cell cycle exit and re-entry in LCN2-null SGZ is normalized after the DFO treatment. (**f**) Representative confocal immunostaining images of BrdU/Ki67 co-labelling (indicated by white arrows; scale bar, 50 μm). (**g**) Impaired contextual retrieval by LCN2-null mice at context A restored upon DFO chronic treatment (n=6 per group). (**h**) Discrimination index is improved after DFO in LCN2-null mice. (**i**) Proposed mechanism for the role of LCN2 in the control of NSCs cell cycle progression through intracellular iron regulation and ROS signalling, for contextual discriminative behaviours. Data are presented as mean ± SEM and are representative of two

independent experiments. (**a, b**) two-tailed Student's *t*-test; *$P \leq 0.05$, **$P \leq 0.01$, ****$P \leq 0.0001$, Wt *versus* LCN2-null; #$P \leq 0.05$, ##$P \leq 0.01$, ####$P \leq 0.0001$, treatment *versus* LCN2-null alone. (**d, e, g, h**) two-way ANOVA analysis with Bonferroni's multiple comparison test; *$P \leq 0.05$ *versus* saline Wt; #$P \leq 0.05$, ###$P \leq 0.001$ *versus* saline LCN2-null mice.

Discussion

This study provides *in vivo* and *in vitro* evidence for a novel role for LCN2 as a modulator of neurogenesis in the adult mammalian brain. It shows that, *in vivo*, LCN2 deletion induces deficits in neural progenitors proliferation, differentiation and maturation, with impact on contextual discriminative behaviours. Specifically, the absence of LCN2 leads to an accumulation of type-1 (radial and non-radial) stem cells, due to a misregulation of the cell cycle, with a higher proportion of progenitor cells exiting the cycle towards apoptotic cell death. Moreover, NSCs cultured *in vitro* allowed to mechanistically disclose that the deletion of LCN2 significantly induces endogenous oxidative stress, cell cycle arrest and death, an effect that is iron-mediated since it is restored by the addition of the iron chelator DFO. Altogether, the data presented imply that LCN2 is required to control NSCs redox status, functioning as an antioxidant agent in the maintenance of a proper oxidative environment for cell cycle progression and stem cells' self-renewal. This mechanism of cell cycle regulation is crucial for hippocampal integrity and function, as the absence of LCN2 impacts on contextual fear discriminative behaviours.

Iron is an essential element required by almost all organisms, playing critical physiological roles that include oxygen transportation, energy metabolism and cellular proliferation. On the other hand, iron is a highly reactive element, having a catalytic role in a multitude of redox reactions [13]. Iron homeostasis dysfunction is the major source of oxidative stress via the generation of ROS by the Fenton's reaction and the aberrant cellular accumulation of iron, ROS, or both, is linked to DNA damage, cell cycle arrest and death [13]. In recent years, LCN2, primarily considered to be exclusively an innate immune protein, was shown to have multifaceted roles in cellular iron transport and homeostasis [19, 21, 23]. Importantly, the isolation and identification of 24p3R by Devireddy and colleagues (2005) paved the way to propose the existence of an iron delivery mechanism that is LCN2-mediated and transferrin-independent [19, 21]. Specifically, it was proposed that upon binding to 24p3R, iron-containing LCN2 is internalized, traffics to the endosomes being iron released from LCN2, thereby increasing intracellular iron [21]. On the other hand, the binding and internalization of iron-free LCN2 chelates intracellular iron and may transfer it to the extracellular milieu, thus reducing intracellular iron [21]. In line with this, herein we propose LCN2 as an iron sequester in NSCs, with impact on cell cycle regulation and cell dead and, ultimately, animal behaviour. Importantly, LCN2-mediated iron delivery to NSCs is most likely occurring by its internalization through the specific 24p3R, shown here to be present in neurospheres and stem cells *in vivo*. In fact, previous studies in kidney cells demonstrated the internalization of LCN2 and its localization in vesicles

scattered throughout the cytoplasm, and in distinct intracellular compartments of that of internalized transferrin [19]. This is a mechanism that should be next further studied in the context of NSCs. Still, altogether, it is increasingly clearer that LCN2 seems to actively participate in cell physiology in several organs.

The regulation of NSCs physiology by LCN2 was primarily observed by the loss of general proliferation rates in the absence of LCN2, when using both BrdU and Ki67 proliferative markers. Moreover, the absence of LCN2 was sufficient to induce the accumulation, expansion and apoptosis of type-1 stem cells (radial and non-radial type of cells), with a subsequent reduction in the proliferation of type-2 and of cell genesis commitment, maturation and integration. Specifically, cell cycle transition from G1 to S phase was compromised in LCN2-null mice, with NSCs being arrested at G0/G1 phase and exiting more the cell cycle. Mechanistically, the current observations support the notion that lack of LCN2 in NSCs leads to endogenous iron accumulation, which in turn increases intracellular ROS, thus creating an oxidative environment that is boosting cell cycle arrest and cell death. Actually, the observation that treatment of LCN2-null mice with the well-known antioxidant agent NAC [29, 33] decreases apoptotic cell death clearly indicates that ROS, increased as the consequence of intracellular iron accumulation by the absence of LCN2, is the likely inducer of cell death in the DG of LCN2-null mice. This is a novel mechanism of NSC commitment that, ultimately, impairs animal behaviour, as shown here (Figure 5i). Consistent with the known ability of LCN2 to traffic iron within the cells, the *in vitro* reversion of the oxidative environment and of cell cycle progression with DFO treatment, along with the *in vivo* prevention of an exacerbated cell cycle exit and apoptotic cell death at the SGZ, as well as the improvement in the contextual behaviour, strengths such hypothesis. This finding is novel as it shows that LCN2 acts in the regulation of endogenous ROS levels in NSCs by controlling its cellular content. Also, and since the addition of rLCN2 restores ROS levels and promotes cell cycle progression, it is possible to assume that this treatment, likewise, restores endogenous levels of iron in LCN2-null neurospheres. Interestingly, and of notice, others have shown that LCN2-null mice have increased intracellular [34] and serum [32] iron levels, which renders them more susceptible to LPS-mediated oxidative stress [32]. In addition, DFO treatment in LCN2-null mice was effective in rescuing LPS-induced toxicity, including the reduction of cleaved caspase-3 levels [32]. It is possible that, also in the brain and specifically in the neurogenic niches, LCN2-null mice accumulate iron in neural progenitors, which is impacting on ROS levels and on the normal progress of cell cycle and adult neurogenesis sequence. Importantly, the fact that the addition of iron to Wt NSCs induced oxidative stress and cell cycle arrest, thus mimetizing the

impairments observed in NSCs of LCN2-null mice, strengths this hypothesis.

Of interest, LCN2 has been extensively described in the brain to be mainly expressed in response to injury or inflammation, so it can engage critical roles in the progression and establishment of inflammation [35]. Particularly, others have shown LCN2 secretion by astrocytes to target and modulate microglia activation and polarization into M1 phenotype, and also to target astrocytes in an autocrine fashion manner for pro-inflammatory activation [35]. Interestingly, astroglial cells can sharply affect neurogenesis, through the release of inflammatory cytokines, by regulating progenitors proliferation, differentiation and the survival of new adult-born neurons [36]. Noticeably, LCN2 could, through this route, and in case of neuroinflammation, impact on neurogenesis by regulating progenitors proliferation and differentiation, and the survival of new adult-born neurons. Still, herein, we believe that, in physiological conditions, regulation of adult neurogenesis by LCN2 may arise from the external systemic environment. While in the physiological brain most reports are unanimous in not detecting LCN2 expression [27, 28, 37], with the exception for a few conflicting reports [24], LCN2 is described to be highly present in the serum at physiological conditions [38], in accordance with our current observations. As so, it is possible that regulation of adult neurogenesis by LCN2 may occur through its delivery by the blood vessels located within the niche and that surround neural progenitors. Of notice, others have shown, particularly in aging, that the systemic milieu and its circulating factors can regulate neurogenesis and cognitive function [39]. Future studies should consider the potential role of the cells at the periphery that produce LCN2 (e.g. neutrophils, hepatocytes) in the regulation of brain cell genesis, and even of the cells in the central nervous system (e.g. neurons, astrocytes), despite the production of LCN2 in the brain, under basal conditions, being at levels below detection with the current available methods.

Stem cells usually reside in an environment of reduced redox status, which favours self-renewal and differentiation [40], highlighting the importance of a proper redox balance. In line with this, and in case of oxidative stress, all cells have a complex antioxidant system to detoxify endogenous ROS [41]. The glutathione peroxidase group of antioxidant enzymes acts in concert to remove ROS and, among them, Gpx4 is considered the primary enzymatic defence system against oxidative damage [41]. In line with this, we assessed the number of neural stem precursors Sox2$^+$ that expressed Gpx4. The fact that significantly less Sox2$^+$ cells in LCN2-null mice DG co-expressed Gpx4, along with the *in vitro* data of increased intracellular levels of ROS in NSCs, confirms the inefficient antioxidant regulatory system of LCN2-null mice. This is, most likely, contributing to further impaired hippocampal function in contextual fear conditioning tasks, as the ultimate consequence of impaired cell differentiation.

This imbalanced redox regulation in LCN2-null mice, resultant of iron trafficking misregulation, can also be the critical mediator of previous observed impairments in hippocampal plasticity and animal behaviour [25]. Specifically, we have previously shown that LCN2-null mice display anxious- and depressive-like behaviours and spatial learning impairments, along with an altered hippocampal neuronal cytoarchitecture and synaptic plasticity [25].

Importantly, herein we cannot exclude the limitation of a full knockout system in disclosing important regulatory mechanisms of adult neurogenesis. LCN2 is certainly not the only key mediator of the process we here describe. The present data supports that a further understanding on whether LCN2 acts in a context- and cellular-dependent manner, through the use of conditional-null models, will provide clues on whether its modulation may impact neurogenesis in health and in disease. Nevertheless, and in summary, the present study provides the detailed sequence of events for the role of LCN2 as a novel modulator of iron-mediated oxidative stress in the orchestration of neurogenesis for hippocampal plasticity and behavioural function. As such, these findings add an important piece into the current knowledge on the mechanisms modulating adult mammalian neurogenesis.

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Competing Financial Interests

The authors have no conflicting financial interests to declare.

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Supplementary information

Supplementary figures

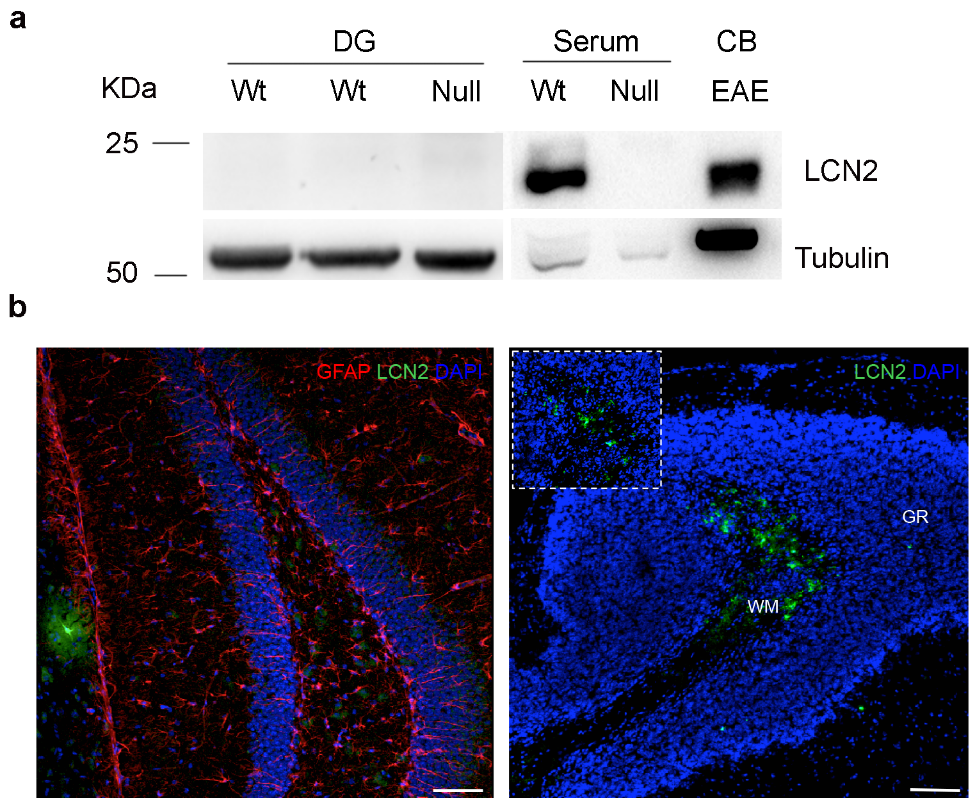

Figure S1. Expression of LCN2 in the DG in physiological conditions. (**a**) Western blot analysis of LCN2 protein expression in DG lysates and in the serum revealed that LCN2 is present only in the serum of Wt mice. Expression in the cerebellum of an animal model of multiple sclerosis was used as positive control, and tubulin as loading control. (**b**) Immunofluorescence labelling for LCN2 (green), and GFAP (red), revealed no staining in the DG under physiological conditions. Staining for LCN2 in the cerebellum of an animal model of multiple sclerosis was used as a positive control, and observed to be restricted to the white matter. Scale bars, 100 μm. CB, cerebellum; DG, dentate gyrus; EAE, Experimental autoimmune encephalomyelitis; GR, granular layer; WM, white matter.

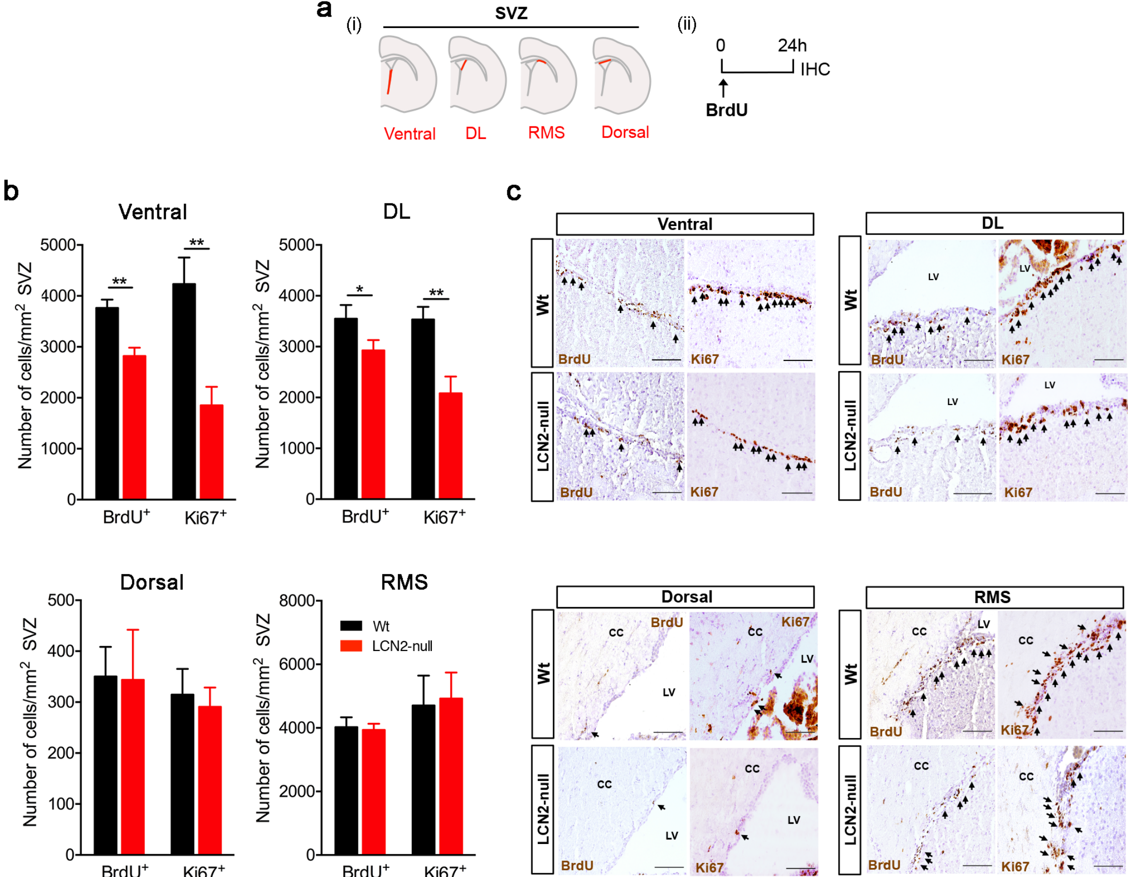

Figure S2. Decreased cell proliferation at the SVZ of LCN2-null mice. (**a**) Schematic diagrams of (i) the sub-divisions considered in the SVZ along the dorsal-ventral axis and (ii) of the BrdU protocol. (**b**) Analysis of the number of proliferating cells, BrdU^{+} and Ki67^{+}, in each sub-division of the SVZ. (**c**) Representative images of the BrdU and Ki67 immunostaining in the different considered sub-divisions of the SVZ, revealing the differences of cell proliferation between the two genotypes (indicated by black arrows; scale bar, 50 μm). Data represented as mean ± SEM and are representative of two independent experiments. Significance was calculated by two-tailed Student's *t*-test. *$P \leq 0.05$, **$P \leq 0.01$. CC, corpus callosum; DL, dorsolateral; IHC, immunohistochemistry; LV, lateral ventricle; RMS, rostral migratory stream; SVZ, subventricular zone.

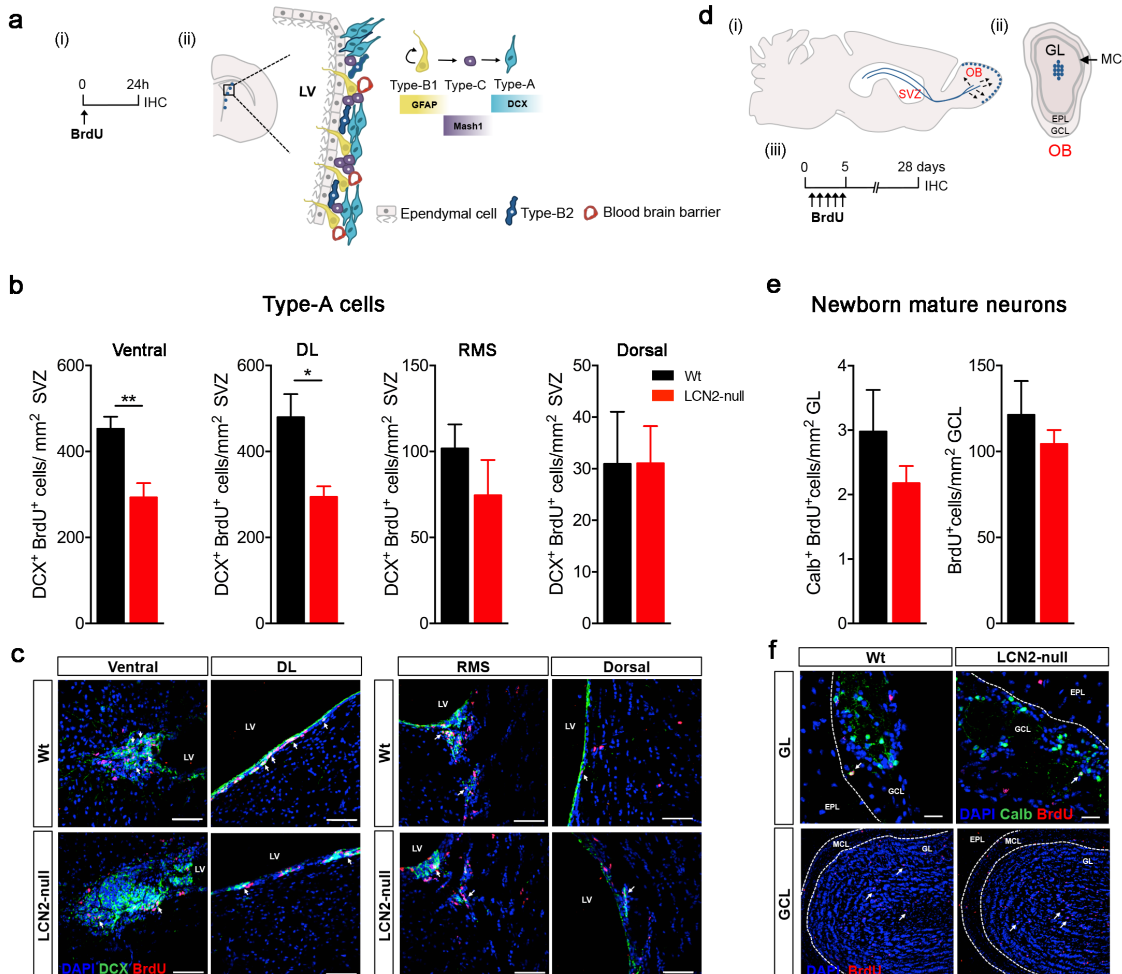

Figure S3. Lack of LCN2 leads to impaired number of proliferating neuroblasts at the SVZ, but not on neuronal integration in the olfactory bulb (OB). (**a**) Representative scheme of the (i) experimental protocol of BrdU used and (ii) illustration of the SVZ neurogenic process, including the cell types and specific cellular markers. (**b**) Analysis of the number of proliferating neuroblasts (DCX$^+$ BrdU$^+$), along the dorsal-ventral axis of the SVZ, showed a significant decrease in LCN2-null mice at the ventral (P<0.01) and DL sub-divisions (P<0.05). (**c**) Representative confocal images of proliferating neuroblasts, considering the sub-divisions of the SVZ along the dorsal-ventral axis, in which the differences of the number of proliferating neuroblasts between the two genotypes are evident (indicated by white arrows; scale bar, 100 µm). (**d**) Schematic illustrations of (i) a sagittal view of the SVZ neurogenesis, from the lateral ventricle through the RMS to the OB, (ii) of the coronal view of the OB layers and (iii) of the BrdU labelling protocol used. (**e**) Quantification of the number of newborn mature neurons in the OB, after migration from the SVZ, as Calb$^+$ BrdU$^+$ cells, per GL area, revealed no major significant differences between genotypes. The number of migratory BrdU$^+$ cells that arrived to the GCL did not significantly differ between Wt and LCN2-null mice. (**f**) Confocal representative images of the terminally differentiated newborn neurons at the GL, and of the migratory BrdU$^+$ cells arriving at the GCL through the RMS (indicated by white arrows; scale bar, 50 µm). Data represented as mean ± SEM and are representative of two independent experiments. Significance was calculated by two-tailed Student's t-test. *P≤0.05, **P≤0.01. DL, dorsolateral; EPL,

external plexiform layer; GCL, granular cell layer; GL, glomerular layer; IHC, immunohistochemistry; LV, lateral ventricles; MCL, mitral cell layer; OB, olfactory bulb; RMS, rostral migratory stream; SVZ, subventricular zone.

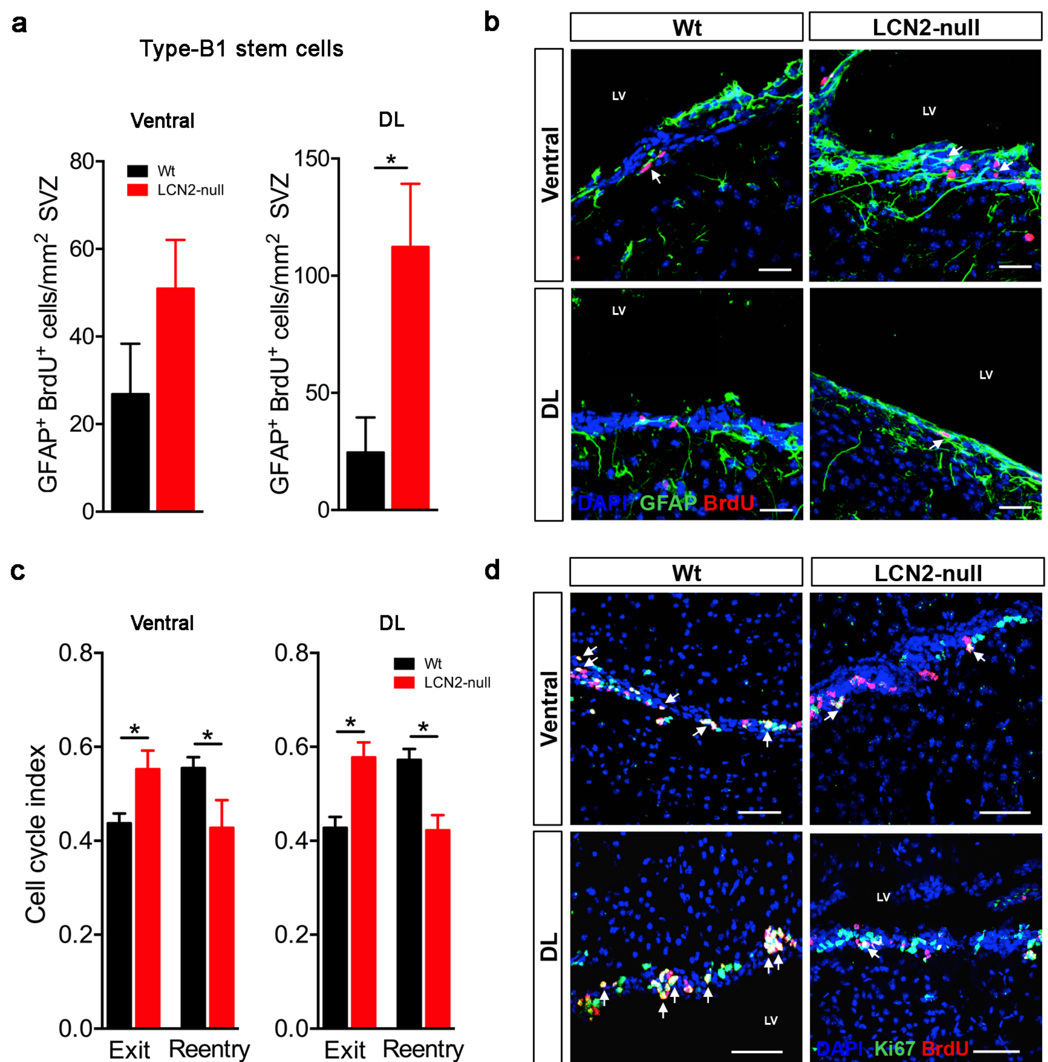

Figure S4. At the ventral and DL sub-divisions of the SVZ, the absence of LCN2 increases the number of type-B1 stem cells and promotes progenitors cell cycle exit. (**a**) Analysis and quantification of the total number of radial glia-like type-B1 stem cells (GFAP+ BrdU+) at the ventral and DL of the SVZ revealed a significant increase in this specific population in the absence of LCN2 ($P<0.05$). (**b**) Representative confocal images of type-B1 stem at the ventral and DL SVZ (identified by white arrows; scale bar, 100 μm). (**c**) The proportion of cells exiting the cell cycle after a 24 h pulse of BrdU is significantly increased in LCN2-null mice ($P<0.05$); accordingly, fewer cells re-entered the cell cycle, both on the ventral and DL sub-divisions of the SVZ. (**d**) Confocal representative images of the BrdU and Ki67 co-labelling at the ventral and DL SVZ, showing the differences in cell cycle indexes between Wt and LCN2-null mice (identified by the white arrows; scale bar, 100 μm). Data represented as mean ± SEM and are representative of two independent experiments. Significance was calculated by two-tailed Student's *t*-test. *$P\leq0.05$. DL, dorsolateral; LV, lateral ventricles; SVZ, subventricular zone.

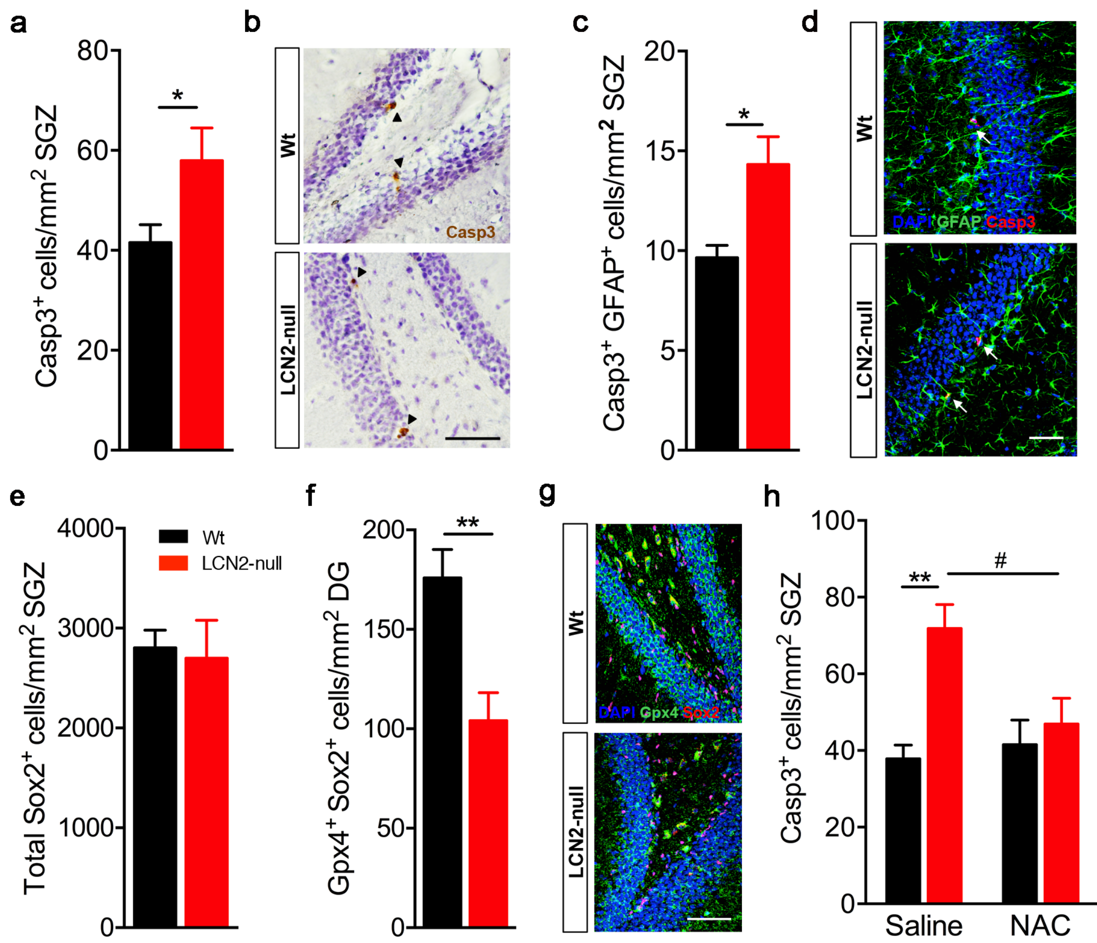

Figure S5. LCN2 deletion increases the rates of apoptotic cell death at SGZ and affects antioxidant regulation in neural stem cells. (**a**) Cellular quantification of the number of activated caspase-3+ cells revealed higher apoptotic cell death in the SGZ of LCN2-null mice, compared to Wt ($P<0.05$). (**b**) Representative images of activated caspase-3 immunostaining in the SGZ of Wt and LCN2-null mice (indicated by black arrowheads; scale bar, 150 µm). (**c**) Co-immunostaining of activated Casp3+ cells with GFAP showed increased apoptosis of type-1 radial glia-like stem cells in the SGZ of LCN2-null mice. (**d**) Representative confocal images of GFAP and Casp3 co-labelling at the SGZ (scale bar, 50 µm). (**e**) Total number of Sox2+ in the SGZ of LCN2-null mice is similar to the Wt littermate controls. (**f**) Quantification of the number of Sox2+ NSCs that co-express the antioxidant enzyme Gpx4 showed a significantly ($P<0.01$) impaired antioxidant regulation in the absence of LCN2. (**g**) Representative confocal images of Sox2 and Gpx4 co-labelling at the DG (scale bar, 50 µm). (**h**) Quantification of Casp3+ cells in the SGZ of Wt and LCN2-null mice revealed a significant decrease in apoptotic cell death after an acute treatment with NAC for 24 h. Data represented as mean ± SEM and are representative of two independent experiments. Significance was calculated by two-tailed Student's *t*-test, *$P\leq0.05$, **$P\leq0.01$; and by two-way ANOVA analysis with Bonferroni's multiple comparison test, *$P\leq0.05$ *versus* saline Wt; #$P<0.05$, *versus* saline LCN2-null mice. Casp3, caspase-3; DG, dentate gyrus; SGZ, subgranular zone.

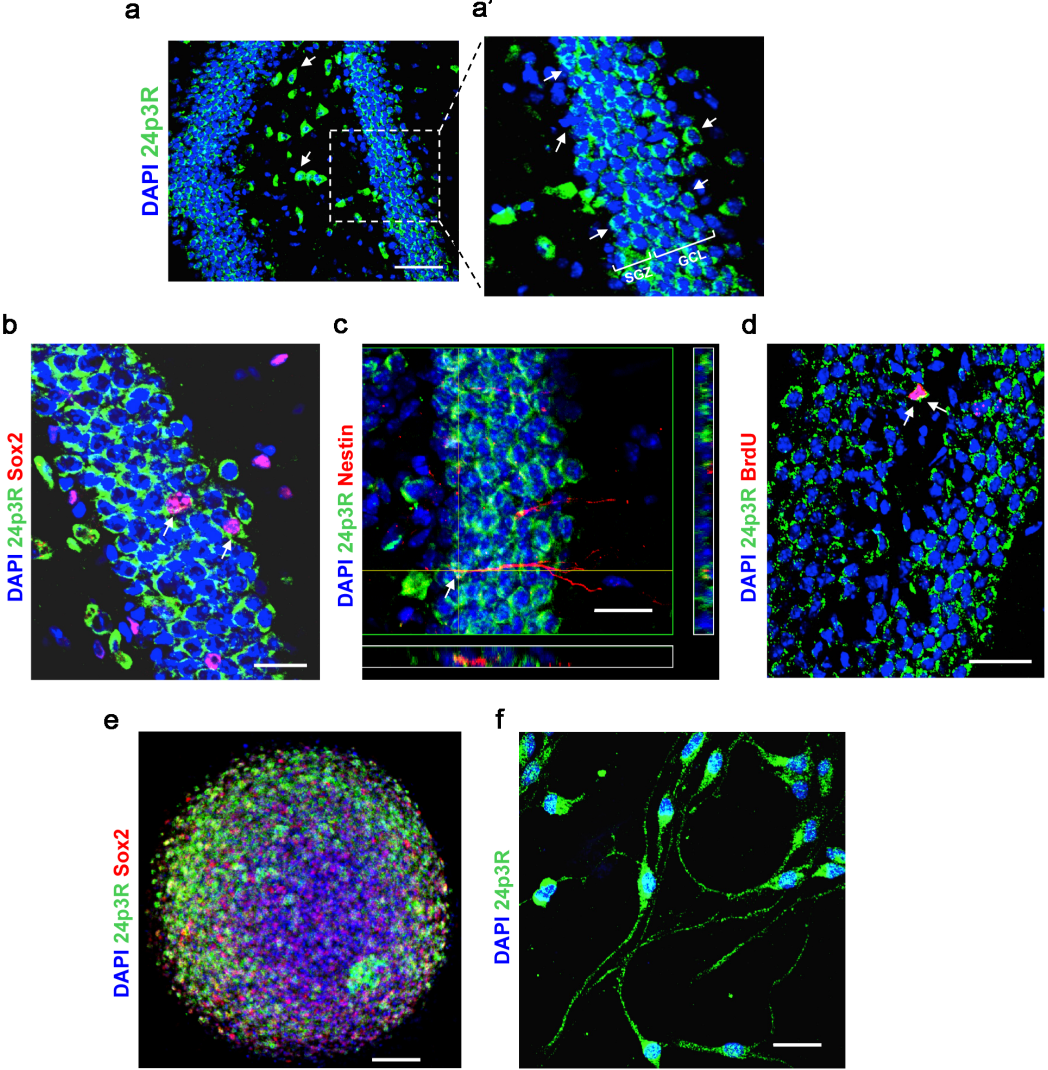

Figure S6. 24p3R is expressed in the DG and by neural stem cells. (**a**) Immunohistochemistry for 24p3R revealed its expression in the DG of Wt animals, at the hilus, SGZ and GCL (indicated by white arrows; scale bar, 150 μm). (**a'**) Detailed expression of 24p3R in the DG. Co-labelling studies demonstrated that NSCs express the LCN2' receptor, specifically Sox2+ (**b**) and Nestin+ cells (**c**). Also, label-retaining BrdU+ cells at the SGZ of the DG express 24p3R (**d**) (indicated by white arrows; scale bar, 150 μm). (**e** and **f**) *In vitro* analysis of 24p3R expression, using Wt hippocampal-derived neurospheres, confirmed the presence of the specific receptor for LCN2 in NSCs cultured under proliferative conditions. GCL, granular cell layer; SGZ, subgranular zone.

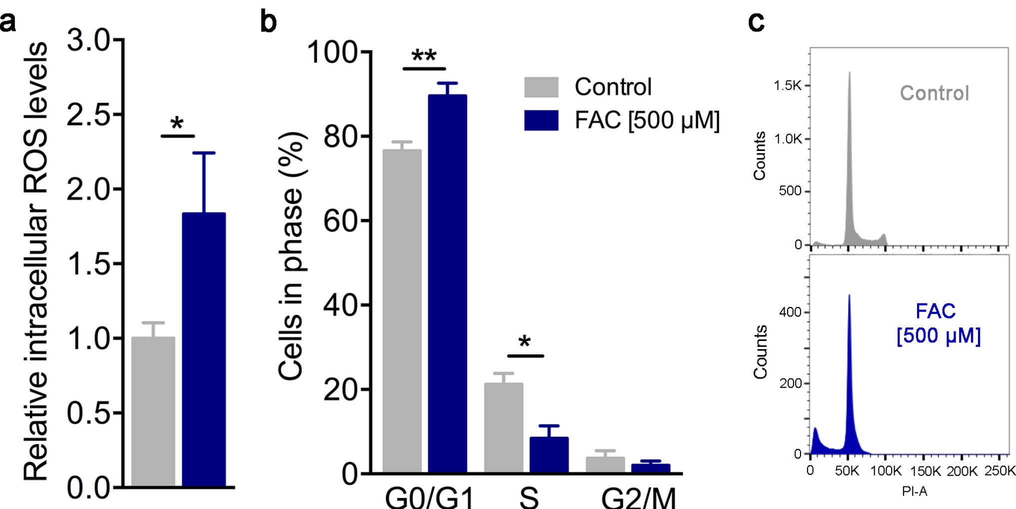

Figure S7. Iron addition to Wt neural stem cells induces oxidative stress and cell cycle arrest. (**a**) Treatment of cultured Wt neurospheres with FAC for 24 h induces almost 2-fold increase in the production of intracellular ROS. (**b, c**) Cell cycle arrest of NSCs induced by treatment with FAC. Data are presented as mean ± SEM, analysed by two-tailed Student's *t*-test and are representative of two independent experiments. *$P \leq 0.05$, **$P \leq 0.01$. FAC, ferric ammonium citrate.

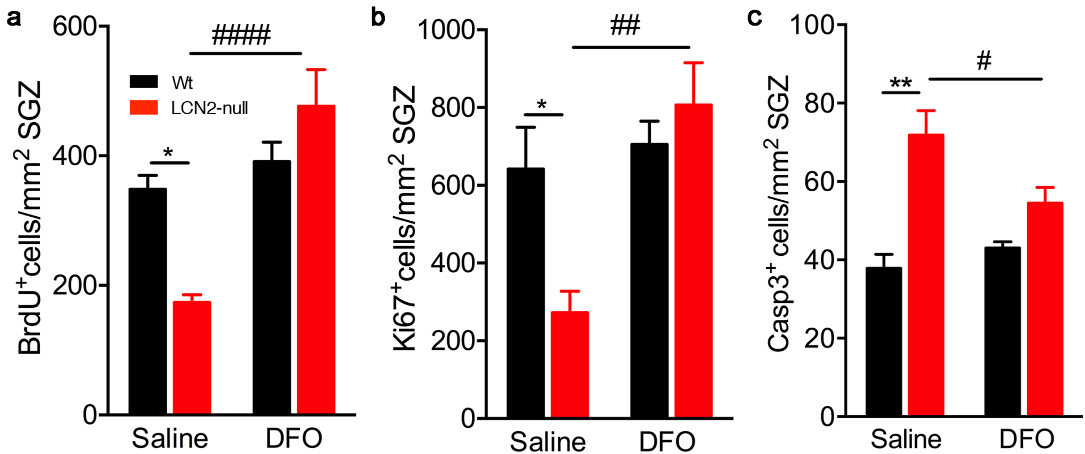

Figure S8. Impaired cell proliferation and death at the SGZ of LCN2-null mice is restored by deferoxamine treatment. (**a**) Treatment of LCN2-null mice with DFO for 24 h normalizes the impaired number of BrdU-proliferating cells, and (**b**) of Ki67+ cells. (**c**) The number of Casp3+ cells in the SGZ of LCN2-null mice is reduced upon DFO treatment. Data represented as mean ± SEM and are representative of two independent experiments. Significance was calculated by two-way ANOVA analysis with Bonferroni's multiple comparison test; $*P \leq 0.05$ *versus* saline Wt; $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P \leq 0.001$ *versus* saline LCN2-null mice. SGZ, subgranular zone; DFO, deferoxamine; Casp3, caspase-3.

Supplementary experimental procedures

Experiments were performed in 2-months old males mice lacking the expression of LCN2 (LCN2-null) and the respective Wt littermate controls in a C57BL/6J mouse background. Mice were housed and maintained accordingly with the guidelines for the care and handling of laboratory animals in the Directive2010/63/EU of the European Parliament and of the Council, in a controlled environment at 22–24°C and 55% humidity, on 12 h light/dark cycles and fed with regular rodent's chow and tap water *ad libitum*. All animal procedures were conducted in accordance with the Portuguese national authority for animal experimentation, *Direção Geral de Alimentação e Veterinária* (ID: DGAV9457).

BrdU labelling for proliferation and label retaining cells assessment

With the purpose to assess proliferation of fast progenitors cells at the SVZ and the SGZ, Wt and LCN2-null mice were intraperitoneally (i.p.) injected once with 50 mg/kg of the thymidine analogue bromodeoxyuridine (BrdU; Sigma Aldrich, St. Louis, MO, USA) and sacrificed 24 h later. Similarly, and in order to label the quiescent pool of stem cells, an additional group of mice of both genotypes were i.p. injected with BrdU twice a day for 5 consecutive days, with an interval of 12 h between injections, followed by a chase period of 28 days. This allows the analysis of the progeny of stem cells that exit the cell cycle and retain the BrdU labelling during the chase period, and the differentiation of the progenitors.

Tissue preparation and immunohistochemistry

Mice were deeply anesthetized with ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg) and perfused with ice-cold 0.9% saline solution, followed by ice-cold 4% paraformaldehyde. Brains were removed, postfixed for 1 h in 4% paraformaldehyde, cryoprotected in a 30% sucrose solution overnight, embedded in optimal cutting temperature compound, snap-frozen and cryopreserved at -20°C before sectioning. Posterior serial coronal sections (20 μm) were cut in a cryostat and collected to slides for immunohistochemistry.

For BrdU imunostaining, a pretreatment of the sections for DNA denaturation with HCl for 30 min at room temperature (RT) was performed. For stereological analysis of the number of proliferating cells in the SVZ and SGZ, primary antibodies diluted in PBS were used: BrdU (1:50, mouse anti-bromodeoxyurine, Clone Bu20a, DAKO, Barcelona, Spain) and Ki67 (1:100, rabbit polyclonal antibody, Novocastra, Newcastle, UK). In parallel, anti-activated caspase-3 (1:200, rabbit polyclonal

antibody, Cell Signalling, Boston, MA, USA) was also used for cell death analysis. Further detection was done using the Ultravision Detection System (Lab Vision, Freemont, CA, USA), and the reaction developed with 3,3'-diaminobenzidine substrate (Sigma); sections were subsequently counterstained with hematoxylin. For immunofluorescence studies, fixed tissue sections were blocked with 10% normal foetal bovine serum (FBS) and primary antibodies incubated in 10% FBS/PBS-0.3% Triton X-100 occurred overnight at RT as following: rat polyclonal anti-BrdU (1:100; #AB6326, Abcam, Cambridge, UK), rabbit polyclonal anti-doublecortin (DCX; 1:200; #AB18723, Abcam), rabbit polyclonal anti-GFAP (GFAP; 1:200; #Z033401-2, DAKO), mouse polyclonal anti-GFAP (1:200; #G3893, Sigma), mouse polyclonal anti-Sox2 (1:200; #AB5603 Abcam), rabbit polyclonal anti-S100β (1:200; #Z031101-2; DAKO), rabbit polyclonal anti-calbindin (Calb; 1:300; #AB11426, Abcam), rabbit polyclonal anti-Ki67 (1:300; #AB9260, Millipore, Billerica, MA, USA), rabbit anti-24p3R (1:200; #SAB3500306, Sigma), rabbit anti-nestin (1:100; #sc-21249; Santa Cruz Biotechnology, Texas, USA), rabbit anti-glutathione peroxidase-4 (Gpx4; 1:50; #ab125066, Abcam) and goat anti-mouse LCN2 antibody (1:50; #AF-1857, R&D Systems, Inc., Minneapolis, MN, USA). Fluorescent secondary antibodies (Alexa 488 and 594; Invitrogen, San Diego, CA, USA), anti-rat, anti-rabbit and anti-mouse, were used to detect the respective primary antibodies at a dilution of 1:500 (in PBS-0.3%Triton X-100) for 2 h at RT. To label the nucleus, incubation with 4',6-diamidino-2-phenylindole (DAPI; #10236276001, Sigma) at a dilution of 1:1000 was performed, after which slides were mounted with Immu-Mount (#9990402, Thermo Fisher Scientific, Waltham, MA, USA).

Confocal imaging and quantitative analysis

Fluorescence images were acquired for SVZ and SGZ using the Olympus Fluoview FV1000 confocal microscope (Olympus, Hamburg, Germany). Fields were acquired using Z-scan with a step of 1 μm between each confocal plane. All sections prepared for comparison were analysed at the same time, using the same acquisition parameters.

To estimate the number of proliferating neuroblasts, on both SVZ and SGZ, tissues were co-labelled for BrdU and DCX, whereas co-labelling with Calbindin and S100 was performed for cell fate, after the cumulative protocol of BrdU, and with GFAP for radial glia-like stem cells identification. Co-labelling experiments for the identification of cells phenotype expressing 24p3R were performed in SGZ sections of Wt animals after the label-retaining protocol along with BrdU, Sox2, NeuN and GFAP. For cell cycle exit experiments, $BrdU^+$ cells within the SGZ were analysed for their co-expression with Ki67.

The rates of proliferation, cell cycle exit and differentiation were estimated, on both neurogenic niches, by analysing 3-4 sections per animal. The number of double positive cells/total number of BrdU positive cells was calculated using Olympus Fluoview FV1000 software (Olympus) and normalized for the respective area (mm^2), which were determined using the StereoInvestigator software (MicroBrightField Bioscience, Magdeburg, Germany) in an Olympus BX51 microscope (Olympus) at low magnification (10x).

Stereology

Cell density estimation of the fast proliferating progenitors at the SVZ and the SGZ was obtained considering the different regions of the SVZ, as described elsewhere [1], and the SGZ as the 3-cell-body-wide zone at the border between the granular cell layer and the hilus. Coronal sections for proliferation analysis comprised SVZ between bregma coordinate 0.86 mm to 0.14 mm (correspondent to the intermediate division of the SVZ as in [1]), and SGZ between -1.46 mm to -2.30 mm coordinates [2]. Every sixteenth section from the two defined regions were blindly analysed and the initial section to be analysed was randomly selected to certify unbiased sampling. The number of $BrdU^+$ and $Ki67^+$ cells was counted using the Visiopharm Integrator system (VIS) software in an Olympus BX51 microscope (Olympus) and results expressed as $BrdU^+$ or $Ki67^+$ per area (in mm^2). The use of the VIS Software allowed delimitation, at low magnification (40x), of the areas of interest and the counting of positive cells within the defined areas at high magnification (400x). A similar stereological approach was performed to assess cell apoptosis in the DG, as the number of activated caspase-3^+ cells.

Western blot

Mouse brain tissue (macrodissected DG and cerebellum) was homogenized in RIPA buffer [containing 250 mM NaCl, 50 mM Tris–HCl, pH 7.6, 2 mM EDTA, 10% glycerol, 1 mM PMSF protease inhibitors (Roche, Switzerland)] with a Teflon pestle. The protein concentration of the obtained homogenates and of the serum samples was determined using the Bradford assay (Biorad, Hercules, CA, USA), and samples were then boiled for 8 min at 98°C in Laemmli buffer (65.8 mM Tris-HCl pH 6.8, 2% SDS, 5% glycerol; Biorad, Hercules, CA, USA), and microfuged for 10 sec before loading. Total protein (15 μg of serum and cerebellum; 50 μg of DG) was loaded into a 15% SDS-PAGE gel, and then transferred onto nitrocellulose membranes (Biorad). Membranes were blocked in Tris-buffered saline containing 5% nonfat milk in TBS-Tween, before incubation with primary

antibodies overnight at 4°C: goat anti-mouse LCN2 antibody (1:1000, R&D Systems) and mouse anti-tubulin (1:2500). Secondary antibodies used for 1 h at RT included anti-goat (1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-mouse (1:10,000; Biorad). Membranes were developed using ECL Clarity reagent (BioRad) and developed in ChemiDoc XRS System (Biorad).

Contextual Fear Conditioning

Contextual fear conditioning (CFC) was conducted in ventilated sound-attenuated chambers with internal dimensions of 20 cm wide x 16 cm deep x 20.5 cm high (SR-LAB, San Diego Instruments, San Diego, CA, USA), with a light mounted directly above the chamber to provide illumination. The floor consisted of stainless grid attached to a shock generator (Coulbourn Instruments, Allentown, PA, USA) for foot shock delivery. A fan mounted on one side of each box provided ventilation and background noise, which was set on only upon context changing. Mice behaviour was recorded by a digital video camera mounted above the conditioning chamber and freezing was manually scored by a blind observer using the Etholog V2.2 software [3]. Freezing was defined as the complete absence of motion for a minimum of 1 sec and each test session was scored continuously in its entirety. The fear conditioning procedure was conducted over 2 days. On day 1, mice were placed in the conditioning chamber (A) and received pairings between light and a cotermination shock (1 sec, 0.5 mA), spared from each other with an interval of 20 sec (Figure 3a). Mice received three light-shock pairings with an intertrial interval of 120 sec between each block and the first light presentation started 180 sec after the mouse was placed into the chamber. Animals returned to home cage 30 sec after the last shock presentation (Figure 3a). On day 2, mice were tested and scored for conditioned fear to the training context for 3 min, but with no presentation of the cue stimulus (Figure 3a). Two hours later, animals were presented to a novel context (B), where the grid was removed, black plastic inserts covered the floor and the walls of the chamber (Figure 3a), that was also scented with a paper towel dabbed with vanilla extract. The session consisted on a 3-min trial and animal freezing was scored for the entire session. Parameters analysed included the percentage of time freezing during the training session, the total percentage of time freezing in the context (A) and (B), and the index of discrimination between contexts as the ratio of percentage of time freezing (contexts A-B)/percentage of time freezing (contexts A+B).

Neural stem cells cultures

SGZ cells were cultured from 1–3 days-old Wt and LCN2-null mice, as an adaptation of the protocol

described elsewhere [4]. Fragments of SGZ were dissected out of 450 μm thick coronal brain sections, digested with 0.025% trypsin (Invitrogen, San Diego, CA, USA) and grown as neurospheres in serum-free medium composed of DMEM-F12/GlutaMAX-I containing penicillin (100 U/ml), streptomycin (100 μg/ml), B27 (1%), epidermal growth factor (EGF; 10 ng/ml) and basic fibroblast growth factor-2 (FGF-2; 10 ng/ml) (all from Invitrogen), at 37°C in a 95% air-5% CO_2 humidified atmosphere.

Cell proliferation assessment

In vitro proliferation studies were conducted using BrdU labelling and upon adhering neurospheres for 2 days onto poly-D-lysine (0.1 mg/ml; Sigma)-coated glass coverslips in 24-well cell culture plates, devoid of EGF/FGF-2. BrdU (10 μM; Sigma) was added to the adhered neurospheres 4 h before fixation with 4% paraformaldehyde. Immunodetection of BrdU included permeabilization with 0.3% Triton X-100 in PBS for 15 min and DNA denaturation in HCl (1 M) for 30 min at 37°C. Nonspecific binding sites blocking occurred for 1 h at RT with 0.2% BSA in PBS-0.3% Triton X-100, followed by incubation for an additional hour at RT with primary rat anti-BrdU antibody (1:20; Abcam) diluted in blocking solution. After rinsing in PBS, the respective fluorescent secondary antibody anti-rat (1:500; Invitrogen) was used for 1 h at RT, after which nuclei was counterstained with DAPI (1:1000; Sigma) for 5 min at RT, and mounted as previously. Fluorescent images were acquired using a fluorescent microscope (Olympus BX61) and a minimum of 25 fields per well was randomly acquired. The proportion of $BrdU^+$ cells was calculated as a fraction of total cells ($BrdU^+$ cells/total $DAPI^+$ cells) using the ImageJ software (1.48 version; NIH, Bethesda, MA, USA).

Self-renewal assay

Cells isolated from the SGZ were plated at 10.000 cells/well in 24-well (0.5 ml/well) uncoated plates in growth medium containing low EGF/FGF-2. The total number of primary neurospheres and their diameter was assessed after 7 days in culture. Then, primary neurospheres were collected, dissociated into single cells (neurocult dissociation kit; StemCell, Grenoble, France) and seeded again in low EGF/FGF-2 medium for additional 7 days. Obtained secondary neurospheres were seeded as before and also analysed for their number and size. Primary and secondary neurospheres obtained were photographed at low magnification (x5) using the AxioVision (Rel. 4.8) software in a Zeiss Observer Z1 microscope (Zeiss, Oberkochen, Germany) and the representative contrast phase images acquired from each genotype analysed using ImageJ software by manually measuring the diameters of neurospheres (assuming a spherical shape) and counting the total number of

neurospheres. Data are represented as the total number of neurospheres counted and the average of their diameter obtained from analysing each well, in triplicate, per independent cell culture.

Neurospheres immunocytochemistry

Fixed cells were permeabilized with 0.3% Triton X-100 in PBS for 15 min and blocked for non-specific binding for 1 h with 0.2% BSA/PBS-0.3% Triton X-100. Cells were then subsequently incubated for 1 h at RT with primary antibodies diluted in blocking solution as following: rabbit polyclonal anti-24p3R (1:200; Sigma) and mouse polyclonal anti-Sox2 (1:200; Millipore). The respective secondary antibodies Alexa Fluor 488 or 546 conjugated, goat anti-rabbit and goat anti-mouse were used for 1 h at RT (all at 1:500; Invitrogen). Nuclei were counterstained by incubating with DAPI (1:1000; Sigma) for 5 min at RT and mounted as above. Fluorescent images were acquired using an Olympus microscope (BX61) and a minimum of 25 fields per well was randomly acquired. The number of positive cells was calculated as a fraction of total cells similarly to cell proliferation evaluation and using the ImageJ software.

Cellular viability

Cell viability of Wt and LCN2-null neurospheres was assessed using the LIVE/DEAD Fixable Dead Cell Stain Kit (Invitrogen), according to the manufacturer's instructions. Briefly, neurospheres with 7 days in culture were dissociated into single cells, washed with PBS and incubated with 1 μl of the fluorescent reactive dye (green fluorescent dye, excitation at 488 nm; Invitrogen) for 30 min at RT, protected from light. Cells were then washed with PBS, fixed for 15 min at RT with 37% formaldehyde and washed again with 1% BSA. The fixed cell suspension was then analysed by flow cytometry (FACSCaliber2 flow cytometer; BD-Biosciences, San Jose, CA, USA) using the appropriate excitation and detection channel to the respective fluorescent reactive dye. Data was analysed with FlowJo 7.6 software (TreeStar Inc., Ashland, OR, USA) as the proportion of total fluorescence staining intensity. Higher fluorescence staining intensity reflects a higher proportion of non-viable cells, since the reactive dye can permeate the compromised membranes and react with free amines both in the interior and on the cell surface.

Annexin V/PI staining

After 7 days in culture, neurospheres were collected, dissociated into single cells and washed in cold PBS and binding buffer (100 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$). FITC-conjugated Annexin V

(5 μl; BD Biosciences) and propidium iodide (50 μg/ml; Invitrogen) were added to the ressuspended cells, and were incubated for 15 min at RT in the dark. Propidium iodide signals were measured using the FACSCaliber2 flow cytometer, as before, whereas Annexin V signal was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic with a 525 nm band pass. Analysis was further performed using the FlowJo 7.6.

Intracellular ROS measurement

Reactive oxygen species (ROS) indicator 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen) was applied to assess endogenous cellular ROS levels in Wt and LCN2-null derived SGZ neurospheres. Briefly, 7-days old neurospheres were cultured on 96-well plates for 48 h without growth factors, followed by incubation with 25 μM of DCFH-DA dye for 10 min at 37°C. After washing with DMEM medium devoid of phenol, cells were further incubated in the same medium for 1 h at 37°C for dichlorodihydrofluorescein (DCFH), the deacetylated product of DCFH-DA by intracellular esterases and oxidation, to react with oxygen species and form dichlorofluorescein (DCF), which is an oxidized fluorescent compound. The amount of intracellular ROS levels was quantified by the detection of DCF using a fluorescence plate reader (SpectraMax Gemini EM, Molecular Devices, Sunnyvale, CA, USA), in triplicates.

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CHAPTER III

The effects of voluntary running and stress on hippocampal neurogenesis and behaviour in the absence of Lipocalin-2

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Manuscript in preparation

The effects of voluntary running and stress on hippocampal neurogenesis and behaviour in the absence of Lipocalin-2

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Abstract

The continuous generation of new neurons in the adult mammalian hippocampus is a form of neural plasticity that modulates learning and memory functions, and also emotion (anxiety and depression). Among the factors known to modulate adult hippocampal neurogenesis and brain function, we have recently described lipocalin-2 (LCN2) as a key regulator of neural stem cells (NSCs) proliferation and commitment, with impact on several dimensions of behaviour. Additionally, voluntary running and stress are strong regulators of cell genesis that are described to, respectively, increase and decrease adult hippocampal cell proliferation and neurogenesis. However, the beneficial effects of voluntary running in the mouse model of LCN2-null-impaired neurogenesis were never explored, neither the relevance of LCN2-regulated hippocampal neurogenesis in response to stress. Here, we show that exercise rescues LCN2-null mice defective hippocampal neurogenesis and behaviour, as it promotes NSCs cell cycle progression and maturation, resulting in reduced anxiety and improved contextual behaviour. On the other hand, a lack of response by LCN2-null mice to the effects of chronic stress exposure was observed, at the cellular and behavioural levels. Together, these findings implicate LCN2 as a relevant mediator of neuronal plasticity and brain function in the adult mammalian brain.

Keywords

lipocalin-2, hippocampal neurogenesis, voluntary running, corticosterone, anxiety

Introduction

In the adult mammalian brain, the continuous generation of new neurons in the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus (Ming and Song, 2011) contributes to modulate local neural plasticity and network dynamics (Bao et al., 2010). The potential significance of this remodelling process has been shown to directly impact on learning and memory (Snyder et al., 2005), in contextual fear conditioning (Saxe et al., 2006), synaptic plasticity (Ming and Song, 2011) and brain function, both in health and disease (Miller and Hen, 2015). In fact, impaired or deficient neurogenesis in the hippocampus has been associated with the development of several neuropsychiatric disorders, including dementia (Latchney and Eisch, 2012; Bellenchi et al., 2013), anxiety (Kempermann et al., 2008) and depression (Santarelli et al., 2003). Noticeably, over the past years numerous studies have identified both intrinsic and environmental factors that regulate adult neurogenesis, and modulate behaviour via neurogenic-dependent mechanisms. Among them, lipocalin-2 (LCN2) was recently reported as a key regulator of adult neurogenesis orchestration (Ferreira et al., 2017) and animal behaviour (Ferreira et al., 2013). Primarily reported as an acute-phase protein in the innate immune response (Flo et al., 2004), LCN2 has recently emerged as an important modulator of brain physiology and disease (Ferreira et al., 2015), required for the maintenance of hippocampal integrity, plasticity and function (Mucha et al., 2011; Ferreira et al., 2013). Specifically, studies with a knockout mouse model for LCN2 (LCN2-null) revealed that LCN2 is involved in emotional and cognitive behaviours (Ferreira et al., 2013), in the control of neuronal excitability and in dentritic remodelling (Mucha et al., 2011; Ferreira et al., 2013). Moreover, LCN2 was shown to be essential for the redox control of adult hippocampal NSCs, with impact on cell cycle progression and death, self-renewal and differentiation, resulting in altered contextual discriminative behaviour (Ferreira et al., 2017).

Of interest, increasing evidence suggests that exercise (i.e. running) improves cognitive functions (O'Callaghan et al., 2007; Creer et al., 2010) and emotional behaviours (Duman et al., 2008), as it promotes synaptic plasticity (Zhao et al., 2006) and hippocampal neurogenesis (van Praag et al., 1999). Particularly, voluntary running was shown to robustly increase neural progenitors proliferation (van Praag et al., 1999; Kronenberg et al., 2003), the generation of new neurons (van Praag et al., 1999), and to enhance spatial pattern separation (Creer et al., 2010) and contextual discrimination (Kohman et al., 2012). In fact, exercise-induced neurogenesis is currently explored as a strategy to overcome and rescue decreased neurogenesis and memory associated with pathology (Patten et al.,

2015) and aging (van Praag et al., 2005). Whether and how voluntary running can impact in the process of hippocampal neurogenesis in the absence of LCN2 is not yet known.

In addition, and contrastingly, stress and its end-effectors glucocorticoids hormones (i.e. corticosterone, CORT) was shown to negatively impact on hippocampal function and structure (Sousa, 2016) and, particularly in the process of neurogenesis, it was described to suppress cell proliferation (Tanapat et al., 2001; Murray et al., 2008) and decrease the survival of newborn cells in the DG (Pham et al., 2003), culminating in the precipitation of anxiety- and depressive-like behaviours (Murray et al., 2008). Of interest, LCN2 has been designated with roles in the mediation of stress-induced responses at the cellular and behavioural levels (Mucha et al., 2011; Skrzypiec et al., 2013), but the contribution of hippocampal neurogenesis in the control of such responses by LCN2 is not recognized.

Considering the described importance of LCN2 in critical steps of NSCs physiology, in adult hippocampal neurogenesis and in animal behaviour, here we evaluated the effects that an external modulation of hippocampal neurogenesis would have in cell proliferation and behaviour in the absence of LCN2. For that, we used voluntary running and chronic CORT administration to, respectively, increase and decrease hippocampal neurogenesis in LCN2-null mice. Here, we show that exercise-mediated increase in neurogenesis improves LCN2-null mice impaired NSCs proliferation and survival, reducing anxiety and promoting contextual discrimination. Contrastingly, LCN2-null mice neurogenesis and behaviour were not affected by the exposure to chronic CORT. Together, these findings highlight the relevance of LCN2 for brain function, particularly in the context of hippocampal neurogenesis external-dependent regulation.

Material and Methods

Animal Experiments

Experiments were conducted in 2-months old males mice lacking LCN2 (LCN2-null), and their respective wild-type (Wt) littermate controls, in a C57BL/6J mice background. Animals were obtained from crossing heterozygous animals and mice genotype was confirmed by polymerase chain reaction. Mice were housed and maintained accordingly with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and of the Council, in a controlled environment at 22–24°C and 55% humidity, on 12 h light/dark cycles and fed with regular rodent's chow and tap water *ad libitum*. All animal procedures were conducted in accordance with the Portuguese national authority for animal experimentation, *Direção Geral de Alimentação e Veterinária* (ID: DGAV9457).

Voluntary running

In order to increase hippocampal neurogenesis, Wt and LCN2-null mice were randomly housed under standard housing conditions (Sedentary group), or with free access to a running wheel (Running group) for 28 days (van Praag et al., 1999). To analyse the effects of exercise on the progeny of NSCs and progenitors survival, animals were intraperitoneally injected with 50 mg/kg of BrdU (Sigma Aldrich, St. Louis, MO, USA) twice a day for 5 consecutive days in the beginning of the running protocol (Figure 1). Behavioural tests were conducted at the end of the voluntary running protocol, next described in detail.

Corticosterone injections

Repeated injection of the major stress hormone CORT in rodents provides a reliable animal model for studying stress-triggered responses (Sousa et al., 2000; Gregus et al., 2005). Thus, to analyse the effect of chronic exposure to glucocorticoids in mediating cell survival and behaviour in the absence of LCN2, both Wt and LCN2-null mice were subcutaneously injected with CORT (20 mg/kg, dissolved in sesame oil; Sigma) daily for 28 days (CORT group) [Figure 4; (Goshen et al., 2008; Zhao et al., 2008)]. An additional group of animals of both genotypes were subcutaneously injected with the vehicle (sesame oil; Vehicle group) for the same period. As described above, experimental groups were intraperitoneally injected with BrdU twice a day for 5 consecutive days at the beginning of the CORT treatment. Animals were weekly monitored for body weight gain during the protocol, and adrenals and thymus weight measured at the time of sacrifice. Behavioural analysis occurred at

the end of the protocol.

Behaviour

Novelty suppressed-feeding

To examine anxiety-like behaviour to a novel environment, animals were assessed in the novelty-suppressed feeding (NSF) paradigm. After 18 h of food-deprivation, animals were placed in an open field arena (Med Associates Inc., St. Albans, VT, USA), where a single food pellet was placed in the center. The latency of time to feed was recorded and used as a measure of anxiety-like behaviour. After reaching the pellet, animals returned to home cage where they were allowed to eat pre-weighted food for 5, 15 and 30 min as a measure of appetite drive.

Elevated plus maze

Anxious behaviour was analysed through the elevated plus maze (EPM) test. The behavioural apparatus (ENV-560; Med Associates Inc.) consisted of two opposite open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm) elevated 72.4 cm above the floor and dimly illuminated. Mice were individually placed in the center of the maze and allowed to freely explore it for 5 min. The percentage of time spent in the open arms, monitored through an infrared photobeam system (MedPCIV, Med Associates Inc.), was used as an index of anxiety-like behaviour, and the total number of entries in the arms maze as an index of general locomotor activity.

Contextual fear conditioning

Contextual fear conditioning (CFC) was conducted in ventilated sound-attenuated chambers with internal dimensions of 20 cm wide x 16 cm deep x 20.5 cm high (SR-LAB, San Diego Instruments, San Diego, CA, USA), with a light mounted directly above the chamber to provide illumination. The floor consisted of stainless grid that was attached to a shock generator (Coulbourn Instruments, Allentown, PA, USA) for foot shock delivery. A fan mounted on one side of each box provided ventilation and background noise, which was only set on upon context changing. Mice behaviour was recorded by digital video cameras mounted above the conditioning chamber, and freezing behaviour was manually scored by a blind observer using the Etholog V2.2 software (Ottoni, 2000). Freezing was defined as the complete absence of motion for a minimum of 1 sec.

The fear conditioning procedure was conducted for 2 days. On day 1, mice were placed in the conditioning chamber and received pairings between light and a cotermination shock (1 sec, 0.5

mA), spared from each other with an interval of 20 sec. Mice received three light-shock pairings with an intertrial interval of 2 min between each block, and the first light presentation started 3 min after the mouse was placed into the chamber. Animals returned to their home cage 30 sec after the last shock was presented. The chambers were cleaned with 10% ethanol between each mouse. On the following day, mice were tested for conditioned fear to the training context and placed in the same chamber (context A), where the training contextual conditions remained from the day before, but no presentation of the conditioned stimulus occurred. Mice were placed into the chambers for 3 min and the entire session was scored for freezing. Two hours after this, animals were presented to a novel context (context B), where the grid was removed and black plastic inserts covered the floor and walls of the chamber. Also, the chamber was scented with a paper towel dabbed with vanilla extract, placed underneath the chamber floor and the ventilation fan was set on. In addition, the experimenter wore a different style of gloves and changed the lab coat. Chambers were cleaned with 10% ethanol between runs and mice were kept in a different holding room before testing. Each mouse was placed into the chamber for 3 min and freezing was scored for the entire session. Parameters analysed included the percentage of time freezing during the training session, the total percentage of time freezing in the context (A) and (B), and the index of discrimination between contexts as the ratio of percentage of time freezing (contexts A-B)/percentage of time freezing (contexts A+B).

Tissue preparation and immunohistochemistry

At the end of the behavioural assessment, mice were anesthetized with an intraperitoneal injection of a mixture of ketamine hydrochloride (150 mg/kg, Imalgene 1000) plus medetomidine (0.3 mg/kg, Dorben), and transcardially perfused with 0.9% saline, followed by perfusion with cold 4% paraformaldehyde (PFA) solution. Brains were removed, postfixed for 1 h in 4% PFA, cryoprotected in 30% sucrose overnight, and then embedded in Optimal cutting temperature compound (ThermoFisher Scientific, Waltham, MA, USA), snap-frozen and kept frozen at -20°C until further sectioning. Posterior serial coronal sections (20 μm) were cut in a cryostat and collected to slides for immunohistochemistry. For BrdU immunostaining, antigen retrieval by heat with 10 mM citrate buffer (Sigma) was performed on the fixed tissue sections, followed by DNA denaturation with HCl (Sigma) for 30 min at room temperature (RT). An additional blocking step with 10% normal foetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) in a solution of PBS 0.3% Triton X-100 (PBS-T; Sigma) was also performed for 30 min at RT. Primary antibodies incubation, diluted in blocking

solution, occurred overnight at RT as following: rabbit anti-glial fibrillary acidic protein (GFAP, 1:200; DAKO, Glostrup, Denmark), rabbit anti-calbindin (1:300; Abcam, Cambridge, UK), rat anti-BrdU (1:100; Abcam), mouse anti-Sox2 (1:200; Abcam), rabbit anti-Ki67 (1:300; Millipore, Billerica, MA, USA) and rabbit anti-glutathione peroxidase 4 (Gpx4, 1:50; Abcam). Fluorescent secondary antibodies (Invitrogen) anti-rat, anti-rabbit and anti-mouse, combined to Alexa 594 or to Alexa 488, were used to detect the respective primary antibodies at a dilution of 1:500 (in PBS-T) for 2 h at RT. To stain the nucleus, sections were then incubated with 4',6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma), after which slides were mounted with Immu-Mount (ThermoFisher).

Confocal imaging and quantitative analysis

Fluorescence images of the DG were acquired using the Olympus FV1000 confocal microscope (Olympus, Hamburg, Germany). Fields were acquired using Z-scan with a step of 1 μm between each confocal plane. All sections prepared for comparison were analysed at the same time, using the same acquisition parameters. The quantification rates of the parameters analysed were estimated by the analysis of 3-4 sections per animal. The number of double positive cells was calculated using the Olympus Fluoview software (Olympus), and normalized for the respective area (mm^2).

Statistical analysis

All experiments were performed and analysed by the same experimenter, blind to the animals' genotype or group treatment under assessment. Variables followed a Gaussian distribution as revealed by the D'Agostino & Pearson normality test. Data are reported as mean ± standard error (S.E.M.). The number of biological replicates (n) is specified in the legend of each figure. Statistical significant differences between groups were determined using two-way ANOVA, followed by Bonferroni's multiple comparison test. Values were considered statistically significant for $p \leq 0.05$ (*, # or δ), $p \leq 0.01$ (**, ## or δδ), $p \leq 0.001$(***, ### or δδδ) and $p \leq 0.0001$(****, #### or δδδδ).

Results

Voluntary running increases cell proliferation and survival

In order to increase hippocampal neurogenesis and analyse its pro-neurogenic effects in the absence of LCN2, Wt and LCN2-null mice were housed in standard cages with free access to a running wheel for 28 days (Figure 1a). First, we quantified cell proliferation in the hippocampus of running Wt and LCN2-null mice and compared to sedentary animals. The number of Ki67^{+} proliferating cells was decreased in sedentary LCN2-null mice, when compared to sedentary Wt (61% less; Figure 1c, d), similarly to what we described before (Ferreira et al., 2017). Four weeks of voluntary exercise robustly increased the number of Ki67^{+} cells in the SGZ (running effect: $F_{1,16}=16.3$, $p=0.001$), specifically promoting a significant increase in cell proliferation of LCN2-null mice (3-fold increase, $p=0.003$ *versus* sedentary LCN2-null mice; Figure 1c, d). In addition, analysis of the total number of BrdU^{+} cells, as a measure of cell survival in the SGZ, revealed that exercise significantly increased this population (running effect: $F_{1,14}=19.9$, $p=0.0005$), both in Wt ($p=0.007$) and LCN2-null SGZ ($p=0.03$), compared to the respective sedentary genotypes (Figure 1c, d).

LCN2-null mice hippocampal quiescent NSCs respond to voluntary running, which promotes progenitors proliferation

We next sought to ascertain whether the observed increased cell proliferation and survival were related to regulatory events in the pool of stem cells. Since we used a label-retaining BrdU protocol at the beginning of the voluntary running protocol, this allowed us to label the quiescent pool of cells and, 28 days later, analyse either the progeny that exited the cell cycle and differentiated, or the pool of cells that retained BrdU and remained quiescent. Quantitative analysis of quiescent type-1 stem cells, that present a radial glia-like morphology and co-express BrdU and GFAP [Figure 1b; (von Bohlen und Halbach, 2011)], revealed a significant increase in sedentary LCN2-null mice (2-fold increase, $p<0.0001$ *versus* sedentary Wt), as described before (Ferreira et al., 2017). After exercise, a significant reduction in the number of radial type-1 stem cells was observed in LCN2-null mice ($p<0.0001$), to numbers similar to sedentary Wt (Figure 1e, f). Of notice, this was consistent with data obtained for type-1 nonradial stem cells in the DG, identified as Sox2^{+} Ki67^{-} cells ($p=0.07$ *versus* sedentary LCN2-null; Supplementary Figure S1a).

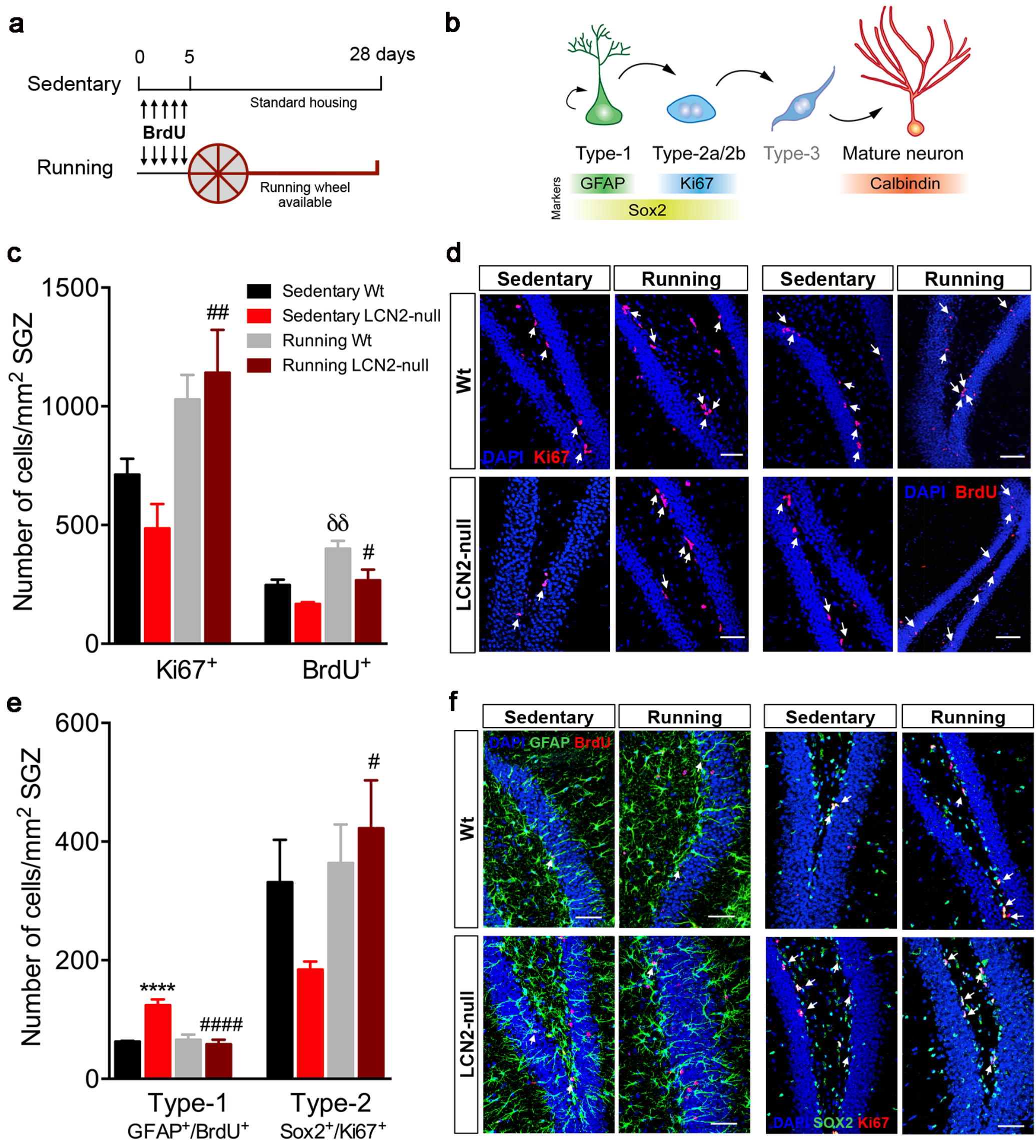

Figure 1: Voluntary running promotes hippocampal cell proliferation and survival, potentiating the transition of quiescent to proliferating neural stem cells in LCN2-null mice. (**a**) Schematic diagram of the experimental paradigm of voluntary running and of the BrdU injection protocol used. For 28 days, Wt and LCN2-null mice were assigned as running, i.e. animals housed to have free access to a running wheel, and as sedentary, housed under standard housing conditions with no running wheel available. (**b**) Representative illustration of the hippocampal neurogenic process, including cellular types and the specific markers used. (**c**) Running robustly increased cell proliferation (Ki67+ cells) and survival (BrdU+ cells) in both Wt and LCN2-null mice (n=4-6 per group). (**d**) Representative confocal images of Ki67 and BrdU immunostaining (indicated by white arrows) in the SGZ of the DG of Wt and LCN2-null sedentary and running mice. (**e**) Quantitative analysis of radial quiescent type-1 (GFAP+ BrdU+) and amplifying type-2 stem cells (Sox2+ Ki67+) after 28 days of running revealed a significant decrease in type-1 population in LCN2-null mice, and a consequent increase in type-2 cells (n=5 mice per group). (**f**) Representative confocal images of GFAP+/BrdU+ and Ki67+/Sox2+

immunostaining in the SGZ of the DG of sedentary and running mice of both genotypes (indicated by white arrows). Scale bars, 50 μm. Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. *Denotes differences between sedentary Wt and LCN2-null mice; $^{\delta}$between sedentary and running Wt; $^{\#}$between sedentary and running LCN2-null mice. $^{\#}p \leq 0.05$, $^{\delta\delta,\#\#}p \leq 0.01$, $^{****,\#\#\#\#}p \leq 0.0001$.

In addition, analysis of amplifying type-2 progenitors, identified as $Sox2^{+}$ $Ki67^{+}$ cells (Figure 1b), showed that running was effective (running effect: $F_{1,16}=4.6$, $p=0.04$) in promoting the transition of type-1 to type-2 stem cells and, specifically in LCN2-null mice, it significantly increased the number of type-2 stem cells ($p=0.03$ *versus* sedentary LCN2-null animals; Figure 1e, f). In fact, voluntary exercise increased the percentage of $Sox2^{+}$ cells in cycle in the SGZ of LCN2-null mice (7% increase, $p=0.08$ *versus* sedentary LCN2-null animals; Supplementary Figure S1b).

Interestingly, we have previously described that LCN2-null mice lack a proper antioxidant regulation in NSCs, which largely contributed to impaired cell cycle regulation and the deficits in the generation of new cells (Ferreira et al., 2017). As we observed that running improved cell proliferation and promoted cell cycle progression of NSCs, we next analysed its effects on the antioxidant regulation of stem cells in LCN2-null mice. Of interest, voluntary running seems to be promoting cell cycle progression from type-1 to type-2 in LCN2-null mice (Figure 1e, f), putatively by re-establishing the antioxidant properties of LCN2-null mice NSCs (running effect: $F_{1,13}=6.87$, $p=0.02$). Analysis of the number of $Sox2^{+}$ cells that express the antioxidant enzyme glutathione peroxidase 4 (Gpx4) showed a significant increase in LCN2-null mice DG, after running ($p=0.01$ *versus* sedentary LCN2-null; Figure 2a, b).

Yet, four weeks of running did not exert any effect in the NSCs population of Wt animals.

Exercise rescues LCN2-null mice behavioural deficits by restoring hippocampal neurogenesis

Since voluntary running effectively rescued LCN2-null impaired cell cycle regulation of NSCs in the SGZ, we next analysed the effect of exercise on behaviour and the generation of new cells in the absence of LCN2. We firstly addressed the effect of exercise in a paradigm that measures anxiety, the NSF test. In line with our previous results (Ferreira et al., 2013), we observed that sedentary LCN2-null mice display an anxious-like phenotype, as they presented a significant increased latency to feed ($p=0.008$; Figure 3a). Of interest, this phenotype was rescued by voluntary running, since

LCN2-null mice decreased the latency of time required to feed after exercise (p=0.02 *versus* sedentary null mice; Figure 3a). Importantly, running had no effect on this behavioural dimension in Wt mice, and no differences were observed between groups in the appetite drive (Figure 3a).

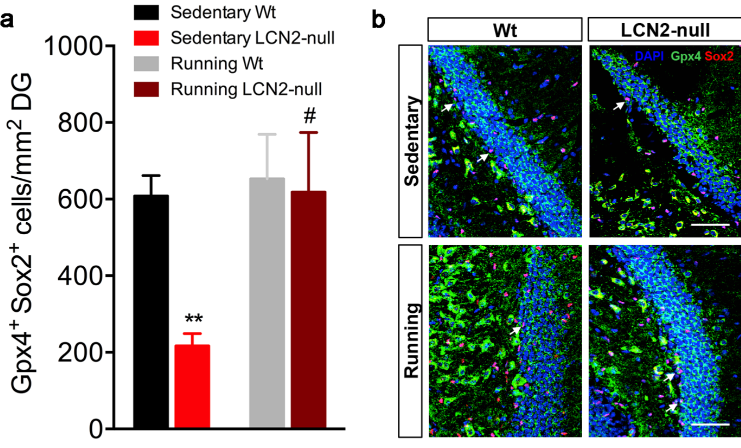

Figure 2: Voluntary running restores antioxidant regulation in LCN2-null neural stem cells. (**a**) Cellular quantification of the number of Sox2+ NSCs that co-express the antioxidant enzyme Gpx4 showed a significantly improved antioxidant regulation in running LCN2-null mice (n=5 mice per group). (**b**) Representative confocal images of Sox2 and Gpx4 co-labelling at the DG (indicated by white arrows; scale bar, 50 µm). Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. *Denotes differences between sedentary Wt and LCN2-null mice; #between sedentary and running LCN2-null mice. #p≤0.05, **p≤0.01.

Next, we tested the animals for their ability to discriminate between overlapping contextual presentations after the voluntary running protocol. While no differences between groups were observed during training (similar learning curve of freezing time along the trials of shock exposure; Figure 3b), running led to an overall significant improvement in contexts discrimination by LCN2-null mice (running effect: $F_{1,20}$=6.07, p=0.02). We observed, as before, that sedentary LCN2-null mice present impaired contextual retrieval (p=0.05) and discrimination index (p=0.01), which was rescued by exercise. The percentage of freezing upon re-exposure to context A (p=0.009; Figure 3c), and the animals' capacity to discriminate between the two different contexts (p=0.02; Figure 3d) was promoted by running in the absence of LCN2.

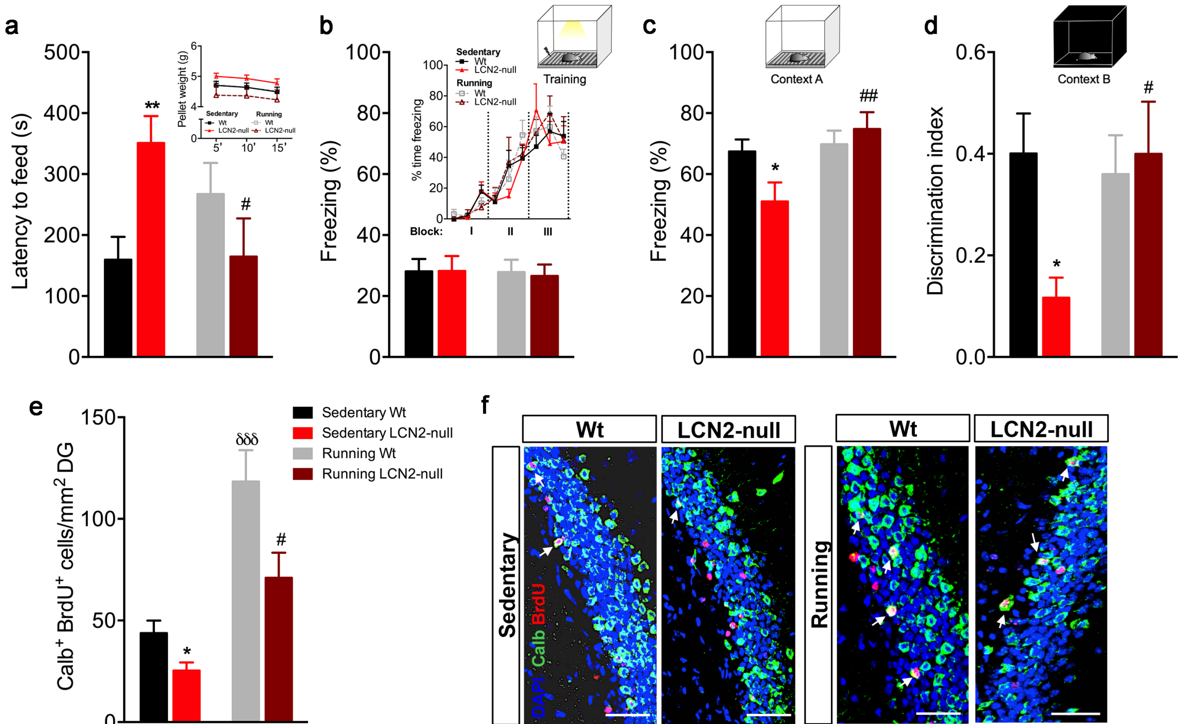

Figure 3: Exercise reduces anxiety and improves contextual discrimination in LCN2-null mice, by increasing the generation of newborn mature neurons. (**a**) Anxiety assessment in the NSF paradigm showed that sedentary LCN2-null mice take longer time to feed, which is decreased after voluntary running (n=6-10 mice per group). No differences between groups in appetite drive were observed. (**b**) Freezing behaviour during the training session, as total percentage of freezing and of freezing acquired along the trials of shock exposure (Block I-III), was similar between groups (n=6-10 mice per group). (**c**) Impaired contextual retrieval in context A by sedentary LCN2-null mice is rescued by exercise. (**d**) Discrimination index affected by the absence of LCN2 is re-established after housing LCN2-null mice with running wheels. (**e**) Quantitative analysis of the number of newborn mature neurons at DG revealed that exercise promoted a significant increase in both Wt and LCN2-null mice (n=5 mice per group). (**f**) Representative images of calbindin and BrdU immunostaining at DG (indicated by white arrows; scale bar, 100 μm). Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. *Denotes differences between sedentary Wt and LCN2-null mice; $^{\delta}$between sedentary and running Wt; $^{\#}$between sedentary and running LCN2-null mice. $^{*,\#}p \leq 0.05$, $^{**,\#\#}p \leq 0.01$, $^{\delta\delta\delta}p \leq 0.001$.

Remarkably, the behavioural improvements observed in LCN2-null mice by exercise were associated with an increase in the generation of newborn mature neurons in the hippocampus. Specifically, we identified the progeny of cells that was labelled in the beginning of the protocol (Figure 1a) and that exited the cycle and differentiated into mature neurons (identified as Calb^{+} BrdU^{+} cells).

Quantification of this population showed that exercise had a strong impact on the generation of new neurons (running effect: $F_{1,17}=30.4$, $p<0.0001$), and that it promoted a significant increase of $Calb^+$ $BrdU^+$ cells in the DG of LCN2-null mice (3-fold increase, $p=0.01$ *versus* sedentary null mice; Figure 3e, f). Particularly for this cell population, housing with running wheels also promoted a substantial increment in Wt animals (3-fold increase, $p=0.0004$) when compared to sedentary controls (Figure 3e, f).

Moreover, a correlation analysis revealed an important association between the number of newborn neurons ($Calb^+$ $BrdU^+$ cells) and the behavioural performance of LCN2-null mice in the NSF and CFC tests (Supplementary Table 1). This analysis shows that the increase in the number of newborn neurons observed in the DG of LCN2-null mice, after exercise, highly contributed to the observed improvements in anxiety ($p=0.07$) and contextual retrieval in the CFC test ($p=0.06$; Supplementary Table 1).

Together, our data reveals that voluntary exercise re-establishes LCN2-null mice behavioural impairments, specifically in anxiety and contextual discrimination, through its pro-neurogenic effects, as it effectively overcome impaired cell proliferation and survival, and promoted type-1 to type-2 stem cells transition.

Effectiveness of chronic CORT administration

In order to decrease hippocampal neurogenesis and analyse its cellular and behavioural effects in the absence of LCN2, we next exposed Wt and LCN2-null mice to a chronic treatment of CORT (20 mg/kg) for 28 days (Figure 4a), and further analysed biological, morphological and behavioural signatures of the stress response.

Firstly, we assessed the biological efficacy of the chronic CORT treatment. For that, we monitored body weight gain once a week during the 4 weeks of CORT injections, and the thymus and adrenals weight at the sacrifice. We observed that CORT exposure significantly affected the percentage of body weight gain along the weeks of treatment (CORT effect: $F_{3,135}=10.4$, $p<0.0001$), with a tendency to a more pronounced effect in CORT Wt animals (Figure 4b). Similarly, CORT treatment altered adrenals ($F_{1,17}=81.4$, $p<0.0001$) and thymus ($F_{1,17}=76.9$, $p<0.0001$) weight (Figure 4c, d), irrespectively of the animals' genotype (adrenals weight*genotype: $F_{1,17}=1.14$, $p=0.30$; thymus weight*genotype: $F_{1,17}=3.09$, $p=0.09$).

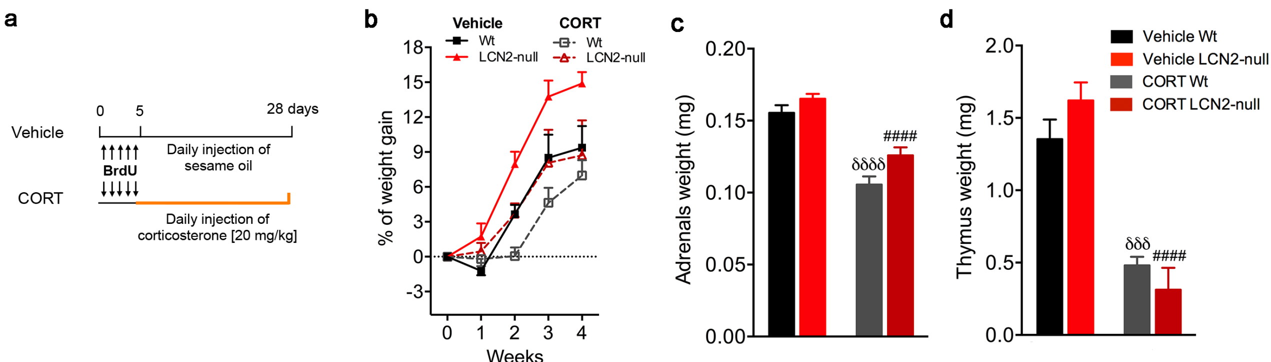

Figure 4: The effects of chronic CORT treatment on biological parameters. (**a**) Representative scheme of the experimental procedure of CORT injections performed. BrdU was injected for 5 days in the beginning of the protocol and, for additional 28 days, Wt and LCN2-null mice were daily injected subcutaneously with corticosterone (CORT group) or sesame oil (Vehicle group). (**b**) Body weight gain in Wt and LCN2-null mice after chronic CORT injection (n=6-10 mice per group) was weekly monitored during the protocol of CORT treatment. (**c**, **d**) CORT treatment significantly decreased adrenals and thymus weight, regardless of the genotype. Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. $^{\delta}$Denotes differences between vehicle and CORT Wt; $^{\#}$between vehicle and CORT LCN2-null mice. $^{\delta\delta\delta}p\leq0.001$, $^{\#\#\#\#,\delta\delta\delta\delta}p\leq0.0001$.

Chronic CORT administration impairs cell proliferation and reduces the NSCs pool

Consistent with the current knowledge on the negative effects of chronic stress and its end-effectors glucocorticoids on adult hippocampal neurogenesis, we next examined the impact of chronic CORT exposure on cell proliferation and survival in the absence of LCN2. We observed that chronic CORT administration impacted the number of proliferating $Ki67^+$ cells at the DG (CORT effect: $F_{1,14}$=3.45, *p*=0.08), by specifically decreasing cell proliferation in Wt animals (*p*=0.0002 *versus* vehicle Wt; Figure 5a, b). Additionally, analysis of cell survival, as the number of $BrdU^+$ cells in the SGZ, revealed that CORT treatment had no significant impact in this parameter (CORT effect: $F_{1,14}$=0.31, *p*=0.58). Of notice, both cell proliferation and survival analysis in LCN2-null mice SGZ, after CORT treatment, revealed no significant effects (Figure 5a, b). We observed a decrease in $Ki67^+$ and $BrdU^+$ cells in vehicle LCN2-null mice, as previously reported (Ferreira et al., 2017), but both populations were not affected by CORT treatment.

We next analysed the effect of chronic CORT exposure in the pool of stem cells. When labelling type-1 radial glia-like stem cells, we observed that the CORT treatment induced a significant depletion on the number of type-1 stem cells (CORT effect: $F_{1,10}$=22.54, *p*=0.0008), regardless of animals' genotype (CORT*genotype: $F_{1,10}$=27.2, *p*=0.0004). Specifically, CORT induced a significant decrease in the number of type-1 $GFAP^+$ $BrdU^+$ stem cells, in both Wt (*p*=0.01 *versus* vehicle Wt) and LCN2-null mice (*p*<0.0001 *versus* vehicle LCN2-null; Figure 5c, d). Importantly, this was consistent with the analysis of nonradial $Sox2^+$ $Ki67^-$ type-1 stem cells (CORT effect: $F_{1,15}$=10.23, *p*=0.006; *p*=0.05 Wt vehicle *versus* CORT, *p*=0.02 LCN2-null vehicle *versus* CORT; Supplementary Figure S2). On the other hand, analysis of type-2 stem cells revealed no major overall effect of stress exposure (CORT effect: $F_{1,15}$=0.90, *p*=0.36), but a significant reduction in the number of $Sox2^+$ $Ki67^+$ proliferating progenitor cells in Wt SGZ (*p*=0.007 *versus* vehicle Wt; Figure 5c, d). In LCN2-null mice, this specific population remained similar to the vehicle group (Figure 5c, d).

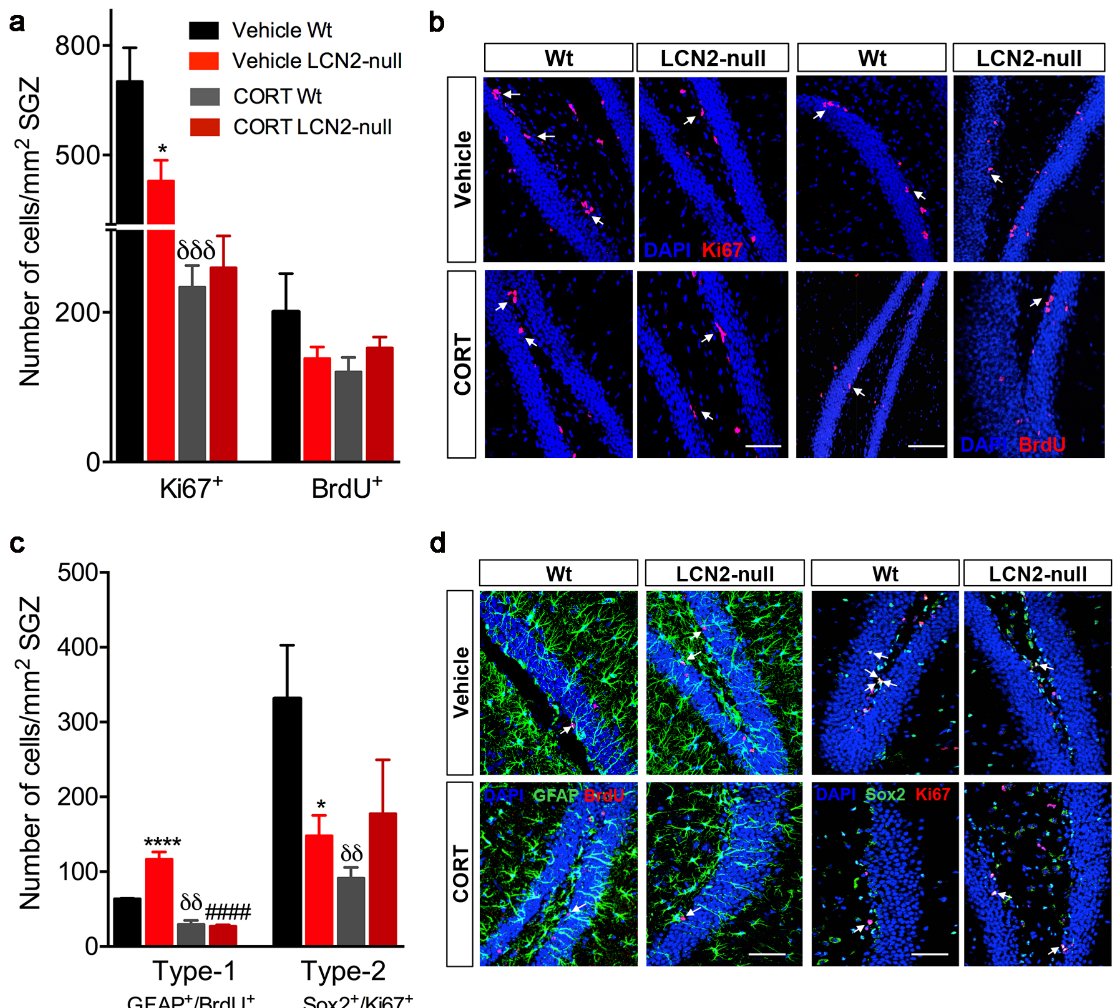

Figure 5: Chronic CORT administration reduces cell proliferation and neural stem cells self-renewal and survival. (**a**) CORT exposure robustly decreased the number of Ki67+ proliferative cells, specifically in Wt animals, whereas it did not significantly impacted on cell survival (BrdU+ cells) (n=4-6 per group). (**b**) Representative images of Ki67 and BrdU immunostaining (indicated by white arrows) at the DG of Wt and LCN2-null mice injected with vehicle and CORT. (**c**) Quantification of the number of radial glia-like type-1 stem cells, as GFAP+ BrdU+ cells at the SGZ, revealed that CORT treatment induced a significant decrease in both Wt and LCN2-null mice (n=4 mice per group). The effect of CORT on type-2 stem cells was only evident in Wt animals (n=5 mice per group). (**d**) Representative images of GFAP+/BrdU+, and Sox2+/Ki67+ immunostaining (indicated by white arrows) at the DG of Wt and LCN2-null control and injected with CORT. Scale bars, 100 μm. Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. *Denotes differences between vehicle Wt and LCN2-null mice; $^{\delta}$between vehicle and CORT Wt; #between vehicle and CORT LCN2-null mice. *$p \leq 0.05$, $^{\delta\delta}p \leq 0.01$, $^{\delta\delta\delta}p \leq 0.001$,****,####$p \leq 0.0001$.

LCN2 is required for the deleterious effects of CORT on behaviour and hippocampal neurogenesis

To disclose the importance of LCN2 in CORT-induced behavioural deficits, as others have described (Skorzewska et al., 2014), we performed behaviour analysis after 28 days of CORT administration, which included assessment of anxiety and contextual discrimination. Specifically for anxiety, and in the EPM paradigm, chronic CORT exposure induced an anxious-like behaviour in Wt mice (p=0.004 *versus* vehicle Wt), as observed by the decreased percentage of time spent in the open arms, whereas LCN2-null mice behaved as in vehicle conditions (Figure 6a). In addition, CORT treatment did not affect general locomotor activity in all groups (Figure 6a). Accordingly, when animals were assessed in the NSF test, CORT Wt animals significantly increased their latency of time to feed (p=0.04 *versus* vehicle Wt), indicating an anxious-like behaviour induced by CORT exposure (Figure 6b), which again was not observed in CORT LCN2-null mice (Figure 6b). No major effects on overall appetite drive were observed (Figure 6b).

Moreover, analysis of animals' capacity to discriminate between contexts in the CFC test, showed that CORT administration influenced contextual retrieval (CORT effect: $F_{1,17}$=3.3, p=0.08; Figure 6c) and discrimination (CORT effect: $F_{1,15}$=5.9, p=0.03; Figure 6d). Particularly, CORT led to a significant decrease in the percentage of freezing upon re-exposure to the training context A in Wt animals (p=0.03 *versus* vehicle Wt; Figure 6c), also decreasing their ability to discriminate between the different contexts (p=0.02; Figure 6d). Again, CORT had no significant effect on LCN2-null mice (Figure 6c, d).

In addition, we observed that impaired neurogenesis induced by CORT requires LCN2 for behavioural outcomes. Specifically, chronic CORT treatment negatively affected the generation of newborn neurons (CORT effect: $F_{1,17}$=5.2, p=0.04). Quantification of the total number of $Calb^{+}$ $BrdU^{+}$ cells showed that CORT promoted a significant decrease in the number of new cells in Wt mice (58% less, p=0.04 *versus* vehicle Wt), whereas it remained unchanged in LCN2-null mice (Figure 6e, f). Together, our data reveals that LCN2 absence blocks glucocorticoids-driven anxiety and cognitive function, as it prevents CORT-promoted hippocampal neurogenesis decline.

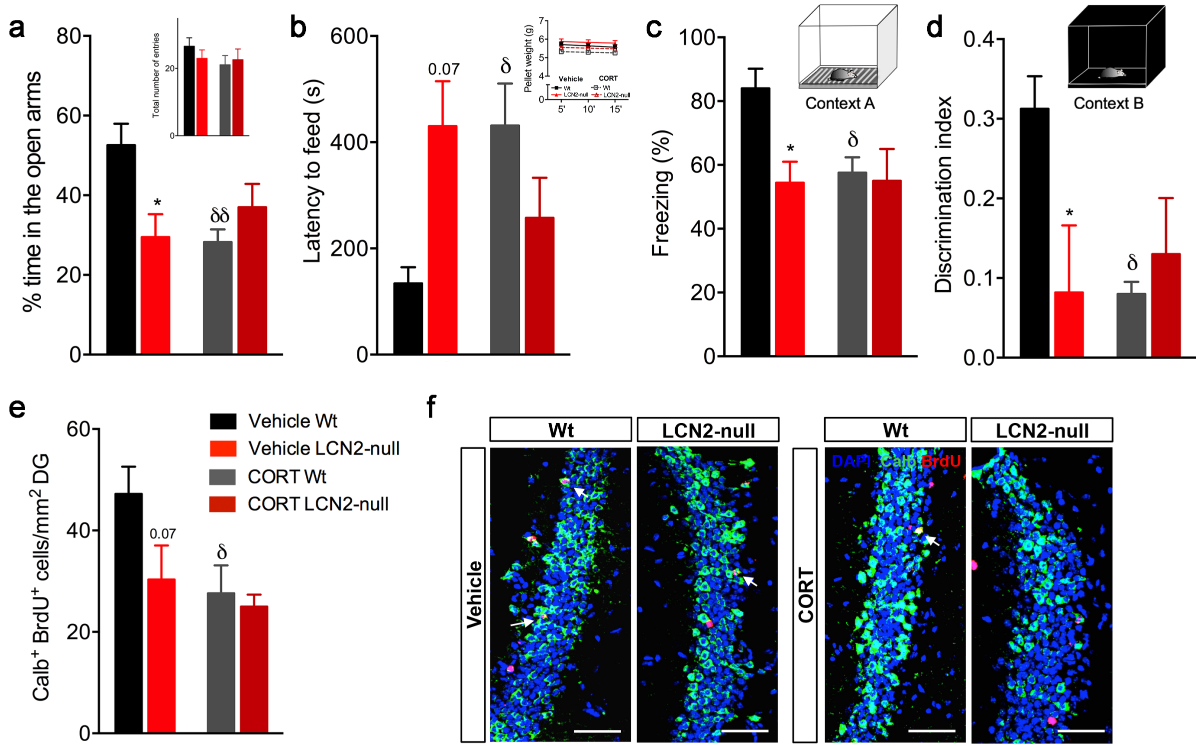

Figure 6: Animal behaviour and hippocampal neurogenesis are differentially affected by CORT in Wt and LCN2-null animals. (**a**) Anxiety assessment in the EPM test, after CORT treatment, showed that Wt mice, but not LCN2-null animals, spent less time in the open arms, with no effect among groups on general motor activity (n=6-10 mice per group). (**b**) In the NSF paradigm, Wt animals increased their latency to feed after the chronic treatment with CORT. No differences were observed between groups in appetite drive (n=6-10 mice per group). (**c**) Contextual retrieval in context A by Wt mice was impaired after CORT treatment (n=6-10 mice per group), but not by LCN2-null mice. (**d**) Discrimination index was affected by the chronic CORT treatment but only in Wt animals (n=6-10 mice per group). (**e**) Quantitative analysis of the number of newborn mature neurons at DG, as $Calb^+$ $BrdU^+$ cells, revealed that CORT treatment induced a significant decrease in Wt mice (n=5 mice per group), with no effect on LCN2-null DG. (**f**) Representative images of calbindin and BrdU immunostaining (indicated by white arrows) at DG of Wt and LCN2-null vehicle and injected with CORT. Scale bar, 100 μm. Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. *Denotes differences between vehicle Wt and LCN2-null mice; $^{\delta}$between vehicle and CORT Wt. $^{*,\delta}p\leq0.05$, $^{\delta\delta}p\leq0.01$.

Discussion

The relevance of LCN2 in the control of adult hippocampal neurogenesis has just recently emerged in the current literature (Ferreira et al., 2017). With well-known roles in cell physiology in other systems (Yang et al., 2002), the lack of LCN2 was recently described to induce deficits in NSCs proliferation and commitment, with impact on hippocampal-dependent contextual fear discrimination (Ferreira et al., 2017) and anxiety (Ferreira et al., 2013). Still, the effect of hippocampal neurogenesis external regulation in the absence of LCN2 is lacking. In here, we show evidence for the effects of neurogenesis modulation by voluntary running and the chronic administration of CORT, in the mouse model of LCN2-null-impaired cell genesis. Specifically, we found that voluntary running-mediated pro-neurogenic effects can overcome the deficits in cell proliferation and differentiation observed in the absence of LCN2, resulting in improved anxiety and contextual discriminative behaviours (Figure 7). On the other hand, a lack of response by LCN2-null mice to chronic CORT administration was observed. We show that CORT differentially affected cell proliferation and survival in Wt and LCN2-null mice, and glucocorticoids-driven anxiety and poor contextual discriminative behaviours were inexistent in the absence of LCN2 (Figure 7). Altogether, our findings add functional relevance to LCN2 for general brain function, particularly in the mediation of hippocampal neurogenesis processes.

Voluntary running-beneficial effects in LCN2-null impaired hippocampal neurogenesis

For decades, the pro-neurogenic benefits of exercise have been described, with reports showing enhanced adult hippocampal cell proliferation and neurogenesis (van Praag et al., 1999; Snyder et al., 2009), and improvements in specific hippocampal-dependent tasks, such as pattern separation (Creer et al., 2010) and contextual fear conditioning (Kohman et al., 2012). In fact, exercise-induced neurogenesis is quite often used as a strategy to overcome and rescue deficits in neurogenesis and memory-associated impairments (van Praag et al., 2005). Remarkably, housing of Wt and LCN2-null mice with running wheels for 28 days effectively improved LCN2-null mice neurogenic and behavioural deficits. Specifically, we observed that exercise was able to recruit LCN2-null quiescent NSCs into the cell cycle, promoting the transition of cells from a quiescent to a proliferative state, a process that was impaired in LCN2-null mice (Ferreira et al., 2017). In accordance, previous reports have shown that voluntary running preferentially recruits quiescent cells for cell division (Brandt et al., 2010), promoting the proliferation of type-2 cells, as well as their survival and maturation within the hippocampus (Bednarczyk et al., 2011).

Moreover, these pro-proliferative cellular effects have been shown to rely on the NSCs cell cycle dynamics. For instance, some authors have shown that running induces quiescent NSCs to enter into the cell cycle (Lugert et al., 2010), while it promotes cell cycle exit of fast progenitor cells (Brandt et al., 2010). It is possible that NSCs from LCN2-null mice, although highly quiescent, present a proliferative potential that is higher than those of Wt mice that can be recruited to increase the neurogenic process in response to external stimulations, such as running. In fact, as a consequence of a decrease in the pool of stem cells, we observed that voluntary running increased the percentage of type-2 progenitor cells present in the active phase of the cycle in the SGZ of LCN2-null mice.

A multitude of intrinsic factors, triggered in response to exercise, are considered to underline the mechanisms of exercise-induced hippocampal neurogenesis. Particularly, vasculature-associated trophic factors, including insulin and vascular-endothelial growth factors, have been implicated as mediators of running-induced neurogenesis, since both are increased in running animals (both serum and hippocampal levels) (Carro et al., 2000; Fabel et al., 2003). In the case of LCN2-null mice, at this point it is not clear through which mechanisms voluntary running is promoting improvements in neurogenesis and behaviour. Of notice, we have previously observed that LCN2-null NSCs present a deficient antioxidant regulation that lead to increased oxidative stress, cell cycle arrest and death (Ferreira et al., 2017). Interestingly, voluntary running improved the antioxidant regulation of LCN2-null NSCs, as quantified by the levels of the antioxidant enzyme Gpx4 expressed by $Sox2^{+}$ cells. This, at least in part, could explain the observed improvements in cell cycle progression of NSCs in LCN2-null mice. In fact, this is in line with other reports showing that regular exercise improves cognitive function and decreases oxidative damage (Radak et al., 2001; Aghel et al., 2010).

The effectiveness of the voluntary running protocol applied was evident by the significant increase in the generation of newborn neurons that it promoted in the DG of both Wt and LCN2-null mice. Importantly, this increase provided additional plasticity that largely contributed to reduce anxiety and restore contextual fear discrimination in LCN2-null mice. In accordance, several authors have shown that voluntary running, by modulating neurogenesis (Nicolas et al., 2015) has anxiolytic effects (Duman et al., 2008; Nicolas et al., 2015). Contrastingly, the increased generation of newborn neurons in Wt animals, promoted by running, did not render better performances in the contextual fear conditioning task. Similarly, others have shown that running-increased neurogenesis does not potentiate contextual fear memory (Akers et al., 2014). Moreover, and in contrast to reports

describing that voluntary running induces anxiety-like behaviour in the open field and light/dark box tests, by increasing hippocampal neurogenesis (Fuss et al., 2010), herein Wt animals presented a normal phenotype when tested for anxiety-like behaviour in the NSF.

Together, our results suggest that adult hippocampal neurogenesis is pivotal in the genesis of anxiety in LCN2-null mice, and that impaired cell genesis, due to the lack of LCN2, is highly responsive to external modulation.

LCN2 in the mediation of chronic CORT-deleterious responses

Stress triggers a variety of adaptive cellular responses that help to control brain homeostasis and shape the adequate behavioural response of an animal, and failure to properly adjust such responses often results in affective disorders. In this context, accumulating evidence also suggests that chronic stress exposure negatively affects hippocampal neurogenesis (Drew and Hen, 2007; Brummelte and Galea, 2010), and highly contributes to anxiety-like behaviours (Lehmann et al., 2013; Skorzewska et al., 2014; Kino, 2015) and memory impairment (Darcet et al., 2014). Noticeably, *Lcn2* has been shown to be up-regulated in the hippocampus after restraint stress, to control neuronal excitability and anxiety behaviour (Mucha et al., 2011) and, in physiological conditions, LCN2-null mice present mood and cognitive alterations (Ferreira et al., 2013) and impaired cell genesis (Ferreira et al., 2017). Taking this into consideration, we evaluated the impact of stress-related hormones in adult hippocampal neurogenesis of LCN2-null mice. For that, we injected daily Wt and LCN2-null mice with CORT for 28 days, which is an alternative to the usage of chronic stress paradigms, and that has been shown to be effective in inducing altered mood behaviours (Lehmann et al., 2013; Skorzewska et al., 2014), in decreasing hippocampal neurogenesis and body weight, and maintain high levels of CORT (Brummelte and Galea, 2010; Levone et al., 2015). In fact, we confirmed the efficacy of the stress protocol here applied since both Wt and LCN2-null mice presented significant alterations in body, adrenals and thymus weight. At the cellular level, we observed that CORT negatively impacted on the NSCs population, as it induced a significant depletion of type-1 and -2 stem cells on both Wt and LCN2-null mice. Also, it affected the generation of new neurons, but only in Wt mice, which translated into an increased anxiety and impaired contextual discrimination. Of notice, this was not observed in LCN2-null mice after treatment with CORT.

Glucocorticoids promote their regulation on both brain and peripheral functions primarily via their two specific receptors, the glucocorticoid and the mineralocorticoid receptors. Noticeably, these

receptors are highly expressed by neural precursor cells, particularly by quiescent type-1 NSCs and type-2a amplifying progenitors, which renders them a higher susceptibility to the levels of circulating glucocorticoids (Egeland et al., 2015). It is, therefore, not surprising to observe a reduction in the pool of stem cells upon CORT injection, which is consistent with other reports (Yu et al., 2010). Still, at this point, it is not clear what is the fate of these cells. Of interest, stress and its related glucocorticoid hormones are known to impact on cell proliferation and neurogenesis either by potentiating apoptotic cell death (Yu et al., 2010), or by promoting the cell cycle arrest of progenitor cells (Heine et al., 2004), which should be further clarified in our model of stress.

The observations that CORT injections had no effect in hippocampal neurogenesis and behaviour in the absence of LCN2 are not surprising if we consider the fact that LCN2-null mice, at physiological conditions, show increased CORT levels (Ferreira et al., 2013). We have previously described that LCN2-null mice present an overactivation of the hypothalamic-pituitary-adrenal axis (Ferreira et al., 2013), translated into a sustained production of CORT, which might explain their incapacity to respond to CORT. Our previous description that LCN2-null mice, in basal states, present anxious and depressive-like behaviours, impaired cognition and long-term potentiation, as well as altered hippocampal cytoarchitecture (Ferreira et al., 2013), largely recapitulate some of the features observed in animals after high glucocorticoid/stress exposure (Pavlides et al., 2002; McLaughlin et al., 2007; Sandi, 2013). As so, we cannot exclude the contribution of the sustained production of CORT in the absence of LCN2 in boosting hippocampal neurogenesis deficits and in promoting behavioural deficits (Ferreira et al., 2017). Certainly, we must consider that the effects of stress largely depend on stress duration and the paradigm used, as others have reported LCN2-null mice to present enhanced stress-induced anxiety after restraint stress (Mucha et al., 2011). Nevertheless, we here show that LCN2 has important roles in mediating the deleterious effects of prolonged chronic exposure to glucocorticoids in neurogenesis and behaviour.

Concluding remarks

This work provides evidence on the effects of hippocampal neurogenesis modulation in a mouse model of LCN2-null-impaired cell genesis. The capacity to modulate neurogenesis and, in the case of voluntary running, to overcome behavioural deficits displayed by LCN2-null mice, but not with CORT, reinforces the importance of LCN2 as a key player in the regulation of neural plasticity, for proper brain functioning.

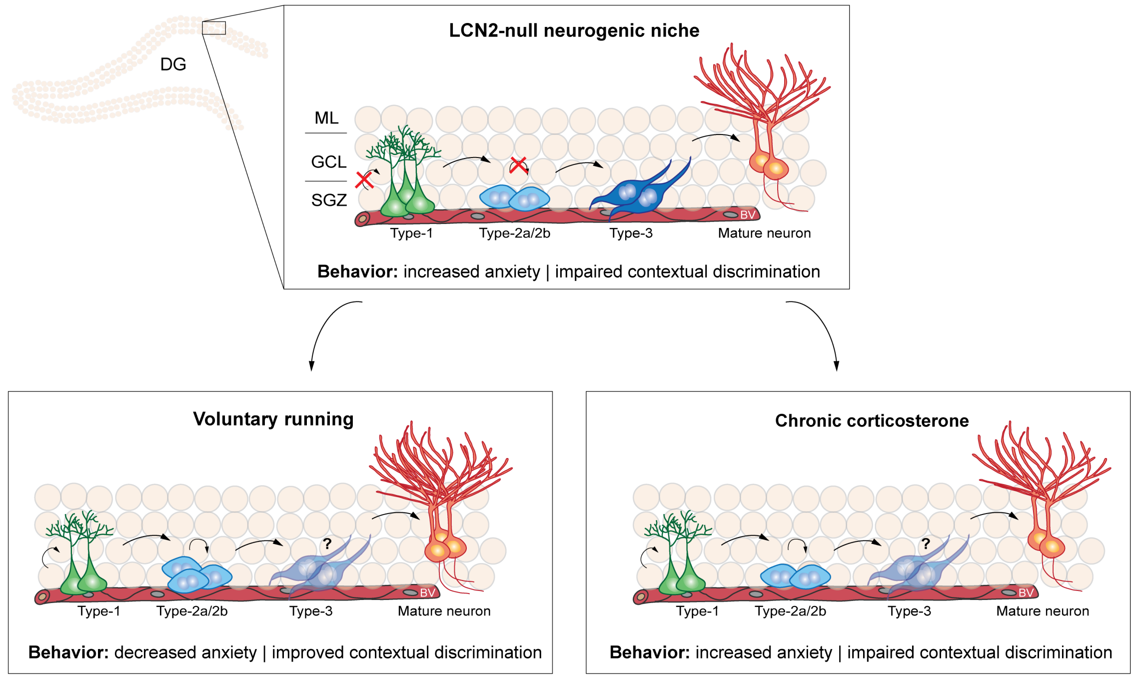

Figure 7: Schematic diagram of the effects of voluntary running and CORT administration on LCN2-null mice hippocampal neurogenesis and behaviour. In LCN2-null DG, voluntary running promotes the transition of type-1 to type-2 stem cells, increasing cell proliferation and survival, and the generation of newborn mature neurons. These positive effects in the neurogenic process translated into decreased anxiety and improved contextual discriminative behaviours. On the other hand, chronic CORT treatment-induced cellular and behavioural deleterious effects were largely dependent on LCN2, as LCN2-null mice after CORT exposure presented similar cellular and behavioural phenotypes as in physiological conditions. BV, blood vessel; DG, dentate gyrus; GCL, granule cell layer; ML, molecular layer; SGZ, subgranular zone.

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Supplementary information

Supplementary figures

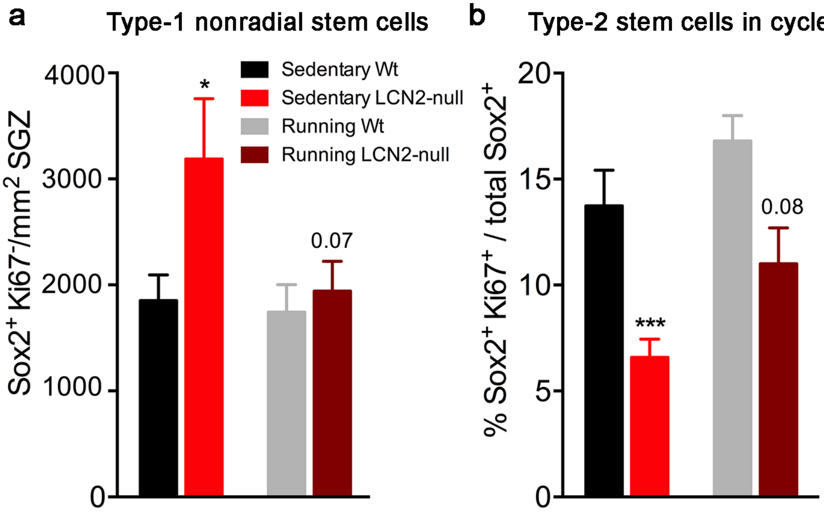

Figure S1: Running promotes the transition of quiescent to proliferating neural stem cells in LCN2-null mice. (**a**) Quantification of nonradial Ki67$^-$ Sox2$^+$ type-1 stem cells after exercise revealed a decrease in LCN2-null mice. (**b**) Analysis of the proportion of type-2 stem cells in cycle showed that exercise promoted the regulation of cell cycle in the absence of LCN2. Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. *Denotes differences between sedentary Wt and LCN2-null mice. *$p \leq 0.05$, ***$p \leq 0.001$.

Table S1: Correlations between neurogenesis and behavioural performances in the NSF and CFC, after voluntary running.

Newborn neurons Calb$^+$/BrdU$^+$	NSF Latency to feed (s)		CFC Freezing (%)	
	Wt	LCN2-null	Wt	LCN2-null
p value	0.26	0.07	0.15	0.06
r	0.5455	-0.8373	0.7484	0.9322

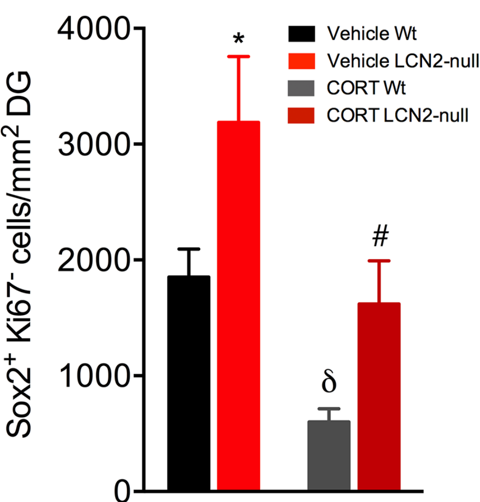

Figure S2: Chronic CORT administration reduces type-1 nonradial stem cells. Quantification of nonradial $Ki67^{-}Sox2^{+}$ type-1 stem cells, after chronic CORT exposure, revealed a significant effect on this population, regardless of the animals' genotype. Data are presented as mean ± SEM and were analysed by two-way ANOVA with Bonferroni's multiple comparison test. *Denotes differences between vehicle Wt and LCN2-null mice; $^{\delta}$between vehicle and CORT Wt; $^{\#}$between vehicle and CORT LCN2-null mice. $^{*,\#,\delta}p \leq 0.05$.

CHAPTER IV

Age-related changes in mice behaviour and the contribution of Lipocalin-2

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Manuscript in preparation

Age-related changes in mice behaviour and the contribution of Lipocalin-2

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Abstract

Aging causes considerable changes in the nervous system, inducing progressive and long-lasting loss of physiological integrity and synaptic plasticity, leading to impaired brain functioning. These age-related changes quite often culminate in behavioural dysfunctions, such as impaired cognition, which can ultimately result in various forms of neurodegenerative disorders. Still, little is known regarding the effects of aging on behaviour. Moreover, the identification of factors involved in regenerative plasticity, in both the young and aged brain, is scarce but crucial from a regenerative point of view and for our understanding on the mechanisms that control the process of normal aging. Recently, we have identified the iron-trafficking protein lipocalin-2 (LCN2) as novel regulator of animal behaviour and neuronal plasticity in the young adult brain. On the other hand, others have proposed LCN2 as a biological marker for disease progression in neurodegenerative disorders such as Alzheimer's disease and multiple sclerosis. Still, and even though LCN2 is well accepted as a regulator of neural processes in the healthy and diseased brain, its contribution in the process of normal aging is not known. Here, we performed a broad analysis on the effects of aging in mice behaviour, from young adulthood to middle and late ages (2-, 12- and 18-months of age), and in the absence of LCN2. Significant behavioural differences between aging groups were observed in all the dimensions analysed and, in mice deficient in LCN2, aging reduced anxiety, while sustained depressive-like behaviour and cognitive impairments observed at younger ages. These behavioural changes imposed by age were further accompanied by a significant decrease in cell survival and neuronal differentiation at the hippocampus. Our results provide insights into the role of LCN2 in the neurobiological processes underlying brain function and behaviour attributed to age-related changes.

Keywords

Aging, behaviour, lipocalin-2, hippocampus, cell survival

Introduction

Aging is a complex process that causes significant structural and physiological changes in the brain, often culminating in behavioural impairments and increased occurrence of neuropsychiatric and neurodegenerative disorders (Perna et al., 2016; Shoji et al., 2016). Several studies have, in fact, explored the impact of aging on brain physiology and behaviour (Botton et al., 2016; Shoji et al., 2016), demonstrating age-related impairments on cognitive functions (Benice et al., 2006), exploratory and locomotor activities (Fahlstrom et al., 2011), sensorimotor behaviour (Lau et al., 2008), and depressive-like states (Malatynska et al., 2012). Still, there are only few reports (Shoji et al., 2016) describing age-related changes in behaviour, from young adulthood to middle and late ages.

Functional changes in the brain imposed by aging include the gradual loss of neural regenerative capacity. Specifically in the hippocampus, morphological and functional changes associated with aging include neuronal loss (Mattson and Magnus, 2006), decreased synaptic density (Bach et al., 1999; Barnes, 2003) and synaptogenesis (Geinisman et al., 1992), increased oxidative (Forster et al., 1996; Hu et al., 2006) and metabolic (Yin et al., 2016) stress, and diminished neurogenesis (Kuhn et al., 1996; Kempermann et al., 2002). These changes strongly correlate with age-associated cognitive decline and memory impairments (Rapp and Heindel, 1994). Particularly, reduced adult hippocampal neurogenesis during aging was shown to be associated with decreased cognitive and learning performances (Lazarov et al., 2010; Mu and Gage, 2011; Merkley et al., 2014), which could be restored by increasing neurogenesis with exercise (van Praag et al., 2005; Merkley et al., 2014; Wu et al., 2015). In this sense, manipulations aimed at increasing neurogenesis constitute a promising approach for alleviating disease- or age-related impairments in brain function and behaviour (Drew and Hen, 2007; Bolognin et al., 2014). Still, the nature of the causal factors responsible for neurodegeneration during aging are poorly understood. The identification of regulators that can control both hippocampal plasticity and function is crucial from a regenerative point of view, and for our understanding on the neurobiological mechanisms that underlie the aging process.

In recent years, the secreted protein lipocalin-2 (LCN2) was identified as a regulator of brain physiological process, by controlling neuronal structural and remodelling, synaptic activity and behaviour at young adulthood (Mucha et al., 2011; Ferreira et al., 2013), with additional described roles in neural stem cells physiology and proliferation (Ferreira et al., 2017).

On the other hand, LCN2 has also been described with important roles in the diseased brain, particularly in mild cognitive impairment (Choi et al., 2011), Alzheimer's disease (Naude et al., 2012), multiple sclerosis (Marques et al., 2012) and, more recently, in Parkinson's disease (Kim et al., 2016). Whether detrimental or protective (Ferreira et al., 2015), the described roles of LCN2 in brain homeostasis, along with its presence in brain regions affected by degeneration, has even proposed the usage of LCN2 as a biological marker for disease progression (Ferreira et al., 2015). Still, the contribution of LCN2 for the process of normal aging is currently unknown.

In this study, we performed mice behavioural analysis during aging, from young adulthood to middle and late ages (2-, 12- and 18-months of age), and further analysed the impact of LCN2 absence in the course of the behavioural effects related to aging. Moreover, we estimated cell survival and neuronal differentiation in the hippocampus as a measure of neural plasticity in the aged brain.

Material and Methods

Ethics statement

All animal procedures were conducted in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and the Council and were approved by the Portuguese national authority for animal experimentation, *Direção Geral de Alimentação e Veterinária* (ID: DGAV9457). Animals were housed and maintained in a controlled environment at 22–24°C and 55% humidity, on 12 h light/dark cycles and fed with regular rodent's chow and tap water *ad libitum*.

Animal model and experimental groups

Experiments were conducted in male mice lacking LCN2 (LCN2-null), and the respective wild-type (Wt) littermate controls, in a C57BL/6J mice background, obtained from heterozygous crossings. Mice were divided into three groups, accordingly to their age: 2-, 12- and 18-months. Both LCN2-null mice and age-matched Wt littermates were used for behavioural and cellular analysis. As a measure of general welfare of animals, body weight was monitored throughout the aging process.

Behaviour

Animals from two different cohorts, and at each selected age, were assessed for their behaviour that included general evaluation of anxiety, mood and cognition (Figure 1a).

Elevated plus maze

Anxious behaviour was analysed through the elevated plus maze (EPM) test. The behavioural apparatus (ENV-560; Med Associates Inc., St. Albans, VT, USA) consisted of two opposite open arms (50.8 cm × 10.2 cm) and two closed arms (50.8 cm × 10.2 cm × 40.6 cm), elevated 72.4 cm above the floor and dimly illuminated. Mice were individually placed in the center of the maze and allowed to freely explore it for 5 min. The percentage of time spent in the open arms, monitored through an infrared photobeam system (MedPCIV, Med Associates Inc.), was used as an index of anxiety-like behaviour, and the number of total entries in the arms of the maze as an indicator of locomotor activity.

Light/dark box test

Anxiety behaviour was also assessed in the light/dark box test, consisting of an open field arena

divided in equal parts. One part was open and brightly illuminated, with the other consisting of a black Plexiglas insert with an entrance at the center of the arena. Each animal was placed at the center of the arena facing the lateral wall and allowed to explore it for 10 min. An infrared automatic system (Med Associates Inc.) allowed monitoring the time spent in each compartment of the arena, and anxiety was calculated by the ratio of time spent in the dark *versus* the light compartment.

Forced-swim test

Learned-helplessness was assessed through the forced-swim test (FST), as a measure of depressive-like behaviour. The test was conducted by placing each animal individually in transparent cylinders filled with tap water (25°C; depth 30 cm) for a 5 min period. The trials were videotaped and manually scored using the Etholog V 2.2 software (Ottoni, 2000). Learned-helplessness behaviour was defined as an increase in immobility time and a decrease in the latency of time to immobility (in sec).

Morris water maze

Cognitive function, by means of spatial reference memory, was evaluated using the Morris water maze (MWM) paradigm. The water maze consisted of a white circular pool (170 cm in diameter, 50 cm in height) filled with tap water (23°C; 25 cm of depth) and placed in a poorly lit room with extrinsic clues. The water tank was divided into four imaginary quadrants and a transparent escape platform (14 cm in diameter; 30 cm high), invisible to the animals, was placed in the center of one of the quadrants. Mice were randomly placed in the water facing the wall in each of the quadrants, and allowed to search for the hidden platform maintained in the same position during the 4 days of the acquisition. The trial was considered as concluded when the platform was reached within the limit time of 120 sec. If failing to reach the platform within this time-period, animals were guided to the platform and allowed to stay in it for 30 sec and an escape latency time of 120 sec was registered. During the 4 days of the acquisition phase, each animal was given four trials per day. Trials were video-captured by a tracking system (Viewpoint, Champagne-au-Mont-d'Or, France) and the time required to reach the platform (latency of time) was recorded for the consecutive trials/days.

Contextual fear conditioning

Contextual fear conditioning (CFC) was conducted for 2 days, as previously described (Ferreira et al.,

2017), to assess fear memory. Briefly, on day 1, mice were placed in the conditioning chamber and received three pairings of light and a terminating shock (1 sec, 0.5 mA), spared from each other with an interval of 20 sec. On the following day, to test for conditioned fear to the training context, animals were placed in the same chamber for 3 min as before, but with no presentation of the conditioned stimulus, and the entire session was scored for freezing. Two hours after, animals were presented to a novel context, with no grid, black plastic inserts covering the floor and the walls of the chamber and scent with vanilla extract. Each mouse was placed into the novel context for 3 min and freezing was scored for the entire session. Freezing behaviour was manually scored by a blind observer using the Etholog V2.2 software and defined as the complete absence of motion for a minimum of 1 sec. Parameters analysed included the total percentage of time freezing in the context (A) and (B), and the index of discrimination between contexts as the ratio of percentage of time freezing (contexts A-B)/percentage of time freezing (contexts A+B).

BrdU injections

In the end of the behavioural assessments, and prior to sacrifice (Figure 1a), animals at the described ages were intraperitoneally (i.p.) injected with BrdU (50 mg/kg; Sigma Aldrich, St. Louis, MO, USA) twice a day for 5 consecutive days, followed by a chase period of 28 days. This allowed the analysis of the effect of aging on the progeny of stem cells and progenitors survival.

Tissue preparation and immunohistochemistry

For tissue processing, brains from anesthetized mice [i.p. mixture of ketamine hydrochloride (150 mg/kg) plus medetomidine (0.3 mg/kg)] were perfused transcardially with 0.9% cold saline, coronally sectioned in a cryostat (20 μm), and further processed for immunohistochemistry. For BrdU immunostaining, antigen retrieval by heat with citrate buffer (10 mM; Sigma) was performed, followed by DNA denaturation with HCl (Sigma) for 30 min. An additional blocking step with a solution of PBS 0.3% Triton X-100 (PBS-T; Sigma) and 10% normal foetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature (RT) was also performed. Primary antibodies incubation, diluted in blocking solution, occurred overnight at RT and included rat anti-BrdU (1:100; Abcam, Cambridge, UK) and mouse anti-NeuN (1:200; Millipore, Billerica, MA, USA). The respective fluorescent secondary antibodies anti-rat and anti-mouse combined to Alexa 594 or to Alexa 488 (Invitrogen) were used to detect the respective primary antibodies at a dilution of 1:500 (in PBS-T) for 2 h at RT. To stain the nucleus, sections were incubated with 4',6-diamidino-2-

phenylindole (DAPI, 1:1000; Sigma), after which slides were mounted with Immu-Mount (ThermoFisher Scientific, Waltham, MA, United States).

Fluorescence images of the dentate gyrus (DG) of the hippocampus were acquired using the Olympus Fluoview FV1000 confocal microscope (Olympus, Hamburg, Germany) and the number of double-positive cells further calculated using Olympus Fluoview FV1000 software (Olympus), and normalized for the respective area (mm^2).

Statistical analysis

All experiments were performed and analysed by the same experimenter, blind to the animals' genotype or group under assessment. Variables followed a Gaussian distribution as revealed by the D'Agostino & Pearson normality test. Data are reported as mean ± standard error (S.E.M.). The number of biological replicates (n) is specified in the legend of each figure. Statistical significant differences between groups were determined using two-way ANOVA, followed by Bonferroni's multiple comparison test. Values were considered statistically significant for $p \leq 0.05$ (*, # or Φ), $p \leq 0.01$ (**, ## or ΦΦ), $p \leq 0.001$(***, ### or ΦΦΦ) and $p \leq 0.0001$(****, #### or ΦΦΦΦ).

Results

Depressive-like behaviours, but not anxiety, persists in aged LCN2-null mice

In order to assess the effects of aging in animal behaviour, and in the absence of LCN2, Wt and LCN2-null mice were aged until 12- and 18-months of age, and evaluated for anxiety, depressive-like and cognitive behavioural dimensions (Figure 1a).

Firstly, we examined age-related changes on the body weight of Wt and LCN2-null mice, and compared to young 2-months old mice. We observed a significant effect of age on body weight ($F_{2,60}$=142.0, p<0.0001), independently of the genotype (genotype*age: $F_{2,60}$=0.53, p=0.59; Figure 1b). Both aged Wt and LCN2-null mice were significantly heavier than younger genotype-matched animals (12- and 18-months > 2-months, p<0.0001; Figure 1b).

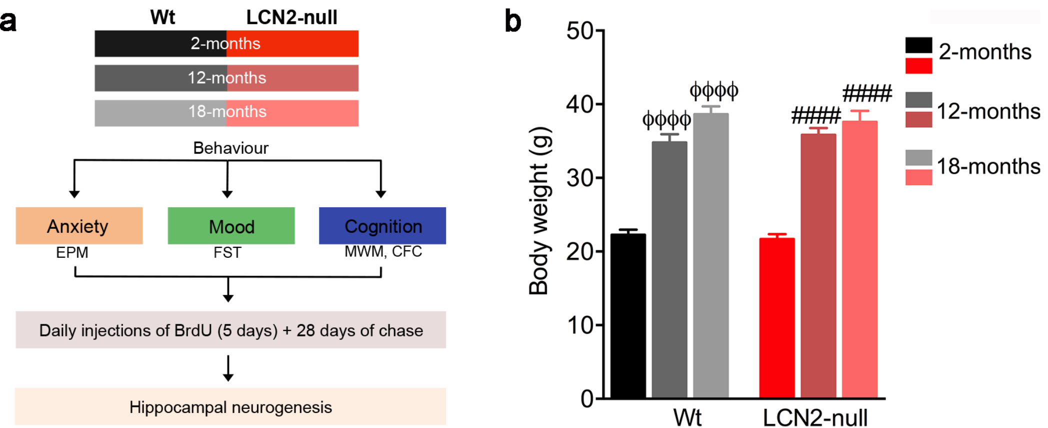

Figure 1: Body weight increases in aged animals, irrespectively of the genotype. (**a**) Schematic diagram of the experimental approach and the group of animals used at the respective ages. (**b**) Body weight measurements at selected ages revealed an age-related effect, with aged animals presenting increased body weight, when compared to genotype-matched 2-months old animals (n=10-12 mice per group). Data are presented as mean ± SEM, analysed by two-way ANOVA with Bonferroni's multiple comparison test. $^{\Phi}$Denotes differences between young and aged Wt; $^{\#}$between young and aged LCN2-null mice. $^{\Phi\Phi\Phi\Phi,\#\#\#\#}p\leq$0.0001. EPM, elevated plus maze; CFC, contextual fear conditioning; FST, forced-swim test; MWM, Morris water maze.

To assess anxiety-like behaviours, we tested the animals of both genotypes and at the different ages in the EPM test. Analysis of the effect of age in the time spent in the open arms revealed that it significantly influenced animal's performance in the maze (age effect: $F_{2,52}$=16.05, p<0.0001). Specifically, Wt 12-months old animals spent less time in the open arms of the maze (12- < 2-

months, p=0.59), which suggested the appearance of an anxious-like behaviour with aging. However, at the oldest age of 18-months, Wt animals exhibited a surprisingly increased percentage of time spent in the open arms (18- > 12-months, p=0.07; Figure 2a). In addition, aging also induced a reduction in the anxiety state presented by LCN2-null mice at 2-months of age. We have previously described that young LCN2-null mice present an increased anxiety-like behaviour in the EPM test (Ferreira et al., 2013), which we confirmed here (Wt: 29%, LCN2-null: 18%; Figure 2a). Analysis of aged LCN2-null mice in the EPM showed that animals significantly increased the time spent in the open arms of the maze, when compared to younger mice (12- > 2-months, p=0.006; 18- > 2-months, p<0.0001), and to age-matched Wt animals (Wt *versus* LCN2-null: 12-months, p=0.05; 18-months, p=0.01; Figure 2a).

In addition, the evaluation of the total number of entries in the EPM maze, as a measure of general locomotor activity, revealed no aging effect in this parameter ($F_{1,29}$=1.16, p=0.29), neither between ages or genotypes (Figure 2b).

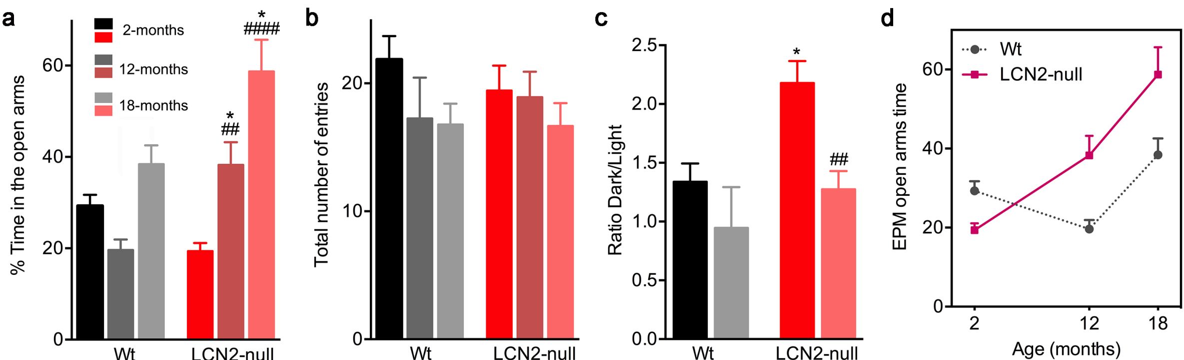

Figure 2: Age-related alterations of anxiety-like behaviours in Wt and LCN2-null mice. (**a**) Assessment of anxiety-like behaviour in the EPM showed an increase in the percentage of time spent in the open arms across age, specifically in LCN2-null mice (n=10-12 mice per group). (**b**) General locomotor activity assessed by the total number of entries in the maze revealed no major aging and genotype effects. (**c**) In the light/dark box test, anxiety-like behaviour of LCN2-null mice at 2-months of old is reduced at 18-months of age. (**d**) Representative progression of EPM performance by the animals during the course of normal aging, evidencing the reduction in anxiety behaviour (as an increased time in the EPM open arms) observed in LCN2-null mice. At 12-months of age, Wt mice become more anxious, a phenotype that was lost at 18-months. Data are presented as mean ± SEM, analysed by two-way ANOVA with Bonferroni's multiple comparison test. #Denotes differences between young and aged LCN2-null mice; *between Wt and LCN2-null mice at each matched age. *p≤0.05, ##p≤0.01, ####p≤0.0001.

In accordance with the observations obtained in the time spent in the open arms of the EPM maze, a similar phenotype was observed when testing 18-months old Wt and LCN2-null mice in the light/dark box test, another commonly used test to assess anxiety-like behaviour. In this test, a decreased ratio time dark/light was observed in older LCN2-null animals, when compared to younger mice (18- < 2-months, $p=0.002$; Figure 2c), confirming the reduction in anxiety by aging in LCN2-null mice. For aged Wt mice, this was not evident (p=0.67; Figure 2c).

Overall, these results suggest that, along with the process of aging, a reduction in anxiety behaviour occurs in the absence of LCN2 (Figure 2d).

Following, we assessed learned helplessness as an index of depressive-like behaviour, by the immobility time and the latency to immobility in the FST. Aging had an overall significant effect on the immobility time ($F_{2,41}=71.96$, $p<0.0001$) and the latency to immobility ($F_{2,37}=33.56$, $p<0.0001$; Figure 3a, b) presented by the animals. Older Wt mice significantly decreased their immobility time in the FST, when compared to 2-months old mice (2- > 12-, 18-months, $p<0.0001$; Figure 3a). In line with this, aged Wt animals also increased their latency of time to immobility (2- < 12-, 18-months, $p<0.0001$), suggesting that aging does not promote a depressive-like behaviour (Figure 3b). Notably, aged LCN2-null mice also decreased the time spent immobile, when comparing to 2-months old null mice (2- > 12-, 18-months, $p<0.0001$; Figure 3a), and increased their latency to immobility (2- > 12-months, $p=0.0005$; 2- > 18-months, $p<0.0001$; Figure 3b). Still, animals presented a depressive-like phenotype when compared to Wt age-matched animals (immobility time - Wt *versus* LCN2-null: 12-months, $p=0.0005$; 18-months, $p=0.09$; latency - Wt *versus* LCN2-null: 12-months, $p<0.0001$; 18-months, $p=0.05$). Together, this suggests that learned helplessness in the FST revealed an age-related decrease in depressive-like behaviour in both group of animals, but still LCN2-null mice sustained a depressive-like behaviour across the aging process (Figure 3c).

Spatial learning and contextual discrimination are affected through the course of aging

To examine age-related changes in spatial learning and memory, we tested the animals, at the different ages, in the MWM paradigm. In this task, all animals, regardless of the genotype, learned to find the position of the hidden platform, as they improved the time required to find it along the 4 days of the test (Figure 4a). The only difference obtained concerned the behavioural performance of 2-months old LCN2-null mice on the day 2 of testing ($p=0.0002$), in accordance to what we have previously reported (Ferreira et al., 2013).

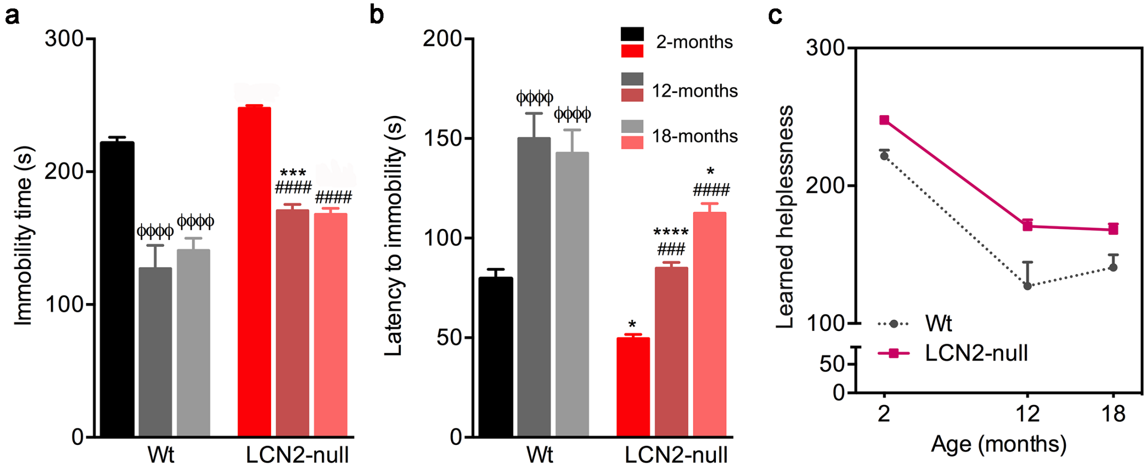

Figure 3: Wt and LCN2-null mice depressive-like behaviour in the course of aging. (**a**) Depressive-like behaviour evaluated as learned helplessness in the FST revealed decreased immobility time in older Wt and LCN2-null mice, with null aged mice continuing to present a depressive-like behaviour (n=10-12 mice per group). (**b**) Mood alterations in older mice were also observed by the latency of time to immobility. (**c**) Depressive-like behaviour in the progress of aging remains in LCN2-null mice, compared to controls, while older Wt animals decreased their immobility time. Data are presented as mean ± SEM, analysed by two-way ANOVA with Bonferroni's multiple comparison test. $^{\Phi}$Denotes differences between young and aged Wt mice; $^{\#}$between differences between young and aged LCN2-null mice; *between Wt and LCN2-null mice at each matched age. *$p\leq0.05$, $^{\#\#}p\leq0.01$, ***$p\leq0.001$, $^{\Phi\Phi\Phi\Phi,\ \#\#\#\#,}$ ****$p\leq0.0001$.

Moreover, the specific analysis of the learning process of Wt animals revealed that it was affected by aging ($F_{5,53}$=3.06, p=0.02). We observed a higher latency of time required by 12- and 18-months old Wt mice to reach the hidden platform, more evident on the second day of testing (12- > 2-months, p=0.02; 18- > 2-months, p=0.005; Figure 4b). On the other hand, the learning profile of LCN2-null mice remained similar across ages ($F_{2,33}$=0.69, p=0.51; Figure 4b): the observed compromised spatial learning of LCN2-null mice at 2-months old persisted throughout the aging process (Figure 4b). Although aging induced memory and learning deficits in Wt mice, LCN2-null animals required always increased time to find the platform in the MWM task (Figure 4c).

Altogether, the increased latency of time in the MWM indicates that spatial learning and memory performances decreased during aging in Wt conditions, remaining poorer in LCN2-null mice (as depicted in Figure 4c).

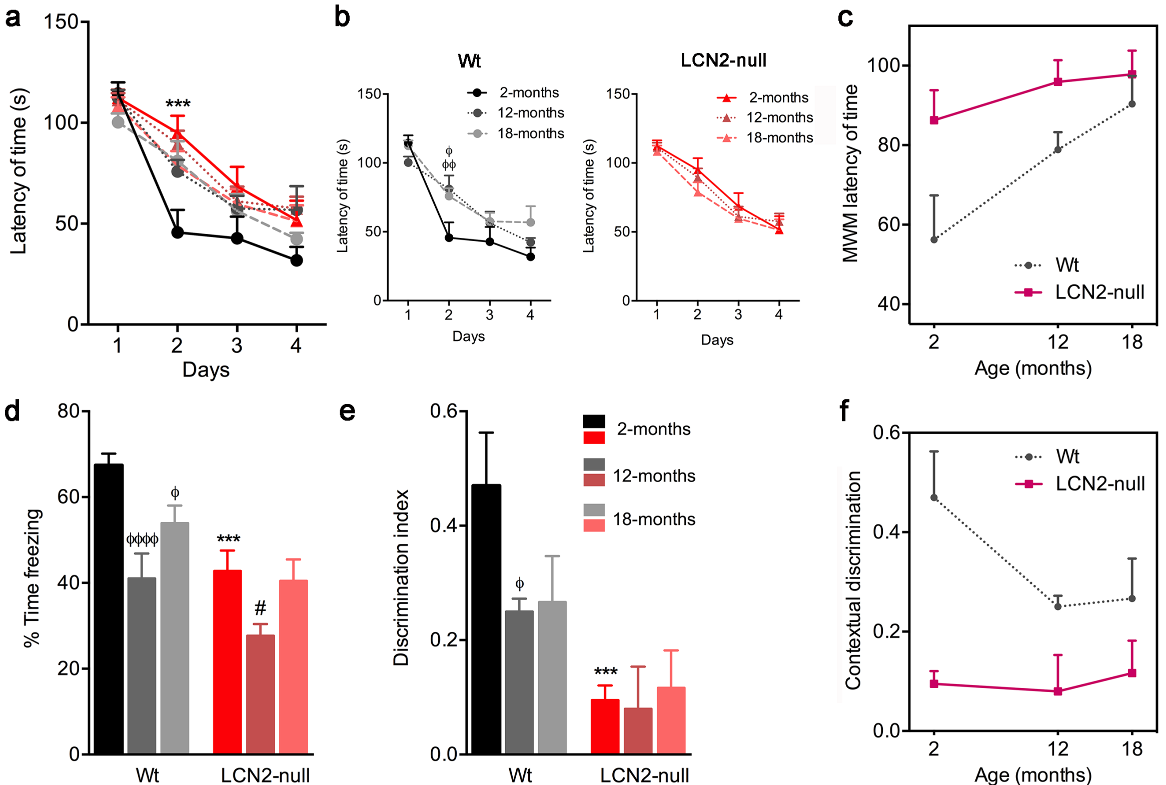

Figure 4: Spatial learning and memory retrieval and consolidation decreases during normal aging. (**a**) Analysis of spatial learning and memory in the MWM test revealed that all groups learned to find the position of the hidden platform across the 4 days of acquisition. (**b**) Older Wt mice required more time to find the platform, while LCN2-null animals presented similar impaired learning curves throughout aging (n=10-12 mice per group). (**c**) Cognitive deficits in the course of normal aging, measured by the reduced latency of time in the MWM. (**d**) Freezing behaviour upon re-exposure to conditioning context revealed a major effect of aging in Wt animals, as freezing behaviour decreased in older animals. (**e**) Impaired memory retrieval and consolidation was confirmed by the decreased ratio of discrimination index in aged Wt mice, which was sustained in LCN2-null mice throughout aging (n=10-12 mice per group). (**f**) Impaired contextual discrimination along aging in Wt and LCN2-null mice. Data are presented as mean ± SEM, analysed by two-way ANOVA with Bonferroni's multiple comparison test. $^{\Phi}$Denotes differences between young and aged Wt mice; $^{\#}$between differences between young and aged LCN2-null mice; *between Wt and LCN2-null mice at each matched age. $^{\Phi,\#}p \leq 0.05$, $^{\Phi\Phi\Phi\Phi}p \leq 0.0001$, $^{***}p \leq 0.001$.

Additionally to the MWM test, we have also used the CFC to assess contextual discrimination, as a measure of fear memory. After training the animals to a cued conditioning task, animals were re-exposed to the conditioning context and freezing behaviour was scored as a measure of memory

contextual retrieval. Analysis of the effect of age in the freezing time revealed that it significantly influenced animal's performance (age effect: $F_{2,34}$=20.83, p<0.0001). Specifically, we observed that older Wt animals significantly decreased their freezing behaviour, in comparison to younger animals (12- < 2-months, p<0.0001; 18- < 2-months, p=0.02; Figure 4d). On the other hand, freezing behaviour of LCN2-null mice remained similar throughout ages. The decreased freezing behaviour observed already at 2-months old (Wt *versus* LCN2-null, p=0.0005) persisted in aged null animals [more pronounced in 12-months animals (12- < 2-months, p=0.02; Figure 4d)]. In addition, analysis of contextual discrimination index, after presentation of a novel context, revealed that aging affected discrimination indexes. Similarly, for contextual retrieval, aging significantly decreased the index of contextual discrimination in Wt animals (12- < 2-months, p=0.05; Figure 4e, f), while LCN2-null mice presented the same ratios of contextual discrimination across ages, similar to younger animals (Wt *versus* LCN2-null: 2-months, p=0.001; Figure 4e). Aging significantly affected the ability of animals to discriminative different contexts presentation, thus influencing fear memory (Figure 4f).

The generation of newborn neurons in the hippocampus decreases with aging

In order to disclose the contribution of hippocampal plasticity to the described age-related alterations in behaviour, in both Wt and LCN2-null mice, we next analysed the generation of adult newborn neurons in the DG of the hippocampus. With this purpose, animals at the described ages were daily injected with BrdU for 5 days, followed by a chase period of 28 days (Figure 5a). Quantification of the total number of $BrdU^+$ cells in the DG, as a mean of cell survival, revealed a significant effect of aging ($F_{1,13}$=246.3, p<0.0001). Animals at 12-months of age presented a significant decrease in the number of labelled survival cells (Figure 5b). Both Wt and LCN2-null mice, in comparison to younger animals, presented a significant decreased in the total number of $BrdU^+$ cells in the DG (Wt: 12- < 2-months, p<0.0001; LCN2-null mice: 12- < 2-months, p<0.0001; Figure 5b). Despite the observed significant decreased cell survival in LCN2-null animals at 2-months (Wt *versus* LCN2-null, p=0.002), it was evident that this cell population was similarly affected during the course of the aging process, as in the Wt animals.

Additionally, analysis of the percentage of newborn neurons generated in the DG, within the 28-day period of labelling, showed that aging significantly impaired the formation of new neurons ($F_{1,14}$=28.50, p<0.0001). Specifically, aged Wt mice presented a significant reduction in the percentage of newborn neurons (12- < 2-months, p=0.0007; Figure 5c), similarly observed in LCN2-null mice at 12-months of age (12- < 2-months, p=0.02; Figure 5c). Again, despite the observed

impairments at 2-months in LCN2-null mice (Wt *versus* LCN2-null, p=0.02), cell differentiation was significantly affected by aging.

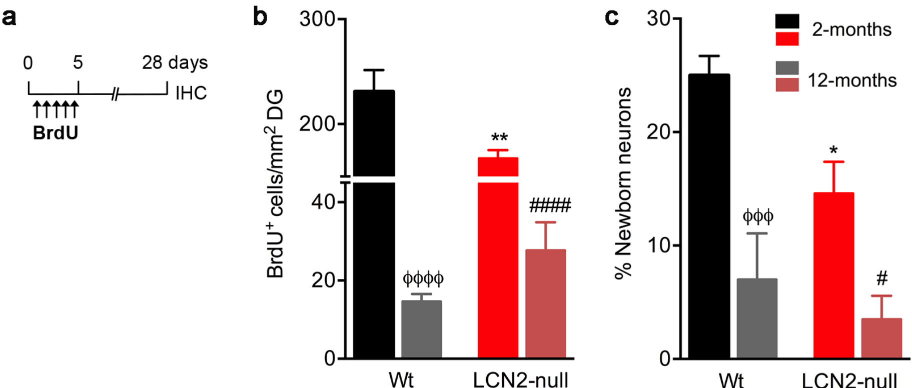

Figure 5: Aging reduces hippocampal neurogenesis in both Wt and LCN2-null animals. (**a**) Schematic diagram of the BrdU protocol used to label cell survival and neuronal differentiation. (**b**) Quantification of total number of $BrdU^+$ cells in the DG of Wt and LCN2-null mice disclosed a significant effect of aging in cell survival (n=4-5 mice per group). (**c**) Percentage of newly born neurons is significantly affected by aging, independently of animal's genotype. Data are presented as mean ± SEM, analysed by two-way ANOVA with Bonferroni's multiple comparison test. $^{\Phi}$Denotes differences between young and aged Wt mice; $^{\#}$between differences between young and aged LCN2-null mice; *between Wt and LCN2-null mice at each matched age. $^{\#,*}p\leq0.05$, $^{**}p\leq0.01$, $^{\Phi\Phi\Phi}p\leq0.001$; $^{\Phi\Phi\Phi\Phi,\#\#\#\#}p\leq0.0001$. IHC, immunohistochemistry.

Discussion

In the present study, we evaluated the impact of aging in several dimensions of animal behaviour, including mood, anxiety and cognition, and upon the absence of LCN2. Moreover, we evaluated brain plasticity in the form of hippocampal neurogenesis as one of the neurobiological mechanisms playing a more determining role in the observed behavioural outcomes across aging. Our results indicate that aged Wt animals presented progressive cognitive deficits, in both contextual discrimination and spatial learning, but not of anxiety and learned helplessness, when comparing to behavioural performances at younger ages (Figure 6). Similarly, the effects of LCN2 absence in learned helplessness and cognition, already observed at 2-months of age (Ferreira et al., 2013), were sustained throughout aging (Figure 6). The exception concerned the anxiety domain, since the time spent in the open arms by LCN2-null mice increased with aging (Figure 6). Moreover, age also significantly impacted on hippocampal neurogenesis, in both Wt and LCN2-null mice, as seen by the prominent decrease in cell survival and in the generation of new neurons.

Evaluation of anxiety-like behaviour along the process of aging revealed that 12-months old Wt mice present an anxious-like behaviour, when compared to younger mice. However, at later ages, a reduced anxiety was observed (Figure 6). This reduction was independent of LCN2, since aged LCN2-null mice, at both EPM and light/dark box tests, decreased the time spent in the open arms and in the light compartment, respectively. Although not so evident in aged Wt mice (Figure 6), our observations are similar to what others have also reported when analysing age-related changes in C57BL/6J mice behaviour (Botton et al., 2016; Shoji et al., 2016). Particularly for the EPM, some authors speculate that the decrease in the time spent in the open arms of the maze, as we demonstrated here for our LCN2-null mice aging cohort, may reflect an increased panic-like escape response to the novel environment (Holmes et al., 2000; Hattori et al., 2011). Moreover, anxiety-like behaviour assessments in aged mice are described to be test-dependent: a same cohort of aged animals, that were less anxious in the EPM, showed aversive behaviour to the center area of the open field test (Botton et al., 2016). These differences have been attributed to the sensitivity of anxiety responses inherent to each behavioural apparatus, which also varies with age (Botton et al., 2016). Nevertheless, here we observed the same phenotype on both EPM and light/dark box test (at least for the 18-months timepoint) in LCN2-null animals.

Regarding depressive-like behavioural domain, while some hypothesis in the literature suggest that aging is associated with an increased risk of depression (Godbout and Johnson, 2006), some inconsistences are also reported on the effects of aging in this type of behaviour (Godbout et al.,

2008; Malatynska et al., 2012; Shoji et al., 2016). The described contradictions have been mainly attributed to the different ages used, animal species and strains, or even to behavioural procedures. While some described a lack of affective deficits in 18-months old C57BL/6 mice in the FST (Malatynska et al., 2012), others reported that immobility time in the FST decreases from young adulthood to middle age [8 – 12-months old; (Shoji et al., 2016)]. In fact, our results are in agreement, as we observed decreased immobility time at 12- and 18-months of age, when compared to the younger ages (Figure 6), in both Wt and LCN2-null mice.

This decrease might be explained by the fact that the same animals also decreased their anxiety across ages (although only evident in Wt mice at 18-months). It is well known that increased anxiety is an important feature of depressive states, and the observed decreased anxiety, at least for the LCN2-null mice, may account to the reduced behavioural despair observed in the FST by aged mice. Still, and even though the immobility time in LCN2-null mice decreased with age, learned helplessness persisted in aged mice (Figure 6) when compared to aged-macthed Wt mice, which may suggest a putative involvement of LCN2 in late depression. The prevalence of depression is elevated in older people (Glaesmer et al., 2011) and, taking into consideration our results, aged LCN2-null mice could constitute an effective animal model to explore putative novel therapeutic approaches in late-life depression. In fact, increased LCN2 plasma levels were observed in depressed older patients, proposing LCN2 as a marker in the pathophysiology of late-life depression (Naude et al., 2013).

One prominent feature associated with aging, and extensively explored and reported in aged animal models, concerns the progressive loss of cognitive functions, especially in certain types of learning and memory (Bach et al., 1999). In line with other reports (Magnusson et al., 2003; Bergado et al., 2011; Shoji et al., 2016), we here observed, in Wt mice, an aged-related spatial learning and memory defect in the MWM task (Figure 6). In contrast, LCN2-null mice performance was sustained throughout aging (Figure 6). The behavioural despair presented by LCN2-null mice at 2-months of age (Ferreira et al., 2013) remained the same, regardless of the aging process. Moreover, when tested for contextual fear memory, a more DG-dependent task, age-related deficits in the ability to discriminate between contexts was observed in both genotypes. With age, Wt mice decreased their contextual discrimination, as observed by the impaired discrimination indexes, while LCN2-null mice, similarly to the MWM performance, presented a constant impaired contextual discrimination across aging (Figure 6). These findings are, in fact, relevant and should be contemplated when considering the usage of LCN2 levels as predictors of cognitive impairments. The description of increased LCN2

plasma levels during mild-cognitive impairment, and the consequent suggestion that it might be helpful in predicting the progression of this state to Alzheimer's disease (Choi et al., 2011), should be considered with caution in light of the present results.

Associated with age-related poor cognitive performances are also the descriptions of functional changes in the hippocampus, the brain region mostly involved in learning and memory (Jarrard, 1995). Alterations include hippocampus structural atrophy (Small et al., 2002) and decreased volume (Mattson and Magnus, 2006), as well as reduced hippocampal neurogenesis (Kuhn et al., 1996) and synaptic plasticity (Barnes, 1994). In fact, deficits in hippocampal long-term potentiation (LTP) imposed by aging have been correlated with defects in spatial memory (Bach et al., 1999; Barnes, 2003). In this sense, it is interest to observe that LCN2-null mice, at 2-months of age, were described to present neuronal atrophy in the dorsal hippocampus and synaptic impairments in hippocampal LTP (Ferreira et al., 2013). Even though we do not known if this is the case, these differences may be accounting to the sustained impaired cognitive function described. We can only speculate that these impairments might persist until older ages and, therefore, contribute for the cognitive decline maintenance in these animals. Nevertheless, further electrophysiological assessments and morphological reconstructions would be required to confirm this.

Other signs of age-associate decline in hippocampal plasticity include hippocampal neurogenesis, which diminishes with aging (Kuhn et al., 1996; Heine et al., 2004). Hippocampal neurogenesis largely contributes to cognition and memory and, in fact, age-related decreased hippocampal neurogenesis has been suggested as the basis for learning and memory decline (Bizon et al., 2004) and impaired contextual discrimination (Moyer and Brown, 2006) during aging. In accordance, our descriptions of impaired learning and cognition and fear memory in aging were further accompanied by a decreased cell survival and neuronal differentiation in the hippocampus of aged mice. This is certainly a great contributor to the observed impaired phenotypes. Moreover, we have recently reported LCN2 to be an important regulator of hippocampal neurogenesis (Ferreira et al., 2017). In young LCN2-null mice, decreased neurogenesis contributes to impaired contextual discriminative behaviours (Ferreira et al., 2017). Still, and although the generation of newborn neurons similarly decreased in aged LCN2-null DG, as in the Wt, this did not translated into a worsen cognitive phenotype. Probably, in this case, is not just a matter of down-regulated neurogenesis but rather of impaired functionality of the new neurons added into the hippocampal circuitry. Moreover, oxidative stress imposed by the absence of LCN2, as we have previously described to impair neurogenesis and contextual discrimination at younger ages (Ferreira et al., 2017), may also account for the

sustained impaired behaviour in aging. Oxidative stress is also considered to underlie aging-related cognitive impairments and degeneration (Nicolle et al., 2001; Hu et al., 2006).

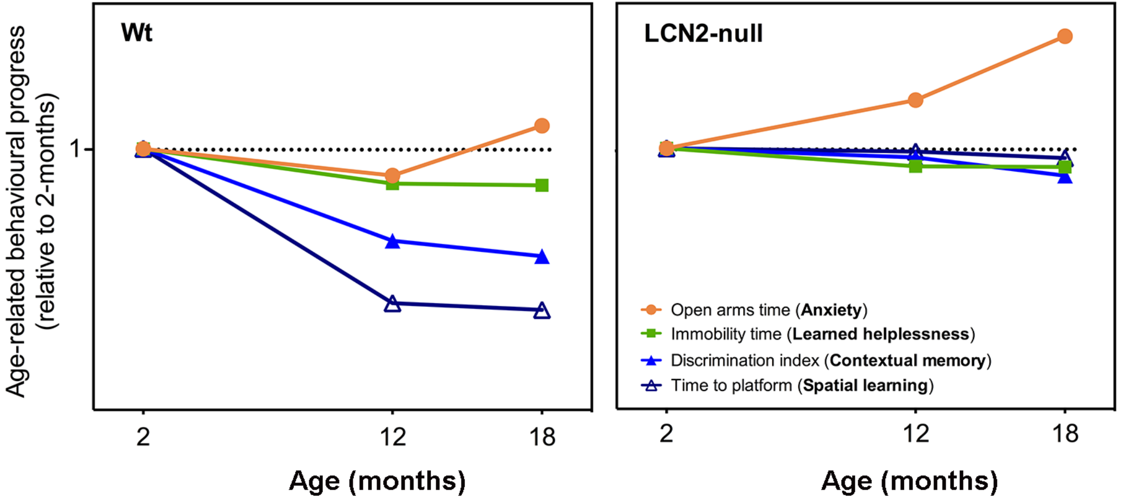

Figure 6: Schematic representation on the progression of the behavioural dimensions assessed throughout aging, in both Wt and LCN2-null mice. Comparison of behavioural performances of aged animals with younger ages revealed that aging, in Wt mice, slightly decreased their anxiety state at 18-months (as observed by the increased open arms time), but did not promoted depressive-like behaviours (immobility time is decreased). Cognitive domains, both spatial learning and contextual discrimination, was significantly impaired with aging. On the other hand, aged LCN2-null mice sustained their impaired behaviour observed already at 2-months of age, specifically in depressive-like behaviour and cognitive domains, with the exception of anxiety that was significantly decreased (represented as the increased in the time spent in the open arms).

Concluding remarks

With the average age of the world's population rapidly rising (United Nations World Population Ageing 2013), the need for studies investigating aging-related cognitive impairments has become increasingly important. In the literature, the usage of animal models of aging has proven useful not only for understanding the aging process, but also for understanding the functioning of the hippocampus. In addition, the plasticity and regeneration capacity intrinsic to the hippocampus, in part explained by the neurogenesis process, opens novel perspectives on the neurobiology of aging. We believe that, with the present report, we have contributed to this field, by describing the behavioural performance of Wt mice during the process of aging, in addition to the elucidation on how a single protein involved in plasticity modulation contributes to such process.

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CHAPTER V

General discussion and future perspectives

Since the discovery that the adult brain is capable of considerable structural and functional plasticity, as the generation of new neurons, the number of studies relying on adult neurogenesis has grown exponentially. In recent years, the demonstration that multipotent NSCs reside in the adult mammalian brain, including in humans, has generated great interest and enthusiasm within the neuroscience community. This is mostly due to the importance of newborn neurons in the maintenance of brain homeostasis [reviewed in (Ming and Song, 2011; Goncalves et al., 2016)], and the recognition of their potential for the development of new therapies in CNS repair (Stoll, 2014). Understanding how new neurons regenerate and incorporate into the networks of the adult brain is important, when considering its broad applications for the treatment of neurodegenerative diseases, cognitive decline, stroke (Vishwakarma et al., 2014), and even depression and anxiety (Benninghoff, 2009). Specifically, this form of brain regeneration has been described as a potential for cell transplantation therapies and/or, more importantly, by the exploration of the underlying mechanisms that support the activation and modulation of endogenous progenitors. In fact, many studies have successfully reported cell transplantation in the restore of neuronal plasticity in the irradiated hippocampus (Acharya et al., 2015), of learning and memory in mouse models of AD (Tong et al., 2014), and in the treatment of Parkinson's disease (Kriks et al., 2011), spinal cord injury (Tarasenko et al., 2007) and MS (Pluchino et al., 2003). Still, the clinical success of cell transplantation therapies in human patients is far from being consensual. Moreover, the adult mammalian brain, *per se*, is suggested to display endogenous regenerative capacity, even though with low efficacy (Okano, 2006; Okano and Sawamoto, 2008). Both adult SVZ and DG neurogenic niches are highly responsive to brain damage and there is evidence, both in animal models and post-mortem tissues from patients, that neural stem and precursor cells of the niches display abnormal proliferative and migratory profiles after brain injury (Sun et al., 2007), possibly to rescue neuronal loss. This regenerative capacity, particularly in the hippocampus, was even described to underlie the functional effect of antidepressant treatment in mood-related disorders (Kempermann and Kronenberg, 2003). As so, this hold great potential and it certainly motivates further investigation on brain endogenous regeneration. Still, it is essential to firstly understand how the process of neurogenesis in health and disease is intrinsically regulated.

Among the factors reported in the literature to serve functions in both the healthy and diseased brain [reviewed in (Ferreira et al., 2015)], with additional reported roles in cell proliferation and survival (Yang et al., 2002), we explored LCN2 as a novel putative regulator of neural plasticity in the adult mammalian brain.

With several and different described functions, across different mammalian organ systems, including the CNS, LCN2' antimicrobial activity is, undoubtedly, the best described one (Flo et al., 2004). However, in recent years, there has been a shift towards the understanding of LCN2 involvement in the regulation of physiological process based on its putative capacity to traffic iron within the cells (Yang et al., 2002; Devireddy et al., 2005). This, in fact, has been opening important links between physiology and pathology. Actually, and particularly for the brain, LCN2 has been attributed both neurodetrimental (Nam et al., 2014) and neuroprotective (Berard et al., 2012) roles in disease progression, particularly in MS, but also in neurodegenerative diseases where iron is a key player, including AD (Naude et al., 2012; Mesquita et al., 2014) and Parkinson's disease (Kim et al., 2016). In fact, the presence of LCN2 in the brain regions associated with AD pathology in human post-mortem brain tissue (Naude et al., 2012), and in the mediation of Aβ cytotoxicity (Mesquita et al., 2014), may relate to the ability of LCN2 to regulate intracellular iron contents. Specifically, LCN2 plays a detrimental role in neural cells in response to Aβ peptides by modulating the expression of iron-metabolism related genes (Mesquita et al., 2014). Nevertheless, the contribution of LCN2 for brain homeostasis, through iron modulation, is scarce and still quite controversial, but important when considering its relevance for disease onset and progression (Ferreira et al., 2015).

As so, in this thesis, we focused on disclosing the role of LCN2 in brain physiology and function. Specifically, we analysed the role of LCN2 on the regulation of neurogenesis in the adult mammalian brain, both at physiological conditions, in the course of aging, and in response to external stimulus. Even though we have considered the regulation of neurogenesis by LCN2 at the two well-described brain regions where cell genesis is known to occur, our major focus in this thesis concerned its regulation at the hippocampal neurogenesis, due to the known importance of this brain region in mediating animal behaviour responses, and in several neuropsychiatric and degenerative diseases (Goncalves et al., 2016).

To explore the regulatory mechanisms engaged by LCN2 in adult neurogenesis, we used, as an animal model, mice with a genetic deletion of the *Lcn2* gene, the LCN2-null mice. These animals are viable, with normal litters, and present no apparent phenotype when housed under normal standard conditions (Flo et al., 2004). For the majority of the experiments performed in this thesis, with the exception for the aging study (Chapter IV), we used young adult LCN2-null animals with 2-months old, as we consider this as a critical period for neural plasticity in the brain and for behaviour in adulthood (Kolb and Gibb, 2011).

In our first approach, we aimed at characterizing the impact of *Lcn2* absence in the maintenance of the pool of NSCs in the neurogenic niches, as well as in the generation of new neuronal and glial cells. Also, using both *in vivo* and *in vitro* methodologies, we disclosed the contribution of LCN2 in the regulation of NSCs cell cycle, by iron-dependent mechanisms, and its impact on hippocampal integrity and function. In the second study, and since we observed a role for LCN2 in the modulation of neuroplastic events in the adult mammalian brain, we further aimed at addressing the impact of the external modulation of neurogenesis in LCN2-null mice neural plasticity and behaviour. For that, we conducted a voluntary running protocol, in order to stimulate hippocampal neurogenesis, while a chronic administration of CORT was applied to induce deficits in neurogenesis. Finally, in our third approach, and considering the relevance of LCN2 in brain functioning during young adulthood, we also proposed to assess the role of LCN2 during the course of aging, specifically in the regulation of aged-related behavioural changes and neurogenesis.
All together, with the work presented in this thesis, we expect to have expanded the current knowledge that exists on the molecular mechanisms regulating cell genesis in the adult brain, and to contribute to a better understanding on the roles of LCN2 in brain functioning and behaviour.

1. Adult neurogenesis orchestration by Lipocalin-2

The concept that LCN2 participates in iron-delivery related processes, at physiological conditions, is quite recent. By controlling intracellular iron contents, LCN2 can serve important homeostatic roles, as it was demonstrated during kidney development (Yang et al., 2002). The identification of LCN2-mediated iron delivery through an endogenous mammalian siderophore (Devireddy et al., 2005; Bao et al., 2010; Devireddy et al., 2010) introduced novel concepts of intracellular iron homeostasis that is conserved from bacteria to humans, and described to be present among several systems of organs [reviewed in (Ferreira et al., 2015)], and in which LCN2 is involved. However, the existence of a fine-tuned mediation of intracellular iron by LCN2, specifically in the brain, is not recognized, which prompted us to ascertain if LCN2 could serve similar homeostatic roles.
In the brain, LCN2 is considered to be a pleiotropic protein that, depending on the cellular origin, the site of action and the levels of production, is able to influence several different cellular mechanisms. Still, there is no consensus regarding to which type of neural cells are able to produce LCN2 and in which conditions [reviewed in (Ferreira et al., 2015)]. While most reports claim that LCN2 is produced only in response to inflammation (Marques et al., 2008) and brain injury (Jin et al., 2014), some studies have described LCN2 expression under physiological conditions by astrocytes (Chia et

al., 2011), epithelial cells of the choroid plexus (Chia et al., 2011), and even by neurons (Mucha et al., 2011). Of relevance, and independently of its cellular origin, LCN2 was shown to play a role in neuronal excitability (Mucha et al., 2011), structural plasticity (Mucha et al., 2011; Ferreira et al., 2013), and behaviour (Mucha et al., 2011; Ferreira et al., 2013). Moreover, LCN2 presence in specific brain regions, such as the hippocampus (Mucha et al., 2011), is postulated to occur so it can serve roles in iron trafficking within the CNS, similarly to what happens in the periphery. In fact, the capacity of LCN2 to regulate hippocampal dendritic spine density and morphology pertains to its ability to bind and transport iron (Mucha et al., 2011). In accordance, we now reveal a novel role for LCN2 in the control of the neurogenic process, since its absence leads to overall deficits in progenitors proliferation, differentiation and survival, with functional impact on contextual discriminative behaviour (Chapter II). In addition, and in order to better understand the role of LCN2 in this process, we have also analysed its expression in the adult physiological brain, giving particular attention to the DG of the hippocampus. In accordance with other reports (Marques et al., 2008; Ip et al., 2011), we were not able to detect the expression of LCN2 under basal conditions at the DG (Chapter II). Instead, we observed LCN2 to be highly present in the serum, as others have also shown (Song et al., 2014), which suggested that regulation of adult neurogenesis by LCN2 might occur extrinsically to the brain, rather than be an intrinsic CNS-derived cue. Of interest, a similar mechanism was recently described, since LCN2 was shown to be released by the bone in a nutrient-dependent manner, enter the bloodstream, cross the blood-brain barrier and bind to receptors in the hypothalamus to suppress appetite (Mosialou et al., 2017). Our current hypothesis is that LCN2 produced at the periphery by, for instance, the liver (Chakraborty et al., 2012), white adipose tissue (Mosialou et al., 2017) and/or neutrophils (Kjeldsen et al., 1993) (Figure 1), as well as osteoblasts (Mosialou et al., 2017), enters the bloodstream and is delivered by the endothelial cells of the blood vessels located within the niche that surround neural progenitors, to act on 24p3R-expressing NSCs (Sox2- and Nestin-positive cells), for the control of intracellular iron cell content (Figure 1). For sure, the potential role of the cells at the periphery that produce LCN2 in the regulation of brain cell genesis warrants further investigation. The conditional deletion of LCN2 at the periphery (Xu et al., 2015), for example, will help to elucidate this interplay.

One possible major drawback in our study might be the fact that we used a full knockout system. For sure, this is not ideal, and can represent only a part of a much more complex effect. Future studies will definitely benefit from a conditional cell type-specific manipulation of LCN2, even to rule out possible developmental influences, and separate the presumably different functions that LCN2

has in controlling and regulating neurogenesis in adulthood. Despite that, our *in vitro* studies were crucial to mechanistically demonstrate that regulation of NSCs is LCN2-specific (Chapter II). More importantly, we showed that this regulation is mediated through its specific cellular receptor 24p3R, in line with what is described for other systems (Yang et al., 2002; Devireddy et al., 2005).

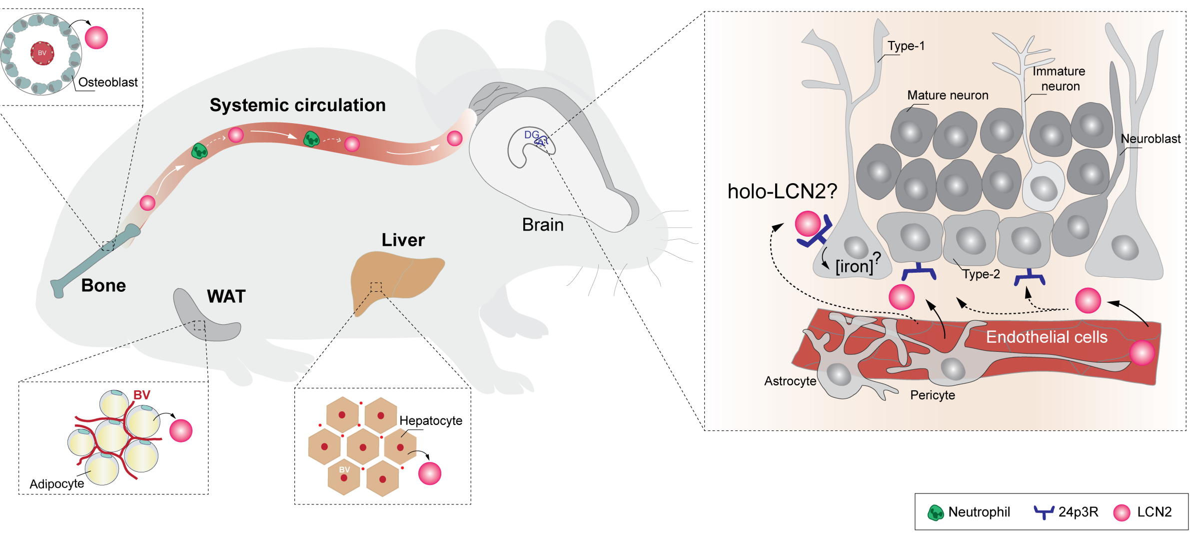

Figure 1: Lipocalin-2 produced at the periphery circulates in the bloodstream and impacts on the hippocampal neurogenic niche to regulate neural stem cells proliferation and self-renewal. Lipocalin-2 delivered by the endothelial cells of the blood vessels that surround the neurogenic niche, binds to 24p3R-expressing NSCs to regulate intracellular iron. Liver, bone, white adipose tissue (WAT) and neutrophils are exemplified tissues/cells at the periphery that are able to express LCN2. BV, blood vessel; DG, dentate gyrus; WAT, white adipose tissue.

The putative mechanism of LCN2-mediated control of neurogenesis revealed to be important, since LCN2-null mice presented a significant accumulation of quiescent NSCs in the hippocampus, due to an iron-mediated increased oxidative stress that impaired stem cells cycle and self-renewal. Precisely, our findings suggest that the lack of LCN2 translates into the accumulation of intracellular iron in NSCs, which imposes the observed oxidative stress environment that boosted apoptotic cell death. We clearly demonstrated, and for the first time in the context of the brain and in NSCs biology, the importance of iron in LCN2-mediated cell proliferation, division and survival. Instead, others have shown roles for LCN2 in iron metabolism for the regulation of cell migration and morphology of neurons (Lee et al., 2012), astrocytes (Lee et al., 2009) and microglia (Lee et al.,

2007). Also, clearly reported are the mechanisms through which LCN2 regulates apoptotic cell death, and that involve the proapoptotic protein BIM (Devireddy et al., 2005). Whether LCN2 mediates NSCs apoptosis through BIM expression, as observed in other cellular contexts (Lee et al., 2009; Lee et al., 2012; Mesquita et al., 2014) was not addressed in here, but should be clarified in future work. Moreover, and contrarily to our findings, some reports showed that, instead, increased levels of LCN2 favours the formation of ROS (Kagoya et al., 2014; Wu et al., 2015), as it regulates intracellular labile iron levels (Kagoya et al., 2014; Wu et al., 2015; Yamada et al., 2016).
Importantly, our results can be easily translated to other organ systems rather than the brain, with certainly important implications for stem cell research. For instance, LCN2 was shown to be an important regulator of the hematopoietic compartment, since LCN2-null mice developed a progressive accumulation of lymphoid, myeloid, and erythroid lineage of cells (Liu et al., 2011). On the other hand, the transgenic overexpression of LCN2 was shown to modify the bone marrow microenvironment by controlling the expression of key secreted factors and cytokines, to regulate and stimulate the reservoir of hematopoietic stem cells and their maturation (Costa et al., 2016). Of interest, we have also characterized cell proliferation in the gut of LCN2-null mice and, although we have not explored it into much detail, we observed a prominent decrease in the number of BrdU-labelled cells present at the proliferation zone of the crypt (data not shown). Nevertheless, the functional significance of this finding warrants further clarification. Probably, LCN2 can serve roles in the maintenance of intestinal homeostasis, as others have already shown in response to gut inflammation (Moschen et al., 2016), by also regulating gut stem cell compartments.

Several studies have previously reported the importance of ROS and redox-signalling mechanisms in the proliferation and cell fate of neural precursors (Yoneyama et al., 2010; Le Belle et al., 2011; Maryanovich and Gross, 2013; Prozorovski et al., 2015). Although the precise molecular mechanisms mediating these effects are not yet fully understood, stem cells maintain a low intrinsic redox state that supports their quiescent state and maintains the normal levels of neurogenesis (Madhavan, 2015). The contribution of iron and iron-traffic proteins in the redox control of NSCs for cellular function, as we describe in this thesis, is unique. The few reports that describe a trophic role for iron in cell proliferation and differentiation in the CNS showed that transferrin mediates oligodendrogenesis in the SVZ (Silvestroff et al., 2012, 2013), supporting evidence for the potential role of transferrin in demyelination. Similarly, our observations that hippocampal neurogenesis is regulated by LCN2, through iron-related mechanisms, also translated into important effects in brain

function, as observed by the deficits presented by LCN2-null mice in synaptic plasticity, neuronal morphology, and behaviour [Chapter II and (Ferreira et al., 2013)]. The importance of iron in this regulation is evident when the treatment of LCN2-null mice with the iron chelator DFO prevented NSCs oxidative stress and cell death (Chapter II), rescued the generation of new neurons (data not shown), and of contextual discrimination behaviour. Moreover, and although we have also characterized adult SVZ neurogenesis in the absence of LCN2, its functional impact in, for instance, odour memory and discrimination (Rochefort et al., 2002) was not explored. Also, the importance of LCN2 in regulating NSCs function and cell genesis in other brain regions recently claimed to present neurogenesis, such as the hypothalamus (Markakis et al., 2004; Kokoeva et al., 2005), and the amygdala (Bernier et al., 2002), can be of interest to further explore, even more if we consider the known roles of LCN2 in metabolism and insulin resistance (Guo et al., 2010; Abella et al., 2015; Mosialou et al., 2017) and in anxiety (Ferreira et al., 2013), respectively.

An important feature of adult hippocampal neurogenesis is that newborn neurons are highly active, integrate into the hippocampal circuitry and contribute to local neural activity for a proper brain functioning (Goncalves et al., 2016). In fact, numerous studies have reported alterations in adult neurogenesis that are associated with the progression and onset of several neurological and neuropsychiatric disorders, thus providing a link between neural plastic events and disease. For instance, the total ablation of neurogenesis precipitates behavioural despair and anhedonia (Snyder et al., 2011), and neurogenesis was also observed to be required for the behavioural effects of antidepressants (Santarelli et al., 2003). It is, therefore, not a surprise to observe how impaired neurogenesis, by the lack of LCN2, decreases neuronal activation in the hippocampus (cFOS countings – not shown), potentiates anxiety and depressive-like symptoms, and possibly accounts for the general decreased LTP observed in LCN2-null mice (Ferreira et al., 2013). In fact, the importance of adult neurogenesis in mediating such processes, through LCN2, became evident when we improved anxiety and cognition by increasing neurogenesis with voluntary running (Chapter III). Our understanding on the molecular factors that control the generation of new neurons in the adult hippocampus, namely at the level of the NSCs, may certainly guide the further comprehension of the events that control brain processes, opening new avenues for the development of more targeted therapeutic strategies. For sure, LCN2 should now be considered as a new and important key player.

2. Lipocalin-2 as mediator of neural plasticity and brain function

The observations that LCN2 is important in the regulation of adult neurogenesis encouraged us to further scrutinize the impact of an external regulation of neurogenesis in the absence of LCN2. For that, we used, in one hand, voluntary running to promote cell proliferation and increase neurogenesis while, in the other, induce deficits in the generation of new cells by the chronic administration of CORT. We decided for this specific type of modulation since these are classically characterized as the most potent environmental regulators of neurogenesis.

Among the different existing forms of enhancing neurogenesis and improving behaviour, which include environmental enrichment (Kempermann et al., 1997) and water-maze learning (Gould et al., 1999), voluntary running is the most powerful and robust regulator (van Praag et al., 1999b). It enhances several aspects of the adult hippocampal neurogenic process, including progenitor cells proliferation and survival (van Praag et al., 1999b), and its beneficial effects in behaviour are well documented in the literature. Exercise-enhanced neurogenesis include improvements in the performance of learning tasks (van Praag et al., 1999a), rescue of behavioural and electrophysiological impairments caused by reduced neurogenesis (Sakalem et al., 2017), amelioration of age-related stress and learning impairments (van Praag et al., 2005), and controlled disease progression or improved cognition in animal models of neurodegeneration (Tapia-Rojas et al., 2016). In fact, regular physical exercise serves as one of the most effective non-pharmacological tool to enhance brain health (Marlatt et al., 2010). Considering these beneficial effects, we decided to positively modulate neurogenesis by voluntary running in LCN2-null mice. For that, we used a chronic protocol of voluntary wheel running (28 days), since this has been shown to be the most effective in increasing neurogenesis (Naylor et al., 2008) and in positively modulate anxiety (Garrett et al., 2012) and cognition (Yau et al., 2012). We observed that exercise-mediated increase in neurogenesis improved LCN2-null mice impaired NSCs proliferation and survival, resulting in reduced anxiety and improved contextual discrimination (Chapter III). Whether this running protocol applied can prime a survival effect on newborn cells in LCN2-null mice, even after cessation of the running protocol, is unclear. There are reports showing that running-induced increase in adult neurogenesis remains even 35 days after the end of the running protocol (Fabel et al., 2009).

In opposition to some reports, which claimed that sustained running maintains high rates of neurogenesis instead of promoting proliferation of dividing cells (Snyder et al., 2009), we observed that running promoted both cell proliferation and survival in the hippocampus of LCN2-null mice, contributing to overall increased neurogenesis (Chapter III). Specifically, the regulation of NSCs

quiescence by running in LCN2-null mice had important implications for long-term modulation of neurogenesis, as demonstrated by the increased production of fast-proliferating stem cells and new neurons. Moreover, this increase was functionally effective, since it was sufficient for LCN2-null mice to adjust their behaviour and improve both anxiety and contextual discrimination. In fact, that is evident in the correlative analysis performed between newborn neurons and behavioural performance after running. One possibility to be considered is whether the re-establishment of NSCs population by running in LCN2-null mice, and subsequent increase in neurogenesis, is related to a reduction of apoptosis occurring in radial glia-like type-1 stem cells. At least in part, this possibility should not be excluded in further analysis, even more if we consider the fact that running was able to promote the rescue of the antioxidant regulation of Sox2-positive stem cells in LCN2-null mice.

One of the mechanisms by which exercise also has profound benefits for brain function regards the dendritic neuronal remodelling and consequent improvements in synaptic plasticity (van Praag, 2009; Leuner and Gould, 2010). In fact, neuronal cell activation (Clark et al., 2010) and LTP enhancement (O'Callaghan et al., 2007) were observed after running. More importantly, it seems to be specific for the DG (van Praag, 2008), thus indicating the importance of neurogenesis in the process. In line with this, we have previously shown that, specifically in the DG, but also in the CA1 region of the hippocampus, LCN2-null mice display neuronal atrophy and decreased synaptic plasticity (Ferreira et al., 2013). Even though newborn neurons represent only a small percentage of the cellular population that compose the granule cell layer, those neurons have a transient increase in LTP, highly contributing to local synaptic plasticity (Schmidt-Hieber et al., 2004; Ge et al., 2007) and, therefore, hippocampal function. While we have no further clear evidence by now, one can associate and imply neurogenesis as central in contributing to the observed impaired neuronal remodelling and synaptic plasticity in LCN2-null mice (Ferreira et al., 2013). Analysis of both neuronal structure and degree of activation (e.g. cFOS analysis) in LCN2-null mice, after running, would contribute to better clarify this. Of course, many other mechanisms mediate the effects of exercise on the brain, including neurotransmitters, neurotrophins and vasculature [reviewed in (van Praag, 2009)], which should be also further taken into consideration.

Moreover, perhaps the major evaluation that we miss in our study concerns the analysis of depressive-like behaviours in LCN2-null mice after the voluntary running, since these animals were described to present increased learned helplessness in the FST (Ferreira et al., 2013). The efficacy of exercise in treating stress-related illnesses, such as depression, is well documented (Lawlor and Hopker, 2001), and believed to occur based on the effects of running on structural and functional

plasticity. In addition, the dysfunction of the HPA axis is a hallmark of stress-related pathologies (Smith and Vale, 2006) and there is increasing evidence that exercise induces extensive changes in the axis, under baseline conditions and in response to stressful challenges. While some studies report a reduction of the HPA axis in response to stressors after running (Campeau et al., 2010), or no running-induced increase in CORT levels (Hare et al., 2014), others describe the activation of the axis upon chronic exercise (Droste et al., 2003; Fuss et al., 2010). This is in fact an intriguing paradox, such that exercise is beneficial for cognition, mood and anxiety, by increasing neurogenesis, but it activates the HPA axis and increases the levels of CORT (Stranahan et al., 2008). This is because, to some authors, activity by voluntary running is considered a stressor for the animals (Droste et al., 2003). Until this point, we have not considered in our analysis the levels of CORT in LCN2-null mice after running, but since cell proliferation remained increased even after the 4 weeks of exposure to running wheels, probably the level of activity reached by our animals was not sufficient to induce stress. Of course, we can only speculate on this, since we have not also controlled for the amount of distance run by the mice. Interestingly, we have previously described that LCN2-null mice, at physiological conditions, present a sustained and increased production of CORT, evidencing a hyperactivation of the HPA axis that culminated in anxiety and cognitive impairments (Ferreira et al., 2013). Precisely how this occurs in the absence of LCN2 is still unclear. The further analysis of the CORT levels in LCN2-null mice, after the protocol of exercise, will, for sure, add more evidence to our current observations. Moreover, and since voluntary running recovered the behavioural impairments observed in the absence of LCN2, the exposure of LCN2-null mice to a mild stressor, immediately after running, would also help to unclear the importance of the HPA axis in LCN2-null animals for the regulation of neurogenesis and behaviour. Rodents with access to a running wheel typically show a more finely tuned response to stress, by improving the control over the HPA axis (Campbell et al., 2009).

In the meantime, we decided to challenge the HPA axis of LCN2-null mice and assess its impact in the regulation of adult neurogenesis. Stress has been reported by many as one of the major psychological factors that reduce the proliferation of hippocampal progenitor cells and the generation of newborn neurons (Santarelli et al., 2003; Murray et al., 2008; Brummelte and Galea, 2010). In this type of regulation, glucocorticoids are the crucial effectors of stress-induced depletion of neurogenesis, since the administration of adrenal hormones negatively affects proliferation (Gould et al., 1998), which can be counteracted by the removal of hormones by adrenalectomy (Cameron and Gould, 1994). There are several animal models to study the effects of stress on hippocampal

plasticity, which include physical restraint (Pham et al., 2003), immobilization (Vollmayr et al., 2003), or foot shock (Malberg and Duman, 2003), among others. However, individual variation in CORT levels between different animals exposed to the same stressor may increase experimental variability (Nestler et al., 2002). In this sense, the protocol of repeated CORT injection in rodents has been validated as a reliable animal model to induce stress in a controllable manner (Sterner and Kalynchuk, 2010) and, therefore, to study the role of stress in hippocampal plasticity and in behaviour (Zhao et al., 2008; Marks et al., 2009). Taking this into consideration, and for our analysis on the effects of stress on hippocampal neurogenesis and behaviour in the absence of LCN2, animals were chronically treated with CORT for 28 days (Chapter III). In accordance with other reports (Brummelte and Galea, 2010; Yau et al., 2012), we showed that chronic CORT treatment significantly decreased hippocampal cell proliferation and survival, resulting in anxiety-like behaviours and deficits in contextual discrimination (Chapter III). However, this effect was only observed in Wt animals; the stress protocol that we applied did not exert any impact on hippocampal plasticity and function in LCN2-null mice. This can be assumed to be, at some point, expected if we consider the hyperactivation of the HPA axis previously described in LCN2-null animals (Ferreira et al., 2013). However, precisely how the specific deletion of *Lcn2* contributes to sustained production of CORT is not understood. Of interest, previous studies have found LCN2 to be up-regulated in the hippocampus after restraint stress, and to contribute to stress-induced neuronal plasticity at the cellular and behavioural levels (Mucha et al., 2011). Specifically, Mucha and colleagues (2011) described LCN2 as a modulator of stress by regulating actin mobility, with effects on dendritic spine density and morphology of hippocampal neurons and, ultimately, neuronal excitability and stress-induced anxiety (Mucha et al., 2011). Noticeably, we reported that LCN2-null mice, in physiological conditions, besides presenting a sustained production CORT (Ferreira et al., 2013), also display increased levels of ROS and a lack of an antioxidant regulation in NSCs (Chapter II). A synergy between the two forms of signalling are likely to exist in LCN2-null mice. For instance, in recent years, stress has been shown to generate ROS to induce oxidative injury in the hippocampus (McIntosh and Sapolsky, 1996; You et al., 2009; Sato et al., 2010), resulting in impaired cognitive functions (Sato et al., 2010). For example, dexamethasone, a synthetic glucocorticoid, was shown to deplete the neurogenic pool by targeting NPCs to apoptosis (Yu et al., 2010), as it generated ROS and compromised cells' antioxidant systems (Yu et al., 2010). On the other hand, chronic oxidative stress *per se* can induce dysfunction of the HPA axis, at least during aging (Kobayashi et al., 2009) or in a disease context (Zhu et al., 2007), resulting in increased serum glucocorticoids levels and

cognitive dysfunction. The further evaluation of CORT levels in LCN2-null mice after the treatment with NAC or DFO (Chapter II), for instance, or even the analysis of ROS levels after treatment of NSCs in culture with a CORT antagonist, for example, should help to better understand the type of regulation that exists in the absence of LCN2. Moreover, an adrenalectomy of LCN2-null mice would also be useful to disclose the importance of glucocorticoids levels in LCN2-mediated neurogenic process.

In addition, the application of different stress protocols (acute *versus* chronic) in LCN2-null mice, and the effect of LCN2 in other different stress-related brain regions, should also help to clarify its importance as a modulator of stress-induced changes. Of interest, *Lcn2* gene has a glucocorticoid response element (Garay-Rojas et al., 1996) and others have already shown *Lcn2* has one of the most highly up-regulated transcripts in the amygdala after acute restraint psychological stress (Skrzypiec et al., 2013). Nevertheless, our current observations that the exogenous administration of CORT had no impact on hippocampal neurogenesis and behaviour of LCN2-null mice strengths the view of the importance that CORT levels also have in the regulation of LCN2-null mice hippocampal neurogenesis, and in the genesis of mood and cognitive alterations. The descriptions of these mechanisms, as we presented in Chapter III, are important if we consider the existent relationship between neurogenesis and affective disorders, such as depression (Yun et al., 2016), and the reported proposal of LCN2 as a biological marker for depression (Naude et al., 2013).

3. Age-related impact of Lipocalin-2 on behaviour and plasticity

In the recent years, the number of reports demonstrating the nature of secreted LCN2 in several diseases has increased. Described to be associated with alterations in brain homeostasis and in specific brain regions known to be affected in neurodegenerative disorders, LCN2 has, in fact, been attributed a promising diagnostic and prognostic value in neurodegeneration [as reviewed in (Ferreira et al., 2015)]. Nevertheless, the precise impact of LCN2 in the CNS physiology is still scarce (Ferreira et al., 2013). With described roles in cognition during young adulthood [Chapter II and (Ferreira et al., 2013)], and also found to be increased in patients with AD (Naude et al., 2012) and mild cognitive impairment (Choi et al., 2011), understanding the role of LCN2 in aging is an important aspect to be considered in order to better understand its role in neurodegeneration, and to help to predict its diagnostic value. Moreover, the identification of biological markers and their association with characteristics of disease is essential with the aim to improve diagnosis and therapeutic approaches.

Since there are only few reports (Shoji et al., 2016) describing the age-related changes in behaviour, from young adulthood to middle and late ages, and also because LCN2 has described roles in behaviour during young adulthood (Ferreira et al., 2013), we primarily focused our analysis on the role of LCN2 in aging by exploring animal behaviour across ages. For that, both Wt and LCN2-null mice were aged until 12- and 18-months of age, and their behavioural performances compared with young adult 2-months old animals. From the dimensions analysed (anxiety, learned helplessness and cognition), our behavioural data revealed that aging is a permanent process that modulates certain aspects of basic behaviour in a continuous manner. Specifically in Wt mice, aging did not promoted anxiety and depressive-like behaviours, while it significantly affected cognitive domains (Chapter IV). Advanced ages were associated with an acceleration of behavioural impairments in memory consolidation at both MWM and CFC tests, but not in locomotion (at least in the EPM maze). In fact, one prominent feature associated with aging, and as we observed, concerns the progressive loss of cognitive functions (Bach et al., 1999). Of relevance, other reports have similarly analysed age-related changes in behaviour, from young adulthood to middle ages, and described decreased immobility in the FST and altered anxiety-like behaviours in older animals (Shoji et al., 2016). However, in this case, they provided an additional broad range of ages in which the animals were tested, which is beneficial from the longitudinal point of view. For sure, our analysis will further benefit from the inclusion of animals at middle ages (e.g. 6-months old).

Contrastingly, in mice deficient for *Lcn2*, aging surprisingly reduced anxiety-like behaviours, but sustained learned helplessness and cognitive impairments observed at younger ages (Chapter IV). While the observed reduction in anxiety can be attributed to the features of the behavioural tests used, as others have pinpointed (Shoji et al., 2016), the observations of a depressive-like behaviour also in advanced ages, and in cognition, highlights the need to better clarify the role of LCN2 in the normal aged brain, even before its association with neurodegenerative diseases. Certainly, many other tests to evaluate the same and/or other behavioural dimensions should be included in further analysis, such as social interaction, locomotor and exploratory activities, among others. Moreover, and although we have monitored for body weight gain throughout the aging process (Chapter IV), assessment of general health (e.g. body temperature), neuromuscular strength and normal pain sensitivity should be also further considered.

Our current knowledge about the neurobiology of aging is still quite limited. Intimately associated with aging, several hallmarks have been described, which represent common denominators of the aging process across species (Lopez-Otin et al., 2013). The present major challenge is the

understanding of the importance of each component in the course of the aging process, with the final goal to improve health care. Among others, cellular senescence, mitochondrial dysfunction and stem cell exhaustion are recognized hallmarks of aging (Lopez-Otin et al., 2013). Of interest, LCN2 is recognized and attributed with functions in some of these biological processes, at least during younger ages. Moreover, there is significant clinical and experimental evidence that inflammation within the CNS increases with age, most likely due to the accumulation of free radical damage over time (Fenn et al., 2015). In fact, another hallmark of the aged brain is the increased oxidative stress and lipid peroxidation (Romano et al., 2010). Importantly, some of these features have equally been described in the brains of LCN2-null mice (Kang et al., 2017; Wang et al., 2017). Nevertheless, we believe that possible recognized roles for LCN2, both in aging and in neurodegeneration, could be attributed to its iron trafficking properties. As the brain ages, iron accumulates in regions that are typically affected by AD and Parkinson's disease (Zecca et al., 2004). Moreover, high concentrations of reactive iron can also contribute to increased oxidative stress (Dixon and Stockwell, 2014) and induce neuronal vulnerability. Of notice, we have shown in this thesis that, during young adulthood, the absence of LCN2 induces an iron-mediated oxidative stress in NSCs, with important effects on brain functioning (Chapter II). As so, further studies on the accumulation and cellular distribution of iron during aging in our animal model (e.g. by Perls' staining) should be able to increase our understanding on the etiology of aging and of neurodegenerative disorders and on the role of LCN2 in such processes. Of course, the ultimate goal is always the development of new therapeutic strategies. In this context, the use of iron chelators, for instance, constitutes a good strategy. In fact, iron chelation in the treatment of diseases such as Parkinson's has gained much attention in the recent years (Ward et al., 2014). We have previously demonstrated the efficacy of the treatment with DFO in preventing oxidative stress by the absence of LCN2, and in the improvement of contextual memory (Chapter II). It would be interesting, in this context, to also evaluate behavioural performances of aged LCN2-null mice after a chronic treatment with DFO.

In the meantime, we focused our analysis on the effects of aging in neural plasticity, specifically in the form of hippocampal neurogenesis. One of the ultimate goals in regenerative medicine, and in stem cell biology, is indeed to overcome the deleterious cellular effects of aging and, ultimately, to be able to reverse them. But, firstly, we have to understand why and how neurogenesis changes during aging, and the further identification of the factors that cause theses changes are crucial to manipulate the system in order to prevent age-related decline. Of notice, the behavioural alterations we described imposed by age were further accompanied by a significant decrease in cell survival

and neuronal differentiation at the hippocampus of both Wt and LCN2-null mice. Nevertheless, and considering the roles of LCN2 in the adult hippocampal neurogenesis (Chapter II), as well as the beneficial effects of voluntary running in overcoming anxiety and contextual memory in LCN2-null mice (Chapter III), it would be interesting to also address if exercise could prevent or even reverse the deleterious effects of LCN2 absence in the aged brain. Many have described that older adults that exercised throughout life had less brain tissue loss, compared to sedentary individuals (Colcombe et al., 2003), and that physically fit aged individuals perform better on cognitive tests (Kramer et al., 1999; Yaffe et al., 2001). Moreover, mice that exercised continuously from young to middle age present significantly less age-related decline in cell genesis, and improved learning (van Praag et al., 2005).

4. Concluding remarks

With the work developed in this thesis, we demonstrate the importance of LCN2 for physiological brain functioning, through the modulation of neural plastic events. Moreover, we identified several novel mechanisms of hippocampal neurogenesis regulation in the adult brain, and our findings open perspectives in understanding the role of iron and iron-related regulators in the (patho)physiology of neuropsychiatric disorders affecting hippocampal neuroplasticity and cognitive function. This, for sure, deserves further clarification. Further insights will come with the development of strategies to, for instance, selectively ablate LCN2, either at the periphery or at neural cells (e.g. astrocytes) in the diseased brain. Such approach will allow to precisely underpin the individual contribution of LCN2 in the generation of new neuronal (and glial) cells and in different brain functions, as well as its role in the onset of chronic conditions, such stress or aging.

In summary, the main achievements of the work developed in this thesis are:

1) LCN2 orchestrates adult neurogenesis, with impact on behaviour;

2) LCN2 is an important mediator of hippocampal neurogenesis in response to exercise and glucocorticoids;

3) LCN2 absence influences age-related behavioural performances and neural plasticity.

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