Universidade do Minho
Escola de Ciências da Saúde

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Tese de Doutoramento em Ciências da Saúde

Trabalho efectuado sobre a orientação da
Prof. Doutora Luísa Alexandra Meireles Pinto
e do
Professor Doutor Nuno Jorge Carvalho Sousa

abril de 2016

DECLARAÇÃO DE INTEGRIDADE

Declaro ter atuado com integridade na elaboração da presente tese. Confirmo que em todo o trabalho conducente à sua elaboração não recorri à prática de plágio ou a qualquer forma de falsificação de resultados.

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Universidade do Minho, 01 de Abril de 2016

Nome completo:
António Maria Restolho Mateus Pinheiro

Assinatura:

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ABSTRACT

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A fundamental hallmark of modern neuroscience lies on the realization that, similarly to what happens in other tissues of our body, the adult mammalian central nervous system is endowed with considerable regenerative potential. This regenerative capacity is reflected in cellular processes defining the so-called neural plasticity, which include rapid and dynamic axo-dendritic remodelling of post-mitotic cells, as well as the ability of restricted brain regions to persistently generate new neuronal and glial cells, a process known as neuro- and gliogenesis, respectively. Together, neural plasticity confers to the mammalian brain a wide adaptive capability to redefine neuro-glial circuits, in response to every-day-life experiences and challenges.

Reflecting the plastic nature of the adult brain, the hippocampus harbors different forms of neural plasticity, which have been implicated in different cognitive and emotional functions. Indeed, besides exhibiting forms of functional and structural dendritic plasticity, the hippocampus constitutes one of the two adult brain regions in which neurons are continuously generated throughout life. The importance of hippocampal neural plasticity extends beyond basal physiological contexts, as it serves as a pathological target in different neurological and psychiatric diseases.

In particular, the hippocampus is considered a major gateway for the translation of the systemic stress-response into deleterious effects on neural plasticity, that are also included in the pathophysiology of diseases in which stress has a major etiological role, such as depression. In fact, not only has depression been linked to impoverished neuronal dendritic arborization and reduced cytogenesis within the hippocampus, but, also, antidepressant therapeutical efficacy has been suggested to be associated with the ability to reverse the aforementioned plastic alterations. Even so, the specific contribution of different forms of hippocampal plasticity in the onset and recovery from depression remains highly controversial. Following the initial reports claiming that hippocampal cytogenesis was fundamental to the antidepressant action, we and others have reported that the short-term improving actions of antidepressants depended on dendritic remodeling, rather than on cytogenesis. However, the longitudinal course of depression, as well as the full extent of the cytogenic process have often been neglected, since several studies have focused their analysis shortly after cytogenesis ablation, when newborn cells were yet to be fully differentiated and integrated in pre-existing circuits.

In the work comprised in this thesis, we have arrested adult cytogenesis in naïve animals through the administration of the cytostatic agent methylazoxymethanol (MAM) and analyzed the long-term behavioral consequences four weeks after. Cytogenesis ablation was sufficient to induce core

symptoms of depression, such as anhedonic behavior, as well as other behavioral traits known to be highly comorbid in depressive patients, namely heightened anxiety and cognitive impairments. Moreover, work with a genetic model of cytogenesis ablation, the glial acidic fibrillary protein (GFAP) - tyrosine kinase (Tk) rat, revealed a time-dependent manifestation of behavioral impairments following cytogenesis ablation. Analysis conducted immediately after ablation in GFAP-Tk animals showed that while cytogenesis suppression can elicit short-term anxiety-like behavior, no alterations were produced in hedonic behavior or cognitive performance. Contrastingly, when conducting behavioral analysis 4 weeks post-ablation, the already present anxiety traits were accompanied with anhedonic behavior, as well as cognitive deficits related with spatial reference memory, behavioral flexibility, contextual fear memory and pattern separation. Interestingly, animals with ablated cytogenesis displayed impaired electrophysiological communication between the hippocampus and the prefrontal cortex, possibly contributing to the described behavioral alterations.

Besides exploring the effects of cytogenesis ablation on the precipitation of depressive-like phenotype, we sought to analyze the importance of adult cytogenesis to the long-term spontaneous and antidepressant-induced behavioral recovery. Hence, we have submitted young-adult rats to an unpredictable chronic mild stress (uCMS) protocol to induce core symptom of depressive-like behavior. During the last two weeks of uCMS, animals were treated with the antidepressants fluoxetine and imipramine, either alone or co-administered with MAM, and allowed to recover during the four subsequent weeks. The long-term behavioral profile of rats suggests that ongoing cytogenesis is essential for long-term recovery from some emotional deficits (anhedonia and anxiety-like signs) and cognitive impairments (working memory and behavioral flexibility). Furthermore, fluoxetine and imipramine antidepressants promoted the generation of new hippocampal cells that, are able to survive and successfully integrate the pre-existing circuits, counteracting the deleterious effects induced by cytogenesis arrest.

Finally, and in light of evidence linking epigenetic DNA methylation with the pathophysiology of depression, we sought to explore whether the recently described pathway of DNA demethylation could be modulated by stress and antidepressant therapy and therefore participate in the pathology of the disease. We have detected decreased levels of DNA hydroxymethylation in the dorsal, but not in the ventral, hippocampus, which was correlated with decreased expression of tet3 in the same region. Strikingly, monoaminergic antidepressants were able to revert these alterations. The dichotomy dorsal vs ventral is also evident in the pattern of genomic locci differentially hydroxymethylated by stress and antidepressants that differs between the dorsal and ventral hippocampal poles.

RESUMO

RESUMO

Uma característica fundamental que timbra aquilo que é a neurociência moderna relaciona-se com facto de reconhecermos que, à semelhança do que acontece noutros tecidos do nosso corpo, o sistema nervoso central é dotado de um considerável potencial regenerativo. Esta capacidade regenerativa reflete-se em processos celulares que definem a chamada plasticidade neural, dentro da qual se incluem a rápida remodelação axo-dendrítica de células pós-mitóticas, assim como a capacidade de áreas restritas do cérebro gerarem continuamente novas células neuronais e gliais, processo este conhecido por neuro- ou gliogénese, respectivamente. Em conjunto, os fenómenos neuroplásticos conferem ao cérebro mamífero uma vasta capacidade adaptativa na redefinição de circuitos neuro-gliais, em resposta às experiências e desafios do nosso quotidiano.

Em particular, o hipocampo assume um papel preponderante na translação da resposta sistémica ao stress em efeitos deletérios ao nível da plasticidade neural. Assim, este comprometimento de diferentes formas de plasticidade neural integra, pois, a patofisiologia de doenças nas quais o stress é um importante factor etiológico, como é o caso da depressão. De facto, a doença depressiva não só foi associada a defeitos atróficos na estrutura dendrítica e a citogénese diminuída no hipocampo, como também a eficácia dos fármacos antidepressivos foi relacionada com a capacidade destes de reverterem aqueles défices neuroplásticos. Contudo, a contribuição específica de diferentes formas de plasticidade hippocampal para o desenvolvimento e recuperação da doença depressiva permanece altamente controversa. Após estudos pioneiros que reportaram que a citogénese hipocampal seria fundamental para a acção dos fármacos antidepressivos, nós demonstrámos que os efeitos terapêuticos a curto-prazo destas drogas dependia antes da remodelação dendrítica, em detrimento do processo citogénico. No entanto, o curso longitudinal da depressão, assim como a completa extensão do processo citogénico têm vindo a ser largamente negligenciados, uma vez que vários estudos focaram a sua análise pouco tempo após a conclusão do tratamento cito-ablativo, numa altura em que as células que seriam formadas teriam ainda que completar o seu processo de maturação e integração nos circuitos pré-existentes.

No trabalho que constitui a presente tese, promovemos a ablação da citogénese adulta em animais naíve mediante a administração do agente citostático metilazoximetanol (MAM) e analisámos as consequências a longo-prazo no perfil comportamental dos animais. A ablação da citogénese revelou-se suficiente para induzir sintomas cardinais da doença depressiva, como é o caso de comportamento anedónico, assim como outros traços comportamentais descritos como

comorbilidades frequentes em doentes depressivos, nomeadamente manifestações de ansiedade e défices cognitivos. A utilização de um segundo modelo genético de ablação de citogénese, ratos glial acidic fibrillary protein (GFAP)-tyrosine kinase (Tk), revelou o desenvolvimento tempo-dependente de défices comportamentais após ablação. Análises efectuadas imediatamente após ablação em animais GFAP-Tk, revelaram que a supressão da citogénese induz a exacerbação de comportamento ansioso a curto-prazo, não se tendo verificado alterações hedónicas ou na performance cognitiva. Em contraste, quando realizada a análise 4 semanas após a ablação citogénica, ao comportamento ansioso associavam-se também anedonia e défices ao nível da memória espacial, flexibilidade comportamental, memória emocional e em tarefas de *pattern separation*. Animais com citogénese suprimida revelaram défices na comunicação electrofisiológica entre o hipocampo e o córtex prefrontal, o que poderá contribuir para as alterações comportamentais identificadas.

Para além do estudo dos efeitos da ablação da citogénese na precipitação do fenótipo depressivo, procurámos ainda analisar a importância da citogénese adulta na recuperação deste fenótipo a longo-prazo, quer espontânea, quer induzida por fármacos antidepressivos. Submetemos ratos adultos ao protocolo de stress crónico imprevisível (uCMS) afim de induzirmos sintomas cardinais da doença depressiva. Durante as últimas 2 semanas de uCMS, os animais foram tratados com os antidepressivos fluoxetina e imipramina, quer isoladamente quer em co-tratamento com metilazoximetanol (MAM) e foram deixados a recuperar durante 4 semanas. O perfil comportamental dos animais sugere que a citogénese adulta é essencial para a recuperação a longo-prazo de défices emocionais (anedonia e comportamento ansioso) e cognitivos (memória de trabalho e flexibilidade comportamental). O tratamento com fluoxetina e imipramina promoveu a geração de novas células hipocampais que se revelaram capazes de sobreviver e integrar nos circuitos pré-existentes, revertendo os efeitos deletérios provocados pela supressão citogénica.

Finalmente, à luz da evidência existente que estabelece uma associação entre a metilação do DNA e patofisiologia da depressão, explorámos se a recém descrita via de desmetilação do DNA poderia ser modelada pelo stress e tratamento antidepressivo e assim participar na patologia da doença depressiva. Detectámos um decréscimo nos níveis de hidroximetilação do DNA no hipocampo dorsal, mas não no ventral, que se correlacionava com uma diminuição na expressão de tet3 na mesma região. Importa notar que antidepressivos monoaminérgicos se revelaram capazes de reverter as alterações mencionadas. A dicotomia dorsal vs ventral parece ser também aqui evidente, uma vez que o padrão de locci genómicos que se detectaram ser diferencialmente hidroximetilados pelo stress e tratamento antidepressivo diferiam entre os pólos dorsal e ventral do hipocampo.

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ABBREVIATIONS LIST

#	5caC	5-carboxylcytosine
	5fC	5-formylcytosine
	5hmC	5-hydroxymethylcytosine
	5mC	5-methylcytosine
A	ACTH	adrenocorticotropic hormone
	AMY	amygdala
	aNE	ammonic neuroepithelium
	AP2γ	activating protein 2 gamma
	AVP	arginine vasopressin
B	BDNF	brain-derived neutrophic factor
	BLA	basolateral amygdala
	BNST	bed nucleus of the stria terminalis
	BrdU	bromodeoxyuridine
C	CFC	contextual fear conditioning
	CNS	central nervous system
	CORT	corticosterone
	CRH	corticotropin releasing hormone
	CS	conditional stimuli
D	DCX	doublecortin
	dDG	dorsal dentate gyrus
	DG	dentate gyrus
	DH	dorsal hipocampus
	dNE	dentate euroepithelium
	DNMT	DNA methyltransferase
E	EC	enthorinal cortex
	ECT	electroconvulsive therapy
	eESP	early elimination-survival phase
	EPM	elevated plus maze

F	fGE	fimbrial glioepithelium
	Flx	fluoxetine
	FST	forced swimming test
G	GCL	granule cell layer
	GCV	ganciclovir
	GFAP	glial fibrillary acidic protein
	GR	glucocorticoid receptor
H	HDCs	head-direction cells
	HPA	hypothalamus - pituitary - adrenal axis
I	Imi	imipramine
L	LMP	late maturation phase
	LTD	long-term depression
	LTP	long-term potentiation
M	MAM	methylazoxymethanol
	MAOi	monoamine-oxidase inhibitor
	MDD	major depressive disorder
	MDP	migration and differentiation phase
	MFP	mossy fiber pathway
	mPFC	medial prefrontal cortex
	MR	mineralocorticoid receptor
	MWM	Morris water maze
N	NAc	nucleus accumbens
	NDRI	norepinephrine-dopamine reuptake inhibitor
	NHD	neurogenic hypothesis of depression
	NRI	norepinephrine reuptake inhibitor
	NSCs	neural stem cells
O	OB	olfactory bulb
	OF	open field

P	PC	pattern completion
	PCP	percursor cell phase
	PerC	perirhinal cortex
	PFC	prefrontal cortex
	PosC	postrhinal cortex
	PP	perforant path
	PS	pattern separation
	PTSD	post-traumatic stress disorder
	PVN	paraventricular nucleus
R	RAM	radial arm maze
	RGLs	radial glial-like cells
	RMS	Rostral migratory stream
S	SEZ	subependymal zone
	SGZ	subgranular zone
	SNRI	serotonin-norepinephrine reuptake inhibitor
	SSRI	Selective serotonin reuptake inhibitor
	TAPs	transient amplifying progenitors
T	TET	ten eleven translocation (enzyme)
	Tk	tyrosine kynase
U	uCMS	unpredictable chronic mild stress
	US	unconditional stimuli
V	vDG	ventral dentate gyrus
	VEGF	vascular endothelial growth factor
	VH	ventral hipocampus

THESIS LAYOUT

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THESIS LAYOUT

Chapter 1 presents a general introduction, covering fundamental aspects of (i) hippocampal structure and function, along with the description of (ii) different forms of neural plasticity occurring within the adult hippocampus. A perspective on both of these topics is fundamental to address the subsequent topic of (iii) stress-induced dysregulation of adult neural plasticity as a pathophysiological element of depression. Finally, (iv) epigenetic alterations in the stressed brain are focused, with special emphasis given to DNA methylation and hydroxymethylation.

Chapter 2 presents the general rationale and major scientific aims of this thesis work.

Chapter 3 comprises the research work focusing the longitudinal impact of hippocampal cytogenesis pharmacological ablation on both the development and remission from depressive-like behavior. This chapter is presented as two sequential original papers published in Translational Psychiatry, in 2013 (Mateus-Pinheiro et al., 2013a) and in Molecular Psychiatry (Mateus-Pinheiro et al., 2013b), subsequently in the same year.

Chapter 4 comprises work in which a transgenic rat model for specific cytogenesis ablation (GFAP-Tk rat model) was used to further explore the longitudinal participation of adult cytogenesis in the development of different short-term and long-term emotional and cognitive impairments. Moreover, the importance of new mature hippocampal cells to specific cognitive domains and to hippocampal-prefrontal cortex communication is also studied.

Chapter 5 presents the most recently produced work, focusing the characterization of epigenetic alterations on DNA demethylation pathways, following chronic stress exposure, in the dorsal and ventral hippocampal dentate gyrus.

Chapter 6 addresses the development of a new behavioral paradigm to assess anhedonic behavior in rodents. This chapter is presented as an original paper published in Frontiers in Behavioral Neuroscience, in 2014 (Mateus-Pinheiro et al., 2014).

Chapter 7 encompasses this thesis general discussion, in which the major findings of the presented research work are debated in an integrated manner. Moreover, research limitations, along with future perspectives in the field are also discussed.

Appendices

Finally, the following appendices are presented:

I) Submitted paper on the regulatory role of the transcription factor AP2gamma in adult glutamatergic neurogenesis (Mateus-Pinheiro et al., submitted).

Chapter I

INTRODUCTION

1.1

On Hippocampal Structure and Function

1.1.1 PRINCIPLES OF INTRINSIC HIPPOCAMPAL ORGANIZATION

The hippocampal formation is a medial temporal lobe structure with an heterogeneous topographical and functional organization. Due to its complex connectivity with numerous cortical and subcortical structures, the hippocampal formation integrates the neural circuitry underlying different cognitive and emotional functions. Besides its elaborate topographical and functional organization and rich connectivity, the adult hippocampal formation is endowed with considerable adaptive and regenerative capacity as it harbors one of the two neurogenic niches of the adult brain, adding an extra level of complexity both to structural and functional aspects of this region, as I will discuss later. Firstly, and in order to further address different functional aspects of hippocampal activity, it is important to briefly specify key notions on hippocampal intra-regional organization, as well as on its relations to other brain structures.

The formation of the hippocampus is generated during embryonic development but most of its neuronal population is only produced post-natally. In fact, this structure has a prolonged developmental window that extends from embryogenesis to the postnatal period which is finely regulated to correct patterning and organization (Rolando and Taylor, 2014). The basic intra-hippocampal circuitry has its foundations during development of the hippocampal neuroepithelium (HNE). The HNE is divided into three morphogenetic components arising from dorsal to ventral: the ammonic neuroepethilium aNE, the primary dentate neuroepithelium (dNE) and the fimbrial glioepithelium (fGE) (Altman and Bayer, 1990). These three contiguous regions constitute the germinal matrix of the intra-regional hippocampal formation circuitry, as their names imply: the aNE (sometimes referred simply as the hippocampal neuroepithelium) is the most dorsally located and the first to differentiate, giving rise to the hippocampal pyramidal neurons that compose the Ammons'horn (also called cornus ammonis; CA) **(Figure 1)**; the

primary dNE will give rise to the dentate gyrus (DG), with the important particularity of retaining a pool of resident adult neural stem cells (aNSC) that will persist throughout adulthood (as further discussed in **Section 1.2**); lastly, a ventrally located fGE will be a source of glial cells that will populate and contribute to the formation of the fimbria, a major information outsource structure of the hippocampus.

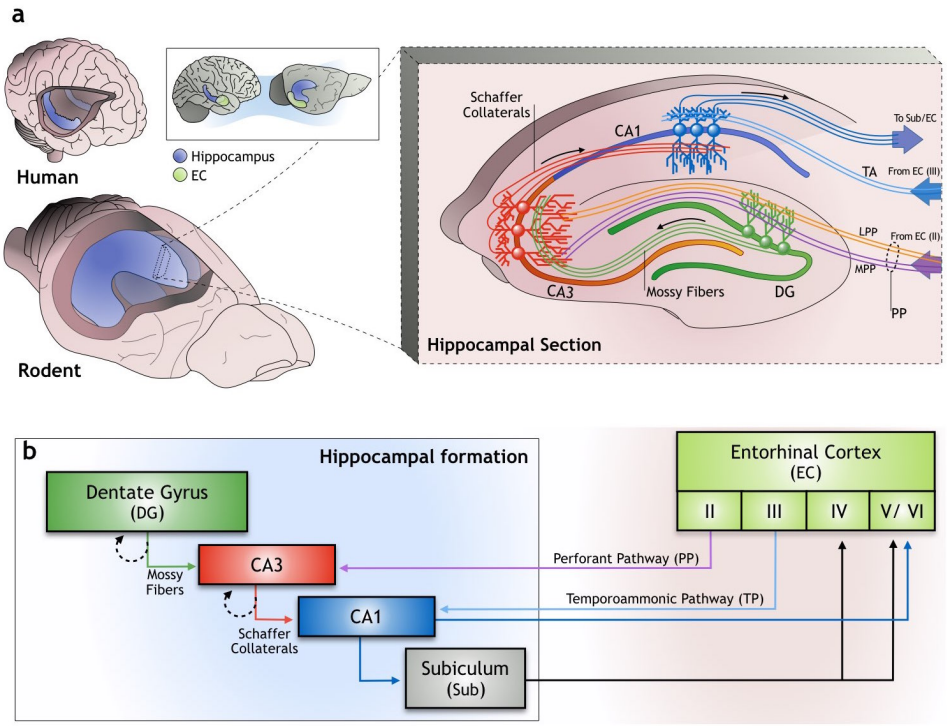

Figure 1. Hippocampal intrinsic organization. **a.** The hippocampal formation is a medial lobe structure, whose intrinsic circuit organization is relatively conserved between humans and rodents. The basic functional unit corresponds to the classical trisynaptic circuit, in which input from the entorhinal cortex (EC) is propagated by recurrent connections within the DG-CA region. **b**. The signal flow follows a DG →CA3 →CA1 sequence, to be then conveyed to the subiculum (sub) that represents a major signal outsource region of the hippocampal formation back to the EC (see main text for further detail).

Once fully formed the hippocampal formation will assume a curved shape with its longitudinal axis extending along the brain septo-temporal axis. The pathways of signal flow of the hippocampal formation[1] (hippocampus proper and dentate gyrus) combine with parahippocampal cortical regions to form the backbone of the traditional hippocampal circuitry, the trisynaptic circuit

[1] Note that the definition of hippocampal formation is not consensual; while some authors define it as comprising the hippocampus proper (CA1, CA2, CA3 and Subiculum) and the dentate gyrus, others extend the definition to include the pre- and parasubiculum, as well as the entorhinal cortex.

(Andersen et al., 1971) **(Figure 1)**. In this loop circuitry, inflow signals from the entorhinal cortex (EC) are propagated by excitatory synaptic relays in the CA and in the DG and then redirected back into the EC. Most external inputs are received from the enthorinal cortex, via long axonal pathways called medial- and lateral perforant paths (or together simply called perforant path, PP), that arise from layer 2 of the EC and terminate in the CA3 and DG (Deng et al., 2010); in parallel to this path, there is a direct path arising from EC layer 3 to CA1, commonly called temporoammonic path. Within the dentate gyrus, granule cells project their axons (the so-called mossy fibers) to the CA3, where pyramidal neurons are interconnected via recurrent connections. These pyramidal neurons will convey signal input towards the ipsilateral CA1, through Shaffer collaterals; CA3 pyramidal neurons also project to the contralateral CA3 and CA1 regions through commissural connections (Neves et al., 2008). To close the loop, CA1 send their axons to the subiculum (or directly to deep layers of EC) where information from the CA1 projection and EC layer III are combined and send along the output pathways of the hippocampal formation (Amaral, 1993). Despite one of the most studied circuits in the brain, current notions on its structuring connections keep being revisited. A recent example was the redefinition of the prevalent notion that dentate granule cells did not send projects to CA2 pyramidal cells. In fact, Kohara and colleagues showed that granule neurons do indeed send abundant monosynaptic projections to CA2 pyramidal cells (Kohara et al., 2014). This alternative trisynaptic circuit has already been shown to be relevant for both context learning (Wintzer et al., 2014) and social memory (Hitti and Siegelbaum, 2014).

The description given above of the basic hippocampal regional organization is obviously a simplification of a much more complex circuit involving many recurrent collaterals and inhibitory interneurons that modulate hippocampal activity (Witter, 1993; McBain and Fisahn, 2001). It is important to bear in mind that the trisynaptic circuit gained a central role in hippocampal research in a time when the prevalent idea was that the hippocampus proper was the major source of subcortical projections from the hippocampal formation. The current well established notions that the subiculum is the main source of subcortical projections and the enthorinal cortex is the main source of neocortical projections, the trisynaptic circuit is now only considered to be a part of the functional circuitry of the hippocampal formation. Nevertheless, and even considering the original "trisynaptic circuit concept" too narrow at light of recent knowledge, this hippocampal intrinsic organization allows to effectively receive, process and relay multimodal information from and to different neural nuclei. As we will see in the next section, the hippocampal formation is one of the

most connected areas of the brain and although commonly referenced as “memory center”, during the last two decades it became increasingly regarded as an integrator of both cognitive and emotional functions (Small et al., 2011; Femenia et al., 2012). The participation of the hippocampal formation in different cognitive and emotional modalities is the functional reflection of its inter-regional connections.

1.1.2 HIPPOCAMPAL INTER-REGIONAL COMMUNICATIONS

The adult mammalian hippocampus its a long curved “banana-shaped” structure that runs across a dorsal-ventral axis in rodents, that corresponds to a posterior-to-anterior axis in humans **(Figure 2)**. As for other species, in rodents the three-dimensional disposition of the hippocampus is relatively complex, as its longitudinal C-shaped axis runs from the septal nuclei rostro-dorsally to the temporal lobe caudo-ventrally (Amaral and Witter, 1989; Squire et al., 2004). For this reason, the longitudinal axis is often referred as the septo (dorsal)-temporal (ventral) axis, while the orthogonal orientation is referred as the transverse axis. As the hippocampus is disposed along a long longitudinal axis, it has the capacity to establish a diverse connectivity with multiple cortical and subcortical structures that I will briefly address, as they are key to further understand hippocampal function.

Afferent Connections

Injections of retrograde tracers in the septal (dorsal) pole of the DG (**dDG**) has revealed that this region receives main afferent connections from the dorsolateral area of the EC (Dolorfo and Amaral, 1998). In turn, the temporal (ventral) DG (**vDG**) receives its main afferences from the ventromedial area of the EC (Dolorfo and Amaral, 1998). The EC receives input from the perirhinal (**PerC**) and postrhinal (**PosC**) cortices: while the dorsolateral EC receives afferent projections preferentially from the PerC, both the lateral and ventromedial areas of the EC receive moderate projections from the PosC originated in layers III and V, and terminating primarily in layers II and III (Burwell and Amaral, 1998). Together, the PerC and PosC are innervate by multiple uni- and polimodal cortical areas, including the retrosplenial, anterior cingulate, prefrontal, somatosensory, temporal, parietal and occipital cortices (Squire and Zola-Morgan, 1991; Cenquizca and Swanson, 2007). However, the connectivity pattern with different brain

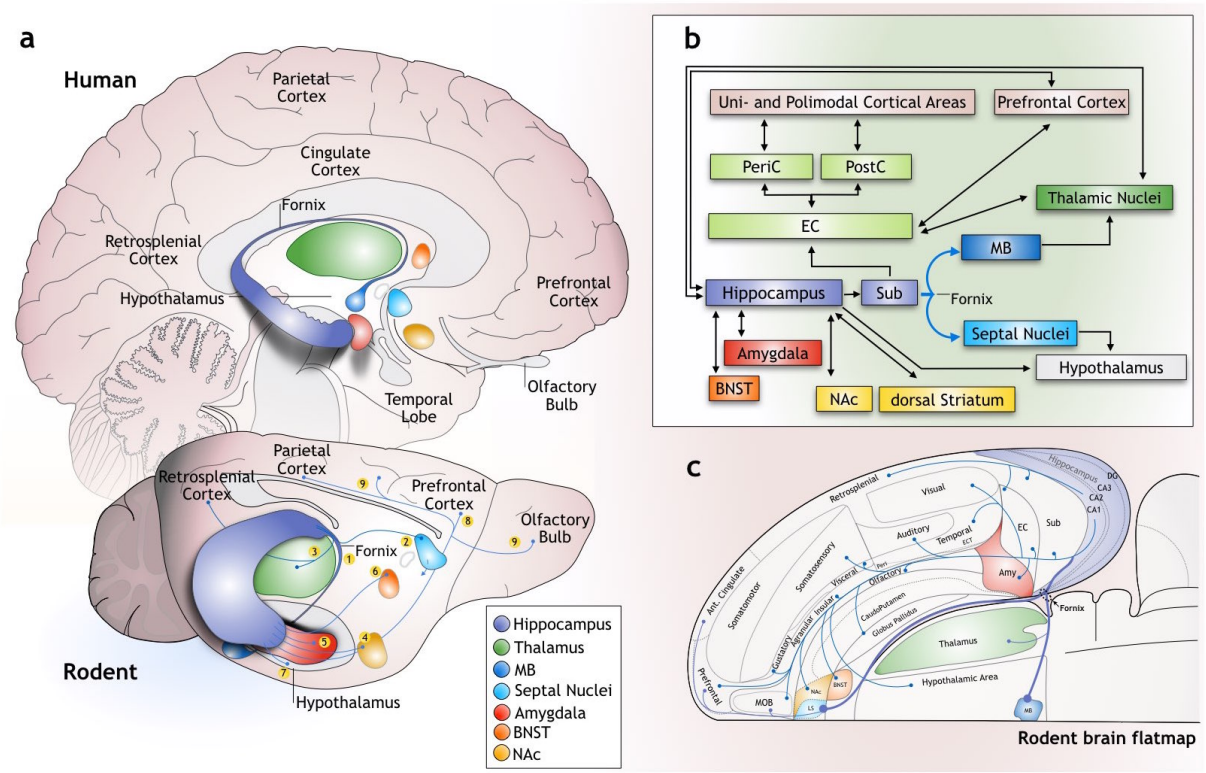

Figure 2. Hippocampal extrinsic connectivity. **a.** Both in the human and rodent brain, the hippocampus displays a rich connectivity with different cortical and limbic regions. **b.** The hippocampus establishes connections with uni- and polimodal cortical areas, mainly through the perirhinal (periC) and postrhinal (posC) cortices. A monosynaptic direct connection is also established between the hippocampus and the prefrontal cortex. In addition, the hippocampus establish bidirectional connections with subcortical regions such as the amygdala (Amy), the bed nucleus of stria terminalis (BNST), the nucleus accumbens (NAc)/ventral striatum, and the dorsal striatum. Moreover, through the fornical system, the hippocampus establishes connections with the lateral septum (LS), the hypothalamic area, the mammilary body and with thalamic nuclei. **c.** Depiction of CA1 (adapted from Cenquizca and Swanson, 2007) and fornical hippocampal projections in rodent brain flat-map (adapted from Swanson, 2004). 1 Through the fornix, to thalamic, hypothalamic and mammilary nuclei; 2 to septal nuclei; 3 to thalamic nuclei; 4 to the NAc; 5 to the Amygdala; 6 to the BNST; 7 to the hypothalamus; 8 to the prefrontal cortex; 9 to other uni- and polimodal cortical areas.

regions differs along the hippocampal septo-temporal axis, in a non-abrupt, but rather gradual manner (Cenquizca and Swanson, 2007; Strange et al., 2014).

In particular, in the rat brain, the perirhinal cortex receives input from primary visual (17) and visual association areas (18a and 18b) (Deacon et al., 1983; Vaudano et al., 1991; Burwell et al., 1995). Although the primary auditory cortex does not project to the PeriC, a small region of auditory association located caudo-ventrally to the primary region, projects to the PeriC (Vaudano et al., 1991; Mascagni et al., 1993; Burwell et al., 1995). Furthermore, somatosensory input also arrives to the hippocampal formation, through more dorsally-located PeriC regions, from the

insular cortex (Deacon et al., 1983). In contrast, the PosrC receives strong visual associational input, some afferent projections from somatosensory regions, but weak or no input both from auditory associational or olfactory areas. In addition, it is also through the PeriC, that input arising from the anterior cingulate and retrosplenial cortices, reaches the dorsolateral EC.

Moreover, ventral areas of the PeriC, along with the PosC also receive projections from polymodal associational cortices including the medial prefrontal (Deacon et al., 1983; Burwell et al., 1995; Conde et al., 1995), ventrolateral prefrontal (Deacon et al., 1983; Burwell et al., 1995), anterior cingulate cortices (Conde et al., 1995), medial precentral and temporal cortices. In addition, PeriC and PosC also receive afferent projections from subcortical areas such as thalamic nuclei, lateral nuclei of the amygdala, supramammilary nuclei and dorsal raphe nuclei (Deacon et al., 1983), as well as from the nucleus accumbens (Burwell and Amaral, 1998), altough also receiving some afferences from caudal portions of neocortical regions, as it is the case of the parietal and occipital cortices. For instance, olfactory input reaches the perirhinal cortex trough the periamygdaloid region and piriform cortex (Deacon et al., 1983; Luskin and Price, 1983) **(Figure 2)**.

Efferent Connections

The hippocampus has regional recurrent efferent connections with the EC, as well as extrahippocampal projections (Cenquizca and Swanson, 2007). Indeed, CA1 extrahippocampal projections to neocortical regions can be described in terms of “three routes”, as previously suggested: a dorsal path, that terminates in the retrosplenial area and caudal end of the anterior cingulate cortex (Vogt and Miller, 1983; Tamamaki and Nojyo, 1990; van Groen and Wyss, 1990; Jones et al., 2005); a ventral path, originating from the ventral 2/3 of CA1 region and extends throught the longitudinal association bundle to the visual, auditory, somatosensory, gustatory, olfactory and visceral cortical areas and agranular insular and orbital areas (as well as to basolateral amígdala) (Cenquizca and Swanson, 2006). A third route exists, consisting in a cortica-subcortical-cortical pathway arising from all the extension of CA1, but progressively increasing in strength from the dorsal to the ventral hippocampus. This pathway goes along the fornix to innervate rostral brain regions, such as the anterior cingulate, prelimbic, infralimbic and orbital cortices (Swanson and Cowan, 1977; Swanson and Kohler, 1986; van Groen and Wyss, 1990; Swanson, 2000; Petrovich et al., 2005). The dorsal hippocampus also sends projections

through the fornix to the mammillary complex, and subcortical projections to the lateral septal nuclei (Swanson and Cowan, 1977); contrastingly, the ventral hippocampus sends projections to the BNST, nucleus accumbens and to the anterior olfactory nucleus (Swanson and Cowan, 1977). Moreover, besides CA1 direct projections and fornical projections, the EC cortex also sends projections to both PeriC and PostC, which also establish a complex map of efferent projections with piriform, frontal, insular, temporal, cingulate, parietal and occipital cortices (Burwell and Amaral, 1998). Regarding subcortical regions, the primary subcortical connections of the periC are sent to the amygdala (to the lateral, basal, accessory basal an the capsular region of the central nuclei of the amygdala), striatum (with strong projection to the caudate nucleus) and thalamus (perigeniculate region and midline thalamic nuclei). The postC also projects to the striatum and thalamus (mainly, with lateral posterior thalamic nuclei), but has little or no connections with the amygdaloid complex. Instead, postC also projects to the claustrum (Burwell et al., 1995). As can be appreciated by its anatomical connections, the perirhinal and postrhinal cortices are areas of major convergence for widespread neocortical efferents, thus playing an important role in linking neocortical areas with allocortical regions.

1.1.3 TOPOGRAPHICAL ORGANIZATION OF HIPPOCAMPAL CONNECTIVITY: THE DORSAL *vs* VENTRAL DICOTHOMY

Despite the fact that the already described intrinsic circuit (see **Section 1.1.1**) is replicated along the entire septo-temporal axis of the hippocampus, the dorsal and ventral hippocampal regions establish different connections with cortical and subcortical areas, thus contributing to an heterogenous functional specialization of the hippocampal longitudinal axis (Witter et al., 1989; Strange et al., 2014) **(Figure 3)**.

Dorsal Hippocampus

When trying to define a set of biological functions that are dependent of the hippocampal formation, spatial processing might come ahead, specially when considering the dorsal hippocampus (DH). In fact, the dorsal pole of the hippocampus harbors a great density of the so-called place cells wich are fundamental for spatial memory encoding (Moser et al., 1995), as they allow us to construct independent spatial representations. On the subject of spatial

representations, it becomes also important to mention grid cells, which reside on the entorhinal cortex, curiously also with greater density in its dorsal region (dorsoventral medial EC), and encode a periodic triangular or hexagonal array that represent the entire animal's environment (Hafting et al., 2005).

Together, place cells and grid cells are the biological encoders employed by the DH, and enthorinal areas in its vicinity, to encode location information in a given environment. However, to navigate from a point A to a point B, one needs not only information on current location, but also directional heading perception. Once again, the ability to perceive such information lies in a particular group of cells residing in the DH, known as head direction cells (HDCs). Indeed, within the subicular complex[2], HDCs have been reported only in the postsubiculum (dorsal presubicular area[3]) and are believed to encode the animal's perceived directional heading with respect to its environment (Taube, 2007). The dorsal subicular area establishes efferent connections with regions integrating Papez Circuit[4] in which HDCs have also been identified, namely the anterior dorsal thalamic nucleus (Taube, 1995), which contains the highest percentage of HDCs (≈60%), the lateral mammillary nuclei (Stackman and Taube, 1998), retrosplenial cortex (Cho and Sharp, 2001), and also the entorhinal cortex (Sargolini et al., 2006). Furthermore, the dorsal hippocampal pole establishes strong connections with the anterior cingulate cortex, that along with retrosplenial cortex, have also been shown to be important for spatial processing and memory (Frankland et al., 2004; Harker and Whishaw, 2004).

The importance of the DH in spatial context processing and navigation, has been further evidenced by several spatial memory and navigation tasks. A test that has and continues to be classically used to assess spatial navigational memory is the Morris Water Maze (MWM), in which animals are placed in a swimming pool and have to find and memorize the position of a hidden submerged platform, with the help of landmarks placed in the test room (Morris, 1984). In fact,

[2] The subicular complex is usually divided into three subdivisions, namely, the subiculum proper, the presubiculum and the parasubiculum (Witter et al., 1989)

[3] Some researchers contend that the dorsal portion of the presubiculum is characterized by cytoarchitectonics and connectivity that differ from the ventral portion of the presubiculum (van Groen & Wyss 1990). These researchers thus prefer to use a different name for the dorsal portion and refer to this area as the postsubiculum. Using this nomenclature, they reserve the term presubiculum for only the ventral portion of this area

[4] Papez circuit: connnects the mammillary nuclei → anterior thalamus → cingulate cortex → hippocampus → mammillary nuclei

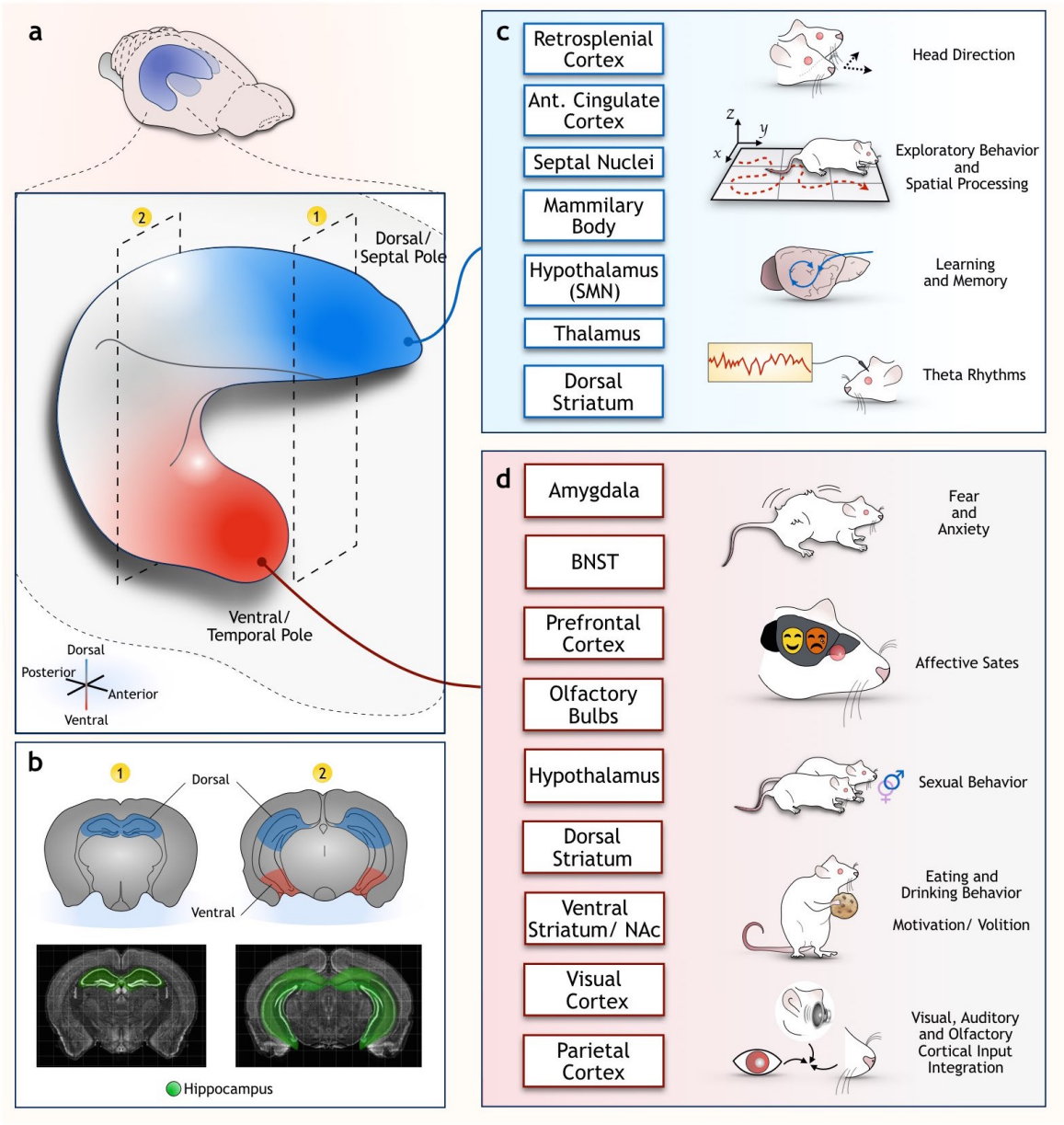

Figure 3. Connectivity and functional correlates of the dorsal and ventral hippocampus. a. The rodent hippocampus has a C-shaped structure, running across its dorsal (septal) pole to its ventral (temporal) pole. **b.** In more rostral coronal sections, (1) only the dorsal hippocampus is identifiable; more caudal sections, run across both dorsal and septal poles (2). **c.** The dorsal hippocampus establishes important connections with the retrosplenial and anterior cingulate cortices, septal and mammilary nuclei, suprammammilary and thalamic nuclei, as well as with the dorsal striatum. It has been shown to participate in head direction perception, spatial navigation, leaning and memory, as well as in hippocampal theta rythms. **d.** The ventral hippocampus establishes important connections with the amygdala and bed nucleus of the stria terminalis (BNST), the hypothalamus and the dorsal and ventral striatum. In addition, it also establishes a singular monosynaptic connection with the prefrontal cortex, as well as with othe uni- and polimodal cortical areas. It has been suggested to participate in anxiety and fear control, in the modulation of affective states, motivation and sexual behavior, among other suggested functional correlates .

lesional studies of one of these regions individually, have provided the first evidence for this notion, as extensively reviewed elsewhere (Moser and Moser, 1998; Fanselow and Dong, 2010). Moser and colleagues showed that ibotenic acid-induced lesion in the septal hippocampus, produced significant spatial learning deficits (Moser et al., 1995). In fact, lesions that preserved at least 26% of the septal pole of the hippocampus did not produced cognitive deficits, demonstrating that as little as this amount of the septal hippocampus is enough to ensure normal spatial memory. In the same line of experiments, it was also demonstrated how ventral hippocampal lesions did not interfere with this type of memory. Such findings have proven to be consistent, as several subsequent studies have endorsed them (Bannerman et al., 2002).

Another test used to evaluate spatial memory, that is chiefly used in rats, is the radial arm maze (RAM), in which animals are required to distinguish positions where they have already been, from new positions, where food is placed (Olton and Samuelson, 1976; Hodges, 1996). In line with findings in water maze tasks, Pothuizen and colleagues found that excitotoxic lesions in the DH severely disrupted spatial reference and working memory, in the RAM test. Here again, lesions confined to the ventral hippocampus (VH) did not interfere with spatial memory function (Pothuizen et al., 2004). However, it was further shown that ventrally-confined lesions increased the return to arms previously associated with food (Ferbinteanu and McDonald, 2001).

Contextual fear conditioning (CFC) is also commonly used to assess hippocampal function (Kim and Fanselow, 1992; Maren et al., 2013). Contexts serve the cognitive function of abstracting situationally informed meaning of the world (Maren et al., 2013). They allow us to match contigencies, spatial cenarios or emotional states to specific cues and memory traces, being for such reason important for our cognitive capabilities of recollection, associative learning, familiarity, anticipation and planning. In a commonly used CFC test setup, conditional stimuli (CS), such as light or a tone, are followed by a footshock (an uncoditional stimulus - US). This CS-US pairings, that occur only in first test context, but not in a second one, will be the basis of a task that tests spatial (context fear) and nonspatial (cued fear memory) and that is, in part, driven by emotion. Lesional studies have showed that lesion in the DH produce impairments in contextual fear memory, but not in cued fear memory (Kim and Fanselow, 1992; Hunsaker et al., 2008). In this test paradigm, the effects produced by ventrally confined lesions is not so linear. In fact, VH lesions, or transient neuronal inactivation (by muscimol infusion) cause severe deficits in cued fear memory, but also interfere with context fear memory (Rogers and Kesner, 2006;

Hunsaker and Kesner, 2008; Fanselow and Dong, 2010). This is in line with the growing notions that the hippocampus, although classically viewed as "memory center", also regulates emotion, what should not be unexpected due to its many reciprocal connections to both neocortical and subcortical regions involved in numerous emotional dimensions. In this CFC paradigm, VH lesion are depriving the amygdala (which participates in mediating fear memory) from hippocampal input, which is only directly conveyed by the VH.

Moreover, the dorsal hippocampus proper has strong bidirectional connections with the medial septum, as well as with the posterior hypothalamic region, namely the supramammilary nucleus (Risold and Swanson, 1996; Fanselow and Dong, 2010), which are important pacemakers of hippocampal theta rythms (Vinogradova, 1995; Kocsis and Vertes, 1997). Hippocampal theta rythms (slow activity in the hippocampus) are selectively present during exploratory behavior and REM sleep and believed to serve a critical role in memory and navigation (O'Keefe and Nadel, 1978; Buzsaki and Moser, 2013).

In sum, the dorsal pole of the hippocampal formation participates in elaborate cognitive functions, related to spatial navigation and exploration, learning and mnemonic functions. As we will see, its ventral pole displays a curiously contrasting functional specialization.

Ventral Hippocampus

The ventral pole of the hippocampus establishes important bidirectional connnections with several amygdalar nuclei (Cenquizca and Swanson, 2007). Importantly, connections with lateral, basolateral and central amygdalar nuclei (Petrovich et al., 2001; Cenquizca and Swanson, 2007) are believed to be critical for the participation of the hippocampus in Pavlovian fear conditioning[5] (Maren and Holt, 2004). Here, the hippocampus is believed to play a critical role in bridging the gap between the conditioned stimulus (CS) and the unconditioned stimulus (US) in situations in which the CS and US presentations are separated by an empty interstimulus interval (i.e., a form of pavlovian conditioning known as trace conditioning). In agreement with this notion, is the report of a particular paradigm of trace conditioning - conditioned taste aversion - to be dependent on the VH (Koh et al., 2009). Together, the circuit composed by the VH, the amygdala and the prefrontal cortex (that also establishes a bidirectional monosynaptic connections with the ventral

[5] Pavlovian fear conditioning: a form of classical conditioning where the CS (e.g. tone or light) is paired with an aversive US (classically, a foot shock) that evokes a CR (e.g. freezing, acoustic startle response or autonomic arousal).

hippocampal pole) (Thierry et al., 2000) compose the neural substrate modulating fear (Mahan and Ressler, 2012). This is, at least in part, the reason why the hippocampus is believed to participate in the manifestation of stress-induced anxiety and other neuropsychiatric disorders (McEwen and Magarinos, 1997).

Another important reciprocal connection of the VH that is worth analyzing, is the one established with the bed nucleus of the stria terminalis (BNST) (Dong and Swanson, 2006). In contrast to other forebrain regions (such as the hippocampus, the amygdala or the medial prefrontal cortex) that modulate hypothalamic neuroendocrine function, the anteromedial area of the BNST is known to have a direct projection to the hypothalamic perviventricular and paraventricular nuclei, where most secretory neurons synthesizing corticotropin-releasing hormone and thyrotropin releasing hormone are found (Markakis and Swanson, 1997). Indeed, BNST has an important neuroendocrine role in stress and anxiety (Walker et al., 2003; Sink et al., 2013; Micioni Di Bonaventura et al., 2014), and the activity of the the PVN is believed to be modulated by the VH, through the BNST (Zhu et al., 2001; Herman et al., 2004; Herman et al., 2005). Reinforcing the role of the VH in stress and anxiety-like behavior, is the study of Kheirbek et al., where optogenetically-induced activation of dentate granule cells in the VH, but not in the DH, promoted a significant suppression of innate anxiety, tested in several test paradigms (Kheirbek et al., 2013). This VH-BNST-Hipothalamic circuit, may also mediate the hippocampal modulation of hypothalamic nuclei functions, as part of what has been designated by Swanson as “behavioral control column", that accounts for the elicitation of neuroendocrine and autonomic responses underlying ingestive (Swanson, 2000) and social behaviors, namely reproductive and defensive behaviors (Dong and Swanson, 2006).

Also within the hypothalamus, the suprachiasmatic nucleus and dorsomedial nucleus receive ventral hippocampal projections (Kishi et al., 2000; Cenquizca and Swanson, 2007). These brain nuclei are involved in depressive behavior and circadian sleep-wake and corticosterone rythms (Bellinger et al., 1976; Chou et al., 2003; Saper et al., 2005; Nollet et al., 2011), thus regulating sleep and affective states (McClung, 2011; McClung, 2013).

Another important connection of the hippocampal ventral pole to be noted is the one established with the ventral striatum. In particular, the ventral hippocampal and entorhinal areas send projections to the nucleus accumbens shell (Naber and Witter, 1998; Voorn et al., 2004), placing the VH in a position to modulate motivation and reward-seeking behavior (Strange et al., 2014), as

hippocampal projections to the NAc have already been shown to be able to control information processing in the NAc (O'Donnell and Grace, 1995; Goto and O'Donnell, 2001).

Finally, and although it might not be a role that we commonly enumerate when referring to hippocampal functions, associations between normal hippocampal activity and motor function has been long known (Vanderwolf, 1969). Globally, studies have demonstrated that stimulation of the VH, but not of the DH, recrute NAc and dopaminergic circuits functions, resulting on increased locomotion (Bardgett and Henry, 1999; Legault et al., 2000; Zhang et al., 2002). Indeed, reward- and goal-directed functions have been attributed to the rodent ventral hippocampus, in light of the view of the ventral striatum and NAc as the interface between the limbic system and motor circuits (Mogenson et al., 1980; Pennartz et al., 2011). It should be noted, however, that the DH may also be important, as it seems to be the locci where encoding occurs, although the major site of projections is indeed in the intermediate and ventral domains of the hippocampal formation (Bast et al., 2009; Strange et al., 2014).

Hence, the ventral hippocampal pole integrates brain circuits responsible for anxiety and fear, stress response, reward-seeking behavior, affective states, neuroendocrine functions and social behaviors with a strong emotional component, functions likely to be attributed to its integration in Papez circuit.

Indeed, this functional segregation has contributed to a classical (and perhaps oversimplistic) view of hippocampal function that confers to the DH the label of “cold hippocampus”, a designation that refers to its role on cognitive functioning (orientation, memory, navigation and exploration), while the ventral “hot” hippocampus is regarded as an emotional processor (motivated behavior, neuroendocrine responses, stress, anxiety and fear). However, although a dorsal-ventral dichotomy do exist, a more in depth analysis of both the anatomical and functional organization of the hippocampal formation reveals that the functional segregation of these two regions is not strictly defined. In fact, it as long been proposed by anatomical studies, and more recently by transcriptomic and functional studies that despite the existence of different hippocampal domains, the transitions are gradual rather than strict, and intermediate hippocampal regions of the hippocampal septo-temporal axis may exhibit neuroanatomical and molecular aspects of both the septal and temporal poles (Strange et al., 2014). In fact, a ill-defined transitional area, called by some authors as the intermediate hippocampus, precludes the delineation of a clear anatomical and functional DH-VH boundary, due to its mixed connectivity and molecular signatures. This

“anatomical and functional hippocampal continuum” is likely to be an important source of what may be taken as contrasting or discrepant results that continue to emerge in several reports seeking to explore the still many unknowns of post-natal hippocampal function.

1.2

Neural Plasticity in the Adult Hippocampus

Similarly to what history has unfold in other fields of science, neuroscience's notions and concepts have mutated and evolved along times. Contrasting to its foundations, a pivotal aspect of modern neuroscience is its appreciation of the extraordinary and complex mutability of the central nervous system (CNS). This so-called plasticity is but the mirror of the CNS's ability to redesign neural pathways function and anatomy in response to everyday life experiences, but also in the advent of injury or disease. This plasticity can occur in many forms, ranging from simply functional adaptations (functional plasticity) to more anatomical and morphological reorganization of circuits (structural plasticity). In this section I will focus on hippocampal neural plasticity and how it contributes to hippocampal circuitry and function.

1.2.1 ADULT HIPPOCAMPAL CELL GENESIS

New cells in the adult brain

In the beginning of the 20th century, Santiago Ramon y Cajal argued that new neurons were only generated during prenatal development (Ramon y Cajal, 1993). One could say that Cajal observations were quite accurate, if considering that at the time there were no tracing methods to label and determine the fate of newborn cells. As a result, Cajal's decree of "fixed and immutable neural paths" prevailed for more then half a century. It was only in the late 60's, with the recent development of [H^3]-thymidine cell labelling, that Joseph Altman and colleagues were able for the first time to describe the generation of new neurons in various regions of the adult rat brain (Altman and Das, 1965; Altman and Das, 1966; Altman, 1969). At that time those studies received little attention, perhaps in favor of the subsequent reports by Rakic failing to detect the generation of new cells in the adult monkey brain (Rakic, 1985). In the late 70's this topic was revisited, with a report showing that some [H^3]-thymidine labelled cells survived for 30 days in the adult hippocampal DG and in the olfactory bulbs, and that these cells exhibited ultrastructural features of neurons (Kaplan and Hinds, 1977; and subsequently in Kaplan and Bell, 1983). These neurons were shown to send

projections along the mossy fibers tract to the hippocampal CA3 region (Stanfield and Trice, 1988). Besides the rediscovery of the generation of new neurons in the adult rat brain, Kaplan and Hinds (1980) also reported the generation of new astrocytes and oligodendrocytes in the adult rat cortex. A major turning point in the strong resistance that adult cell genesis (a process also referred as adult cytogenesis) has encountered, was provided by Paton and Nottebohm (1984), in a study conducted in adult canaries, showing that newly generated neurons incorporated pre-existing functional circuits and exhibited vigorous electrophysiological responses to auditory stimuli. This study provided the first evidence of the functional relevance of adult generated neurons, and perhaps most importantly, emphasized the importance of studying and characterizing the true potential of these cells (Nottebohm, 2004[6]). Following these breakthroughs, in the 90's adult neural stem cells (NSCs) were successful isolated from the rodent brain (Reynolds and Weiss, 1992) and subsequently from the human brain (Kukekov et al., 1999). Eventually, the description of Bromo- and Iododeoxyuridine (thymidine analogs) as a new approach for detection of DNA replication (Gratzner, 1982) fueled the growth of the field, and soon neurogenesis was reported in several mammal species (Ming and Song, 2005;Gould, 2007), including in humans (Eriksson et al., 1998).

Adult Hippocampal Neurogenesis

Today it is well established that adult cytogenesis, a process defined as comprising the generation, differentiation and integration of new cells in the pre-existing brain neuronal networks, occurs in the adult brain (Gage, 2000). The generation of new neurons - **neurogenesis** - prevails throughout life in discrete brain areas (Doetsch et al., 1999; Gage, 2002). Such spatially defined brain regions where neurogenesis occurs, known as neurogenic niches, display a permissive microenvironment for the maintenance and differentiation of neural stem cells and to their proliferation. Up to now, only two regions of the brain have been consensually labelled as neurogenic niches in the mammalian brain: the subependymal zone (SEZ) lining the lateral ventricles[7] and the subgranular zone[8] (SGZ) of the hippocampal DG. I shall focus my attention on the latter. Within the hippocampal DG, newly-born cells are generated throughout the SGZ and after becoming committed to the neuronal lineage, they migrate towards the granule cell layer

[6] Nottebohm (2004), provides a comprehensive view of the serie of elegant works developed in songbirds, that contributed to elucidate the process of adult neurogenesis and its role in auditory processing in these animals.

[7] In the SEZ the precursor cells are mostly found in the anterior segment of the walls of the lateral ventricles. Here, newly-born precursor cells generate neuroblasts that will migrate along the rostral migratory stream (RMS), reaching the olfactory bulb (OB), where they fully differentiate mostly into granule inhibitory interneurons (Chumley et al. 2007; Wu et al. 1999).

[8] The hippocampal subgranular zone (SGZ) corresponds to a narrow band of about 2-3 cell layers wide, lying between the granule cell layer and the hilus.

(GCL), where they mature to become granule neurons, predominantly excitatory glutamatergic neurons (Seri et al., 2004; Brill et al., 2009) **(Figure 4)** .

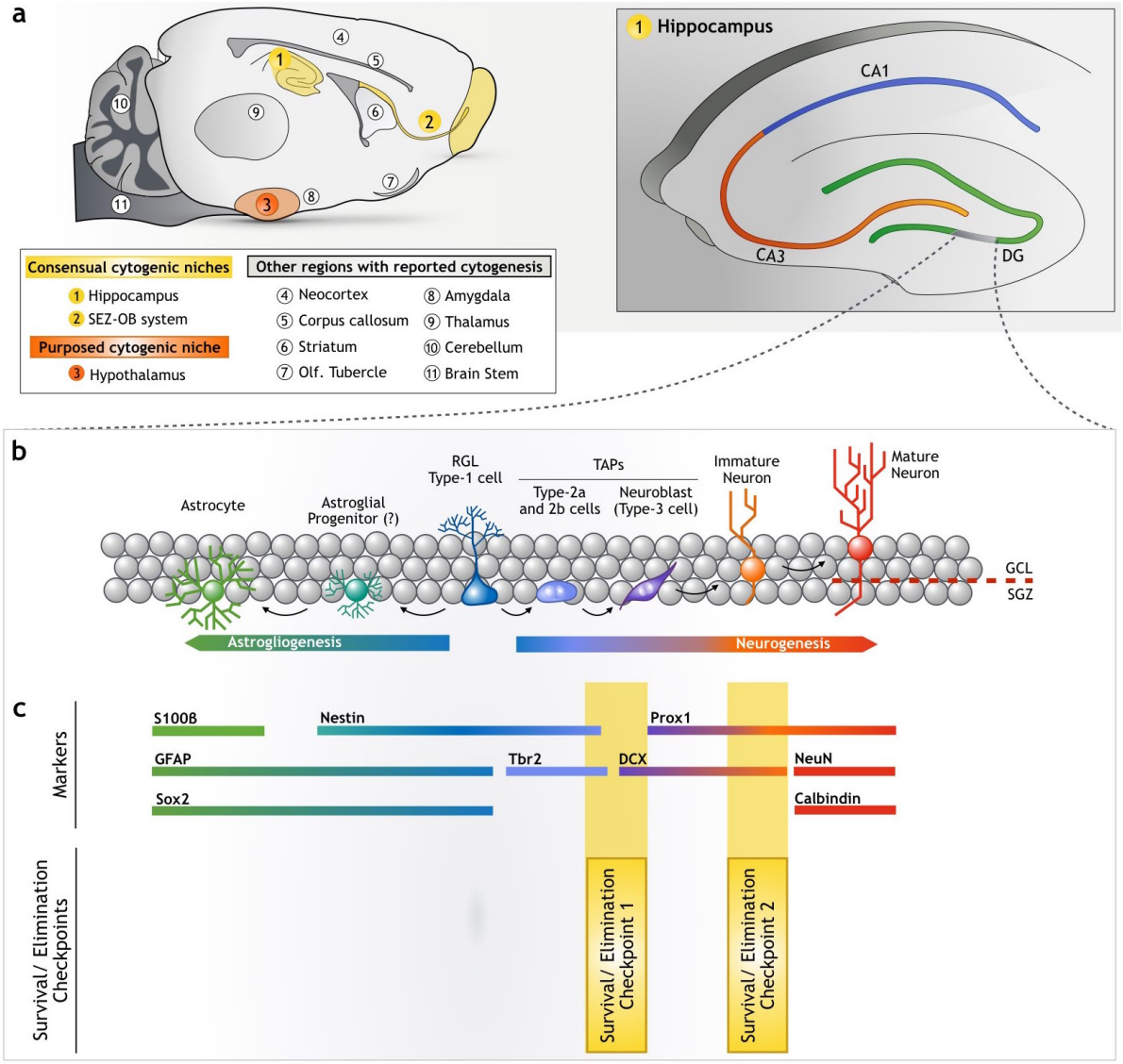

Figure 4. Cytogenesis[9] **in the adult mammalian brain. a.** Two cytogenic niches are consensually accepted to exist in the adult mammalian brain, and have been identified in several species: the hippocampal dentate gyrus (DG) and the subpendymal zone (SEZ) - olfactory bulb (OB) system (SEZ-OB system). The hypothalamus is increasingly accepted as the third cytogenic niche of the adult brain. Although adult cytogenesis has been reported in many different areas, several contradictory reports exist. **b.** Within the hippocampal DG, neural stem cells (radial glia-like cells) are present in the subgranular zone (SGZ). From these cells, new progenitor cells are formed and migrate towards the granular cell layer (GCL) where they fully differentiate into mature granule neurons (**neurogenesis**). Alternatively, progenitor cells may become commited to the glial lineage, giving rise to new astrocytes (**astrogliogenesis**). **c.** Both cytogenic processes occur though a series of sequential steps (less well characterized in the astrogliogenic process), that can be staged using specific immunohistochemical markers (see main text for further detail). RGLs- radial glia-like cells. TAPs- transient amplifying progenitors.

[9] Here, we use an inclusive definition of "**cytogenesis**", encompassing the multi-potent capacity to generate new cells from both neuronal and glial lineages. We refer to the process of generation of new cells specifically from neuronal, astroglial or oligodendroglial lineages as neurogenesis, astrogliogenesis or oligodendrogliogenesis, respectively.

The process of adult hippocampal neurogenesis, which in many aspects largely recapitulates embryonic neurogenesis (Esposito et al., 2005), can be described in 4 sequential phases: i) a percursor cell phase, ii) an early elimination-survival phase, iii) a post-mitotic migration and differentiation phase and iv) a late maturation phase. The percursor cell phase (PCP) corresponds to the expansion of the pool of multipotent cells residing in the hippocampus. The hippocampal SGZ harbors an heterogeneous precursor cell population, distinctly identifiable through particular cell markers that each cell type expresses (Ming and Song, 2005) (some of them listed in **Figure 4**). Following similar observations made in the SEZ (Doetsch et al., 1999), Seri et al. described that neural stem cells (NSCs) residing in the adult hippocampus had astrocyte-like properties, displaying radial morphology (Seri et al., 2001). These radial glia-like cells (RGLs, alternatively called type-1 cells) express the glial fibrillary acidic protein (GFAP) and neural stem cell protein (nestin), two intermediate filament proteins (Seri et al., 2004). They present different modes of division, either displaying symmetric divisions to expand the type-1 progenitor cell pool, or undergoing asymmetric divisions to give rise to intermediate progenitor cells (type-2 cells, also known as transiently amplifying neural progenitors or simply TAPs). TAPs have higher proliferative activity and within this cell population, two subsets have been identified: type-2a cells no longer display radial morphology, but still display radial glial markers (GFAP and Nestin); type-2b cells, in the other hand, are negative for GFAP and start to present the first marks of neuronal lineage choice, namely the transcription factors NeuroD1 and Prox1 (the latter, specific from granule neurons of the dentate gyrus) (Oliver et al., 1993; Steiner et al., 2006). TAPs receive the first synaptic input (GABAergic input), becoming able to respond to external stimuli (Tozuka et al., 2005) and increase cell proliferation to promote pool expansion. Among type-2b, expression of the microtubule associated protein doublecortin (DCX) is the first indication of neuronal lineage commitment. Its expression is maintained in type-3 cells, that no longer express GFAP or nestin, and is extended during approximately 2-3 weeks after cell-cycle exit, in a post-mitotic phase (Francis et al., 1999; Rao and Shetty, 2004; Plumpe et al., 2006).

Following the PCP, comes one of the most critical steps of adult neurogenesis, here designated as the early elimination-survival phase (eESP). In an initial survival checkpoint, Sierra and colleagues (2010) have shown that the large majority of newborn cells undergo death by apoptosis, within the first 4 days since birth. The surviving cells, that had been tonically activated by ambient GABA released from local inter-neurons (Bhattacharyya et al., 2008), will then start

receiving GABAergic synaptic inputs (Ming and Song, 2011). Latter, this is also the phase were new cells establish synapses to the target area CA3, and when they start to receive the first glutamatergic synaptic input (Tozuka et al., 2005; Christian et al., 2014). In fact, a latter elimination-survival checkpoint occurs around week three, when survival of new neurons is dependent on NMDA-related synaptic activity (Tashiro et al., 2006). Quite surprisingly, at the end less than 25% of newborn neurons survive and integrate pre-existing circuits (Christian et al., 2014).

In the post-mitotic migration and differentiation phase (MDP), cells that have exited cell-cycle undergo a serie of developmental processes, including axonal elongation, dendrite extension and increased synaptic plasticity, while migrating into the GCL. During this period they start to express markers such as the neuronal nuclei epitope NeuN and the calcium-binding protein calbindin (Brandt et al., 2003). Eventually newborn neurons attain structural integration around weeks 4-5 (Esposito et al., 2005; Zhao et al., 2006). Finally, in late maturation phase (LMP) new neurons undergo a phase of maturation of dendritic spines and increased synaptic plasticity, and according to some authors, only become electrophysiologically indistinguishable from the remaining pre-existing mature neuronal population some weeks later (van Praag et al., 2002; Ambrogini et al., 2004). Importantly, all above mentioned steps are finely tuned by a serie of epigenetic and transcriptional mechanisms, extensively reviewed elsewhere (Mateus-Pinheiro et al., 2011; Hodge et al., 2012).

In addition to the hippocampal DG and the SEZ-OB system, some authors have presented evidence for adult mammalian neurogenesis to occur in other brain regions, including cortex (Gould et al., 2001; Tamura et al., 2007), hypothalamus (Kokoeva et al., 2005; Perez-Martin et al., 2010), the striatum (Dayer et al., 2005; Cho et al., 2007), amygdala (Rietze et al., 2000; Bernier et al., 2002), cerebellum (Ponti et al., 2010), substantia nigra (Zhao et al., 2003), brainstem (Bauer et al., 2005), among others (see Bonfanti and Peretto, 2011 for review). Indeed, many of these studies provide compelling evidence supporting the idea that adult neurogenesis may be more regionally widespread than what was initially believed. However, this is still a matter of debate, as many conflicting reports exist and caution is needed in the interpretation of some findings that might be the direct result of experimental manipulations. In humans, hippocampal neurogenesis occurs in an comparable extent in middle-age individual and rodents (Eriksson et al., 1998; Spalding et al., 2013) and, curiously, appears to show a much less pronounced decline

with aging (Spalding et al., 2013). In addition, human neuroblasts are also formed in the lateral ventricles, but do not populate the olfactory bulbs (Bergmann et al., 2012). Instead, it has been quite recently shown, by a carbon-14 dating approach, that these neuroblasts integrate in the human striatum (Ernst et al., 2014).

Adult Hippocampal Gliogenesis

While neurogenesis prevails throughout life in discrete brain areas, the generation of new glial cells (a process known as **gliogenesis**) is not regionally restricted in the adult brain (Kriegstein and Alvarez-Buylla, 2009; Ninkovic and Gotz, 2013). Most of the proliferating progenitors detected outside the neurogenic niches, are NG2 and Olig2 (Dimou et al., 2008; Simon et al., 2011), although they have also been found in the hippocampal SGZ (Encinas et al., 2011). They generate glial cells, mostly from the oligdendroglial lineage, a gliogenic process therefore called oligodendrogliogenesis. Curiously, while most of Olig2-positive cells generate mature myelinating oligodendrocytes in white matter regions of the adult cerebral cortex, in the gray matter they give rise mostly to NG2-positive glia, that persists in these regions (Dimou et al., 2008). Reports of widespread NG2/Olig2-positive proliferating cells were also reported in humans (Geha et al., 2010), but functional correlates of adult oligodendrogliogenesis are still scarce.

Another form of gliogenesis occuring in the adult hippocampus proceeds from the duality of cell fate choices of hippocampal RGLs. In fact, RGLs generate not only new neurons through TAPs, but also astrocytes - astrogliogenesis (Bonaguidi et al., 2012) (**Figure 4)**. Indeed, through *in vivo* clonal analysis it has been shown that RGLs undergo multiple rounds of self-renewal and differentiation to produce both neurons and astrocytes (Bonaguidi et al., 2011; Dranovsky et al., 2011; but see Encinas et al., 2011 for a contrasting RGL proliferation-differentiation model). The process of adult hippocampal astrogliogenesis and its regulation has merited much less attention when compared with its neurogenic counterpart. Tanigaki et al. (2001) proposed that Notch1 and Notch3 promoted the irreversible commitement of progenitor cells towards astroglial fate. More recently, using adult DG derived neural percursor cells, it has been suggested that glial cell line-derived neurotrophic factor (GDNF) can promote astrogliogenesis, through the activation of the signal transducer and activator of transcription 3 (STAT3; Boku et al., 2013).

In respect to the functional importance of hippocampal astrogliogenesis, until now there are no studies specifically addressing the role of this form of adult plasticity in physiological and behavioral responses. However, and also according to data that I will present and discuss later on this thesis, hippocampal RGLs can be the source of both newborn neurons and astrocytes, supporting the consequent notion that the existing studies using cytogenic ablation approaches are likely to affect unselectively these two cell lineages. Hence, it becomes licit to believe that the presumably "neurogenesis functional correlates" described so far are (with few exceptions) more likely to be cytogenesis functional correlates, encompassing the contribution of both newborn neuronal and astroglial populations.

Functional correlates of adult hippocampal cytogenesis

Understanding how new cells in the adult hippocampus can reshape brain circuits and affect different behavioral functions is a remarkably complex and demanding task. This complexity emerges primary from the difficulty to precisely segregate the function of newborn cells from the preexisting neuron-glia cell network in which they are integrated. When attempting to construct an integrative view of evidence gathered so far, the second major source of entropy lies in the diversity of models and approaches that have been used to study this topic. In fact, there is sufficient evidence to support the notion that cytogenesis functional roles are variable according to **(i)** factors that are intrinsic to the studied animals (eg. young adult vs old; female vs male), **(ii)** experimental interference (eg. physiological/basal conditions vs disease states or pharmacological interventions) and **(iii)** the time-window of the analysis (eg. newly-formed cells vs 6-weeks old, fully integrated new cells). Hence, I will begin by summarizing key findings associating hippocampal cytogenesis with cognitive functions in the mammalian brain, focusing mainly on evidence gathered by studies that have used cytogenesis ablation approaches **(Figure 5)**.

Memory and Forgetting. Impaired hippocampal cytogenesis has been implicated in spatial memory deficits in several studies (Snyder et al., 2005; Dupret et al., 2008; Imayoshi et al., 2008; Garthe et al., 2009; Lemaire et al., 2012). In particular, Snyder and colleagues (2008) have shown that irradiated animals presented deficits in the Morris Water Maze (MWM) task. However, irradiation just before testing did not produce memory deficits, again supporting the current view that the reported implications of hippocampal cytogenesis are highly variable according to the adopted experimental timeframe. The importance of considering a longitudinal perspective was again

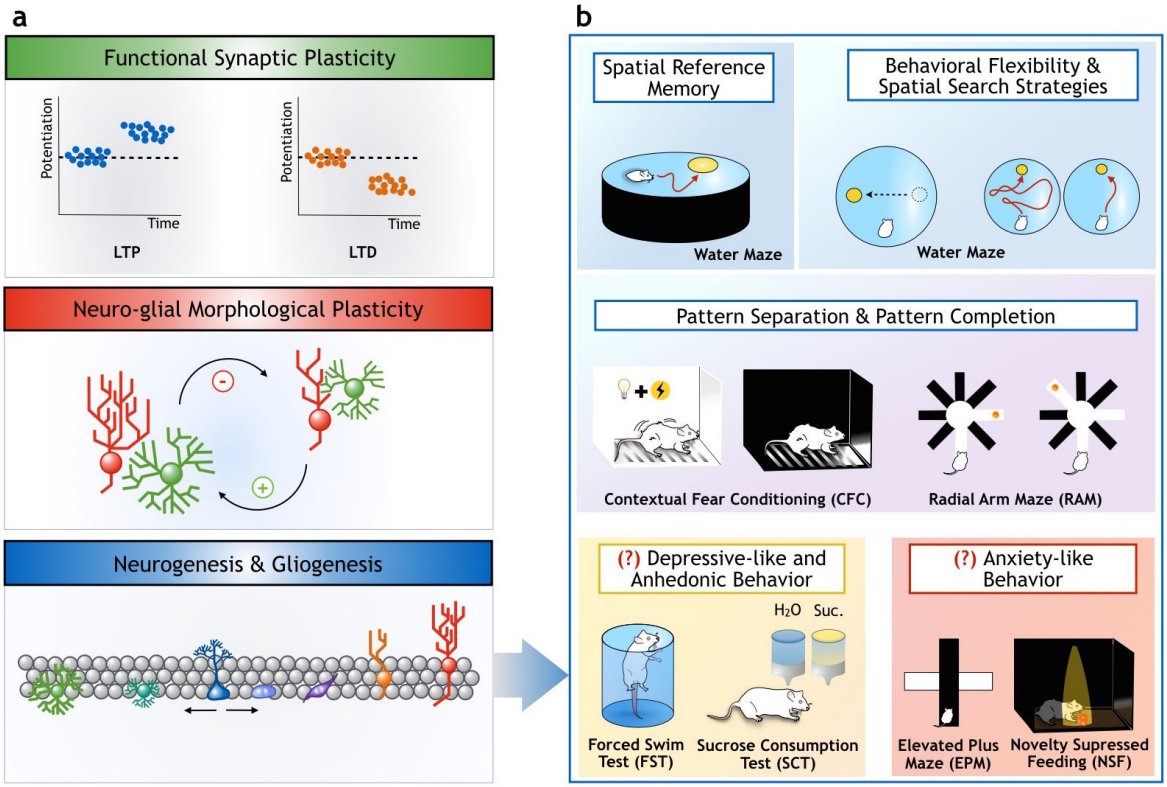

Figure 5. The *plastic* hippocampus and functional correlates of hippocampal cytogenesis. **a.** The hippocampus harbors different forms of neural plasticity, ranging from functional synaptic plasticity (e.g. long-term potentiation or long-term depression) to different types of structural plasticity, namely neuro-glial morphological remodeling and hippocampal cytogenesis. **b.** Adult hippocampal cytogenesis has been implicated in different cognitive domains, such as reference memory, behavioral flexibility and pattern separation/completion. The participation of adult hippocampus in emotional behavior (e.g. depressive- and anxiety-like behavior) have also been suggested, although this is still a topic of controversy.

recently supported by showing that cytogenesis ablation 1 week prior testing produced no effects on spatial memory, whereas the same analysis 2 and 4 weeks post-ablation revealed significant deficits post-ablation (Denny et al., 2012). Importantly, cytogenesis ablation has been suggested to impact specifically in spatial reference memory in the water maze, without having effects on the cue-guided or egocentric orientation versions of the test (Dupret et al., 2008). Although still controversial, the diversity of approaches and time-points of analysis may account for the reason why some authors describe no alterations in spatial memory upon hippocampal cytogenesis ablation (Shors et al., 2002; Saxe et al., 2006; Wojtowicz et al., 2008; for discussion see Leuner et al., 2006). Moreover, it was recently proposed that hippocampal cytogenesis is also relevant in forgetting (Akers et al., 2014). The authors propose that increasing neurogenesis after memory formation, either by voluntary running or by memantine or fluoxetine administration, is sufficient to induce forgetting in adult animals.

Behavioral Flexibility and Spatial Search Strategies. In behavioral neuroscience, behavioral flexibility is the adaptive ability to favor or ignore the use of familiar associations when either intrinsic or extrinsic contingencies change (Burghardt et al., 2012). As it was also proposed before through computational network simulations (Wiskott et al., 2006;Gage et al., 2008), several authors have reported impaired behavioral flexibility after cytogenesis ablation (Garthe et al., 2009; Burghardt et al., 2012; Garthe et al., 2014). Indeed, 12 weeks after irradiation mice showed no deficits in learning the position of the shock zone in an active place avoidance task. However, animals presented deficits in learning to avoid the shock zone if its position was changed over trials, when compared with animals with active cytogenesis (Burghardt et al., 2012). Another form of cognitive process that has been suggested to be dependent on ongoing hippocampal cell genesis, is related to the adopted search strategies in the classical water maze (Garthe et al., 2009; Gu et al., 2012; Garthe et al., 2014). In fact, it has been suggested that although typically analyzed parameters (such as path length or latency to escape) reflect how efficiently spatial memory is consolidated, information related with the animal's cognitive spatial representation and the consequent efficiency of spatial navigation is usually overlooked (Garthe and Kempermann, 2013). Through a series of works, the authors advocate and present evidence showing that performance in the water maze depends on the successful integration of egocentric route-base information into allocentric representations, the latter depending on the ability to encode new information through the generation of new neurons (and presumably also astrocytes) in the adult hippocampus (Garthe and Kempermann, 2013; Garthe et al., 2014).

Pattern Separation and Pattern Completion. The concept of pattern separation (PS) and how it relates to underlying neural networks is still controversial. In computational and behavioral terms, PS corresponds to the neural mechanism of storing successive patterns of input stimuli into distributed networks. It is considered a form of counteracting catastrophic memory interference[10], thereby avoiding that recent memory traces conflict with and degrade previously consolidated ones (Wiskott et al., 2006; Abrous and Wojtowicz, 2015). In both animals and humans, hippocampal cytogenesis deficits have been associated with the long-term manifestation of pattern separation impairments (Leutgeb et al., 2007; Leutgeb and Leutgeb, 2007; Bakker et al., 2008; Clelland et al., 2009; Arruda-Carvalho et al., 2011; Denny et al., 2012; Niibori et al., 2012).

[10] Catastrophic interference: is the phenomenon in which successive patterns of incoming stimuli overwrite the existing patterns to the point of the latter being unrecognizable (McClelland et al. 1995; Abrous and Wojtowicz, 2015)

Interestingly, Nakashiba and colleagues (2012) have proposed that adult born neurons in different maturation stages are important not only to PS but also to Patter Completion (PC). Contrastingly to PS, PC is the neural mechanism in which a degenerated recent input pattern is resolved through the retrieval of an intact previously stored pattern (Leutgeb and Leutgeb, 2007). In fact, the authors show that while young newborn neurons contribute to pattern separation, older newborn granular neurons switch their function, becoming important for pattern completion (Nakashiba et al., 2012).

Before discussing how hippocampal cytogenesis has been related to emotional behavior, a conceptual disambiguation is needed. Growing evidence has been supporting the prevalent notion that rodents can express different emotional states (Ben-Ami Bartal et al., 2011; Wohr and Schwarting, 2013; Decety et al., 2016). However, these are naturally extremely difficult to characterize or quantify with currently available methods, and impossible to precisely model in rodents (Cryan and Holmes, 2005). In this perspective, it is impossible to be sure wether we are (or will ever be) able to evaluate mood and anxiety in animals, as this evaluation depends chiefly from the individual self-examination of his inner state and how he conveys the information to the observer. However, within the fields of pharmacological and behavioral neurosciences, there is a large bulk of evidence supporting the construct and face validity of behavioral test paradigms that are believed to translate "emotional status" of the animals. Such examples include mood-related dimensions such as behavioral despair or anhedonic[11] behavior or anxious-like behavior, as for instance neophobic[12] behavior (see **Section 1.3**). Hence, I will briefly summarize findings relating hippocampal cytogenesis impairments with deficits found in those test paradigms (for a more in depth review see Petrik et al., 2012 and Tanti and Belzung, 2013).

Mood-related behavior. One of the first hints on the potential association between adult cytogenesis and mood disorders, were provided by the demonstration that corticosterone suppressed the generation of new neuronal and glial cells in the adult DG (Gould et al., 1992). This notion was further supported by later studies showing that activation of serotonergic receptors promoted hippocampal cytogenesis (Gould, 1999), while serotonin depletion in the adult rat brain was associated with decreased cytogenesis (Brezun and Daszuta, 1999).

[11] Anhedonia: the inability to enjoy pleasure from a formerly pleasurable experience. It is core symptom in the clinical presentation of depressive spectrum disorders.

[12] Neophobia: is the persistent or heightened fear/anxiety in face of something new. In rodents, neophobic behavior is typically assessed in test paradigms where animals approach a new and unknown environment.

In cytogenesis ablation studies it is difficult to establish whether cytogenesis ablation per se is sufficient to induce behavioral deficits in emotional dimensions such as anhedonic behavior. In fact, anhedonic behavior has been reported after cytogenesis suppression in rodents (Snyder et al., 2011) and also in non-human primates after bilateral hippocampal irradiation (Perera et al., 2011). However, other works, including from our lab have found no association (Bessa et al., 2009; Jayatissa et al., 2009; Surget et al., 2011). The same holds true for increased immobility in the forced swim test (FST), after cytogenesis ablation in naïve animals. While Snyder and colleagues (2011) report deficits in FST, several other authors present contrasting results (Holick et al., 2008; Bessa et al., 2009; Revest et al., 2009). Another critical aspect is the question of whether hippocampal cytogenesis is required for the action of pharmacological or somatic treatments, such as antidepressants or for instance electroconvulsive therapy (ECT). I will tackle this topic in the next section.

Anxious-like behavior. The participation of hippocampal cytogenesis in the control of anxiety-related behavior is another topic of much controversy. Anxiety-like behavior after cytogenesis ablation in naïve animals has been reported in several test paradigms, namely the novelty supressed feeding (NSF) (Bessa et al., 2009), the elevated plus maze (EPM) (Revest et al., 2009) and the light/dark test (Revest et al., 2009; Fuss et al., 2010). Again, several studies that followed various ablation approaches and experimental timeframes presented apparently discordant results (Santarelli et al., 2003; Saxe et al., 2006; Surget et al., 2008).

To conclude, several ablation studies have implicated hippocampal cytogenesis in different cognitive and emotional behavioral dimensions, in naïve animals. When reviewing current literature, contrasting results and views are evident, but the major source of incongruence lies on the still debatable participation of adult hippocampal cytogenesis in emotional behavior control. In **section 1.3**, I will further explore this topic adding evidence gathered from animal models of disease and pharmacological treatments.

1.2.2 ADULT HIPPOCAMPAL DENDRITIC PLASTICITY

Although adult cytogenesis encloses great potential to modulate neural circuits and brain functions, another much massive phenomenon, occurring virtually all over the brain, is also responsible for the plasticity of the adult CNS. Such phenomenon refers to the highly dynamic dendritic plasticity that, theoretically, has the potential to occur in every single neuronal unit in the adult brain. Structural dendritic plasticity, along with dendritic functional plasticity[13], have been intensively studied both in the developing and mature brain, and a systematic review of this topic is not within the scope of this introductory preamble. Instead, I will briefly highlight the importance of this form of plasticity in the adult brain, giving special emphasis to dendritic plasticity occurring within the hippocampus and its functional repercussions.

Dendritic Plasticity in the Adult Brain

Rewiring of neural networks is achieved either by collapsing (Bastrikova et al., 2008) and/or creating new synapses (Knott et al., 2006). This redesign of the synaptic scheme can be the result of the highly dynamic dendritic spines turnover (Trachtenberg et al., 2002) and re-routing of axonal projections (De Paola et al., 2006; as reviewed in Butz et al., 2009). Indeed, neural networks are profoundly plastic in the adult brain (Majewska et al., 2006) and have raised the interest of both experimentalists, but also computational neuroscientists (Verzi et al., 2005). Furthermore, adding to the long known notion that axon terminals contribute to structural plasticity in the adult brain (Wolf et al., 1989), the simplistic classical view that dendrites function as synaptic input collectors and conveyers of information to the soma has been revisited (Branco and Hausser, 2010). In fact, recent years have provided evidence demonstrating that the single dendritic branch may act as a fundamental unit of signaling in the mammalian brain (Branco and Hausser, 2010; Jia et al., 2010). This view exponentially increases the complexity and potential of this form of plasticity, as it massively increases the computational capacity of a single neuron in normal physiological conditions. Furthermore, within the mammalian brain dendritic structural plasticity can assume even a greater proportion in reactive conditions. In fact, even areas that are considered relatively stable, such primary sensory cortices (Grutzendler et al., 2002; Mizrahi and Katz, 2003) can undergo profound reactive neuroplastic changes in face of extrinsic insults (Jain

[13] Disambiguation - Structural Dendritic Plasticity vs Functional Dendritic Plasticity. Theoretically, the concept of functional plasticity considers synapses as variable amplification factors (synaptic strengths) within a hardwired network structure. In contrast to any forms of functional plasticity that change synaptic strengths without changing the anatomical connectivity between neurons, structural plasticity comprises changes in synapse numbers, axonal fibre densities, axonal and dendritic branching patterns, synaptic connectivity patterns (Butz et al., 2009).

et al., 2000). This also holds true for the hippocampus. In fact, it has long been known that the hippocampal formation can express alternative forms of structural plasticity as a response to a structural lesion (Lynch, 1974; Butz et al., 2009). Indeed, the hippocampus seems highly prone to experience-dependent axonal and dendritic structural reconfigurations, such as environmental enrichment or voluntary exercise (Redila and Christie, 2006), as well as it is vulnerable to structural reconfiguration induced by deleterious stimuli, such as stress (Watanabe et al., 1992; Magarinos et al., 1996), or pathological conditions such as schizophrenia (Garey, 2010), autism spectrum disorders (Raymond et al., 1996; Durand et al., 2012) or depression (Duman et al., 1997; Bessa et al., 2009).

Hippocampal Dendritic Plasticity in Learning and Memory

Early studies using the sea slug Aplysia have provided the grounding evidence for the remodelling of synaptic architecture after non-associative learning and long-term facilitation (Bailey and Chen, 1983; Glanzman et al., 1990). Today, there are several lines of evidence associating structural alterations within the hippocampus and its role in learning and memory formation (Lamprecht and LeDoux, 2004). A paradigmatic example is the correlation between the mossy fiber pathway (MFP) configuration and behavioral performance in several test paradigms (Rekart et al., 2007). This is shown by the positive correlation between the amount of CA3 that is innervated by the MFP in the rat hippocampus and performance in MWM (Prior et al., 1997). The association between the structural complexity of the MFP with learning and memory in a radial arm maze task (Crusio et al., 1987) and a reversal learning task have also been reported (Schopke et al., 1991). Curiously, this association is bidirectional, as besides being possible to predict some dimensions of cognitive behavior from the structural richness of intrahippocampal circuits, these same circuits seem to be reshaped by experience-dependent mechanisms during cognitive tasks (Crusio et al., 1987; Lipp et al., 1988; Prior et al., 1997). Indeed, learning-induced growth of intra-hippocampal circuits has been reported in several studies (Ramirez-Amaya et al., 2001; Holahan et al., 2006). It is important to emphasize that in many aspects structural plasticity is indissociable from functional plasticity. Learning and memory consolidation are one of such examples, where many evidences exist showing the association between memory formation and a Hebbian[14] strengthening of existing synapses (Melamed et al., 2004; Massey and Bashir, 2007; Raymond, 2007). In this context, structural

[14] The most frequently postulated mechanism for synaptic plasticity is based on Hebb's rule, which states that strengthening of a connection between two neurons occurs, if both cells are simultaneously and repeatedly active (Hebb, 1949).

dendritic plasticity may hardwire those functional changes, as in the case of the generation of new synapses in close apposition induced by long-term potentiation (LTP), or when synapses are lost as a result of long-term depression (LTD) (Raymond, 2007; Becker et al., 2008; Butz et al., 2009).

The participation of hippocampal structural dendritic plasticity in the control of emotional behavior is still an open question. However the correlation between the neuronal atrophy occurring in different cortico-limbic areas following chronic exposure to stress with mood- and anxiety-related deficits, together with the correlation between the reestablishment of structural dendritic morphology by antidepressant treatment and the improvement in the aforementioned behavioral deficits (Pittenger and Duman, 2008; Bessa et al., 2009) are one of the most consistent findings in literature that support the role of axonal and dendritic plasticity in emotional homeostasis.

What about astrocytes?

I will conclude this chapter with a final remark whose major purpose is to invigorate the need to revisit concepts and views in post natal plasticity, that have been classically neuron-centric. When referring to dendritic plasticity we are implicitly referring to neuronal cells, but these cells are not alone in the adult brain when it comes to the ability to rapidly adapt and change their morphological architecture. In fact, astrocytes, that best neurons in the adult brain in the proportion of 3 to 1, are also key players in redesigning adult neuroglial networks (Slezak et al., 2006). This is achieved via two general mechanisms. First**,** astrocytes are in close contact with synapses and control neuronal morphology by releasing factors that enhance or inhibit synaptic transmission (Zhang et al., 2003). Under the principles that we have already discussed, this can lead to the activity-dependent formation or elimination of new synapses. The same outcome is also achieved by the astrocytic secretion of a variety of factors that regulate dendritic and axonal growth, such as laminins (Liesi et al., 1983), neurotrophins (Althaus and Richter-Landsberg, 2000) or S100β (Donato, 2001). Second, astrocytes themselves are highly plastic and have the ability to change their morphology in a variety of contexts. There is evidence showing that astrocytes show altered morphology in response to environmental complexity (Jones and Greenough, 2002), as well as hypertrophy in relation to motor skills learning in rats (Anderson et al., 1994). Hippocampal LTP-induction has also been shown to promote morphological alteration in hippocampal astrocytes (Wenzel et al., 1991). Insight on how these astrocytic morphological alterations are molecularly regulated has been growing, and the association of impaired learning and memory performance with defects in astrocytic cytoarchitecture supports the hypothesis that astrocytes operate conjointly with neuronal assemblies to encode engrams in the adult brain (Slezak et al., 2006; Gibbs et al., 2008).

1.3

Dysregulation of Adult Neural Plasticity in the Stressed Brain: a road to Depression?

"Neither the prestige of your subjects and the power of your instruments, nor the extent of your planning can substitute for the originality of your approach and the keenness of your observation."

Hans Selye

So far, we have seen how hippocampal structure and topographical organization allow this brain region to integrate the neural circuits responsible for different cognitive processes and emotional behaviors. In addition, I have highlighted evidence supporting the importance of different forms of neuro- and glioplasticity, persisting in the adult brain, as the structural and functional substrates of some behavioral outputs. Understanding hippocampal function and how it is modulated by neuro-glial plasticity is the comprehensional basis to explore the question of how disruption of adult neural plasticity may integrate mechanisms of disease and recovery in the CNS.

1.3.1 STRESS, NEURAL PLASTICITY & DEPRESSION: JOINING THE DOTS

Starting point: Stress and the HPA axis

The mundane use of the word "stress" in current days has its origins in the wealth of scientific work that Hans Selye's pioneer research has propelled, in the beginning of the 20's century. Using Selye's own words, stress may be defined as "the non-specific response[15] of the body to any demand for change" (Selye, 1936) and, in its physiological and adaptive essence, serves to promote the constancy of the "milieu intérieur" (as Selye would write as a reference to the work of the french physiologist Claude Bernard), that is to say, to maintain homeostasis, as we now prefer to term it (Selye, 1950). With the prolonged over-recruitment of the adaptive mechanisms elicited by stress exposure, the adaptive response is worn-out, triggering multi-systemic maladaptive

[15] Selye originally alluded to the sum of all non-specific, systemic reactions of the body which ensue upon exposure to stress as the **general adaptation syndrome**, later to be gradually replaced by the now common **stress response.**

by stress exposure, the adaptive response is worn-out, triggering multi-systemic maladaptive effects (Selye, 1976; Tsigos and Chrousos, 2002). Such effects are related to both central and peripheral immune (Chrousos, 1995), metabolic and endocrine (Benker et al., 1990) disturbances, as well as the eventual installation of different systemic, neurodegenerative and psychiatric conditions (Herman and Cullinan, 1997; Dinan, 2005; Baumeister et al., 2014).

In both adaptive and maladaptive responses, endogenous corticosteroids are one of the major effectors of the stress response. These steroid hormones are produced by the adrenal cortex and participate in many different systemic physiological processes (de Kloet, 2003). Their predominant role in either kidney water and ion transport, or carbohydrate metabolism allows for their classification in either mineralocorticoids or glucocorticoids, respectively. Aldosterone is the major endogenous mineralocorticoid, and although regional production of aldosterone in the brain has been reported, its functional repercussions in the CNS, beyond hemodynamic regulation, remain to be fully comprehended (Gomez-Sanchez et al., 2005). Hence, in light of current knowledge, circulating glucocorticoids - cortisol in humans, and corticosterone (CORT) in rodents - are the prime effectors of stress response.

Corticosteroids, as lipophilic steroids, are long known to act on the brain (McEwen et al., 1968; Reul and de Kloet, 1985) and the harmful effects that ensue upon prolonged or excessive levels of corticosteroids, such as neuronal degeneration and cell loss, have also been early reported (Muhlen and Ockenfels, 1969; Sapolsky, 1985). Indeed, high secretion of corticosteroids is until now considered the major molecular signature of stress response (Sapolsky, 1996; Sapolsky, 2015).

A key neuroendocrine system that translates an environmental threat or challenge into a series of coordinated physiological responses that are part of the stress response is the so-called HPA axis (from hypothalamus-pituitary-adrenal axis) (Herman and Cullinan, 1997; Lupien et al., 2009) **(Figure 6)**. Briefly, HPA axis stimulation upon stress exposure leads to the engagement of stress-responsive brain regions, that include the hypothalamus, the hippocampus, the septal nuclei, the amygdala, the BNST and the PFC, among others (Cullinan et al., 1995; Lopez et al., 1999). Activation of these areas will then prompt neurons in the medial parvocellular region of the hypothalamic paraventricular nucleus to release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). Within the pituitary gland, CRH will trigger the secretion of adrenocorticotropic hormone (ACTH), leading to the production of corticosteroids by the adrenal

The HPA axis, also elicits the activation of the sympathetic nervous system, the release of inflamatory cytokines and metabolic responses, all of which also encompass the overall stress response (de Kloet, 2003; Leonard, 2006). Importantly, the responsiveness of the HPA axis is largely determined by the ability of corticosteroids to regulate ACTH and CRH secretion, by binding to both glucocorticoid (GRs[16], also called type II corticosteroid receptors) and mineralocorticoid receptors (MRs, also called type I corticosteroid receptors) in key areas such as the hippocampus and the PFC (Jacobson, 2005). In fact, once the stressor has subsided, negative feedback regulatory loops executed by glucorticoids in these areas will promote HPA downregulation. By contrast, brain regions such as the amygdala and some brainstem locci act as HPA activators when facing a challenge (Lupien et al., 2009).

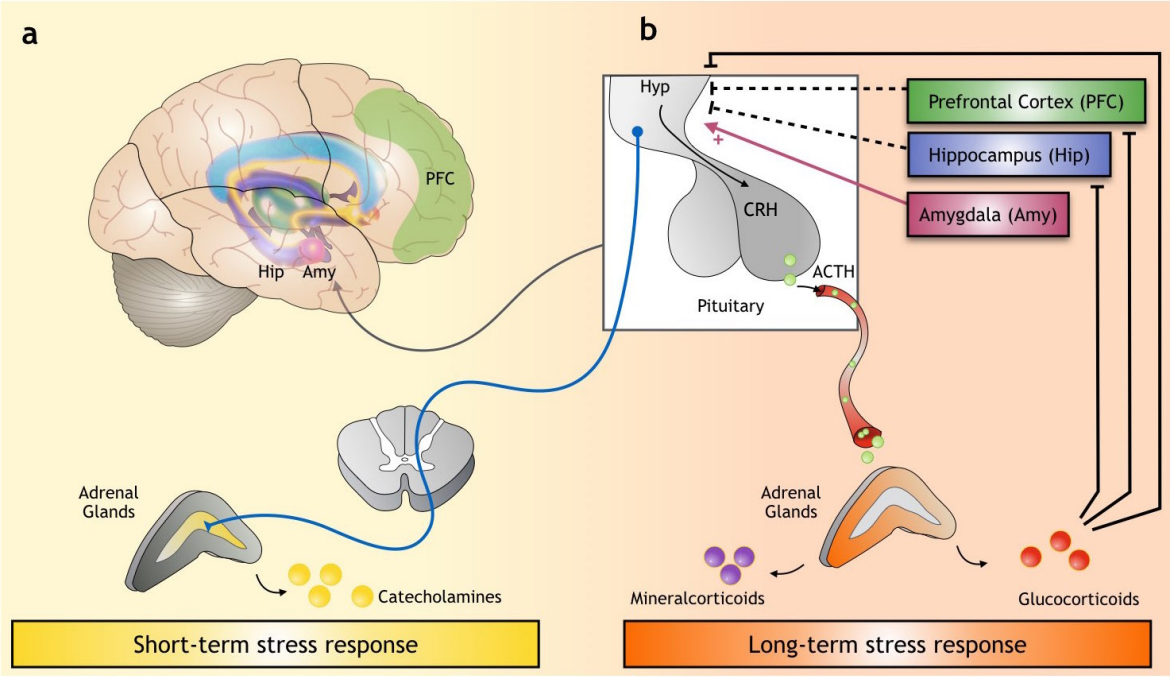

Figure 6. Stress response: the sympatho-adrenomedulary system and the hypothalamic-pituitary-adrenal (HPA) axis. **a.** Stress triggers a response by the sympathetic nervous system, which will stimulate the adrenal medulla to produce cathecolamines (adrenaline and noradrenaline) and inflammatory cytokines , that in turn will mediate the most immediate physiological alterations of stress response. **b.** In turn, the HPA axis will be responsible of triggering a long-term stress response. Briefly, limbic brain areas, such as the amygdala, promote the activation of the hypothalamic paraventricular nucleus to release corticotropin-releasing hormone (CRH). Within the pituitary gland, CRH will trigger the secretion of adrenocorticotropic hormone (ACTH), leading to the production of corticosteroids (mineralcorticoids and glucocorticoids). Corticosteroids will impact both peripherally and in the central nervous system, exerting a negative feedback control on the hypothalamus and pituitary, as well as in brain areas suggested to participate in the "shutting-down" of the HPA axis (e.g. hippocampus and prefrontal cortex).

[16] GRs have about 10 times lower affinity than MRs for corticosterone, but display the highest affinity for the synthetic glucocorticoid dexamethasone, which is often use to study the selective activation of GRs.

The physiological activity of the HPA axis in many vertebrate follows a circadian rhythm (Herman et al., 1993), with a diurnal secretion peak coinciding with the begining of the active phase of the organism's sleep-wakefulness cycle (light-phase in human, dark-phase in rodents), after which circulating corticosteroids rapidly decay to basal levels. As pointed before, this circadian pattern of activity is often disrupted in the context of maladaptive stress, leading to several deleterious effects in the brain. In relation to what we have been discussing in previous chapters, different forms of adult structural plasticity are often compromised following HPA axis dysregulation and hypercorticosteronemia.

From stress to impaired plasticity

As summarized above, there is a group of cortical and limbic regions, sometimes referred as stress-responsive areas, whose topographical organization and functional properties will determine their action in HPA axis regulation (as reviewed in Herman et al., 2005). Areas such as these include the amygdala, the hippocampus, the PFC, the BNST or the Striatum and structural and functional alterations in these regions are thought to be associated with glucocorticoid hypersecretion (or in some contexts, hyposecretion) seen upon chronic stress exposure or different neuropsychiatric disorders (Jacobson and Sapolsky, 1991; Pittenger and Duman, 2008). It is today also widely recognized that the mediation of synaptic remodeling and cytogenesis alterations in these brain regions is not a solo agent mission carried out by corticosteroids. Instead, they operate jointly with excitatory amino acids (Venero and Borrell, 1999), as well as with trophic factors such as brain-derived neurotrophic factor (BDNF) (Patterson et al., 1996; Govindarajan et al., 2006; Revest et al., 2014) and vascular endothelial growth factor (VEGF) (Cao et al., 2004), signaling molecules, as for instance protease tPA (Pawlak et al., 2005) and synaptic remodeling proteins such as neural adhesion molecule (NCAM) and synapsin (Syn) (Wellman, 2001; Cook and Wellman, 2004). This complex regulatory and inter-dependent pathways are likely the source of contrasting plastic alterations observed in different brain regions upon chronic stress exposure, that will be summarized next **(Figure 7)**.

Hippocampus. Initial studies revealing how chronic stress and CORT induced loss of dendritic complexity in hippocampal CA3 were the spearhead of research that led to our current understanding on how stress has the ability to induce structural remodeling events in the adult brain (Watanabe et al., 1992; McEwen, 1999; Sousa et al., 2000). Within the hippocampus, stress exposure has also been shown to produce dendritic atrophy in apical dendrites of CA1 and granular

dentate neurons (McEwen, 1999). Besides dendritic shortening, spine density and spinogenesis have been reported to be decreased in hippocampus following stress exposure (Chen et al., 2008; Chen et al., 2010) **(Figure 7)**. Moreover, in a recent study, Maras and colleagues showed that while the number of CA3 synapses was reduced both in the septal and temporal hippocampal poles, in CA1 this effect was restricted to the septal hippocampus (Maras et al., 2014). These effects of chronic stress on structural hippocampal neural plasticity are largely reversible, as the mentioned morphological alterations are resettable to basal levels after the cessation of stress (McEwen et al., 2016). As highlighted before, structural plasticity alterations are many times coupled with changes in functional plasticity. Indeed, never is this more true than in the hippocampus. Both *in vitro* and *in vivo* studies have demonstrate that stress and CORT impair LTP, and that repeated stress could mediate the switch from LTP to LTD (Diamond and Rose, 1994; Kim and Diamond, 2002). Notably, and supporting the functional heterogeneity of the hippocampal longitudinal axis (see **Section 1.2**), a recent work from our lab showed how stress produces contrasting effects on dendritic plasticity in the dorsal and ventral hippocampal poles. In fact, whereas in the DH, chronic stress triggered a volumetric reduction as a result of atrophy of CA3 and CA1 apical dendrites, in the VH there was an increase in hippocampal volume concurrent with the increase of CA3 apical dendrites (Pinto et al., 2015). Furthermore, in the hippocampus, brain-derived neurotrophic factor (BDNF) has been the most widely studied molecular regulator of neural plasticity. Stress has been linked to reduced levels of BDNF, which in turn have not only been linked to impoverished dendritic arborization, but also to reduced hippocampal cytogenesis (Schaaf et al., 2000; Sairanen et al., 2005; Magarinos et al., 2011; Lakshminarasimhan and Chattarji, 2012). Indeed, the DG appears to be highly vulnerable to the deleterious effects of stress, as impaired generation of new granule neurons (impaired neurogenesis), has been shown to be accompanied by loss of older pre-existing granule neurons (Pham et al., 2003), as also mimicked by CORT administration (Sousa et al., 1999). In addition, hippocampal glioplasticity appears also to be targeted by stress, as chronic stress was shown to decrease both the number and somal volume of astroglia (Czeh et al., 2006). These findings in animal models correlate and provide a possible explanation for the reduced hippocampal volume found in patients with disorders often accompanied by hypercortisolemia, such as PTSD or Cushing's disease (Starkman et al., 1992; Bremner et al., 1995; Starkman et al., 1999).

mPFC. Similarly to what happens in the hippocampus, chronic stress significantly reduces both structural and functional plasticity in the medial PFC (mPFC) **(Figure 7)**. Stress exposure has been shown to reduce spinogenesis and dendritic complexity in layer II/III and layer V pyramidal neurons (Cook and Wellman, 2004; Radley et al., 2004; Cerqueira et al., 2007). These alterations have also

been reproduced by CORT administration (Watanabe et al., 1992; Wellman, 2001; Cerqueira et al., 2005) and, as in the hippocampus, are reversible after subsiding stress exposure (Radley et al., 2005; Liston et al., 2006). Molecular regulators mediating these alterations include TrkB and excitatory amino-acids (Martin and Wellman, 2011), while it is still difficult to conciliate inconsistent findings regarding BNDF expression with the observed morphological alterations. Moreover, structural alterations are associated with functional plasticity effects, namely with the blunting of LTP response (Goldwater et al., 2009), as well as decreased LTD elicitation in brain slices of animals that had been exposed to stress (Zhong et al., 2008), the last being an apparent difference between the hippocampus and the PFC, whose functional importance remains to be unveiled. In addition, glial cells are also targeted by stress, as shown by the CUS-induced decrease in GFAP mRNA levels and impaired glial metabolism (Banasr et al., 2010). Accordingly, in humans the severity of PTSD symptoms is inversely correlated with the volumetric variation of the anterior cingulate region of the PFC (Woodward et al., 2006), and loss of gray matter has been documented as a result of exposure to adverse experiences (Ansell et al., 2012).

Amygdala. The amygdala paradigmatically reflects how the effects of stress in adult plasticity are region-specific. In contrast to other limbic structures, chronic stress exposure is able to enhance synaptic plasticity in the amygdala (Vyas et al., 2002; Vyas et al., 2003; Drevets et al., 2008; but see also Banasr and Duman, 2007) **(Figure 7)**. In fact, expanded dendritic branching and increased spinogenesis have been reported in spiny neurons of the basolateral amygdala (BLA) after prolongued stress (Roozendaal et al., 2009). These hypertrophic effects have been reported to be long-lasting, as they persist several weeks after stress (Vyas et al., 2004), which represents an unique feature of the temporal dynamic of structural plastic changes promote by stress, as in other areas these alterations are reversed some time after stress cessation. Interestingly, the levels of dendritic spine density in the BLA have been shown to be a good predictor of the anxiety state of animals (Rao et al., 2012). Stress-induced neuro-structural alterations have been recently shown to be accompanied by enhanced LTP in lateral amygdala (Suvrathan et al., 2014). Although the molecular regulation of structural plasticity in the amygdala is yet to be fully understood, studies have linked glucocorticoids action, the neurotrophic factor BDNF (Govindarajan et al., 2006) and the proteinase tPA (Bennur et al., 2007) to the increased dendritic spines densitiy and synaptic connectivity in this region. These neuroplastic alterations found in rodents are the likely effect of the over activation of the neural circuit responsible for fear and anxiety, and are translatable to humans, where both volume and activation have been found associated to stress-related disorders such as PTSD (Rauch et al., 2000; Shin et al., 2005). In addition, studies have found decreased glial cell

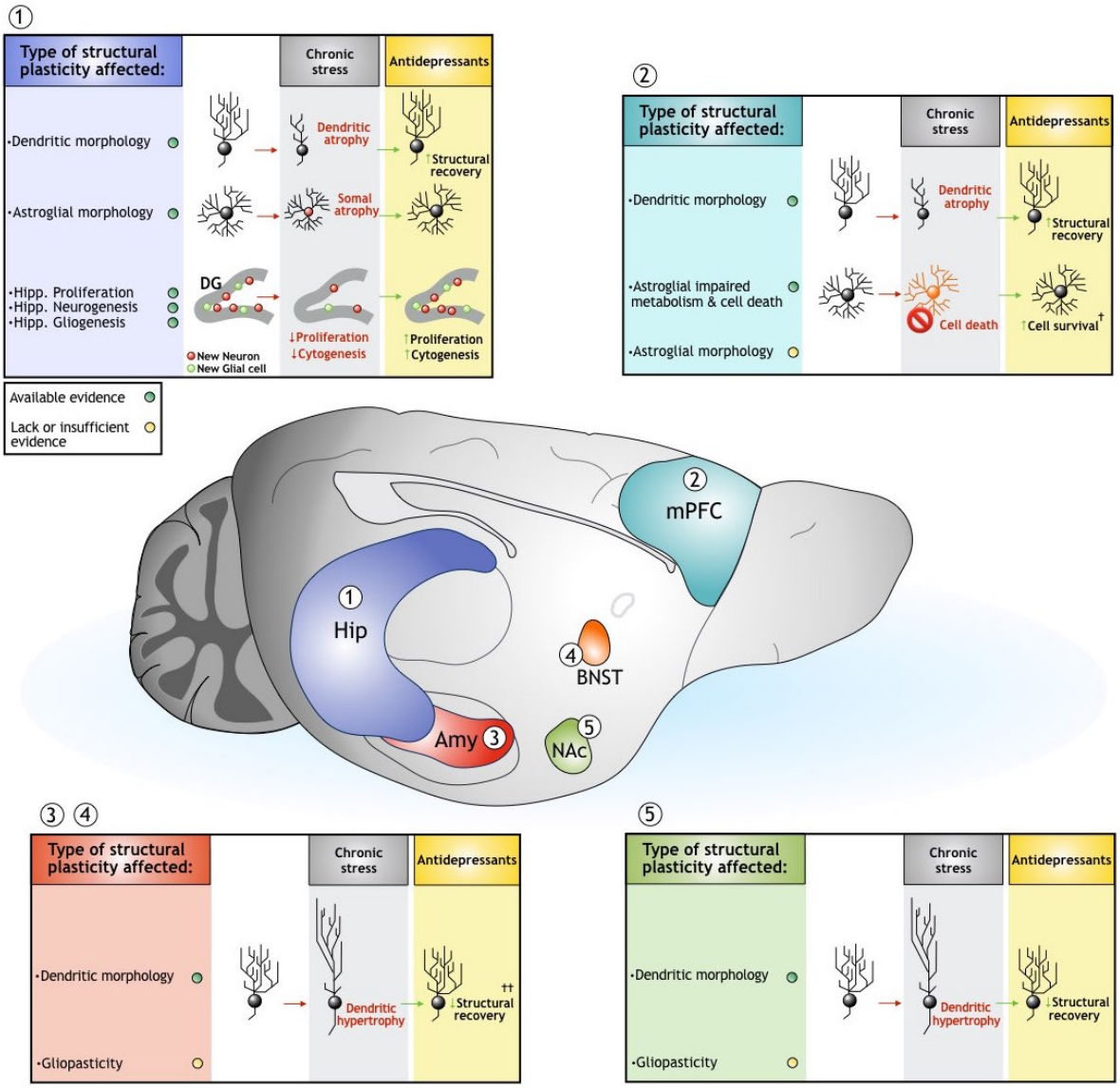

Figure 7. Stress, antidepressants and adult structural plasticity. Chronic stress has been shown to impact different forms of structural plasticity in the adult brain. In the hippocampus (Hip), chronic stress promotes dendritic atrophy, along with astroglial somal atrophy and cytogenic impaiments. Different classes of antidepressant drugs were shown to revert these neuroplastic alterations. In the medial prefrontal cortex (mPFC), stress-induced dendritic atrophy is accompanied by alterations in glial metabolism and glial cell death. Drugs with antidepressant action have been shown to revert these neuro-glioplastic deficits. Contrastingly, in the amygdala (Amy), the bed nucleus of the stria terminalis (BNST) and the nucleus accumbens (NAc), chronic stress was shown to elicit hypertrophic effects on dendritic neuronal structure, that are reverted by antidepressants. The modulation of glioplasticity by stress or antidepressants in these regions remains unknown. .

numbers in the amygdala of patients with PTSD, and depression, making glioplasticity a likely target.

BNST. The activation of the BNST has been described to side with the amygdala in the response to stress and anxiety behavior (Walker et al., 2003; Hammack et al., 2009). Indeed, similarly to what happens in the amygdala, both CORT and chronic stress have been shown to increase dendritic lenght in anteromedial division of the BNST, as well as increased volume (Vyas et al., 2003; Pego et al., 2008) **(Figure 7)**. These alterations have been associated to increased BDNF and TrkB receptor expression (Hammack et al., 2009). The effects of stress in functional plasticity have also been shown to be pronounced, as in the BNST stress has been shown to trigger the transition of LTD to LTP (Glangetas et al., 2013). To the best of our knowledge there are no reports addressing gliopasticity alterations in the BNST, or studies studying volumetric changes in this brain region in humans.

Striatum. Although not typically included in the "stress-response neurocircuitry", a brief reference to the striatum is mandatory, as striatal neuroplasticity have also been reported following stress exposure (Pittenger and Duman, 2008; Dias-Ferreira et al., 2009). Briefly, several lines of evidence have provided insights on how chronic stress can produce neuronal remodelling in corticostriatal circuits, affecting decision-making and habit formation (in the dorsal striatum), as well as reward-related behavior (in the ventral striatum) (Dias-Ferreira et al., 2009; Sousa and Almeida, 2012) **(Figure 7)**. Regarding the latter, chronic stress can modulate synaptic strength of synapses in midbrain dopaminergic neurons (Saal et al., 2003), possibly leading to NAc dysregulation.

Impaired neural plasticity as pathophysiological connector to depression

Major depression (or Major Depressive Disorder, MDD) is a highly prevalent chronic psychiatric disorder, estimated to affect 350 million people worldwide (WHO, 2012), with a pooled anual incidence of 3.0% (Ferrari et al., 2013). Depression alone accounts for 4.3% of the global burden of disease and is among the largest single causes of disability worldwide (11% of all years lived with disability globally) (World Health Organization, 2013). According to the Diagnostic and Statistical Manual of Mental Disorders V (DSM-V; American Psychiatry Association, 2013), major depression symptoms include depressed mood and anhedonia, both considered core features of depression (Nelson and Charney, 1981) often accompanied by neurovegetative symptoms (such as asthenia and anorexia), feelings of despair, guilt and suicidal ideation (Hasler et al., 2004; American Psychiatry Association, 2013). The complexity of its clinical presentation is also reflected in the high comorbidity with anxiety, which has led to the suggestion of a common

pathophysiology for both conditions (Hettema, 2008). Also, cognitive dysfunction, namely changes in executive function, attention deficits, and memory impairments, have been reported in depressed patients (Castaneda et al., 2008; Gonda et al., 2015). The complexity and high heterogeneity of this clinical entity probably accounts for the fact that MDD neurobiological basis remains a difficult conundrum.

Several lines of evidence gathered both from clinical and preclinical studies have associated different pathophysiological mechanisms in the aetiology of the disease (see **BOX 1**), ranging from the more classical neurochemical unbalances (Castren, 2005), to include neuroimmunological responses (Maes, 2011), and impairments in neural plasticity (McEwen et al., 2016).

BOX 1. Pathophysiological elements suggested to be associated with Depression

Neurochemical Systems	Monoaminergic depletion has classically been associated with depression (Berton and Nestler, 2006). In addition, increased glutamate levels in depressed patients have also led to the assumption of glutamate excitoticity as a contributing factor to the neurobiological basis of the disease (Kucukibrahimoglu et al., 2009). Both views are supported by the observations that antidepressants can counteract these neurochemical unbalances (Milan et al., 2015).
Immune System	Inflammation and cell-mediated immune activation have been linked to depression (Maes, 1995, 2011). This neuroimmune etiological component of depression has been associated with immune response mediators such as interleukin-4 (IL-4) interleukin-6 (IL-6) and tumor necrosis factor alfa (TNF-α) (Dowlati et al., 2010).
Cell Signalling & Neurotrophins	Neurotrophins are important regulators of cell growth, proliferation and differentiation. Decreased levels of neurotrophins have been associated to stress exposure and depression (Duman, 2004, Duman and Monteggia, 2006). Moreover, neurotrophins, such as BDNF or VEGF have been associated with antidepressant action (Warner-Schmidt and Duman, 2007), further supporting the so-called *neurotrophic hypothesis of depression*.
Neural Plasticity	Different forms of neuroplasticity, such as dendritic plasticity and hippocampal cytogenesis, have been linked to the pathogenesis of depression (McEwen et al., 2015). Moreover, glioplasticity has been also implicated as pathological target of the disease (Banasr et al., 2008). Several antidepressant classes have been shown to reverse impairments in adult neural plasticity (Patricio et al., 2015), substantiating the view o adult neural plasticity as fundamental pathological pilar of depression (see main text for further detail).

Among the different ethiological factors of major depression, chronic stress exposure has been widely associated to the precipitation of the disease symptomatology (Pittenger and Duman, 2008). Similarly to the deleterious effects of chronic stress exposure on structural and functional neural plasticity, different imaging and post-mortem studies report volumetric, neuromorphological and cytogenic alterations in patients diagnosed with major depression (Lorenzetti et al., 2009). In the amygdala, MRI studies suggest that amygdala size may vary with illness duration (Lange and Irle, 2004; Weniger et al., 2006; Monkul et al., 2007) and while in earlier stages of the disease patients tend to present increased amygdalar volumes (Lange and Irle, 2004; Weniger et al., 2006), with the progression and recurrence of depressive episodes, patients exhibit a reduction in amygdala volumes (Caetano et al., 2004; Hastings et al., 2004; Monkul et al., 2007), but different studies reporting volumetric variations in opposite direction are still difficult to conciliate (Campbell et al., 2004). Also, in the PFC, imaging studies suggest negative volumetric variations in MDD patients (Lacerda et al., 2004; Monkul et al., 2007), although other studies have failed to found this correlation (Bremner et al., 2000). Supporting neuroimaging studies, histopathological data shows lower cortical thickness and decreased neuronal size and density in prefrontal cortical regions (Rajkowska et al., 1999). Furthermore, the neuroplastic alterations caused by stress in the ventral striatum that affect the excitatory input into the NAc are likely to modulate the function of this brain region, whose dysfunction in depression is believed to be associated with anhedonic behavior (as reviewed in Nestler and Carlezon, 2006).

The hippocampus is the most extensively studied region in the context of MDD and the associated structural alterations. Albeit some contradictory findings, several studies support the association between hippocampal volumetric reductions and MDD (Bremner et al., 2000; Shah et al., 2002; Sheline et al., 2003; Caetano et al., 2004; Frodl et al., 2004; Lange and Irle, 2004; Weniger et al., 2006). In addition, illness duration and severity appear to influence the reported finding (Lorenzetti et al., 2009), as decreased hippocampal volumes have been more frequently described in patients with history of multiple episodes, longer illness or more severe symptomatology (Sheline, 1996; Vakili et al., 2000; MacQueen et al., 2003; Caetano et al., 2004). Accordingly, hippocampal neuronal atrophy have also been described in post-mortem brains of MDD patients (Stockmeier et al., 2004; Pittenger and Duman, 2008), but again some studies report no alterations in cell integrity or neuronal morphology in both MDD or bipolar disorder patients (Lucassen et al., 2001; Muller et al., 2001). In addition, as it happens following chronic stress exposure, post-mortem studies revealed that MDD appears to be not only

associated with dendritic/ neuromorphological remodeling but also with decreased adult hippocampal cytogenesis (Jacobs et al., 2000; Miller and Hen, 2015). The theory that propounds that hippocampal cytogenesis is associated with the precipitation and treatment of depression has been named neurogenic hypothesis of depression (NHD) (Jacobs et al., 2000). Initially, this theory has stemmed from two main correlations relating hippocampal cytogenesis and depressive illness. First, the already discussed reduction of hippocampal volume in depressed patients and the ability of high levels of glucocorticoids to reduce hippocampal cytogenesis (Cameron and Gould, 1994). Second, the observation that serotonergic antidepressants can re-establish normal levels of hippocampal cytogenesis in the mammalian brain (Malberg et al., 2000), as further discussed bellow.

In humans, due to technical limitations of directly labelling new cells, the full extent of the hippocampal cytogenic process has not been analyzed, but three studies have evaluated hippocampal neural progenitors in post-mortem brains of patients with depression, as a proxy measurement. While Reif and colleagues described no differences (Reif et al., 2006), a study by Boldrini et al. found a non-significant trend towards a decrease in hippocampal NSCs in depressed patients (Boldrini et al., 2009). In contrast, Lucassen et al., reported a significant reduction of precursor cells in non-treated depressed patients, comparing to age and sex-matched controls (Lucassen et al., 2010).

In light of the limitations to directly measure the birth of new cells in humans, animal models of depression have been widely used to test the arguments in which the neurogenic hypothesis of depression is grounded. In most cases, the link between chronic stress and impaired neuroplasticity is used to model a depressive-like phenotype in rodents (Willner et al., 1992; Willner, 2005; Chattarji et al., 2015). Chronic stress exposure appears to recapitulate the association between psychosocial stressors and the precipitation of depression in susceptible individuals (Kendler et al., 1999; Caspi et al., 2003; Heim and Binder, 2012). The unpredictable chronic mild stress (uCMS)[17] protocol, originally developed by Paul Willner and colleagues, has been commonly used and refined for more than 25 years to model depressive-like behavior in rodents (Willner, 1997). The uCMS protocol induces multi-dimensional behavioral deficits in rodents, that are arguably translatable to the human disease (Bessa et al., 2009; Hill et al.,

[17]Consists on the continuous exposure to a set of mild stressors in an unpredictable fashion, over a relatively prolonged period of time. The duration of the protocol is variable and usually ranges from 10 days to 8 weeks (Hill et al., 2012). A diversity of stressors is used to prevent habituation of the animals; this has been reported to occur when a single stressor is used over time (Willner, 2005). Unpredictability is another important feature of this model, acting as a reinforcing factor of the stress effect.

2012). Not only they display mood-related deficits, such as anhedonic behavior, as they do present anxiety-like phenotype and different cognitive impairments (Bessa et al., 2009). Furthermore, the behavioral phenotype is accompanied by several neurobiological alterations, in HPA activation, neurotransmitter systems, neurotrophin levels and in neural plasticity, alike those that have been reported in patients with MDD (Willner, 2005; Hill et al., 2012).

The use of these preclinical models of Depression has allowed to address two fundamental questions concerning the putative role of hippocampal cytogenesis in depression, whose answers remain debatable even if we stand more than 15 years past the initial proposal of the NHD. First, to explore the importance of hippocampal cytogenesis in etiology of the disease, which must still be clarified. In spite of several studies reporting the correlational findings of decreased proliferation in the hippocampus of both patients and animal models of depression, many contradictory reports exist (Tanti and Belzung, 2013). As discussed previously (see **Chapter 2**), cytogenesis ablation in rodents has been shown to produce cognitive deficits, which can be translatable to the cognitive dysfunctions often observed in MDD patients (Saxe et al., 2006; Clelland et al., 2009). However, in spite of studies reporting anxiety-like behavior and anhedonia following cytogenesis suppression, these results are apparently at odds in many other works that have failed to find this association (see the following works for contrasting results, Santarelli et al., 2003; Bessa et al., 2009; David et al., 2009; Revest et al., 2009; Fuss et al., 2010; Snyder et al., 2011). In a previous work from our lab, treatment with the cytostatic drug methylazoxymethanol (MAM) in naïve animals to ablate cytogenesis produced short-term anxiety-like behavior, while not producing depressive-like behavior (Bessa et al., 2009). The different cytogenesis ablation procedures together with different experimental time-frames and analysis time-points are likely to account for the observed discrepancies, thus precluding the formulation of an unifying view of evidence gathered so far. Second, the putative relationship between the pro-cytogenic effect of antidepressants and their therapeutic efficacy must be further explored.

1.3.2 MODULATION OF HIPPOCAMPAL PLASTICITY BY ANTIDEPRESSANTS

The second pillar supporting the importance of neural plasticity in depression derives from the reported effects of most antidepressant classes tested to date in both functional and structural adult plasticity **(Figure 7)**. Neuroimaging studies conducted in MDD patients have shown that

treatment responders have larger hippocampal volumes than non-responders, although this was only observed in female patients (Vakili et al., 2000; Vythilingam et al., 2004). In another study, Sheline and colleagues (2003) found an inverse correlation between hippocampal volume in depressed patients and the duration of untreated illness, again supporting the role of a successful antidepressant treatment in the reversal and/or protection from hippocampal atrophy observed in unipolar depression. To the best of our knowledge, no post-mortem studies in humans have reported the correlation between increased hippocampal volume and the increase variation in neuronal dendritic lenght.

Contrastingly to the lack of data available in humans, there is substantial evidence showing that chronic, but no acute, administration of antidepressants to animal models of depression do enhance both functional and structural hippocampal plasticity (McEwen et al., 2016). Indeed, several classes of antidepressants have been described to increase LTP in the DG (Stewart and Reid, 2000; Levkovitz et al., 2001). A similar enhancement of LTP has been described in CA1 upon antidepressant treatment, blocking the blunt of LTP and the promotion of LTD both induced by stress (Vouimba et al., 2006;Holderbach et al., 2007). Moreover, the effects of antidepressants in functional plasticity appear not to be regionally confined to the hippocampus, but to modulate the communication of the hippocampus with other brain regions. Accordingly, the administration of the Selective Serotonin Reuptake Inhibitor (SSRI), Fluoxetine, and the atypical tricyclic, Tianeptine, are able to reverse stress-induced deficits in PFC LTP upon stimulation of hippocampal outflow (Rocher et al., 2004). At the structural level, different antidepressant classes have been shown to increase the number of synapse, spines and dendrites in the hippocampus, such as SSRIs (e.g. Fluoxetine, Escitalopram) (Bessa et al., 2009; Li et al., 2015), Tricyclics (e.g. Amitriptyline, Imipramine) (Norrholm and Ouimet, 2001; Bessa et al., 2009; Chen et al., 2010), NRIs (e.g. Reboxetine) (Baj et al., 2012), MAOIs (e.g. Pirlindol) (Morais et al., 2014) and atypical antidepressants (e.g.Tianeptine, Vortioxetine) (Watanabe et al., 1992; Chen et al., 2016). Accordingly, we have recently conducted a comparative study, to test different classes of antidepressants in hippocampal plasticity. Interestingly, all antidepressants, except a more recently developed atypical antidepressant, Agomelatine, rescued the stress-induced dendritic atrophy in granule neurons (Patricio et al., 2015). In addition, antidepressants have been shown to also promote dendritic structural recovery in other stress-responsive areas of the brain (Pittenger and Duman, 2008)**(Figure 7).**

Furthermore, chronic treatment with antidepressants also impact on the generation of new hippocampal cells. In fact, the ability of chronic antidepressant treatment to bolster hippocampal cytogenesis is now well recognized and, notably, this pro-cytogenic effect is promoted by most pharmacological classes tested to date, such as SSRIs (e.g. Fluoxetine, Paroxetine)(Qiu et al., 2007; Bessa et al., 2009), Tryciclics (e.g. Amytriptiline, Imipramine)(Caldarone et al., 2004; Surget et al., 2008; Bessa et al., 2009), MAOIs (e.g. Tranylcypromine, Moclobemide)(Malberg et al., 2000; Li et al., 2004), Tetracyclics (e.g. Maprotiline) (Jhaveri et al., 2010), SNRIs (e.g. venlafaxine)(Mostany et al., 2008), NRIs (e.g. reboxetine) (Malberg et al., 2000) and NDRIs (e.g. Bupropion) (Onoue et al., 2014). It is important to note that it is still not clear whether this pro-cytogenic action affects differentially the DG along its septo-temporal axis, as different reports exist describing increased proliferation only in the dorsal, only in the ventral or in both hippocampal poles (as reviewed in Tanti and Belzung, 2013). Nevertheless, the fact that this pro-cytogenic action is shared by a multitude of antidepressant classes was one of the arguments supporting the hypothesis of hippocampal cytogenesis being a key form of neural plasticity necessary to the therapeutical effects of these drugs. A second compelling argument lies on the fact that antidepressants commonly used in clinical practice, typically take 2 to 4 weeks to manifest therapeutical efficacy (Miller and Hen, 2015), a lag period that is somewhat coincident with the period of mammalian neurogenesis.

Curiously, increased hippocampal cytogenesis is not an effect solely achieved by antidepressant drugs, as other therapeutical interventions have evidenced parallel action. This is the case in electroconvulsive therapy (ECT), where a single ECT seizure has been shown to be sufficient to increase the number of newborn cells in the DG (Madsen et al., 2000).

However, and as in the case of the pathophysiology of depression, the role of adult cytogenesis in the therapeutical action of antidepressants is a matter of much controversy (Petrik et al., 2012). In humans, studies have been restricted to post-mortem analysis, where correlational analysis are possible but to establish causality between cytogenesis and therapeutic actions is currently unfeasible. Lucassen and colleagues found no effect of different antidepressant treatments in the number of progenitor cells, despite observing a significant decrease in non-treated patients (Lucassen et al., 2010). Other studies revealed increased numbers of neural progenitors in antidepressant-treated patients (Boldrini et al., 2009), as well as an increase in the total number of granule cells in treated patients (Boldrini et al., 2013), although the latter finding may be related to non-cytogenic effects. In animal models of depression, the most substantial results have

emerged from cytogenesis ablation studies. In 2003, Santarelli and colleagues published the first evidence of blunted antidepressant response following cytogenesis ablation by x-ray irradiation (Santarelli et al., 2003). However this initial proposal was subsequently challenged in several works that have either failed to observe an association between cytogenesis supression and antidepressant efficacy, or supported the notion that ongoing cytogenesis was not responsible for all the behavioral improving effects of antidepressants (Holick et al., 2008; Surget et al., 2008; David et al., 2009). Accordingly, Bessa et al. have showed that cytogenesis supression by MAM did not preclude short-term monoaminergic antidepressant efficacy, rather suggesting the initial mood-improving effects to rely more on the recovery from hippocampal and PFC neuronal dendritic atrophy (Bessa et al., 2009).

Hence, as in the few existing human studies, animal studies appear to have conflicting data regarding the participation of hippocampal cytogenesis in the onset of depression, but also in the therapeutical action of antidepressants. As discussed in the previous chapter in relation to studies seeking to characterize the functional roles of adult cytogenesis in naïve animals, also in this research context many sources of potential variability become clear when reviewing available literature. First, although we tend to analyze data emerging from rats and mice indiscriminately, there are several indications at the neuroanatomical level (Andersen, 2007) and even in terms of hippocampal cytogenesis (Snyder et al., 2009) that differ between these two species, and that may therefore account for some discrepancies. Second, the chosen paradigm to model depression, and factors such as duration and severity may differentially affect neural plasticity, so that the potential of recovery by antidepressant treatment through non-cytogenic plasticity may vary. Third, different cytogenesis ablation procedures have specific limitations, such as exacerbated immunological response (irradiation) or generalized anti-mitotic action (cytostatic drugs), that may undermine a clearer interpretation of the therapeutic efficacy of the tested antidepressants. Finally, a major factor that is been largely undervalued is the temporal dimension of experimental planning and analysis time-points. Indeed, and due to the different structural and functional properties characteristic of progenitor, immature and mature cells, it should be expectable that studies that analyze both consequences of cytogenesis ablation or treatment efficacy at different time-points post-ablation yield distinct results.

To sum up, the triad stress-plasticity-depression is well supported by both animal and human studies, but a closer look into available literature points out how the specific role of different forms

of plasticity to the pathophysiology and treatment of depression remains highly debatable. Most likely such complex, but exhilarating, debate may only be solved by combining a renewed and critical interpretation of available data with additional studies, designed to address this matter in a longitudinal perspective, while also controlling the possible confounding effects emerging from the sources of discrepancy, already discussed above.

1.4

New forms of epigenetic regulation in the adult brain: a focus on DNA hydroxymethylation

Part of the work included in the present thesis has focused on epigenetic mechanisms in the context of the stressed brain, latter to be discussed (see **Chapter 5**). This section, rather than configuring an exhaustive review on epigenetic regulatory mechanisms within the CNS (for review, see Mehler, 2008; Mateus-Pinheiro et al., 2011), is intended to provide a brief introductory overview on epigenetic regulation, with emphasis given to DNA methylation and hydroxymethylation, as the work here presented has focused on these epigenetic marks.

1.4.1 DNA METHYLATION AND HYDROXYMETHYLATION IN THE CNS

DNA methylation

The field that we now call epigenetics stemmed from the early observations that heritable mechanisms could regulate phenotypic expression without implicating alterations on DNA sequence. Globally, epigenetic regulation is accomplished through four cardinal and interdependent mechanisms: (i) DNA methylation, (ii) histone modifications and higher order chromatin remodeling, (iii) non-conding RNAs and (iv) RNA and DNA editing/recoding (Mehler, 2008). All of these have been implicated in multiple molecular and cellular processes essential for higher nervous system functions (Feng et al., 2007; Mehler and Mattick, 2007; Taniura et al., 2007; Tsankova et al., 2007; Mehler, 2008). In particular, DNA methylation in the fifth position of the cytosine residues (5mC) is a well known epigenetic mark that has been widely studied. Originally, DNA 5mC was thought to occur specifically within CpG context and act exclusively as a transcriptional repressive mark (Jones, 2012). This classical view has been revisited following the description of DNA 5mC occurring at non-CpG regions in embryonic stem cells (ESC) and neurons, where it can lead, instead, to transcription activation (Jones, 2012). This epigenetic modification is achieved through the transfer of methyl groups from S-adenosyl methionine to DNA

cytosine residues, which is catalyzed by a family of DNA methyltransferases (DNMTs) responsible for both the "maintenance" of methylation (DNMT1) or de novo methylation (DNMT3A, DNMT3B, DNMT3L) (Klose and Bird, 2006). The functional repercussions of DNA 5mC on the CNS have long become evident, with the early descriptions of the importance of this epigenetic mark in the adult brain for learning and memory (Day and Sweatt, 2010). Indeed, DNMT3A and DNMT1 deletion in the mouse forebrain impairs synaptic plasticity and compromises learning and memory (Feng et al., 2010). Accordingly, pharmacological inhibition of these enzymes in the hippocampus has been shown to produce memory deficits (Miller and Sweatt, 2007). Moreover, profiles of DNA methylation display an intricate relation with patterns of histone protein post-translational modifications conducted by methyl CpG-binding proteins (MBDs) that are recruited to methylated DNA and, in association with large protein complexes, regulate gene repression and orchestrate DNA replication and repair (Klose and Bird, 2006; Mehler, 2008). Several studies addressing the role of DNA methylation and the associated MDBs, such as MeCP2 and MDB1, have provided important insights into how these epigenetic mechanisms are modulated by different physiological or pathological states (Klose and Bird, 2006; Ooi et al., 2007). In fact DNA methylation has been linked to neuronal identity and neuronal functional specialization (Ladd-Acosta et al., 2007), neuronal survival, plasticity and also stress response (Klose and Bird, 2006; Ooi et al., 2007). Furthermore, the process of astrocyte differentiation has also been shown to be tuned through DNA methylation mechanisms (Kondo, 2006).

DNA hydroxymethylation

Until recently the reverse mechanism of DNA methylation was poorly comprehended (Wu and Zhang, 2010). In 2009 a novel DNA mark was unveiled at the fifth position of cytosine - the 5-hydroxymethylation (5hmC) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009). DNA 5hmC was found to be an alteration catalyzed the TET family of methylcytosine dioxygenases (TET1/TET2/ TET3) which oxidizes 5mC to 5hmC and subsequently to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), in a reaction that is both Fe(II) and 2-oxyglutarate dependent (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009; Ito et al., 2010) **(Figure 8)**. These 5fC and 5caC are substrates of the thymine-DNA glycosylase (Tdg), representing what are believed to be transient epigenetic marks that will be converted in non-methylated cytosine (Maiti and Drohat, 2011; Piccolo and Fisher, 2014).

Most differentiated tissues present very low levels of TET1 and high levels of TET3, with the exception of the zygote, while undifferentiated embryonic stem cells show the opposite pattern

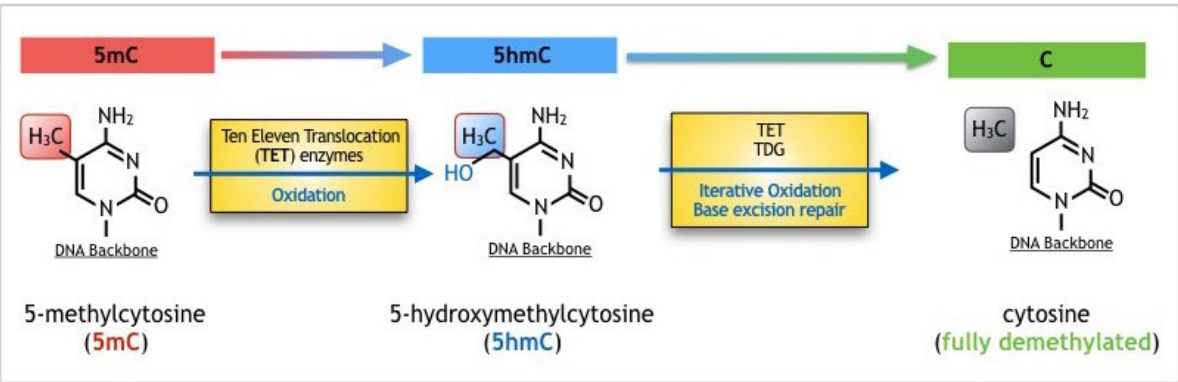

Figure 8. TET enzymes and DNA hydroxymethylation. The conversion of 5-methylcytosine to 5-hydroxymethylcytosine is catalyzed by the TET family of methylcytosine dioxygenases (TET1/TET2/TET3) which oxidize 5mC to 5hmC and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), in a Fe(II) and 2-oxyglutarate dependent reaction. 5fC and 5caC can subsequently operate as substrates for thymine-DNA glycosylase (Tdg), which eventually results in the generation of a non-methylated cytosine, representing an active DNA demethylation pathway. Adapted from Nabel and Kohli, 2011 .

(Szwagierczak et al., 2010). Importantly, the high levels of TET3 expression in several brain regions indicate that it might play an important role in post-mitotic neurons (Santiago et al., 2014). Even so, TET1 function in the central nervous system has been the most studied so far. It has been shown that Tet1-KO mice display a decrease in hippocampal progenitor cells, while also having impairments in spatial reference memory test in the Morris Water Maze (Zhang et al., 2013). Moreover, mice lacking Tet1 have been showed to have memory extinction defects, accompanied by an abnormally enhanced hippocampal LTD. These studies also revealed that NSCs isolated from the DG of Tet1-KO mice present an enrichment of hypermethylated genes that are downregulated, many of which participate in neurogenesis regulation. TET2 and TET3 functions in the adult CNS are less characterized. In particular, the study of TET3 function in the brain has been delayed due to the neonatal lethality of the Tet3-KO mice. Recently, a study revealed a dramatic genomic redistribution of 5hmC within the infralimbic PFC in response to fear extinction learning, and that fear extinction leads to TET3-mediated accumulation of 5hmC (Li et al., 2014). Altogether these studies highlight that TET proteins may play critical roles in the adult CNS which remain to be elucidated. This aspect might be of particular interest in a stress exposure context, which is generally associated to hypermethylation alterations in the CNS. Therefore a better characterization of TET enzymes function and the demethylation pathways could provide important cues on the mechanisms underlying cognitive and emotional disabilities after chronic stress exposure.

1.4.2 DNA 5mC/5hmC, STRESS AND MENTAL HEALTH: IS THERE A LINK?

Different studies have already linked epigenetic mechanisms to the molecular and cellular basis of stress and depression, as well as to the action of antidepressants (Schroeder et al., 2007; Tsankova et al., 2007; Krishnan and Nestler, 2008).

Regarding DNA 5mC, there are several indications supporting the role of this epigenetic mark in stress response and the concomitant development of depressive behavior. Among these, early-life stress in mice has been shown to control DNA 5mC levels, and MeCP2 DNA binding, in post-mitotic neurons in the hypothalamic PVN, leading to increased arginine vasopressin (AVP) expression (Murgatroyd et al., 2009). Epigenetic-induced changes in AVP expression were associated with neuroendocrine and behavioral responses typical from those following stress exposure or seen in animal models of depression, such as corticosterone hyper secretion or increased latency to immobility in the FST. In the other hand, a latter work has suggested that variations in DNA 5mC of the corticotropin releasing-factor (CRF) could mediate resilience to chronic social stress in adult mice (Elliott et al., 2010). Also, Nuber et al. used a Rett syndrome mouse model and put forward evidence supporting the role of MeCP2 as a modulator of glucocorticoid function, a finding that is of particular relevance to stress-induced psychiatric disorders, such is the case of depressive spectrum disorders (Nuber et al., 2005; Klengel et al., 2014).

Another argument favoring the view of DNA 5mC epigenetic regulation as a key pathological mark in depression is the translational validity of the stress-induced increase in DNA 5mC in the hippocampus and other brain regions in rodents (Miller and Sweatt, 2007; Zhang et al., 2010), as increased DNA 5mC levels have also been described in specific genomic locci at the hippocampus of suicide completers (McGowan et al., 2008; Poulter et al., 2008; McGowan et al., 2009). Also important, is the evidence provided by two works were antidepressant action has been linked to the modulation DNA 5mC levels within the NAc (LaPlant et al., 2010) and the hippocampus (Sales et al., 2011).

Although the lines of evidence presented above allow to establish a connection between 5mC and the pathophysiology of stress-related disorders, such as depression, the role of active DNA demethylation remains to be unveiled. Although still sparse, some works reported data supporting

the need to better explore this form of epigenetic regulation in psychopathological contexts: Wei et al. reported decreased TET1 expression in a genetic rat model of depression and a decrease in hydroxymethylation of bdnf (Wei et al., 2015). The administration of sodium butyrate had antidepressant-like effects, reversing the depressive-like behavioral phenotype, while also restoring normal levels of TET1 expression, and bdnf hydroxymethylation. Furthermore, a recent study has put forward a new rat model of Gulf War, combining stress with neurotoxin exposure (Pierce et al., 2015). Although it is impossible to dissociate the environmental effects of stress from the neurochemical consequences of neurotoxin exposure, the authors report variations in global levels of 5mC and 5hmC, differentially affected according to the brain region analyzed. Accordingly, a recent report also found variation in genomic 5hmC levels following acute stress, again proposing that this epigenetic mark may constitute an important mediator in the translation of environmental effects into physiological and pathological responses (Li et al., 2016).

Together, data implicating DNA 5mC in stress response, depression pathology and antidepressant action, combined with the yet sparse indication that hydroxymethylation and the associated enzymatic regulators are targeted in the exact same biological contexts, are compelling arguments to further investigate the role of this epigenetic mark in both the development an treatment of stress-related disorders.

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Chapter II

RESEARCH AIMS

2. RESEARCH AIMS

Despite the correlated findings of stress-induced impaired adult neural plasticity and the development of different emotional and cognitive deficits evidenced by animal models of depression, the importance of hippocampal plasticity for the development and remission of this disease remain highly controversial, as several authors presented what appears to be contradictory data.

In this thesis we sought to explore the longitudinal participation of hippocampal cytogenesis in the pathophysiology and treatment of depressive-like behavior, as the neglected aspect of the temporal dynamics of the disease course and the cytogenic process are likely source of discrepancy between several reported studies. For that, we used an unpredictable chronic mild stress (uCMS) animal model of depression in association with different cytogenesis ablation strategies. Furthermore, we treated animals with two monoaminergic antidepressants (ADs), fluoxetine and imipramine, to ascertain the modulatory effect of these drugs on hippocampal plasticity. Complementarily, we also characterized dendritic structural alterations occurring in hippocampal granule cells.

Within this research context, 3 major research aims were defined:

1. Evaluate the importance of both immature and mature newly-formed hippocampal cells for the development and remission from depressive-like behavior (initially addressed in **Chapter 3**, and latter in **Chapter 4**).

2. Explore how uCMS exposure and chronic AD treatment modulate epigenetic alterations in the adult hippocampus, specifically at the level of the more recently described DNA demethylation pathways (addressed in **Chapter 5**).

3. Refine current methods to characterize and measure hedonic behavior in rodents, as the lack of sensitivity and high output variability of current methods may contribute to aforementioned incongruences (**Chapter 6**).

Chapter III

HIPPOCAMPAL CYTOGENESIS IN THE ONSET & REMISSION FROM DEPRESSION

Sustained remission from depressive-like behavior depends on hippocampal neurogenesis

A. Mateus-Pinheiro, L. Pinto, J.M. Bessa, M. Morais, N.D. Alves, S. Monteiro, P. Patrício, O.F.X. Almeida and N. Sousa.
Translational Psychiatry, 3: e210. (2013)

Cell genesis and dendritic plasticity: a neuroplastic pas de deux in the onset and remission from depression.

A. Mateus-Pinheiro, P. Patrício, J.M. Bessa, N. Sousa and L. Pinto
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Sustained remission from depressive-like behavior depends on hippocampal neurogenesis

A Mateus-Pinheiro[1,2,4], L Pinto[1,2,4], JM Bessa[1,2], M Morais[1,2], ND Alves[1,2], S Monteiro[1,2], P Patrício[1,2], OFX Almeida[3] and N Sousa[1,2]

Impairment of hippocampal neurogenesis has been associated with the expression of depressive-like symptoms and some studies have suggested neurogenesis as a critical factor in the normalization of behavior by antidepressant (AD) drugs. This study provides robust evidence that ongoing neurogenesis is essential for the maintenance of behavioral homeostasis and that its pharmacological arrest precipitates symptoms commonly found in depressed patients. Further, the incorporation of newly born neurons and astrocytes into the preexisting hippocampal neurocircuitry is shown to be necessary for the spontaneous recovery from the adverse effects of stress and for long-term benefits of AD treatments.
Translational Psychiatry (2013) **3,** e210; doi:10.1038/tp.2012.141; published online 15 January 2013

Introduction

Major depression is a highly prevalent disorder that imposes a significant social burden. While the pathophysiology of the disease is still poorly understood, growing evidence suggests that impaired neuroplasticity may be a key underlying mechanism. Surrogate markers of impaired neuroplasticity, such as reduced neuroproliferation and impoverished dendritic arborization in the hippocampus, have been implicated in the onset, progression and remission of depressive symptoms.[1–4] Several antidepressants (ADs) tested to date stimulate hippocampal neurogenesis,[5–7] but it is unclear as to whether these pro-neurogenic effects are responsible for their mood-, emotional- and cognitive-improving actions. Recent studies indicate that ADs exert their short-term therapeutic effects by inducing remodeling of dendrites and synapses in mood-regulating limbic brain regions rather than by stimulating neurogenesis *per se*;[8–11] however, considering the longitudinal course of depression, the debate on whether the long-term benefits of AD treatments result from altered hippocampal neurogenesis and gliogenesis remains open.

In this study, we examined whether and how the neurogenic process, from cell birth to integration of newly born cells into the existing circuitry, influences the development of and remission from depressive-like symptoms. Astrogliogenesis was included in the present analysis in light of evidence that astroyctes have a role in preventing of the expression of depression-like behaviors in laboratory animals.[12–14] A validated unpredictable chronic mild stress (uCMS) paradigm was implemented to induce core symptoms of depressive-like behavior in rats; during the last 2 weeks of the uCMS protocol, animals were administered imipramine or fluoxetine. To assess the role of hippocampal neurogenesis and gliogenesis in the long-term effects of ADs, cell proliferation was artificially blocked through the coadministration of methylazoxymethanol (MAM) with the ADs. After 1 month of recovery from the experimental procedures, the behavioral dimensions commonly affected on depression were assessed and correlated with hippocampal neuroplastic alterations.

Materials and methods

Animals and treatments. Male Wistar rats (200–250 g, aged 2 months; Charles-River Laboratories, Barcelona, Spain) were maintained under standard laboratory conditions (12 h light: 12 h dark cycles, 22 °C, relative humidity of 55%, *ad libitum* access to food and water). Groups of rats ($n = 10$–12 per group) were randomly assigned to the following eight experimental groups: non-stress control + saline or MAM; stress (uCMS) + saline or MAM; uCMS + fluoxetine or imipramine alone, or coadministered with MAM. All procedures were carried out in accordance with EU Directive 2010/63/EU and NIH guidelines on animal care and experimentation.

A validated uCMS protocol was applied for 6 weeks as previously described.[8,15] The ADs fluoxetine (10 mg kg^{-1}; Kemprotec, Middlesborough, UK) and imipramine (10 mg kg^{-1}; Sigma-Aldrich, St Louis, MO, USA) were administered intraperitoneally (1 ml kg^{-1}); MAM (7 mg kg^{-1}; MRIGlobal Chemical Carcinogen Repository, Kansas City, MO, USA) was administered subcutaneously (0.45 ml kg^{-1}). All drugs were dissolved in dimethyl sulfoxide (5%) and saline (0.9%) and administered daily, during the 2 last weeks of the uCMS protocol. Pilot studies showed that MAM did not adversely influence general health parameters, such as locomotor activity (ambulation in an open field, swimming speed in a water maze) and fur quality.[8,16,17] Here, we found no significant alterations induced by MAM in appetite drive (Supplementary Figure S1), in swimming performance in the water maze (Supplementary Figure S2), as well as in weight

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal; [2]ICVS/3B's—PT Government Associate Laboratory, Guimarães, Portugal and [3]Neuroadaptations Group, Max-Planck Institute for Psychiatry, Munich, Germany
Correspondence: Dr N Sousa, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, Braga 4710-057, Portugal.
E-mail: njcsousa@ecsaude.uminho.pt
[4]These authors contributed equally to this work.
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variation (Supplementary Figure S3). BrdU was injected intraperitoneally (50 mg kg^{-1}) for 5 days at the cessation of uCMS in order to evaluate neurogenesis by immunocytochemistry (see below).

Subsequently, animals were allowed to recover for 4 weeks and behavioral analyses were carried out at weeks 11 and 12, during the daily light phase (09:00–18:00 h).

Behavioral analysis

Sucrose consumption test. Anhedonia was assessed on a weekly basis by the sucrose consumption test, throughout the experimental procedures. Baseline sucrose preference values were established during a 1-week habituation period during which animals were presented with two pre-weighed drinking fluid bottles, containing water or 1% (m/v) sucrose. Before each recording of sucrose preference, rats were food- and water-deprived for 20 h and exposed to the test drinking solutions for 1 h. Sucrose preference was calculated as described previously.[8]

Elevated-plus maze. Anxiety-like behavior was examined through the elevated-plus maze (EPM) test, in a 5-min session, as previously described.[15] The percentage of time spent in the open-arm was used as an index of anxiety-like behavior and the number of entries in the closed-arms was taken as an indicator for locomotor activity (Supplementary Figure S4).

Novelty suppressed feeding test. Anxiety-like traits were further assessed through the NSF paradigm. After a 18-h period of food-deprivation, animals were placed in an open-field arena, as previously described,[8] where a single food pellet was positioned in the center. After reaching the pellet, animals were individually returned to their home cage, where pre-weighted food was available, and were allowed to feed during 10 min. The latency to feed in the open-field arena was used as an anxiety-like behavior measurement, whereas the food consumption in the animal home cages provided a measure of appetite drive. No differences were observed in the appetite drive between the experimental groups that could lead to a misinterpretation of the results (Supplementary Figure S1).

Forced swimming test. Learned-helplessness was assessed through the forced swimming test. Assays were conducted 24 h after a 5-min pretest session, by placing the rats in transparent cylinders filled with water (25 °C; 50 cm of depth) during 5 min. Trials were video-recorded and the immobility time, as well as the latency to immobility were measured using an automated video tracking system (Viewpoint, Champagne au mont d'or, France). Learned-helplessness was considered as an increase in the immobility time. Results were complemented with the analysis of latency to immobility, as a second measure of learned-helplessness (Supplementary Figure 5).

Cognitive assessment. Cognitive function was evaluated in a spatial working memory task and in a behavioral flexibility task, performed in a black circular tank (170 cm diameter) filled with water (23 °C; 31 cm of depth) placed in dimly lit room with extrinsic clues. The water tank was divided in four quadrants by imaginary lines and a black platform (12 cm diameter; 30 cm high), invisible to rodents, was placed in one of the quadrants. Trials were video-captured by a video-tracking system (Viewpoint). Animals were habituated to the test room, during the 2 days preceding the tests, being kept in the room for 1 h in each day. These tests were conducted as it follows:

Working memory task

The working memory task was used to evaluate the cognitive domain that relies on the interplay between the hippocampal and prefrontal cortex functions.[18] The goal of this task, a modification of the original spatial reference memory test, is to assess the ability of animals to learn the position of the hidden platform and to retain this information during four consecutive trials. An escape platform was placed in one of the quadrants, and was maintained in the same position during the four daily trials. The test was performed during four days, and in each day the platform was repositioned in a new quadrant in a clockwise-fashion. In each of the daily trials animals were positioned in a different starting point (north, east, west and south) and a trial was considered as concluded when the platform was reached within the time-limit of 120 s. If the animals were unable to find the platform during the trial time they were guided to the platform and allowed to stay in it for 30 s. The time of escape latency was recorded for each trial.

Behavioral flexibility task. Following working memory assessment, tests were conducted for 4 days maintaining the platform in the same quadrant. At the fifth day, the behavioral flexibility performance of animals, a prefrontal cortex-dependent function, was tested by positioning the platform in a new (opposite) quadrant. Animals were tested in four trials according to the same procedure previously described. Besides the time of escape latency, the time spent in both new and old quadrants were recorded. Analysis of memory acquisition in the 4 days preceding was conducted in order to assess whether the different animal groups had equivalent memory for the old platform position (Supplementary Figure 6).

To confirm that differences observed in the escape latency were not due to distinct locomotor performance, we measured the average swimming velocities during trials. No differences were found among the groups in this parameter (Supplementary Figure S2).

Immunostaining procedures. Animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and were transcardially perfused with cold 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde.

Coronal cryosections (20 μm) and vibratome sections (40 μm) were firstly stained for BrdU (1:50; Dako, Glostrup, Denmark), followed by staining for DCX (for neuroblasts, 1:500; Abcam, Cambridge, UK), NeuN (for mature neurons; 1:100; Chemicon, Temecula, CA, USA) or GFAP (for glia; 1:200; Dako). Finally, all sections were stained with 4',6-diamidino-2-phenylindole (1 μg ml^{-1}). For each animal, BrdU-positive cells within the subgranular zone of the dentate gyrus were analyzed after double staining with neuronal (DCX or NeuN) or glial (GFAP) markers and cell counts were

performed by confocal microscopy (Olympus FluoViewTM FV1000, Hamburg, Germany). Hippocampal proliferation at the end of the recovery period was assessed by Ki67 staining (1:200; Leica Microsystems, Wetzlar, Germany). Estimation of cell density in the dentate gyrus was obtained by crossing the cell number values with the corresponding dentate gyrus areas, determined using a Olympus BX51 optical microscope and the Newcast software (Visiopharm, Horsholm, Denmark).

Three-dimensional morphological analysis. To assess the three-dimensional dendritic morphology of characterized hippocampal neurons, we developed a new technique that combines Golgi-Cox impregnation with immunofluorescence staining. Briefly, brains were immersed in Golgi-Cox solution for 21 days and then transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated and xylene cleared. An adapted optimized BrdU immunostaining procedure was then applied in order to identify newly born neurons. Dendritic arborization, spine numbers and shape were, therefore, analyzed specifically in newborn $BrdU^+$ cells and compared with non-$BrdU^+$ neurons. The detailed method has been described in detail elsewhere.[19]

Statistical analysis. Statistical analyses were done using SPSS software (SPSS, Chicago, IL, USA). After confirmation of homogeneity, data was subjected to appropriate statistical tests. Analysis of variance repeated measures was used to analyze cognitive-learning tasks performance. One-way analysis of variance was used to evaluate the remaining behavioral and molecular results. *F*-values and *P*-values derived from the between groups analysis of variance analyses are properly indicated along the text. Differences between the groups were determined by Bonferroni's *post-hoc* multiple comparison test, and the corresponding *P*-values are indicated in the figures. A *t*-test was used two evaluate differences among the two groups where appropriate. Statistical significance was accepted for $P<0.05$.

Results

Blockage of hippocampal proliferation triggers depressive-like symptomatology in naive rats. We first analyzed the long-term behavioral effects of neuro- and gliogenesis pharmacological suppression in naive animals (non-stressed animals), 4 weeks after the cessation of MAM treatment. Administration of MAM to naive rats, severely decreased the generation of neurons ($BrdU^+/NeuN^+$ cells, $t_8=6.024$; $P=0.0003$) and astrocytes ($BrdU^+/GFAP^+$ cells, $t_8=2.889$; $P=0.020$) (Figures 1a and b) and induced sustained deficits in hippocampal proliferation (Ki-67^+ cells, $t_8=8.229$; $P<0.0001$) (Figure 1c). As all neurons had matured 4 weeks after BrdU injections, we did not find $DCX^+/BrdU^+$ cells. Treatment with the antimitotic drug MAM produced increases in two surrogate measures of depressive-like behavior (reduced sucrose preference, a reflection of an anhedonic state, $t_{18}=1.941$; $P=0.034$, Figure 1d; increased immobility in the FST, $t_{18}=3,889$; $P=0.001$, Figure 1e). MAM administration also elicited signs of increased anxiety, as measured in the EPM ($t_{18}=4.069$; $P=0.0007$, Figure 1f) and in the NSF ($t_{18}=4.324$; $P=0.0004$, Figure 1g and Supplementary Figure S1), an interesting finding in light of the fact that a sizeable subpopulation of depressed human subjects exhibit hyperanxiety. In addition, MAM treatment was associated with impaired spatial working memory ($F_{1,22}=5.726$; $P=0.026$, Figure 1h and Supplementary Figure S2) and behavioral flexibility ($t_{18}=4.158$; $P=0.0006$, Figure 1i). Interestingly, new neurons ($BrdU^+$ neurons), that escaped mitotic blockade, were found to have markedly reduced spine densities ($t_{28}=6.412$; $P<0.0001$, Figure 1j) and altered spine morphology (Supplementary Figure S7), as compared with neurons that had matured before the experimental manipulations (Figure 1j).

Hippocampal neurogenesis and gliogenesis are fundamental for sustained spontaneous and pharmacological recovery from depressive-like behavior. The importance of active neurogenesis in the precipitation of depressive-like behavior in animals exposed to uCMS, a validated animal model of depression,[15,20] was examined next. While most studies only report on immediate, possibly transient, recovery from stress, we here assessed 'extended recovery' by evaluating the display of depressive-like behavior 4 weeks after the cessation of stress (Figure 2a). In these experiments, MAM was administered during the last 2 weeks of AD treatment, allowing the examination of whether uninterrupted neurogenesis is necessary for long-term—spontaneous and AD treatment-associated—recovery from stress-induced depressive-like behavior. Like MAM, stress attenuated hippocampal neurogenesis and gliogenesis ($F_{6,28}=17.35$, $P<0.0001$, *post-hoc* $P<0.001$ for neurons; $F_{6,28}=6.079$; $P=0.0004$, *post-hoc* $P<0.01$ for glia; Figures 3a–d) and elicited signs of anhedonia in an AD-reversible manner. However, the AD actions occurred independently of ongoing neuroproliferation (Figures 2b and c). Animals exposed to uCMS only showed partial spontaneous recovery, as measured by the sucrose consumption test, but such behavioral recovery was absent in animals exposed to uCMS and MAM ($F_{6,63}=4.005$; $P=0.0019$, *post-hoc* $P<0.001$, Figures 2b and c). The latter animals showed significantly reduced levels of neurogenesis ($F_{6,28}=26.80$; $P<0.0001$, *post-hoc* $P<0.001$, Figure 3b) and proliferation ($F_{6,28}=26.80$; $P<0.0001$, *post-hoc* $P<0.001$; Figures 3e and f) for up to 4 weeks after cessation of uCMS and MAM treatment. Strikingly, recovery during AD treatment was insensitive to the arrest of neurogenesis (Figures 2b and c). When tested in the forced swimming test (a test which measures reversal of learned helplessness within 24 h of AD treatment[21]), rats showed spontaneous and pharmacologically-induced recovery from the effects of uCMS, independently of ongoing neurogenesis (Figure 2d).

Anxiety traits frequently form part of the symptomatic profile of depressed patients and animal models of depression.[8,22–24] Ablation of hippocampal neurogenesis was here found to prevent the spontaneous reinstatement of baseline emotional states in animals previously exposed to uCMS; this was evident in two tests of anxiety-like behavior, EPM

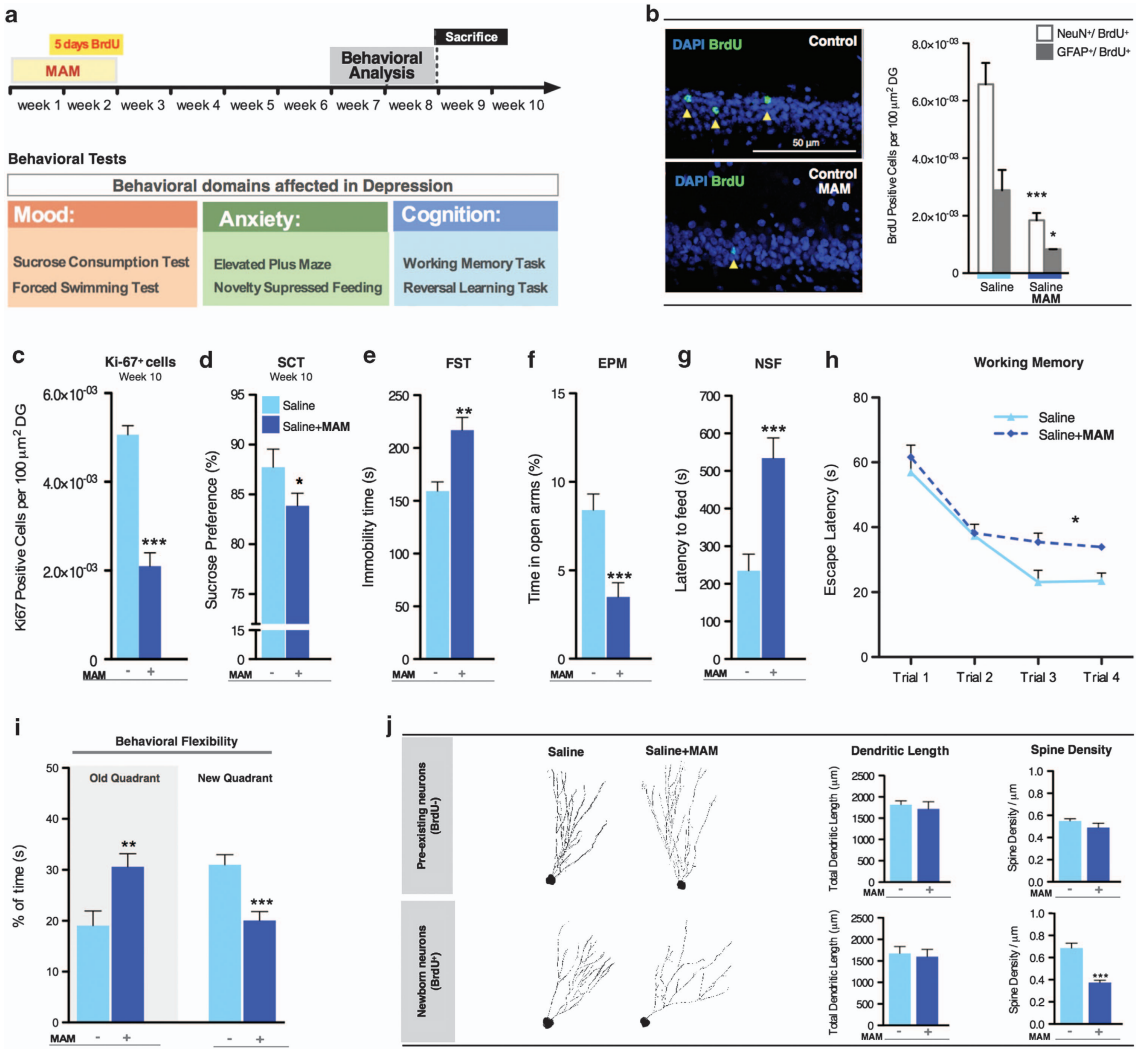

Figure 1 Neurogenesis arrest induces long-term emotional and cognitive changes typical of depression. (**a**) Neurogenesis was arrested by methylazoxymethanol (MAM) administration and the effects on behavior were assessed after 4 weeks. MAM treatment decreased the number of BrdU-positive cells in the hippocampal dentate gyrus (**b**), that underwent neuronal (BrdU/NeuN) and astroglial (BrdU/GFAP) differentiation. (**c**) Deficits in proliferation were sustained 4 weeks after MAM treatment cessation. Behavioral phenotype was evaluated using a battery of tests to assess distinct behavioral domains affected in depression. (**d**, **e**) Long-term mood impairments were observed in the sucrose consumption test (SCT) (**d**), and in the forced swimming test (FST) (**e**) 4 weeks after MAM treatment. (**f**, **g**) Increased anxiety-like behavior was detected in the elevated plus maze test (EPM) (**f**) and in the novelty-suppressed feeding test paradigm (NSF) (**g**) in animals previously treated with MAM. (**g**, **h**) Cognitive performance was also affected 4 weeks after neurogenesis arrest, as both (**h**) working memory and (**i**) behavioral flexibility were impaired 4 weeks after MAM administration. MAM treatment did not affect the dendritic length of neither preexistent or newly born granule neurons (**j**), but there was a decrease in spine density in the dendrites of newly born neurons after MAM exposure. Error bars denote s.e.m. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; $n=10$–12 per group.

($F_{6-63}=4.122$; $P=0.0015$, *post-hoc* $P<0.01$, Figure 2e) and NSF ($F_{6-63}=8.932$; $P<0.0001$, *post-hoc* $P<0.001$, Figure 2f). Both imipramine and fluoxetine normalized the anxious phenotype induced by uCMS, but whereas the therapeutic effects of fluoxetine were compromised by MAM administration ($t_{18}=1.739$; $P=0.0495$ in the EPM, Figure 2e; and $t_{18}=1.893$; $P=0.0373$ in the NSF, Figure 2f), those of imipramine were not ($P>0.05$ in both tests, Figures 2e and f).

Complaints of cognitive impairment are common among depressed subjects. Stress is known to dysregulate a number of cognitive functions that depend on the structural integrity of the hippocampus, prefrontal cortex and reciprocal connections between these two regions. Here, we show that arrest of neurogenesis prevents spontaneous improvements in spatial behavioral flexibility ($F_{6-63}=2.309$, $P<0.0445$, *post-hoc* $P<0.05$, Figure 2g) and spatial working memory

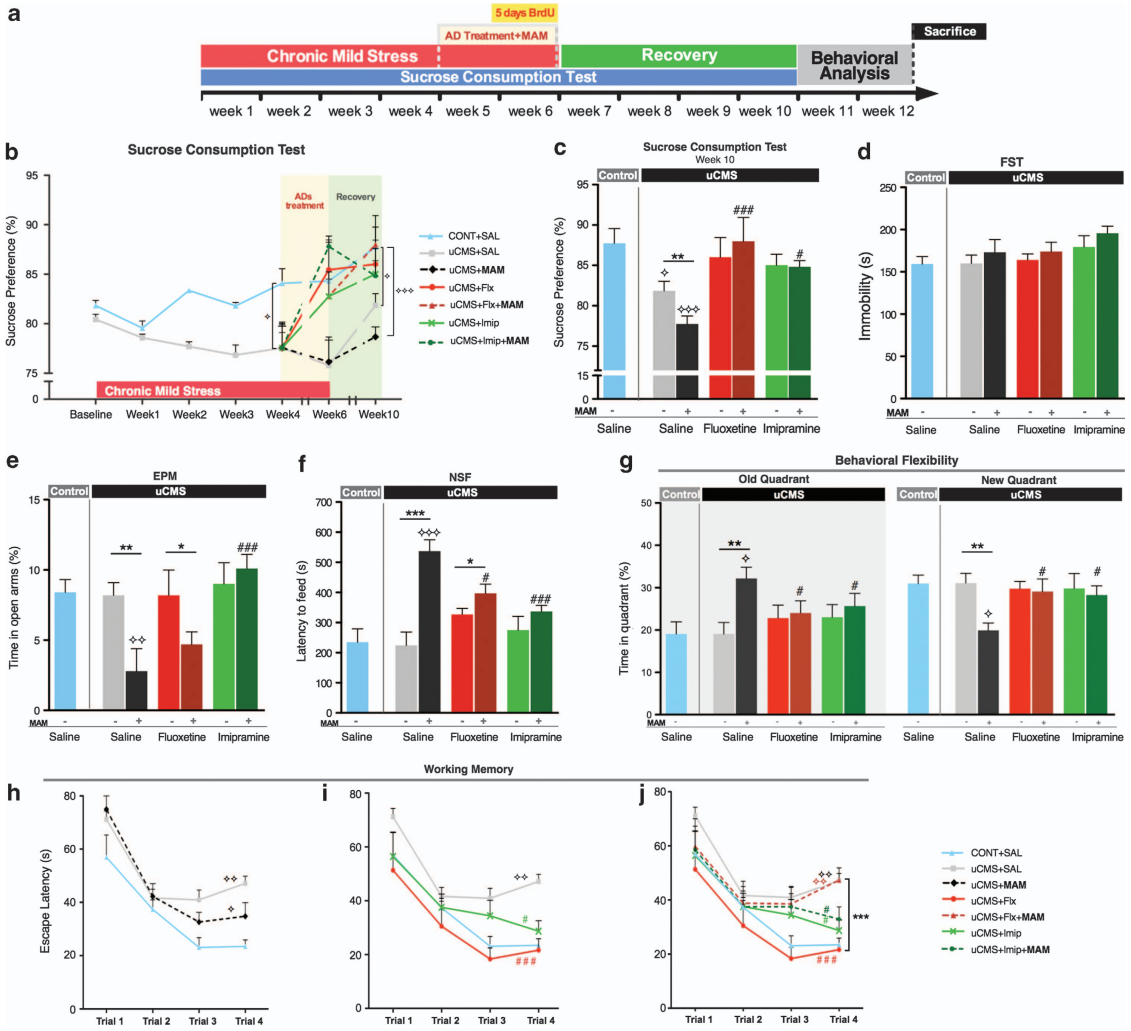

Figure 2 Neurogenesis arrest prevents long-term recovery from depression. (**a**) The relevance of neurogenesis for long-term recovery from depression was evaluated in animals exposed to an unpredictable chronic mild stress (uCMS) protocol 4 weeks before behavioral assessment. (**b**, **c**) Stress exposure triggers anhedonic behavior, that was reverted by fluoxetine and imipramine. Neurogenesis arrest with methylazoxymethanol (MAM) in stressed animals precluded recovery from anhedonic signs in the sucrose consumption test (SCT). (**d**) Learned helplessness behavior, assessed in the forced swimming test (FST), was normalized after 4 weeks of spontaneous or antidepressant-induced recovery from stress. (**e**, **f**) The long-term recovery from anxiety-like behavior was prevented by neurogenesis arrest; fluoxetine anxiolytic effects were attenuated by MAM administration, while imipramine action remained unaffected. (**g**–**j**) Neurogenesis arrest prevented the recovery form cognitive deficits in (**g**) behavioral flexibility and in (**h**) working memory of animals exposed to uCMS; (**i**, **j**) the therapeutical action of fluoxetine on working memory was suppressed by MAM administration, while imipramine effect was maintained after neurogenesis arrest. Error bars denote s.e.m. *Denotes the effect of MAM; $^{\diamond}$Denotes the effect of uCMS; $^{\#}$Denotes the antidepressants effect, by comparison of the antidepressants-treated animals with uCMS animals. $^{*,\diamond,\#}P<0.05$, $^{**,\diamond\diamond,\#\#}P<0.01$, $^{***,\diamond\diamond\diamond,\#\#\#}P<0.001$; $n=10$–12 per group. EPM, elevated plus maze test; NSF, novelty-suppressed feeding test paradigm.

($F_{2-33}=3.768$, $P=0.034$, *post-hoc* $P<0.05$, Figure 2h) for up to 4 weeks after cessation of the uCMS paradigm. Impairments induced by uCMS were reversed by both imipramine and fluoxetine (*post-hoc* $P<0.05$ in both tests; Figures 2g and i), but interestingly, whereas fluoxetine failed to restore working memory when it was coadministered with MAM, the cognition-improving efficacy of imipramine did not depend on active neurogenesis ($F_{3-31}=3.081$, $P=0.041$ *post-hoc* $P<0.05$, Figure 2j). Imipramine treatment elicited a strong pro-gliogenic effect ($F_{6-28}=6.079$; $P=0.0004$, *post-hoc* $P<0.01$, Figure 3b), as compared with fluoxetine which was more effective at promoting differentiation of newly born cells into neurons rather than astroglia ($F_{6-28}=17.35$, $P<0.0001$, *post-hoc* $P<0.001$). Consistently, blockade of neurogenesis with MAM did not interfere with the ability of imipramine to improve working memory (*post-hoc* $P>0.05$, Figure 2j).

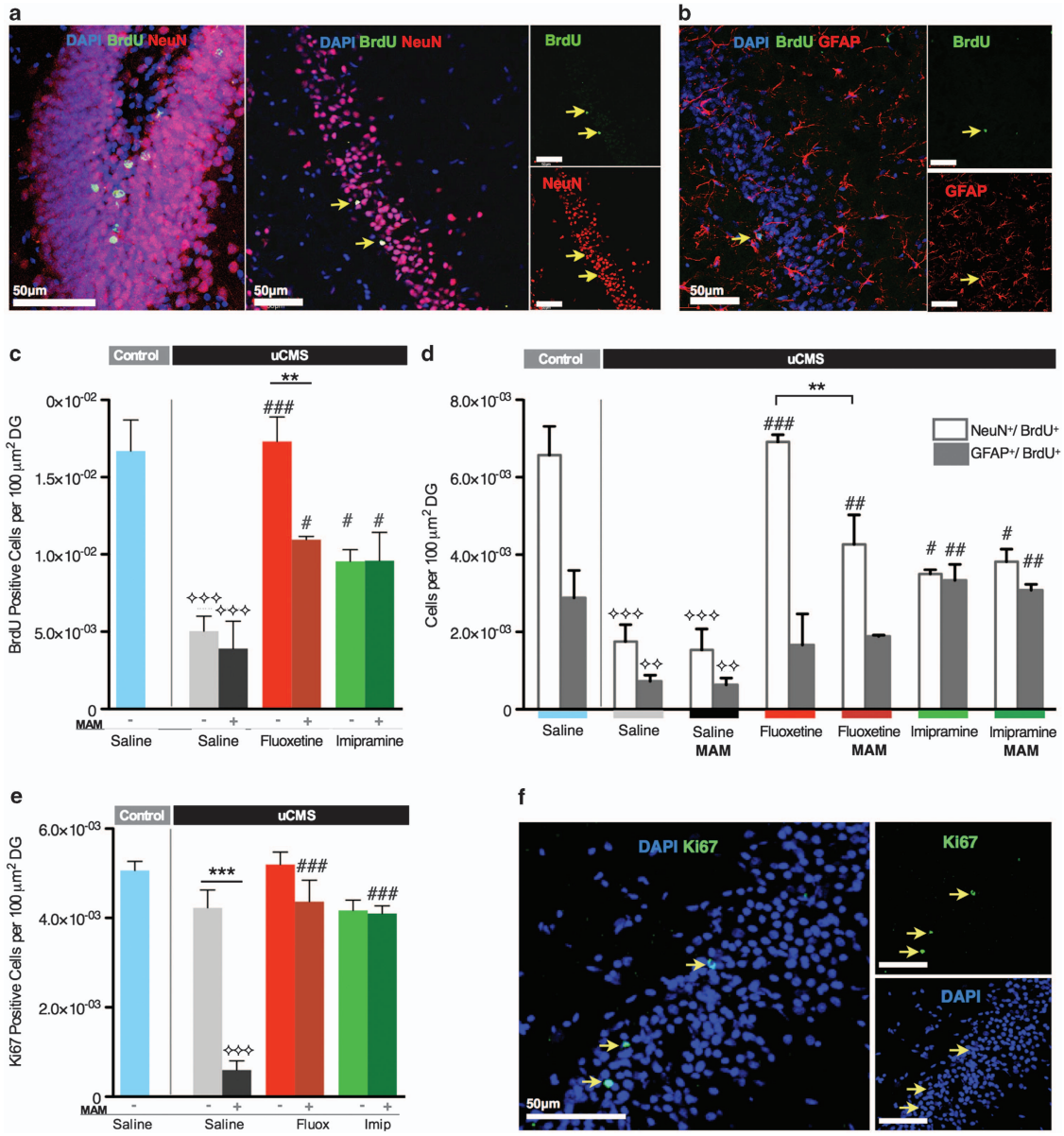

Figure 3 Pro-neurogenic and pro-gliogenic actions of the antidepressants fluoxetine and imipramine are associated with emotional and cognitive long-term recovery from depression. (**a**, **b**) Micrographs depict examples of (**a**) BrdU/NeuN double-labeled neurons and (**b**) BrdU/GFAP double-labeled glial cells in the hippocampal dentate-gyrus (DG) of control animals. (**c**, **d**) Graphs show the density of (**c**) total BrdU labeled cells and of (**d**) BrdU/NeuN, to evaluate neurogenesis, and BrdU/GFAP double-labeled cells, to evaluate gliogenesis, in the hippocampal DG. Unpredictable chronic mild stress (uCMS) animals, with or without methylazoxymethanol (MAM) treatment, have significantly lower levels of neurogenesis and gliogenesis when compared with control animals. Fluoxetine and imipramine treatment leads to a recovery in the levels of neurogenesis and gliogenesis, respectively. (**e**) Proliferation analyses, assessed at the end of the 4-weeks recovery period, by Ki67 staining, showed that uCMS and antidepressant-treated animals have similar proliferation levels to control animals. The uCMS effect on proliferation was prevented by neurogenesis arrest with MAM administration. (**f**) Micrograph depicts Ki67 labeled cells in the hippocampal DG of a control animal. *Denotes the effect of MAM; ◇Denotes the effect of uCMS; #Denotes the antidepressants effect, by comparison of the antidepressants-treated animals with uCMS animals. $^{*,\diamond,\#}P<0.05$, $^{**,\diamond\diamond,\#\#}P<0.01$, $^{***,\diamond\diamond\diamond,\#\#\#}P<0.001$; $n=5$ per group. DAPI, 4',6-diamidino-2-phenylindole.

uCMS induces sustained neuromorphological changes in newly born hippocampal neurons. Mood, anxiety and cognitive performance are regulated by dynamic alterations in synaptic and dentritic structure and function.[8,25,26] The present study shows that the neuromorphological changes that we previously observed immediately after uCMS

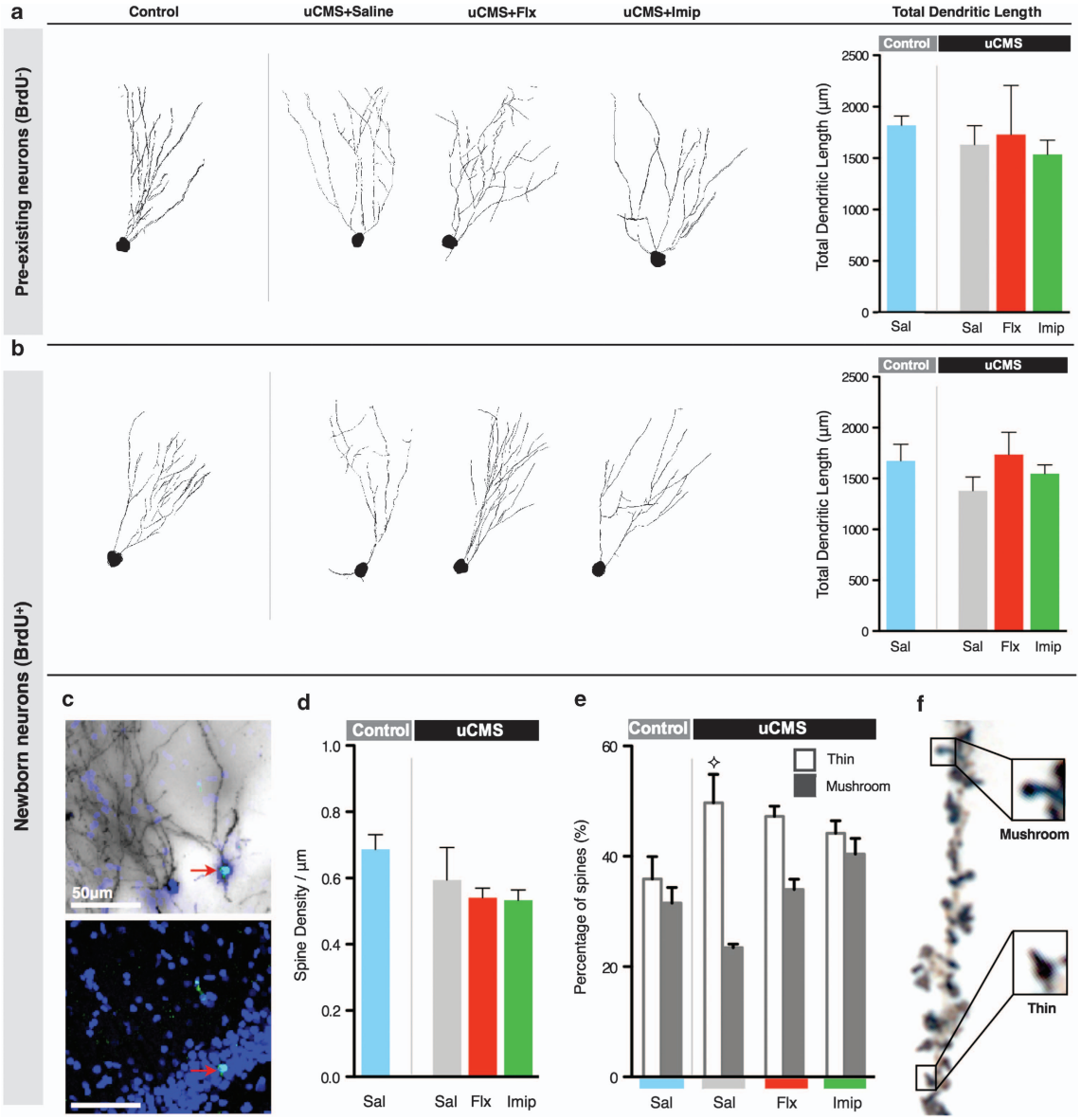

Figure 4 Exposure to unpredictable chronic mild stress (uCMS) causes long-term neuromorphological alterations in newborn granule neurons. (**a**) Structural changes in the dendritic arborization of preexisting granule neurons in the hippocampal dentate gyrus (DG), analyzed with the Golgi-Cox impregnation method, induced by uCMS exposure are reverted at long-term (4 weeks after uCMS exposure). (**b**–**d**) uCMS exposure does not have long-term impact on the dendritic arborization (**b**, **c**), neither in the global spine density of newborn neurons in the hippocampal DG (**d**), analyzed with the Immuno-Golgi method (co-labeling of BrdU and Golgi-Cox staining). (**e**, **f**) Newborn granule neurons of rats exposed to uCMS 4 weeks before, present increased percentage of thin spines; these alterations are attenuated in the animal groups treated with both antidepressants (**e**, **f**). $^{\diamond}$Denotes the effect of uCMS; $^{\diamond}P<0.05$; $n=10$–15 per group.

exposure[8] are fully reversed after a 4-week period of recovery (Figure 4a). In fact, no differences were observed in the dendritic morphology and spine density of mature dentate gyrus neurons between controls and animals previously submitted to uCMS (Figure 4a). However, using the novel Immuno-Golgi method to distinguish between newly born and preexisting neurons,[19] we observed that uCMS affects neither dendritic extension (Figures 4b and c) nor overall spine density (Figure 4d) in newborn neurons; rather, our analysis revealed that new cells generated during spontaneous and AD-induced recovery from uCMS have an unusually greater density of thin spines ($F_{4-70}=5.103$,

$P = 0.0011$, *post-hoc* $P < 0.05$; Figures 4e and f). As thin spines are a common feature in conditions of reduced cognitive function,[27,28] these findings indicate that uCMS leaves persistent morphological and behavioral scars. Interestingly MAM treatment mimicked sustained spines alterations in $BrdU^+$ neurons produced by uCMS exposure, which were reversed by ADs coadministration (Supplementary Figure S7).

Discussion

Overall, the present results show that the appropriate incorporation of newly born hippocampal cells in preexisting neural networks is essential for spontaneous recovery from depression-associated emotional and cognitive impairments in rats; moreover, these processes are important for long-term maintenance of the behavioral homeostasis achieved through the use of ADs.

Indeed, hippocampal cell proliferation arrest by MAM on untreated animals caused a 70% reduction on neurogenesis and gliogenesis, similarly to the anti-proliferative effects produced by uCMS exposure, which were causally associated with long-lasting emotional and cognitive disabilities. Remarkably, MAM treatment in naive animals produced behavioral deficits resembling those manifested after chronic exposure to stress. In this regard, it is important to note that such emotional and cognitive impairments emerged only 4 weeks after cessation of MAM treatment and were not manifested shortly after neurogenesis ablation, as we have showed previously.[8] Considering the late manifestation of these behavioral disabilities, it is inferable that continuous proliferation and complete circuitry integration of new neurons and glial cells, a process that takes 4–6 weeks in rodents, is necessary for the maintenance of emotional and cognitive homeostasis.

In line with the idea that continuous hippocampal cell proliferation is relevant for the spectrum of depressive symptoms, is the present observation that the ability of ADs to reverse emotional and cognitive impairments relies on their potential to restore hippocampal neuro- and glio-genesis. An interesting finding is that different classes of ADs have distinct impact on newborn cells-fate, that are reflected in different behavioral effects. As data suggests, noradrenaline reuptake inhibitors (imipramine) can ameliorate anxiety and cognitive deficits induced by stress independently of ongoing neurogenesis, whereas the anxiolytic and pro-cognitive efficacy of serotonin reuptake inhibitors (fluoxetine) require uninterrupted neurogenic processes.

These findings are consistent with earlier demonstrations of the essential role of neurogenesis in the regulation of anxiety behavior[8,9,24,29] and cognitive function[30,31] although the distinct contributions of serotoninergic and noradrenergic pathways to this regulation have not been previously reported. In light of recent data suggesting that astrocytic dysfunction and glial pathology have an important role in the regulation of emotional and cognitive behavior,[12,32,33] our observation that imipramine treatment promotes the generation and differentiation of new hippocampal cells into astrocytes may explain its strong ability to counteract MAM treatment; this is consistent with the fact that, norepinephrine, in contrast to serotonin, can directly activate the resident pool of progenitor cells and stimulate neurogenic, as well as gliogenic processes.[34]

In addition, the present results indicate that reestablishment of synaptic connections in hippocampal networks is a likely prerequisite for the spontaneous and pharmacological restoration of normal emotional and cognitive states. Studies demonstrating the fast behavioral actions of ketamine, an NMDA receptor antagonist, support this view.[35] However, we also reveal, for the first time, the occurrence of persistent alterations in spine morphology in neurons that escaped the stress- or MAM-induced hippocampal arrest that might be of relevance for future exposures to stressful conditions. Overall, our findings suggest that slower neuroplastic changes, involving neurogenesis and complex dynamic remodeling of neuro-glial networks, appear to have an important role in determining the extent of recovery from, and eventual relapse of, depressive symptoms.

In summary, this work suggests that cytogenic alterations are relevant but cannot account for the entire behavioral phenotypic recover after the remission from depressive-like behavior, as some behavioral alterations do not correlate with variations in neuro- and glio-genesis. Thus, most likely the incorporation of newly born cells into preexistent circuits impact, in combination with complementary neuroplastic and neuroendocrine alterations, on cortico-limbic circuitries involved in the remission from depressive symptomatology.

Conflict of interest

The authors declare no conflict of interest.

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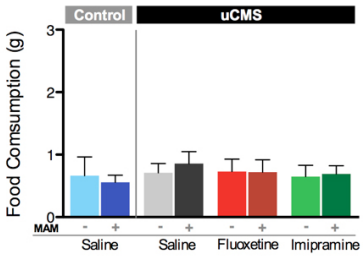
Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)

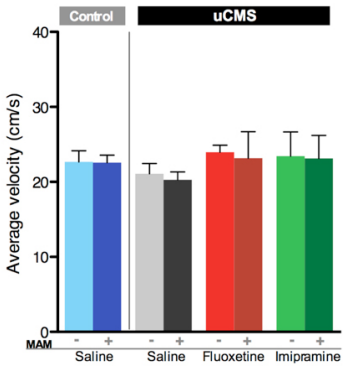
Sustained remission from depressive-like behavior depends on hippocampal neurogenesis

A. Mateus-Pinheiro, L. Pinto, J.M. Bessa, M. Morais, N.D. Alves, S. Monteiro, P. Patrício, O.F.X. Almeida and N. Sousa.
Translational Psychiatry, 3: e210. (2013)

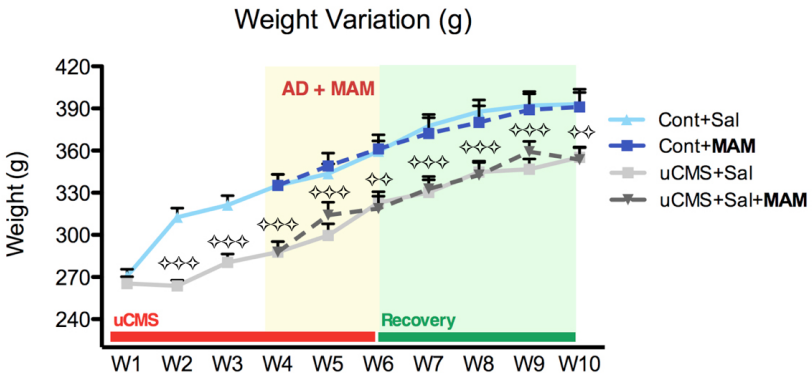
- SUPPLEMENTARY INFORMATION -



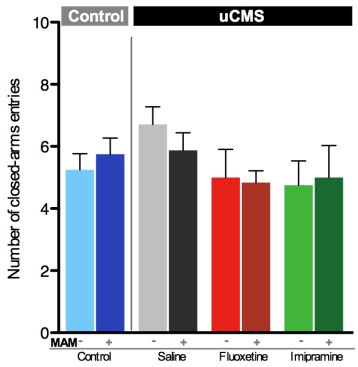
Supplementary Figure S1. Appetite drive (in a 10min period) in the novelty suppressed feeding test. Error bars denote SEM. *P<0.05, **P<0.01, ***P<0.001; n=10 to 15 per group.



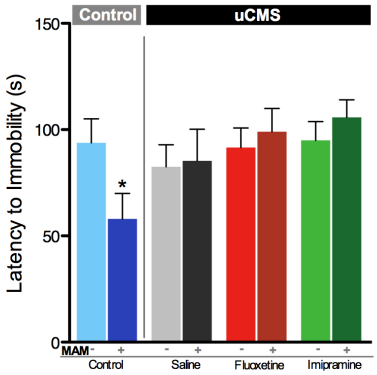
Supplementary Figure S2. Average swimming velocity in the water maze trials. Error bars denote SEM. *P<0.05, **P<0.01, ***P<0.001; n=10 to 15 per group.



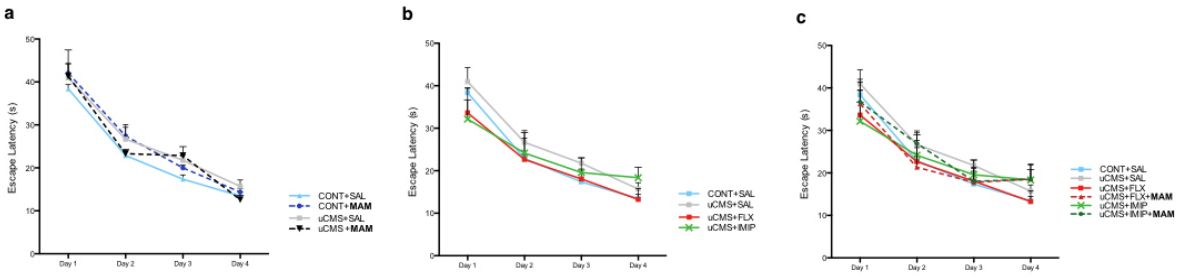
Supplementary Figure S3. MAM effect on weight variation. Error bars denote SEM. *P<0.05, **P<0.01, ***P<0.001; n=10 to 15 per group.



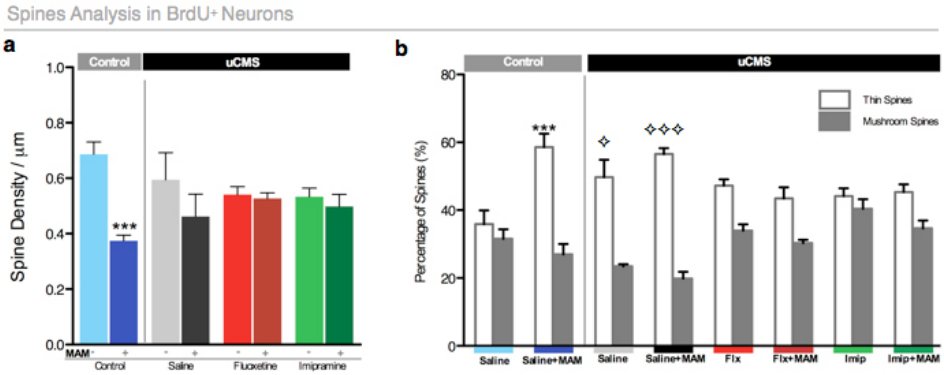
Supplementary Figure S4. Closed-arms entries in EPM test. Error bars denote SEM. *P<0.05, **P<0.01, ***P<0.001; n=10 to 15 per group.



Supplementary Figure S5. Latency to immobility in FST. Error bars denote SEM. *P<0.05, **P<0.01, ***P<0.001; n=10 to 15 per group.



Supplementary Figure S6. Performance on memory acquisition trials on the 4 days preceding the behavioral flexibility test (5th day). (a) Depicts the effect of MAM in memory acquisition performance of control and uCMS rats; (b) depicts the ADs effect; (c) depicts the effect of MAM co-administration along with ADs. Error bars denote SEM. *P<0.05, **P<0.01, ***P<0.001; n=10 to 15 per group.



Supplementary Figure S7. Effects of MAM on density and morphology of spines from newborn neurons. (a) Effect of MAM administration on spine density in newborn neurons (Golgi/ $BrdU^+$ cells); (b) MAM administration mimics the increase of thin spines produced by uCMS exposure, which is reversed by ADs administration. Error bars denote SEM. ✧*P<0.05, ✧✧; **P<0.01, ✧✧✧;***P<0.001; n=10 to 15 per group.

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Cell genesis and dendritic plasticity: a neuroplastic *pas de deux* in the onset and remission from depression

A Mateus-Pinheiro[1,2], P Patrício[1,2], JM Bessa[1,2], N Sousa[1,2] and L Pinto[1,2]

Brain neuroplasticity is increasingly considered to be an important component of both the pathology and treatment of depressive spectrum disorders. Recent studies shed light on the relevance of hippocampal cell genesis and cortico-limbic dendritic plasticity for the development and remission from depressive-like behavior. However, the neurobiological significance of neuroplastic phenomena in this context is still controversial. Here we summarize recent developments in this topic and propose an integrative interpretation of data gathered so far.

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Keywords: antidepressants; cell genesis; depression; neuroplasticity; stress

INTRODUCTION

The potential to respond to environmental stimuli through dynamic rearrangements of synapto-dendritic networks, as well as by regulating the generation of new neuronal and glial cells, renders the brain highly mutable. These phenomena, collectively known as neuroplasticity, are critical to promote neuronal adaptations; its failure is now increasingly considered to be a major component in many neuropsychiatric conditions. Among these, depressive spectrum disorders are a paradigmatic example of the importance of neuroplastic alterations in the adult brain. Recent studies provide a comprehensive picture of the effects of stress, a major trigger factor in depression, in the (de)regulation of neuroplasticity;[1–4] the latter is, in turn, related to the emergence of physiological and behavioral alterations comprised in the symptomatic profile of depressive disorders. Although these molecular and physiological mechanisms regulating neuroplastic processes are relevant for the onset of depressive symptoms, they have also been implicated in the action of antidepressants (ADs). So far, and although there is still much to be elucidated, it is becoming evident that the triad stress-neuroplasticity-depression constitutes fertile ground for new findings.

NEW CELLS AND DENDRITES: IMPORTANCE FOR THE TREATMENT AND REMISSION FROM DEPRESSION

Although different forms of neuroplasticity are affected in depression, a debate endures concerning the exact neurobiological significance of postnatal hippocampal cell genesis, both for the development of depressive pathology and for the therapeutic action of ADs. From the bulk of evidence gathered so far, it is increasingly appreciated that alterations in cell genesis are involved in the pathology and treatment of depression; however, there are several conflicting reports regarding its relevance. Three factors are likely to contribute to these controversies. First, there is a 'necessary' difficulty to approach this question in humans suffering from depression; postmortem studies in humans and animal models of depression have, nevertheless, provided important insights. Second, it seems to exist a major prevalence of studies focusing on the functional implications of neurogenesis, in disregard of gliogenesis, a parallel cell-genesis process likely to be of relevance in this context. Lastly, because these events are highly dynamic, the adoption of different experimental models and time frames when analyzing the participation of cell genesis in the pathology and treatment of depression is critical to have a complete perspective of the topic. On account of these experimental dissimilarities, an integrative, and careful, interpretation of data published in the last years is required when attempting to put the pieces of the puzzle together.

Suppression of hippocampal cell proliferation in naive animals through irradiation, pharmacological approaches or through the use of transgenic models of cytogenesis ablation has been shown to be associated with the development of deficits in different behavioral dimensions commonly affected in depression.[2,5,6] Strikingly, most of the studies in which analyses were performed shortly after cytogenesis ablation did not reveal significant deficits in most behavioral domains normally assessed in the characterization of animal models of depression (Figure 1).[7,8] However, recent reports in which abrogation of cytogenesis is maintained for long periods (over 4 weeks)[6] or in which the behavioral analysis was conducted only 4 weeks after the cessation of cytogenesis suppression,[4] reported multidimensional behavioral deficits that emerged only weeks after the antiproliferative insult. Importantly, the specific late manifestation of depressive-like behavior and cognitive disabilities in animals in which cytogenesis had been suppressed illustrates how manipulating lengthy neuroplastic phenomena is associated with the non-immediate development of behavioral impairments, which are only fully manifested once newborn cells are expected to be incorporated in local neuroglial circuits. This view has been recently supported by the demonstration that the specific inhibition of 4-week old new hippocampal neurons causes deficits in memory retrieval in mice; remarkably, inhibiting the activity of either younger or less-plastic older neurons does not produce effects in this cognitive domain.[9]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal and [2]ICVS/3B's—PT Government Associate Laboratory, Braga/Guimarães, Portugal. Correspondence: Professor N Sousa or Dr L Pinto, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga 4710-057, Portugal.
E-mail: njcsousa@ecsaude.uminho.pt or luisapinto@ecsaude.uminho.pt
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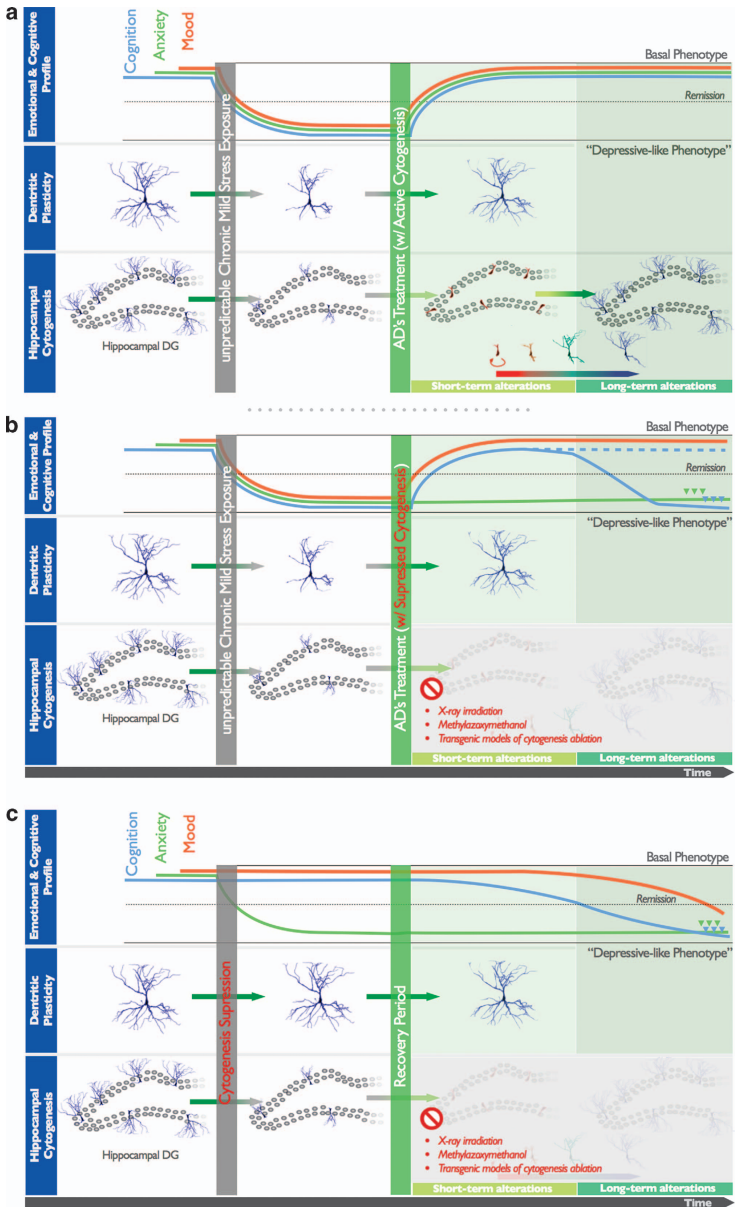

Figure 1. The participation of dendritic plasticity and hippocampal cell genesis in the development and treatment of depression. (**a**) In animal models of depression, chronic exposure to stress, one major trigger factor of depression, leads to the dendritic atrophy of hippocampal and prefrontal cortex neurons and compromises the generation of new cells. These neuroplastic impairments are associated with the development of multidimensional deficits manifested in behavioral dimensions such as mood, anxiety behavior and cognitive performance. Treatment with typical SSRIs allows for the fast reestablishment of dendritic neuronal architecture, which is associated with the fast remission from depression-like behavior. The slow generation of new neuronal and glial cells, potentiated by antidepressant (AD) treatment will be fundamental to maintain this remission and to promote a sustained long-term recovery from depression. (**b**) Suppression of hippocampal cell genesis, using X-ray irradiation, the cytostatic agent methylazoxymethanol or transgenic animal models of cytogenesis ablation, does not seem to have an impact on the mood-improving effects of ADs, but rather to preclude AD's efficacy in reversing anxiety behavior and impairments in some cognitive modalities (blue line); other cognitive functions remain unaffected (dashed blue line). (**c**) Ablation of cytogenesis in naive animals, while maintaining dendritic morphology intact, leads to the short-term manifestation of hyperanxiety behavior; deficits in mood and cognitive modalities are only evident later on, when newborn cells are expected to be fully matured and incorporated in the local neurocircuitry. SSRIs, selective serotonin reuptake inhibitors.

An exception must be made in respect to anxiety behavior, because disruption of hippocampal cytogenesis is associated to the immediate development of heightened anxiety, which is commonly comorbid in depressed patients. In fact, it has been demonstrated that immature newborn neurons display a major role in anxiety behavior control.[10–12]

In contrast with the slow reconfiguration of neuroglial networks promoted by the addition of new cells in the adult brain, are the rapid synaptic and dendritic morphological changes. These underlie the short-term impacts on distinct emotional and cognitive processes observed in the onset of depressive symptoms. Indeed, defects on neuronal cytoarchitecture have been documented in postmortem analysis of brain tissue from depressive patients, which seem to be ameliorated by chronic AD treatment.[13,14] These structural defects include cortico-limbic dendritic atrophy in depressed subjects,[15] accompanied by abnormalities in glial cell structure. Importantly, in animal models of depression, these changes are associated with hallmarks of depressive behavior, such as anhedonic behavior, behavioral despair and cognitive disabilities.[2]

Besides this common participation of slow cytogenesis processes and rapid dendritic rearrangements in the pathology of depressive spectrum disorders, these two neuroplastic phenomena, with very different temporal dynamics, constitute the substrate by which ADs (partially) exert their therapeutic actions. Thus, the reestablishment of normal neuroglial networks seems to be achieved in a biphasic manner (Figure 1): in a short-term context, AD actions rely on rapid modulatory effects upon genes involved in the restructuring of the synaptic network; later on, the generation of new fully matured neuronal and glial cells will have an impact on the long-term remission from emotional and cognitive disabilities manifested during a depressive episode. In fact, despite triggering an immediate pro-proliferative response, this early effect corresponds only to the onset of a slow neuroadaptation whose neurobiological importance can only be fully appreciated later on, once new cells attain complete maturation and functionality, and are integrated in the local neurocircuitry. Taken together, and because mammalian neurogenesis is described to take 4–6 weeks, the overall outcome of AD's therapeutic action upon neuroplasticity may only be entirely manifested after this period. Remarkably, this period correlates with the time latency that typically prescribed ADs take to fully manifest their action in depressive patients.

Although the neuroplastic alterations occurring during the onset, treatment and remission from depression are being increasingly characterized, comprehension of the processes (namely genetic and epigenetic programs) that orchestrate these alterations is still limited. Future research focusing on these processes should also be extended to the still underexplored glioplastic component of this disorder. Furthermore, local neuroplastic adaptations are likely to occur in articulation with systemic neuroendocrine and immunological alterations, which are still to be integrated in the complex puzzle of mechanisms implicated in depression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter IV

HIPPOCAMPAL CYTOGENESIS ABLATION IMPAIRS INTER-REGIONAL COMMUNICATION BETWEEN THE HIPPOCAMPUS AND PREFRONTAL CORTEX AND PROMOTES BEHAVIORAL DEFICITS

Hippocampal cytogenesis ablation impairs inter-regional communication between the hippocampus and prefrontal cortex and promotes the time-dependent manifestation of behavioral deficits

A. Mateus-Pinheiro, P. Patricio, N.D. Alves, V. Sardinha, A.R. Machado-Santos, J. Correia, J. Flint, J. Oliveira, N. Sousa and L. Pinto.

To be submitted

Hippocampal cytogenesis ablation impairs inter-regional communication between the hippocampus and prefrontal cortex and promotes the time-dependent manifestation of emotional and cognitive deficits

António Mateus-Pinheiro[1,2], Patrícia Patrício[1,2], Nuno Dinis Alves[1,2], Vanessa Morais Sardinha[1,2], Ana Rita Machado-Santos[1,2], Joana Silva-Correia[1,2], Jonathan Flint[3], João Filipe Oliveira, Nuno Sousa[1,2] and Luisa Pinto[1,2#]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[2]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[3]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom
#Correspondence to: luisapinto@ecsaude.uminho.pt

ABSTRACT

Impaired ability to generate new cells in the adult brain has been linked to deficits in multiple emotional and cognitive behavioral domains. However, the mechanisms by which the abrogation of post-natal neural stem cells impacts on brain homeostasis and function remains controversial. Here, we used one of the first transgenic rat lines available, the GFAP-Tk line, to selectively eliminate neural stem cells and assess the repercussion on different behavioral domains. We adopted two parallel experimental timeframes, to study both short-term and long-term effects of cytogenesis ablation (1 week post-ablation and 4 weeks post-ablation, respectively). Moreover, we conducted *in vivo* electrophsyiological analysis to assess the effects of cytogenesis ablation on the electrophysiological signatures of the hippocampal and prefrontal cortex regions. Our results show that the short-term repercussions of post-natal cytogenesis ablation are restricted to anxiety behavior. Contrastingly, cytogenesis abrogation promoted the late manifestation of anhedonic and anxiogenic deficits, along with multi-dimensional cognitive impairments. Furthermore, we found that cytogenesis ablation impaired electrophysiological function between the hippocampus and the prefrontal cortex, which are likely to contribute to the described cognitive alterations. Altogether, we describe a progressive time-dependent manifestation of emotional and cognitive impairments following cytogenesis ablation, supporting a differential role of immature vs mature cells in the modulation of different behavioral dimensions within the adult brain.

Keywords: Post-natal cytogenesis; GFAP-Tk; Hippocampus; Anhedonia; Anxiety; Memory; LTP

INTRODUCTION

In mammals, new cells are generated, throughout life, in the hippocampal subgranular zone (SGZ). Importantly, these cells have been shown to be able to differentiate, migrate radially towards external granular cells layers and to functionally integrate the local neuro-glial circuitry[1-3]. It is now known that this ability of generating new cells after birth, a process called post-natal cytogenesis, is influenced by a myriad of every-day-life experiences[4,5] and affected as pathological target in some neuropsychiatric disorders, as well as by different classes of drugs[6-8]. Such observations led to the early assumption that these newly-generated cells could participate in pivotal brain functions, but the initial inability to experimentally dissociate the contribution of new cells from the post-mitotic cell population undermined the attempt to understand the specific role of these cells. During the last 15 years, three types of strategies have been adopted to abrogate post-natal cytogenesis and study its impact on brain homeostasis: **i)** the use of x-ray irradiation[9], which is a regionally restricted procedure, but that lacks cell-specificity as it affects also mature cells and elicits a significant inflammatory response; **ii**) the use of cytostatic drugs[6,7], which are able abrogate cytogenesis in all cytogenic niches of the brain, but that again do not spare other mitotic cell populations and may trigger dose-dependent collateral effects difficult to control; iii) the use of transgenic mice lines[10,11], that allow for the specific (promoter-directed) ablation of neural stem cells, without interfering with other cell types or having known systemic effects. This last approach has proven to be useful during the last years in order to elucidate the neurobiological role of cytogenesis in the adult brain.

Indeed several studies that used either Glial Fibrillary Acidic Protein (GFAP)-Thymidine kinase (Tk) mice or Nestin-Tk mice lines, that allow for specific ablation of progenitor cells upon chronic administration of ganciclovir, have provided evidence for the requirement of new cells for a specific subset of high-level neural processes that rely on hippocampal function. In particular, ablation of cytogenesis using both GFAP-tk and Nestin- tk mice has been shown to impair contextual fear conditioning, but not cued conditioning memory[10,12]. The fact that other behavioral parameters, such as the overall spatial reference memory performance in the Morris water maze (MWM) and Y-maze were kept intact, demonstrates how restrict and specific the role of newly-born cells is[10,12]. Interestingly, although the latency to reach the escape platform in the MWM is not significantly affected upon cytogenesis ablation, a more detailed analysis has shown that Nestin-Tk mice display long-term retention deficits on subsequent probe trials, showing no preference for

the quadrant in which the platform was positioned[12]. The findings that LTP induction in DG is compromised in mice without hippocampal cytogenesis, has led to the hypothesis that the observed deficits are related with deficits in hippocampal synaptic plasticity[10]. In addition, It has been reported that in GFAP-Tk mice, cytogenesis ablation promoted a reduction in the efficacy of perforant path inputs, thus leading to an increase in the magnitude of γ-frequency bursts in the attempt to overcome this impairment[13]. Moreover, behavioral flexibility has also been shown to be impaired in GFAP-tk mice, reflected as impaired avoidance in an active place avoidance task[14]. More recently, a GFAP-Tk rat model was generated[15]. Curiously, authors did not found alterations in spatial working memory in radial maze, neither on the learning or reversal phases of spatial reference memory task in the water maze[15]. These findings conflict with previous data, although several studies exist where no alterations were found upon cytogenesis ablation, rendering this topic quite controversial.

Hence, in this work we promoted cytogenesis ablation after chronic ganciclovir (GCV) administration (18 days). To address the importance of both immature and mature new hippocampal cells, we adopted two analysis time points: one "short-term" analysis, in which behavioral tests were conducted immediately after the cessation of GCV treatment; a second "long-term" analysis was performed in second subset of animals, only 4 weeks post-ablation.

METHODS

Animals

GFAP-Tk transgenic rat line was perviously generated as described before[15]. 2 female GFAP-Tk founders were used to establish an in-house colony, since male rat were shown to be infertile and thus breeding is restricted to female heterozygous rats and wt animals. Genotyping identified 18 transgenic pups and 20 wt littermates, that were used in this study. Animals were grouped-housed in polypropylene cages (2 animals per cage) under 12h light: 12h dark cycles, at 22°C, relative humidity of 55% and with food and water ad libitum.

All procedures were carried out in accordance with EU Directive 2010/63/EU and NIH guidelines on animal care and experimentation and were approved by the Portuguese Government/Direcção Geral de Alimentação e Veterinária (DGAV) with the project reference 0420/000/000/2011 (DGAV 4542).

Ganciclovir (GVC) treatment and experimental timeline

Ganciclovir (GCV) (Kemprotec, UK) was dissolved in hydroxyethilcellulose at 20 $mg.ml^{-1}$, and 10 $mg.Kg^{-1}$ was injected intraperitoneally once a day, for 18 consecutive days, in 2 month-old animals. Both hemizygous (+/-) and littermate wilde-type controls (wt) were divided in two subgroups and subjected to behavioural testing either in the day following GCV treatment cessation ("short-term analysis"), or 28 days after the end of treatment ("long-term analysis"). In all animals, 5-Bromo-2-Deoxyuridine (BrdU), a thymidine analog, was injected daily (50 $mg.Kg^{-1}$/ day; i.p.), during the last 5 days of GCV treatment.

Behavioral analysis

Sucrose Consumption Test (SCT)

Baseline sucrose preference values were established during a 1-week habituation period (1 week prior the beginning of GCV treatment) during which animals were presented with two pre-weighed drinking fluid bottles, containing water or 1% (m/v) sucrose. A single SCT trial was performed in each established analysis time-point. Before each recording of sucrose preference, rats were food- and water-deprived for 12h and exposed to the test drinking solutions for 1h. Sucrose preference was calculated as described previously.

Sweet Drive Test (SDT)

The SDT test was additionally used to measure anhedonic behavior, as previously described (Mateus-Pinheiro et al, 2014)[1]. Briefly, each animal was allowed to explore the SDT box for 10 min, where sweet (Cheerios, Nestle) or regular pellets (Mucedola 4RF21-GLP) were available. After each trial, preference for sweet pellets was calculated as already described. Three SDT trials were conducted during the last week of the uCMS protocol (1 trial every 48h). The number of entries into each chamber was used as a measure of exploratory behavior. Data from the last trial is shown.

Forced Swimming Test (FST)

Depressive-like behavior was assessed through the forced swimming test. A FST trial was conducted 24h after a 5-min pretest session, by placing the rats in transparent cylinders filled with water (25 1C; 50 cm of depth) during 5 min. Trials were video-recorded and the immobility time, as well as the latency to immobility were measured using an automated video tracking system (Viewpoint, Champagne au mont d'or, France). Learned-helplessness was considered as an increase in the immobility time.

[1] Please see **Chapter 7**, for details on the Sweet Drive Test (SDT).

Novelty Suppressed Feeding (NSF) test

Anxiety-like traits were further assessed through the NSF paradigm. After a 18-h period of food-deprivation, animals were placed in an open- field arena, as previously described where a single food pellet was positioned in the center. After reaching the pellet, animals were individually returned to their home cage, where pre-weighted food was available, and were allowed to feed during 10min. The latency to feed in the open-field arena was used as an anxiety-like behavior measurement, whereas the food consumption in the animal home cages provided a measure of appetite drive. No differences were observed in the appetite drive between the experimental groups that could lead to a misinterpretation of the results (not shown).

Water Maze Tests

Water maze tests were used to test the performance in both **spatial reference memory** and **working memory** tasks as described previously[16]. Briefly, these tests were conducted in a circular black pool (170 cm diameter) filled with water at 22°C to a depth of 34 cm in a room with extrinsic clues (triangle, square, cross and horizontal stripes) and dim light. An invisible platform was placed in one of four quadrants. Trials were video-captured by a video-tracking system (Viewpoint, Champagne au mont d'or, France). Animals were habituated to the test room, during the two days preceding the tests, being kept in the room for 1h in each day.

Working Memory Task. The working memory task was used to evaluate the cognitive domain that relies on the interplay between the hippocampal and PFC functions. An escape platform was placed in one of the quadrants, and was maintained in the same position during the four daily trials. The test was performed during four days, and in each day the platform was repositioned in a new quadrant in a clockwise-fashion. In each of the daily trials animals were positioned in a different starting point (north, east, west and south) and a trial was considered as concluded when the platform was reached within the time-limit of 120s. The time of escape latency and the path described to reach the platform (distance swam) were recorded for each trial.

Reference Memory Task. With the reference memory task the hippocampal- dependent cognitive function was evaluated. For five days the platform remained on the same quadrant and animals were tested in four daily trials according to the same procedure previously described. The time of escape latency and the path described to reach the platform (distance swam) were recorded for each trial.

Swimming Strategies Analysis

Swimming analysis was performed specifically in the long-term analysis time-point. Data collection and analysis of Morris Water Maze spatial reference trials were performed using a video tracking system (Viewpoint, Champagne au mont d'or, France). Search strategies were defined as previously described[17]. Quantitative analysis and strategy classification was performed with data collected by the Viewpoint software, using an algorithm that we developed for automatic strategy attribution based on pre-defined parameters. For strategy blocks analysis, we defined two blocks of strategies: Block1, comprising the "non-hippocampal dependent strategies" (Thigmotaxis, Random Swim and Scanning) and Block2, comprising the "hippocampal dependent strategies" (Directed Search, Focal Search and Direct Swim); "chaining" was considered a transitional strategy and thus was not included in any of the blocks. Strategy blocks were defined as a sequence of at least three trials with the strategies from the same class. For block lengths, a maximum of two-trial interruptions were tolerated but not counted.

Contextual Fear Conditioning (CFC)

CFC behavioral analysis were conducted specifically in the long-term analysis time-point. Contextual Fear Conditioning was conducted in white acrylic chambers with internal dimensions of 20 cm wide, 16 cm deep and 20.5 cm high (Med Associates), with an embedded light bulb mounted directly above the chamber to provide illumination. Each chamber contained a stainless steel shock grid floor inside a clear acrylic cylinder, where animals were placed. Animals were subjected to two probes, a **context probe** and a **cue (light) probe**. The CFC procedure was conducted over 3 days (please refer to **Fig. 5**), as follows:

Day 1. Rats were placed in the conditioning white chamber (Context A) and received 3 pairings between a light (20 s) and a coterminating shock (1 s, ≈0.7 mA). The interval between pairing was set as 180 s, and the first tone presentation commenced 180 s after the rat was placed into the chamber. Freezing was defined as the complete absence of motion, including motion of the vibrissae, for a minimum of 1s. At the end of the three pairings, rats remained in the chamber for a further 30 s before being returned to their home cage. The chambers were cleaned with 10% ethanol solution between each trial.

Day 2. For the context probe, animals were placed in the white chamber (Context A), where they were originally shocked, 24h after the light-sock pairings. Freezing behavior was measured

during 3 minutes. Two hours later, animals were put in a modified version of the chamber (Context B) that was sheeted with a black plasticized cover that was previously sprayed with vanilla oil, in order to alter both spatial and odor references; the ventilation fan was not operated; the experimenter wore different colored gloves and lab coat. Again, freezing was measured during 3 minutes.

Day 3. For the cued probe, animals were replaced in Context B, and at the end of variable period of 2 to 3 minutes a light was turned on (20s). Freezing was measured in the subsequent minute.

Novel Object Recognition Task (*modified version to assess pattern separation*)

We developed a variation of the classical Novel Object Recognition (NOR) task, in which memory assessment is based on the rodents object recognition is based on pattern separation; all used objects were made of the same material (plastic foam), had the same shape and were only distinguishable by different black and white patterns on their surface (please refer to **Fig. 5)**. Briefly, in day 0 animals were placed in an empty arena and allowed to freely explore it for 5 minutes, for context habituation. In the following day (day 1), animals were exposed to two identical objects, with the same patterns, (object A) and allowed to explore the arena during 10 minutes (acquisition phase) . One hour after (short-term retention assessment), one of the objects was replaced by an object with a different surface pattern (object B). Twenty-four hours later (day 2; long-term retention assessment), animals were exposed to the original object A along with a new object with a different pattern (object C). To increase the level of interference on pattern separation, the walls of the arena were covered with different black and white patterns, in order to increase the requirements of intact pattern separation to recognize the familiar object. Sessions were video-recorded and exploration times were assessed automatically with Ethovision software (Noldus).

Immunohistochemical Analysis of Cell Proliferation and Long-term Survival

Animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and were transcardially perfused with cold 0.9% NaCl. Brains were carefully removed, frozen and preserved at -20°C. Coronal cryosections (20 mm) were firstly stained for BrdU (1:50; Dako, Glostrup, Denmark), to assess short-term hippocampal cell proliferation and survival both in the dorsal and ventral dentate gyrus (dDG and vDG, respectively). For long-term cell survival analysis, sections from animals injected with BrdU 4 weeks before were co-stained with BrdU (1:50; Dako, Glostrup,

Denmark), followed by sequential staining for NeuN (for mature neurons; 1:100; Chemicon, Temecula, CA, USA) or GFAP (for glia; 1:200; Dako). Finally, all sections were stained with 4',6-diamidino-2-phenylindole (1 mg.ml $^{-1}$). For each animal, BrdU- positive cells within the subgranular zone of the dentate gyrus were analyzed after double staining with neuronal (NeuN) or glial (GFAP) markers and cell counts were performed by confocal microscopy (Olympus FluoViewTM FV1000, Hamburg, Germany). Estimation of cell density in the dentate gyrus was obtained by crossing the cell number values with the corresponding dentate gyrus areas, determined using a Olympus BX51 optical microscope and the Newcast software (Visiopharm, Horsholm, Denmark).

Electrophysiological Recordings

Surgery

Experimental procedures for implantation and recording extracellular field potentials were performed according to previously described protocols[16]. Testing animals were anesthetized using sodium pentobarbital (selected since it acts through GABA receptors activation, thus minimizing the impact on the glutamatergic transmission). After anesthesia induction, animals were placed over a homoeothermic blanket (Stoelting, Ireland) and the rectal temperature kept at 37°C. Animals head was fixed to a stereotaxic frame, with a gas anesthesia platform linked to a rat mask (Stoelting, Ireland). From their coordinates (rostrocaudal, mediolateral and dorsoventral), the localization of the areas of interest, mPFC (coordinates, 1.94 mm anterior to bregma, 0.4 mm lateral to the midline, 2.54 mm below bregma), and ventral hippocampus (vHIP) (coordinates, 3.8 mm posterior to bregma, 3.3 mm lateral to the midline, 3.4 mm below bregma) was determined according to the rat brain atlas (Paxinos, 2001). A platinum/iridium recording electrode (Science Products, Germany) was placed in the mPFC and a concentric bipolar tungsten/stainless-steel electrode (WPI, USA) positioned into either the ipsilateral CA1/subicular region of the dHIP or vHIP.

A set of 10 animals (5 GFAP-Tk transgenic and 5 wt) were used to perform the electrophysiological recordings of local field potentials (LFPs) and synaptic plasticity, over a period of 6 days.

Synaptic plasticity evaluation

Synaptic plasticity between the PFC and the vHIP was measured as previously described (Cerqueira et al., 2007). Briefly, LTP was induced through the electrode inserted in the vHIP promoting a characteristic monosynaptic field excitatory postsynaptic potential (fEPSP) in the PFC.

Every 30 seconds it was invoked a test pulse (100 ms) at an intensity enough to promote a potential around 70% of its maximum amplitude (250–500 μA; S88X Grass Stimulator, Astro-Med, Germany). The evoked potential to such stimulation is likely to reflect summated post-synaptic potential (PSP). The induction of LTP was obtained through a high frequency stimulation (HFS) protocol, performed by two series of 10 trains (250 Hz, 200 ms) at 0.1 Hz, 6 min apart, delivered at test intensity. Signals were recorded every 30 seconds during 30 minutes before (basal recordings) and 45 minutes after stimulation.

LTP response was assessed by measuring the differences in fEPSP slopes between responses prior (baseline) and post HFS. fEPSP slopes were analyzed using Signal software (CED, UK; sampling rate, 10 KHz) and expressed as a percentage change of the mean responses to normal stimulation before and after HFS.

At the completion of the electrophysiological protocols, a biphasic 1 mA stimulus was delivered to both electrodes in order to mark the local of recording. Afterwards, rats were deeply anesthetized with sodium pentobarbital and brains carefully removed and emerged in paraformaldehyde (PFA) 4%.

Brain sectioning and electrodes position confirmation

Rat brains were processed in the vibratome (50 μm thick sections), and the resulting sections were stained with Cresyl Violet to confirm electrodes positioning.

Data analysis and Statistics

Statistical analyses were done using SPSS and Graphpad softwares. After confirmation of homogeneity, data was subjected to appropriate statistical tests. ANOVA repeated measures was used to analyze spatial reference memory, working memory and LTP data. Unpaired T-test was used in the remaining analysis. F-values and P-values are properly indicated along the text. Statistical significance was accepted for $P<0.05$.

RESULTS

Ganciclovir treatment significantly abolished short-term hippocampal proliferation, and long-term neuronal and glial survival in GFAP-Tk animals

In the short-term analysis (following a period of 7 days of behavioral analysis, after the cessation of GCV treatment) we analyzed hippocampal proliferation in both the dorsal and ventral hippocampus (**Fig. 1a, b**). GCV treatment promoted a significant reduction in the number of BrdU-positive cells in both the dDG (t_4=3.340, P=0.014) and the vDG (t_4=2,147, P=0.049). Regarding 4 weeks cell survival, GCV effectively abrogates total cell (total BrdU-positive cells) in both hippocampal poles (dDG: t_4=8.055, P< 0.0001; vDG: t_4=2,578, P=0.031) (**Fig. 1c**). Specifically, both hippocampal neurogenesis and gliogenesis were markedly supressed, as shown by the reduced survival of new neuronal (BrdU/NeuN double-positive cells; dDG: t_4=6,209, P=0.002; vDG: t_4=5,330, P=0.003) and glial cells (BrdU/GFAP double-positive cells; dDG: t_4=4,395, P=0.006; vDG: t_4=3,646, P=0.003) (**Fig. 1d**).

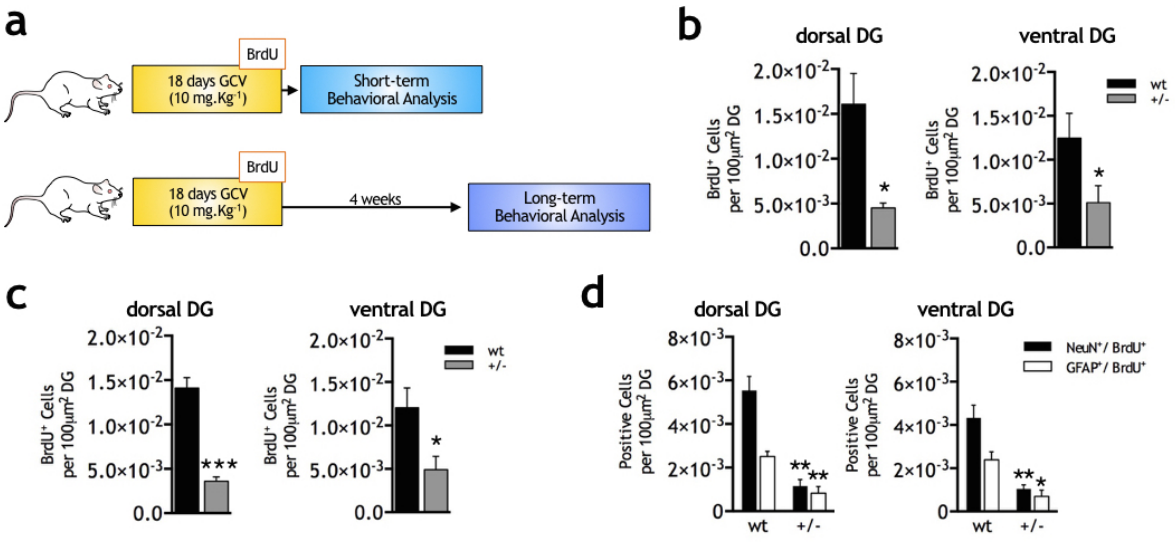

Figure 1. Short-term proliferation and long-term cell survival in the ventral and dorsal dentate gyrus, following GCV treatment. (**a**) Depiction of the experimental timeline for the "short-term analysis" (behavioral tests conducted immediately post-ablation and sacrificed 7 days later) and the "long-term analysis" (behavioral analysis conducted 4 weeks post-ablation). (**b-d**) BrdU was administered at the end of GCV treatment for subsequent analysis of hippocampal 7-day **(b)** and 4-weeks cell survival **(c). (d)** Hippocampal neuro- and gliogenesis were assessed 4 weeks post-ablation. Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; n = 3 per group.

Adult hippocampal cytogenesis ablation promotes short-term anxiety-like behavior, but has no effect on depressive/ anhedonic behavior or spatial reference memory

Immediately after the cessation of GCV treatment, GFAP-TK$^{+/-}$ animals did not present signs of anhedonic behavior in the SCT (P=0.39; **Fig. 2a**) or SDT (P=0.41; **Fig. 2b**). Moreover, cytogenesis ablation did not produce the short-term precipitation of depressive-like behavior, as both GFAP-TK$^{+/-}$ and wt animals presented similar immobility time in the FST (P=0.19; **Fig. 2c**). Interestingly, suppression of adult cytogenesis was sufficient to induce short-term anxiety-like behavior, manifested as a increase in the latency to feed in the NSF paradigm (t_{16}=3.696, P<0.001; **Fig. 2d**). In this time-point, we also tested animals in spatial reference memory task and found no interference of cytogenesis ablation in the animals performance (P=0,52; **Fig. 2e**).

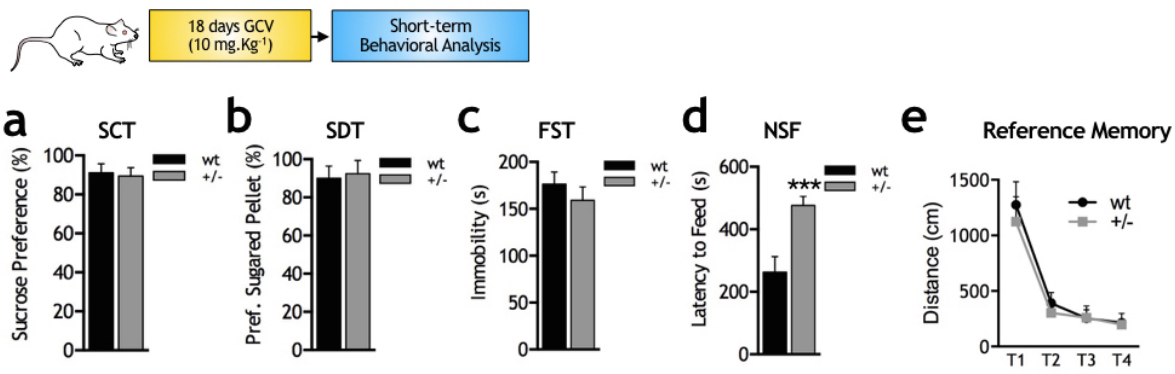

Figure 2. Short-term impact of adult cytogenesis ablation on rodent behavior. (**a**,**b**) Anhedonic behavior was measured in the (**a**) SCT and in the (**b**) SDT. **(c, d)** The presence of depressive-like behavior was assessed by the (**c**) FST, while the (**d**) NSF paradigm was used to measure anxious-like behavior. **e.** Cognitive performance was evaluated in a spatial reference memory task. Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; n = 9-10 per group.

Deficits in hedonic behavior emerge 4 weeks after adult cytogenesis ablation, along with heightened anxiety

While we have not found short-term behavioral deficits upon cytogenesis ablation, beyond those of heightened anxiety, the long-term behavioral profile of GFAP-Tk$^{+/-}$ animals encompasses additional impairments. In particular, GCV treatment elicited the long-term manifestation of anhedonic behavior, manifested as decreased consumption preferences in both the SCT (t_{16}=1.796, P=0.045; **Fig. 3a**) and the SDT (t_{16}=2.020, P=0.030; **Fig. 3b**). While no differences were observed in the FST (P=0.34; **Fig. 3c**), this anhedonic profile was further accompanied by anxious-like signs, evidenced in the NSF (t_{16}=2.454, P=0.013; **Fig. 3d**). Together with the short-

term behavioral characterization, this behavioral pattern suggests that adult cytogenesis suppression elicits a time-dependent manifestation of anxiety and anhedonic impairments.

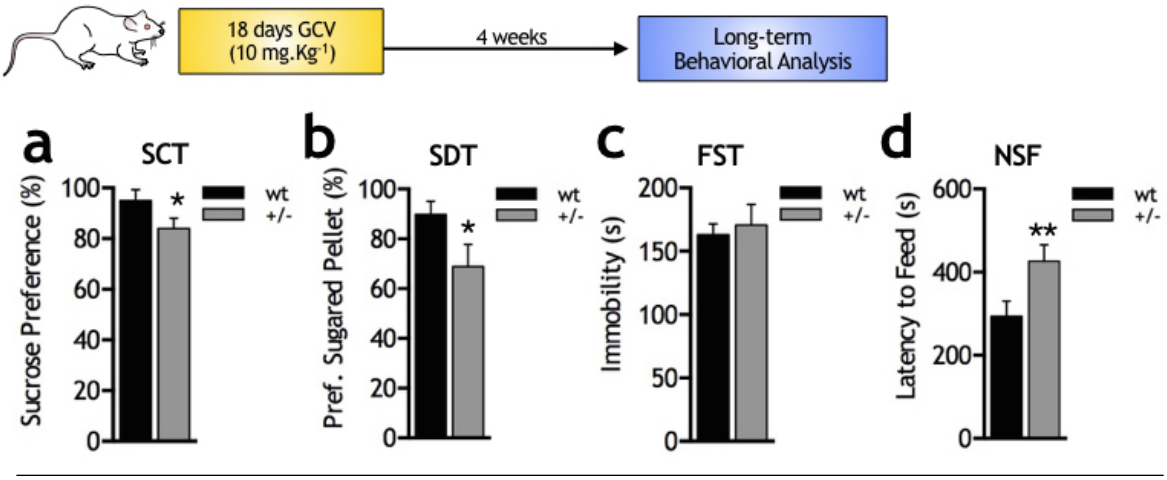

Figure 3. Long-term impact of adult cytogenesis ablation in emotion-related behavioral categories . (**a**,**b**) Anhedonic behavior was measured in the (**a**) SCT and in the (**b**) SDT. (**c, d)** The presence of depressive-like behavior was assessed by the (**c**) FST, while the (**d**) NSF paradigm was used to measure anxious-like behavior. Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; n = 9-10 per group.

Adult cytogenesis does not impact on spatial reference memory overall performance, but produces alterations in the adopted cognitive strategies and working memory

Our results show that chronic GCV abolishment of cytogenesis did not produce significant alterations in the distance taken to reach the escape platform during the learning phase of the spatial reference memory task in the water maze (**Fig. 4a).** However, analysis of the strategies adopted to reach the escape platform showed that GFAP-Tk rats are able to maintain test performances comparable to wt littermate controls by delaying the switch from non-hippocampal dependent strategies ("Block 1") to hippocampal dependent strategies ("Block 2") (**Fig. 4b**). In fact, the majority of wt animals initiates strategies Block 2 by test day 2, while GFAP-Tk rats do it so mainly during days 3 and 4, presenting an increased mean duration of Block 1 in relation to wt rats (**Block1:** wt = 6.0 ± 0.5 vs GFAP-Tk$^{+/-}$ = 11.7 ± 1.1; t_{18} = 4.616; P = 0.0001; **Block2:** wt = 11.6 ± 1.8 vs GFAP-Tk$^{+/-}$ = 4.0 ± 2.0; t_{18} = 1.970; P = 0.0032; **Fig. 4c - f**).

Moreover, 4 weeks post-ablation we also measured working memory in a water maze test paradigm in which the position of the escape platform was repositioned in a new quadrant in a clockwise-fashion. Hence the ability of animals to retain the platform position in each trial along 4 days was assessed. Ablation of cytogenesis in GFAP-Tk$^{+/-}$ rats produced long-term deficits in this

cognitive domain, reflected as a significant increase in the overall distance traveled to reach the escape platform ($F_{(1, 64)}$= 5.927; P= 0.017; **Fig. 4g**).

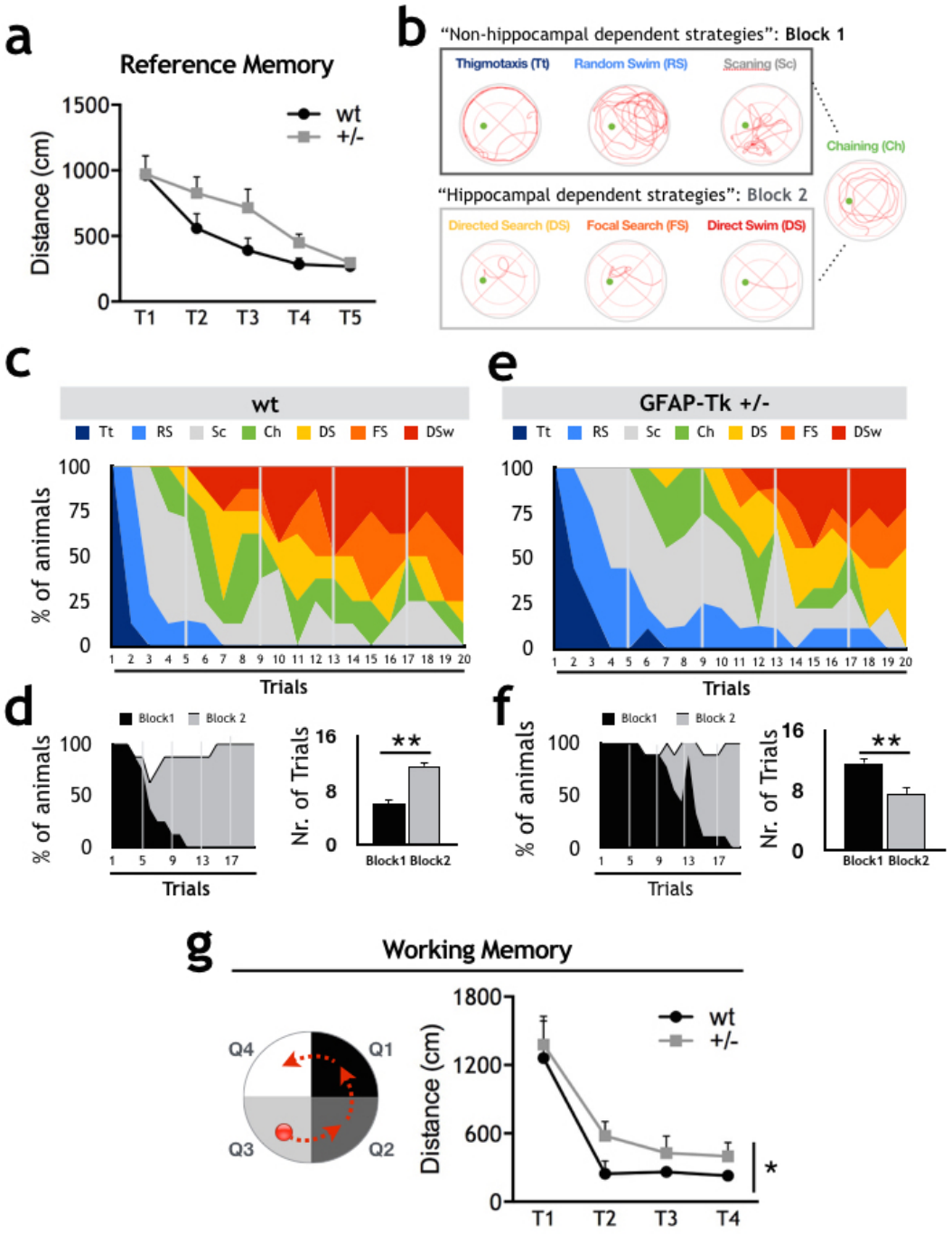

Figure 4. Long-term impact of adult cytogenesis ablation in water maze cognitive tasks. **(a)** Animals were evaluated in a spatial reference memory task 4 weeks post-cytogenesis ablation. (**a-f**) The overall test performance was complemented with the analysis of the swim strategies (**b**) adopted by each animal to reach the escape platform; the trial distributions of individual strategies and strategy block prevalence are shown both for wt (**c, d**) and GFAP-Tk$^{+/-}$ animals (**e, f**). Furthermore, the long-term impact of cytogenesis ablation was also tested in a working memory task (**g**). Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; n = 9-10 per group.

Adult cytogenesis abrogation affects pattern separation in a variation of the classic NOR task

The NOR task is a simple behavioral assessment of memory that relies primarily on a rodent's innate exploratory behavior in the absence of externally applied rules or reinforcement. With the applied protocol, exploratory behavior of a novel object relied on intact pattern separation hippocampal function. Our results show that both animal groups presented identical total times of object exploration, discarding any possible effects of cytogenesis abrogation in exploratory behavior (**Fig. 5a**). In the short-term memory assessment, GFAP-Tk$^{+/-}$ displayed decreased percentage of exploration of the new object (wt: object A = 43.70 ± 8.42 vs object B = 63.59 ± 5.96; t_{18} = 1.909; P = 0.0362; GFAP-Tk$^{+/-}$: object A = 56.71 ± 4.15 vs object B = 47.18 ± 4.79; t_{18} = 1.502; P= 0.0152, **Fig. 5b**), in comparison with wt animals. In the long-term (24h) memory assessment, neither wt or GFAP-Tk$^{+/-}$ rats were able to effectively identify the novel object, as reflected by evenly matched exploration times among both objects, **Fig. 5c**).

Adult cytogenesis abrogation impairs contextual fear conditioning, but not cued-conditioning

In the contextual fear conditioning (CFC) task, animals were submitted to a context probe, aimed to test hippocampal-dependent memory, and a light-cued probe, aimed to assess the integrity of memory circuits relying at lesser extent in hippocampal function.

First we assessed wether both animal groups presented similar percentages of freezing after the light-shock pairings day, so that potential divergent values in this parameter would undermine a clear interpretation of the results obtained in the following days. In fact, both groups had similar average freezing percentages after the conditioning trials (**Fig. 5d**). In the context probe, GFAP-Tk animals presented a reduction in the percentage of freezing, while wt animals presented higher freezing percentages when exposed to a familiar context (Wt = 78.83 ± 4.27% vs GFAP-Tk = 67.32 ± 4.46%; t_{18} = 1.866; P = 0.0392, **Fig. 5e**). Switching to a new environment, decreased freezing times in similar fashion for both groups (Wt = 22.66 ± 2,7% vs GFAP-Tk = 22.15 ± 2.14%; t_{18} = 0.7245; P = 0.2390, **Fig. 5e**).

In the light probe, both animal groups presented higher percentages of freezing after exposure to the light cue (Wt = 80.07 ± 2.72 vs GFAP-Tk = 77.13 ± 2.38; t_{18} = 0.8112; P = 0.2139, **Fig. 5f**). Overall, CFC results show that GFAP-Tk rats display long-term specific deficits in contextual

hippocampal-associated memory, while cytogenesis ablation leaves associative non-hippocampal dependent memory intact.

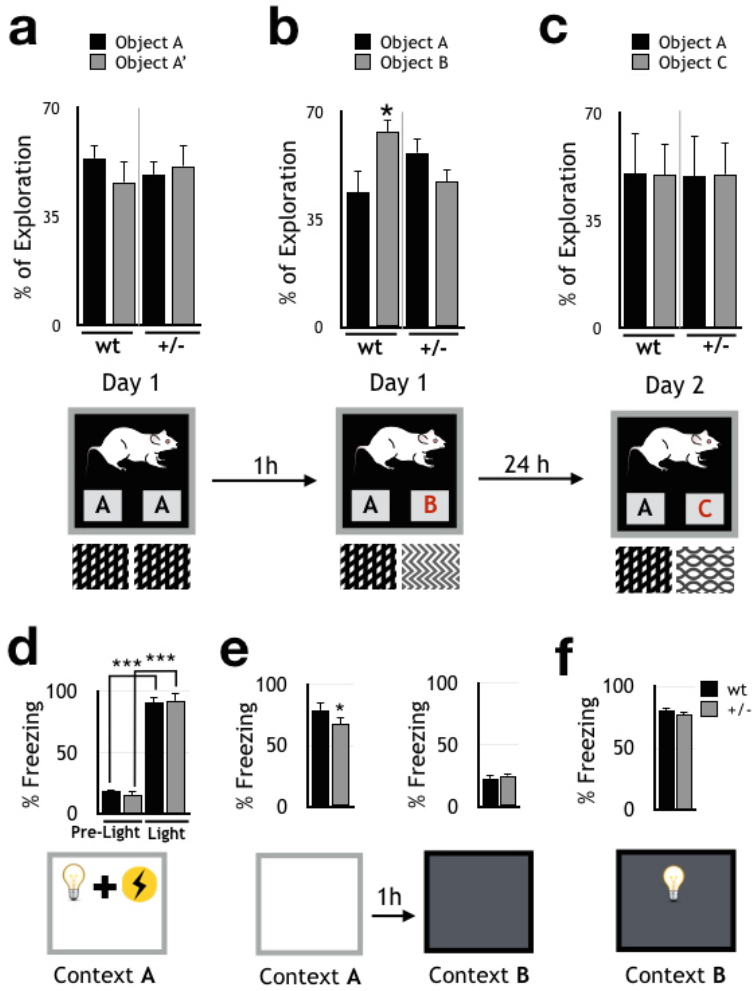

Figure 5. Long-term impact of adult cytogenesis ablation in pattern separation and contextual fear memory . **(a-c)** Animals were evaluated in modified version of the NOR test to assess pattern separation. **(d-f)** Furthermore, contextual fear memory was tested in CFC paradigm. Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; n = 9-10 per group.

Adult cytogenesis abrogation produces synaptic plasticity impairments affecting vHIP-mPFC communication

To assess wether the lack of generation of new hippocampal cells could determine long-term defects on interregional synaptic plasticity, we used an LTP paradigm to evaluate synaptic plasticity between the vHip and mPFC. Our results show that after HFS in the ventral hippocampal region, GFAP-Tk rats showed decreased LTP induction on the mPFC (P=0.0167; **Fig. 6**).

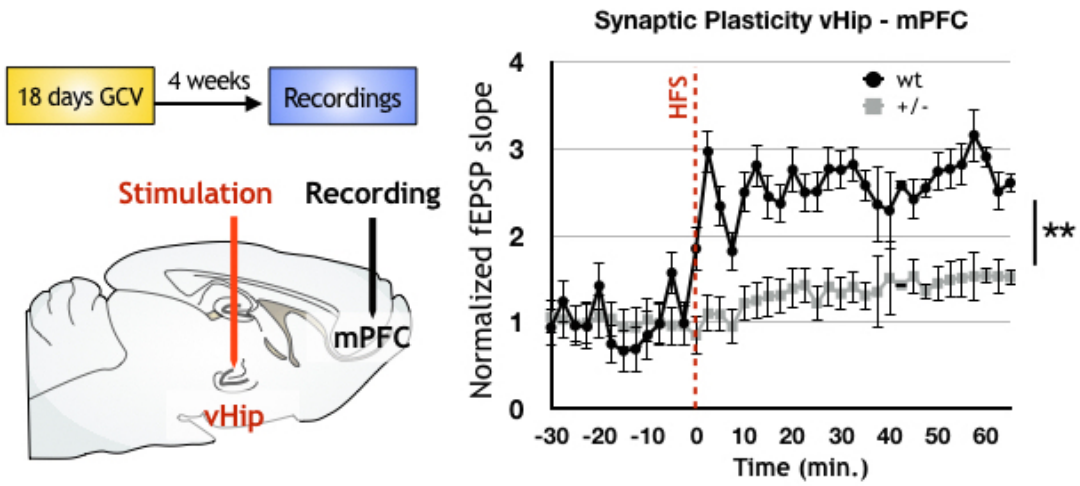

Figure 6. Cytogenesis ablation blunts hippocampal-PFC LTP response. Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; n = 5 per group.

DISCUSSION

In this work, we have administrated GCV through daily i.p. injections, to abrogate adult cytogenesis. Our immunhistological analysis confirmed that this approach is effective in ablating subgranular cytogenesis. Similar to what other authors have found[6,15], our short-term analysis revealed no association between the lack of new immature hippocampal cells with the manifestation of anhedonic or depressive-like behavior. However, immature cells seem to have an important role in the modulation of anxiety, as cytogenesis ablation is sufficient to produce quasi-immediate manifestations of anxious-like behavior.

Following reports that show that 3 to 5 weeks old neurons, but not younger or older newborn cells, have an important impact on different hippocampal-associated cognitive domains[12,18], we also chose to perform behavioral analysis 4 weeks post GCV administration. Interestingly, and in line with what we have found before using a pharmacological cytogenesis-ablation approach[7], we report the late manifestation of anhedonic behavior. This time-dependent emergence of anhedonic behavior may be in favor of the importance of the integration of new mature hippocampal cells for hedonic behavior control, while supporting the neurogenic hypothesis of depression premise of the participation of hippocampal cytogenesis in the pathophysiology of depression. However, we failed to detect alterations in the FST, which has been used to characterize rodent's "depressive-like behavior"[9]. Nevertheless, and along with the abnormalities found in the expression of anxious-like behavior, our data supports the time-dependent participation of adult cytogenesis in the modulation of different emotion-related behavioral dimensions.

Furthermore, we found that cytogenesis abrogation spared the overall performance of GFAP-Tk rats in a spatial reference memory and a reversal learning tasks. This is in line with previous studies conducted in Nestin-Tk mice[12], GFAP-Tk mice[10,13] and more recently, in the same GFAP-Tk rat model that we used[15]. In recent years, however, attention has been paid to the cognitive strategies that animals employ to reach the escape platform, as well as to its relation to adult hippocampal cytogenesis[19]. Indeed, our data shows that cytogenesis blockage imposes a long-term switch from hippocampal-dependent to non-hippocampal dependent processes during spatial reference memory trials. Therefore, this is a likely adaptive process that occurs in animals with impaired hippocampal cytogenesis in order to compensate eventual spatial memory deficits. Moreover, working memory was also found to be affected 4 weeks post GCV administration. At this respect it is interesting to note that the test paradigm relies on the interplay between the hippocampal and PFC functions. Besides local field potentials alterations, and data described by

other authors showing impaired local LTP elicited in the hippocampal DG after cytogenesis ablation, we now show that synaptic communication betweeen the hippocampus and the PFC is compromised after cytogenesis ablation. It is possible that altogether, these defects on synaptic plasticity may account for the memory deficits that we observed.

Furthermore, abrogation of cytogenesis 4 weeks before testing, produced long-term impairments in contextual fear conditioning and pattern separation. Although these findings are in line with many preceding works using similar mice models[10-14], our data conflicts with a recent report using the same rat model that we use, in which the authors report no alterations in these domains[15]. In relation to pattern separation assessment, the employed methods differed between these two works, and authors hypothesize that the used radial maze paradigm could be too demanding to detect differences in a so particular cognitive function. In fact, we were able to detect deficits in pattern separation in memory retrieval test session conducted 1 hour after the acquisition trial. When performing the same retention test 24 hours later, a task that would imply greater pattern separation and retention ability, the paradigm has no longer sensibility to detect pattern separation alterations; indeed, neither GFAP-Tk rats or wt animals are able to recognize the new objects, implying their inability to separate two very similar memory inputs acquired 24 hours before.

In conclusion, our work supports the view that adult cytogenesis does not participate in every hippocampal neural process and plays specific roles in a only subset of emotional and cognitive domains. Moreover, different functional roles of new immature and mature cells likely account for the observed time-dependent manifestation of behavioral deficits that we have described. It is important to note that the current ablational approach impacts on the generation of both neuronal and glial cells[20,21] and thus do no allow to discriminate the individual contributions of these different cell populations for the cognitive domains studied. The detected alterations were found in basal conditions. However, several authors support the idea that under particular conditions, as for instance in conditions of stress and increased glucocorticoid levels, newly born cells may be recruited at a greater extent to play a role on different emotional and cognitive domains[22,23]. Therefore, future studies of stress exposure could contribute to a better understanding of the role of new cells in the adult brain at its full exten. Finally, designing experimental approaches that allow for the selective ablation of either neuro- or gliogenesis will also pave the way towards a comprehensional view on the differential impact of these cell lineages to higher neural processes.

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Chapter V

STRESS-INDUCED ALTERATIONS OF DNA DEMETHYLATION PATHWAYS IN THE DORSAL AND VENTRAL HIPPOCAMPAL DENTATE GYRUS

Effects of chronic stress and antidepressant treatment in DNA demethylation pathways in the dorsal and ventral hippocampal dentate gyrus

A. Mateus-Pinheiro, P. Patricio, J. Marinho, M. Santiago, N.D. Alves, A.R. Machado-Santos, J. Silva-Correia, J. Marques, M. Branco, N. Sousa and L. Pinto.

To be submitted

Effects of chronic stress and antidepressant treatment in DNA demethylation pathways in the dorsal and ventral hippocampal dentate gyrus

António Mateus-Pinheiro[1,2], Patrícia Patrício[1,2], Joana Marinho[1,2], Mafalda Santiago[1,2], Nuno Dinis Alves[1,2], Ana Rita Machado-Santos[1,2], Joana Silva-Correia[1,2], Joana Marques[1,2], Miguel Branco[3], Nuno Sousa[1,2] and Luisa Pinto[1,2#]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[2]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal
[3]Centre for Genomics and Child Health, Blizard Institute, Queen Mary University of London, United Kingdom
#Correspondence to: luisapinto@ecsaude.uminho.pt

ABSTRACT

Exposure to chronic stress promotes several neuroplastic alterations in the adult brain, that contribute to emotional and cognitive unbalances. However, the molecular triggers by which stress exerts its neuropathological effects are still to be fully understood. Recent studies have put forward the hypothesis that the impact of stress on epigenetic machinery in the brain could be an important mechanism by which neuroplastic processes become compromised. Here, we show that the enzyme TET3 is downregulated in the hippocampal dentate gyrus (DG) of rats exposed to chronic stress, and that this alteration is accompanied by a reduction in DNA 5-hydroxymethylation (5hmC) levels. This effect was restricted to the dorsal DG and not observed in the ventral DG. The reduction of this epigenetic mark is also observed within the resident mitotic cell population, as 5hmC is significantly lost in proliferating neural progenitor cells of the subgranular zone (SGZ) after unpredictable chronic miild stress exposure (uCMS), suggesting a possible role for DNA hydroxymethylation in the maintenance of the neural progenitor cell pool in the dorsal DG. Furthermore, we conducted a genomic analysis by oxidative reduced representation bisulfite sequencing (oxRRBS) in both hippocampal poles. Our first results support the loss of 5hmC levels specifically in the dorsal dentate gyrus and the promotion of DNA hydroxymetylation upon chronic antidepressant treatment, targeting different locci in the dorsal and ventral DG.

Keywords: Stress; 5-hydroxymethylation; TET3; dentate gyrus; cytogenesis; dendritic plasticity; antidepressant

INTRODUCTION

In DNA hydroxymethylation, 5-methylcytosine (5mC) is oxidized to 5-hydroxymethylcytosine (5hmC) in a reaction catalyzed by the TET family of methylcytosine dioxygenases (TET1, TET2 and TET3). Through a set of additional oxidizing steps, 5hmC will be subsequently converted into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC)[1-5], to eventually be transformed in fully-demethylated cytosine residues[6,7].

Until recently, the function of 5hmC within the adult brain has been poorly comprehended. However, reports of high levels of TET3 expression in different brain regions raised the early hypothesis of a putative important role of this enzyme in post-mitotic neurons[8]. Even so, TET1 function in the central nervous system has been the most studied so far. It has been showed that Tet1-KO mice display a decreased number of hippocampal progenitor cells, while also having impairments in a spatial reference memory test in the Morris Water Maze[9]. Moreover, mice lacking Tet1 have been shown to have memory extinction defects, accompanied by abnormally enhanced hippocampal long-term depression. These studies also revealed that NSCs isolated from the DG of Tet1-KO mice present an enrichment of hypermethylated genes that ate downregulated, many of which participate in neurogenesis regulation.

TET2 and TET3 functions in the adult central nervous system (CNS) are less characterized. In particular, the study of TET3 function in the brain has been delayed due to the neonatal lethality of the Tet3-KO mice. Recently, a study revealed a dramatic genomic redistribution of 5hmC within the infralimbic prefrontal cortex in response to fear extinction learning, and that fear extinction leads to TET3-mediated accumulation of 5hmC[10]. Altogether these studies highlight that TET proteins may play critical roles in the adult CNS which remain to be elucidated. This aspect might be of particular interest in a stress exposure context, which is generally associated to hypermethylation alterations in the CNS. Therefore, a better characterization of TET enzymes function and demethylation pathways could provide important cues on the mechanisms underlying cognitive and emotional disabilities after chronic stress exposure.

Hence, we have submitted animals to a 6-weeks prevalidated unpredictable chronic mild stress (uCMS) protocol and evaluated several cognitive and emotional domains. We then analyzed the expression of TET enzymes in both the dorsal and the ventral hippocampal dentate gyrus (dDG and vDG, respectively). Moreover, we performed immunhistochemical analysis to assess hydroxymethylation levels in these two regions, which were subsequently complemented with data from genome-wide analysis of 5hmC and 5mC by oxRRBS.

MATERIALS AND METHODS

Animals and treatments

Male Wistar rats (200–250g, aged 2 months; Charles-River Laboratories, Barcelona, Spain) were maintained under standard laboratory conditions (12 h light: 12 h dark cycles, 22°C, relative humidity of 55%, ad libitum access to food and water). Four groups of rats were used: a control group (Ct) and three groups exposed to stress, and treated with either saline (sal), fluoxetine (flx) or imipramine (imi). A validated uCMS protocol was used, as previously described[15]. Subsets of these animal groups (with a final n=9) were tested behaviorally and sacrificed for subsequent studies at three established time-points: after 4 weeks of stress, and before antidepressant treatment; after 6 weeks of stress and immediately after antidepressant treatment conclusion; and, finally, 4 weeks after stress and the cessation of antidepressant administration. All procedures were carried out in accordance with EU Directive 2010/63/EU and NIH guidelines on animal care and experimentation.

Antidepressant Drugs and BrdU administration

During the last two weeks of uCMS, animals were treated with either saline solution (0.9% NaCl), the selective serotonin reuptake inhibitor, fluoxetine (10 $mg.Kg^{-1}$ per day), or the tricyclic drug imipramine (10 $mg.Kg^{-1}$ per day). To perform hippocampal proliferation and cell survival analysis, 5-Bromo-2-Deoxyuridine (BrdU), a thymidine analog, was administered to animals, in the following manner: in the subset of animals sacrificed after 4 weeks of stress exposure, a single BrdU injection (100 $mg.kg^{-1}$, i.p.) was administered 24h before sacrifice in order to evaluate hippocampal proliferation; the following animals groups were submitted to 5 days of BrdU administration (50 $mg.kg^{-1}$/ day, i.p.) at the end of the uCMS protocol, in order to perform both early survival and long survival studies.

Behavioral analysis

Sucrose Consumption Test (SCT)

Baseline sucrose preference values were established during a 1-week habituation period (1 week prior the beginning of uCMS exposure) during which animals were presented with two pre-weighed drinking fluid bottles, containing water or 1% (m/v) sucrose. A single SCT trial was performed in each established analysis time-point. Before each recording of sucrose preference, rats were food- and water-deprived for 12h and exposed to the test drinking solutions for 1h. Sucrose preference was calculated as described previously[15].

Sweet Drive Test (SDT)

The SDT test was additionally used to measure anhedonic behavior, as previously described[11]. Briefly, each animal was allowed to explore the SDT box for 10 min, where sweet (Cheerios, Nestle) or regular pellets (Mucedola 4RF21-GLP) were available. After each trial, preference for sweet pellets was calculated as already described. Three SDT trials were performed at the end of experimental time-point (1 trial every 48h). The number of entries into each chamber was used as a measure of exploratory behavior. Data from the last trial is presented.

Forced Swimming Test (FST)

Depressive-like behavior was assessed through the FST. A FST trial was conducted 24h after a 5-min pretest session, by placing the rats in transparent cylinders filled with water (25°C; 50 cm of depth) during 5 min. Trials were video-recorded and the immobility time, as well as the latency to immobility were measured using an automated video tracking system (Viewpoint, Champagne au mont d'or, France). Learned-helplessness was considered as an increase in the immobility time.

Novelty Suppressed Feeding (NSF) test

Anxiety-like traits were further assessed through the NSF paradigm. After a 18-h period of food-deprivation, animals were placed in an open- field arena, as previously described where a single food pellet was positioned in the center. After reaching the pellet, animals were individually returned to their home cage, where pre-weighted food was available, and were allowed to feed during 10min. The latency to feed in the open-field arena was used as an anxiety-like behavior measurement, whereas the food consumption in the animal home cages provided a measure of appetite drive. No differences were observed in the appetite drive between the experimental groups that could lead to a misinterpretation of the results (not shown).

Novel Object Recognition Task

We used the Novel Object Recognition (NOR) task, to assess memory function, as previously described. Briefly, in day 1 animals were placed in an empty arena and allowed to freely explore it for 5 minutes, for context habituation. In the following day (day 1), animals were exposed to two identical objects and allowed to explore the arena during 10 minutes (acquisition phase). We then performed a single long-term retention assessment, twenty-four hours later, in which animals were exposed to the original object along with a novel unfamiliar object. Sessions were video-recorded and exploration times were assessed automatically with Ethovision software (Noldus).

Immunostaining procedures

Animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and were transcardially perfused with cold saline solution (0.9% NaCl). Four animal brains of each experimental group were randomly assigned to be macrodissected, so that both the ventral and the dorsal hippocampal dentate gyrus could be isolated as described bellow; the remaining 5 brains were processed in the cryostat. Coronal cryosections (20 mm) were rehydrated in phosphate buffered saline (PBS) and post- fixed with 4% paraformaldehyde. For BrdU, 5mC and 5hmC analyses, sections were then treated with 2N HCl in PBS (1 hour/RT). Incubation with primary antibodies (ABs) was done overnight at 4°C. Primary ABs used were BrdU at 1:100 (Abcam), followed by sequential staining for 5hmC at 1:1000 (Active Motif), NeuN (for mature neurons; 1:100; Chemicon, Temecula, CA, USA) or GFAP (for glia; 1:200; Dako). Finally, all sections were stained with 4',6- diamidino-2-phenylindole (1 mg ml^{-1}). Appropriate Alexa-Fluor-conjugated secondary ABs (from Molecular Probes) were used at 1:1000, incubated for 2 hours at RT. Images and cell counts were obtained by confocal microscopy (Olympus FluoViewTM FV1000, Hamburg, Germany). Estimation of cell density in the dentate gyrus was obtained by crossing the cell number values with the corresponding dentate gyrus areas, determined using a Olympus BX51 optical microscope and the Newcast software (Visiopharm, Horsholm, Denmark).

RNA extraction and qPCR analysis

Dorsal and ventral DG were macrodissected and collected 24h after the last stressor (n=5 animals/group). Immediately after dissection, tissues were frozen and stored at - 80°C. Total RNA from dorsal and ventral DGs from the different time points and experimental groups was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. The ND-100 UV-visible light spectrophotometer (NanoDrop Technologies®) was used to evaluate the purity of RNA/DNA samples, by measuring the OD 260/280, and to determine the RNA and DNA concentration of each sample. cDNA was generated by reverse transcription from 500ng of RNA with the qScript cDNA SuperMix (Quanta Biosciences). For qPCR, oligonucleotide primers for the selected genes of interest were designed using NCBI Primer BLAST software. Sense and antisense sequences were used: Tet1 Fw. 5'ACAATGGAAGCACTGTGGTTTG3'); Tet1 Rev. 5'CAGTGTCTGCAAGCCGGTAT3'; Tet2 Fw.5'GTCGAGTTTGAACACCGAGC3'; Tet2 Rev. 5'GTGACCACCACTGTACTGCC3';Tet3 Fw. 5'AGAACCAGGTGACCAATGAGG3'; Tet3 Rev. 5'CAGTGCACCCATTGTAGAGGT.3'

Oxidative reduced representation bisulfite sequencing (oxRRBS)

Genomic DNA extracted from both dorsal and ventral DGs from control, uCMS+saline and uCMS+fluoxetine animals (2 to 3 animals per group) was used to conduct a first genome-wide study of 5mC and 5hmC distribution, through oxRRBS, as described elsewhere[21].

Statistical Analysis

Statistical analyses and graphic data representations were done using GraphPad Prism and SPSS®22 software. After appropriate normality tests, student's t-test was used to evaluate differences between Control and uCMS groups. Multiple-comparison one-way ANOVA, with Bonferroni post-hoc analysis was used to evaluate antidepressant action; ANOVA F and P values are displayed in the main text, while post-hoc P values are depicted in the figures. Statistical significance was accepted for $p<0.05$. Test statistics and p-values are shown for each test.

RESULTS

uCMS promotes persistent anhedonic-behavior, short-term transient anxiety-signs and long-term memory impairments.

We have conducted a longitudinal behavioral study, in which analysis were conducted early after 4 weeks of uCMS exposure, at the conclusion of uCMS and latter on, 4 weeks post-stress (**Fig. 1a**). In summary, at the end of uCMS week 4, stressed animals already evidenced anhedonic behavior in both the SCT (t_{16}=5.296; P<0.001) and SDT (t_{16}=3.502; P=0.002) paradigms and depressive-like behavior in the FST (t_{16}=4.287; P=0.003), as well as anxious behavior in the NSF test (t_{16}=2.320; P=0.017) (**Fig. 1b,c**). No memory deficits were yet installed (**Fig. 1c**). At the end of the 6 weeks of the uCMS protocol, anhedonic (SCT: t_{16}=2.691; P=0.008; SDT: t_{16}=5.354; P<0.001), depressive (t_{16}=3.132; P=0.003) and anxious behavior (t_{16}=3.122; P=0.003) were accompanied by memory retention deficits in the NOR task (t_{16}=3.845; P<0.001) (**Fig. 1d**). Both fluoxetine and imipramine effectively reverted the installed emotional deficits in the SCT/SDT (SCT: $F_{2,24}$=4.686; P=0.019), in the FST ($F_{2,24}$=6.490; P=0.006) and in the NSF ($F_{2,24}$=7.648; P=0.003) (**Fig. 1d**). In addition, both antidepressants (ADs) prevented the development o cognitive impairments evidenced in NOR ($F_{2,24}$=12.29; P<0.001) (**Fig. 1d**). Moreover, 4 weeks after the cessation of uCMS, untreated stressed animals presented long-term anhedonic behavior (SCT: $F_{2,24}$=7.933; P=0.002), while deficits in the FST and NST tests had already remitted (**Fig. 1e**). Along with anhedonic deficits (t_{16}=3.146; P=0.003), untreated animals

also presented persistent memory deficits in the NOR test (t_{16}=7.417; P<0.001)(**Fig. 1e**). Both ADs mediated the successful remission from anhedonic (SCT: $F_{2,24}$=7.933; P=0.002) and cognitive impairments ($F_{2,24}$=11.07; P=0.0004)(**Fig. 1e**).

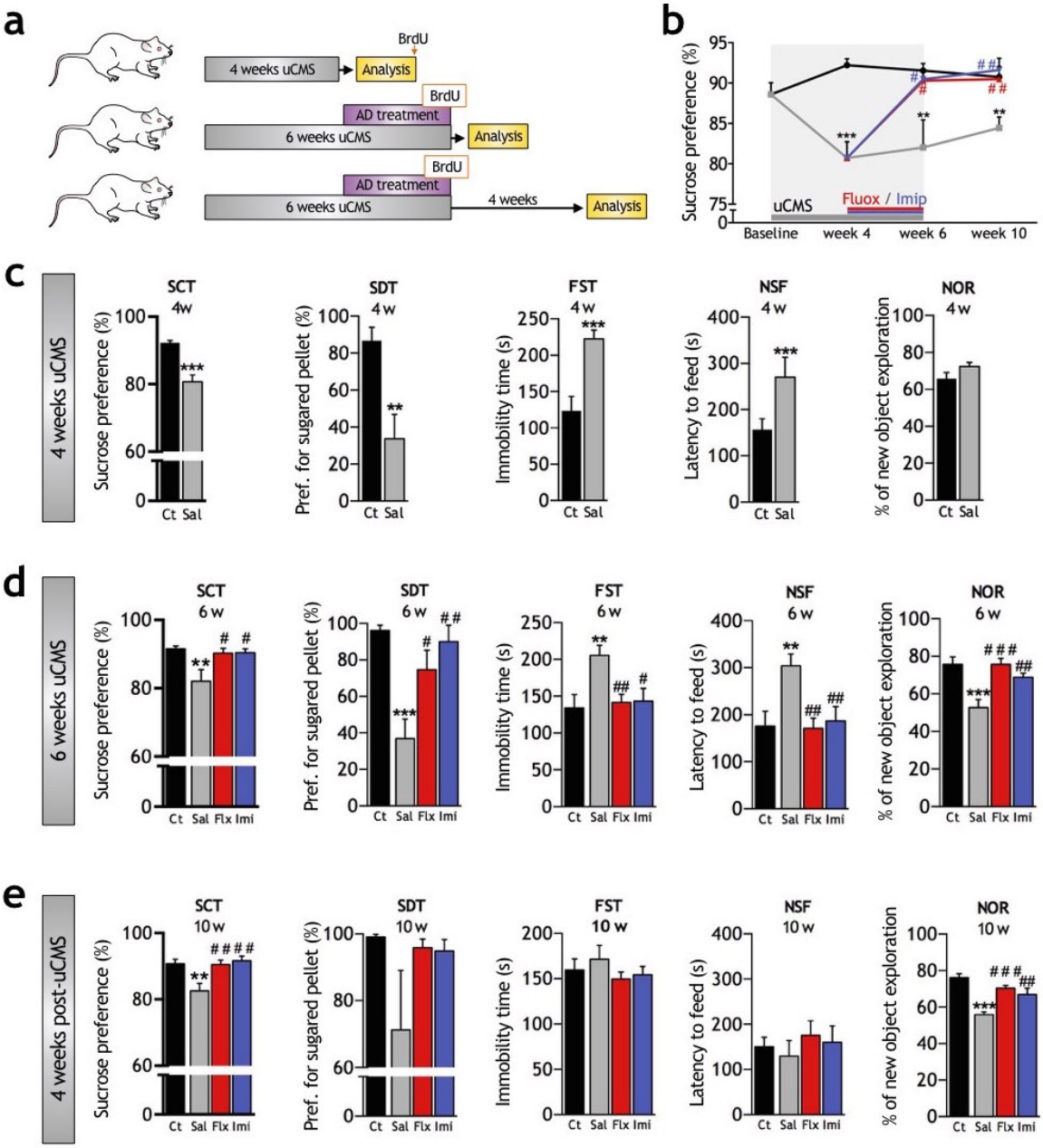


Figure 1. Longitudinal behavioral Profile of uCMS exposed animals. a. Animals subsets were analyzed at three different experimental time-points. **b.** Anhedonic behavior was longitudinally evaluated in the sucrose consumption test (SCT). **c-e.** Animals ere evaluated in the SCT, sweet drive test (SDT), forced swimming test (FST), as well as in the novelty supressed feeding (NSF) and novel object recognition (NOR) paradigms. A battery of behavioral tests was conducted in all animal subsets, specifically at the end of uCMS week4 (**c**), at the of uCMS week6 (**d**) and 4 weeks after uCMS exposure (**e**). Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; *denotes the effect of uCMS, #denotes antidepressant effect; n = 8-9 per group.

Stress has opposite effects in dendritic structural plasticity of granule neurons, in the dorsal vs ventral DG

Besides the previously discussed behavioral phenotype, uCMS induced short-term body weight loss (**Fig. 2a**) and dysregulation of the circadian corticosterone secretion pattern (**Fig. 2b**). Both physiological alterations were remitted 4 weeks after stress (**Fig. 2 a,b**). Furthermore, uCMS exposure promoted neuromorphological alterations in the dendritic length of granule cells, which differ between the dorsal and ventral DG (dDG and vDG, respectively). While, at the end of uCMS week 4 and 6, dDG neurons display significant dendritic atrophy (week 4: t_{18}=2.959; P=0.004 ;week 6: t_{18}=4.141; P<0.001), those from the vDG show hypertrophic alterations, significantly evidenced at uCMS week 6 (t_{18}=1.760; P=0.048)(**Fig. 2 c,d**). While in the dDG, AD-mediated recovery of these structural alterations was evident ($F_{2,27}$=11.73; P=0.0002), in the vDG this action was not so evident (**Fig. 2d**). Moreover, untreated animals are able to spontaneously revert this alterations, 4 weeks after ceasing stress exposure (**Fig. 2e**).

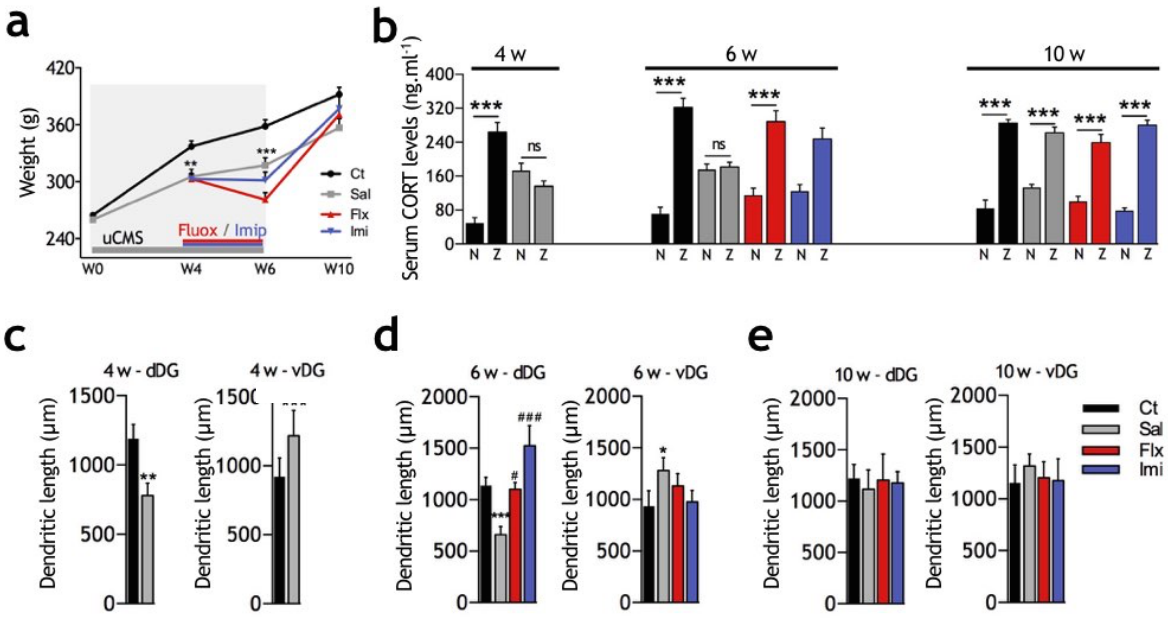


Figure 2. Stress induced alterations in physiological parameters and in hippocampal dendritic plasticity. a-b. Animal weight was monitored longitudinally (**a**), along with the serum corticosterone levels (**b**). **c-e** Furthermore, dendritic length of dentate granule neurons was evaluated, in the dDG and vDG, at the end of uCMS week4 (**c**), at the of uCMS week6 (**d**) and 4 weeks after uCMS exposure (**e**). Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; *denotes the effect of uCMS, #denotes antidepressant effect; n = 8-9 per group. N - nadir; Z - zenit.

Antidepressants restore stress-induced impairments in hippocampal proliferation, neurogenesis and gliogenesis

To evaluate the short-term effects of uCMS in hippocampal proliferation, we administered a single BrdU pulse, 24h before sacrifice, at the end of week 4 (**Fig. 3a**). Our results show that at this time, hippocampal proliferation is already significantly decreased in both the dDG (t_8=4.159; P=0.002) and vDG (t_8=2.230; P=0.028) (**Fig. 3b**). Moreover, after 6 weeks of stress, uCMS also impacted on the early survival of newborn hippocampal cells, reflected by the abrupt drop on BrdU-positive cells labelled in both regions (dDG: t_8=22.90; P<0.001; vDG: t_8=4.790; P<0.001) (**Fig. 3c**). Interestingly, while both ADs were able to revert these alterations in the dDG ($F_{(2,12)}$=127.7; P<0.001; post-hoc P<0.001, for both, their action on vDG was non-significant (P=0.062) (**Fig. 3c**). Four weeks after stress cessation we evaluated long-term survival, focusing either hippocampal neuro- or gliogenesis (**Fig. 3 d,e**). Within the dDG, uCMS significantly compromised the generation of new neurons (t_8=2.597; P=0.016) and glial cells (t_8=2.459; P=0.020) (**Fig. 3 f,g**). While both ADs were able to restore normal levels of neurogenesis in the dDG ($F_{(2,12)}$=15.12; P<0.001), only imipramine showed the ability to reverse gliogenic impairments ($F_{(2,12)}$=12.82; P=0.0011; post-hoc $P_{FLX\ vs\ SAL}$= >0.05 and $P_{IMI\ vs\ SAL}$= <0.01) (**Fig. 3 f,g**). Contrastingly, the effects of AD treatment were less profound in the vDG, where only imipramine was able to revert neurogenic deficits ($F_{(2,12)}$=4.574; P=0.033), while no effects were promoted by either stress or AD treatment in gliogenesis (**Fig. 3 f,g**).

Stressed animals present low transcriptional levels of TET3 in the dorsal DG, but not in the vDG, correlated with a decrease on labelling intensity for 5hmC

Next, we sought to explore whether uCMS induced epigenetic alterations related with DNA demethylation pathways within the hippocampal DG. Moreover, to explore if this putative modulatory effect was also mediated by ADs, we started by analyzing the effects promoted by fluoxetine treatment. Transcriptional analysis of TET enzymes in the hippocampus showed that uCMS exposure did not impact on the expression levels of *tet1* or *tet2*, both in the dDG and the vDG (**Fig. 4 a- c**). However, specifically at the end of the 6-weeks uCMS exposure, we found a reduction on the mRNA expression levels of *tet3* in the dDG (t_5= 6,191; P< 0.001; **Fig. 4b**). Moreover, fluoxetine was able tor revert *tet3* expression to basal levels (t_5= 2,615; P= 0.024; **Fig. 4b**). Contrastingly, in the vDG TET3 mRNA was comparable to control animals (**Fig. 4c**). We were also able to correlate the animals that showed higher levels of stress, either measured by the presence of increased levels of corticosterone or by poor performance in behavioral tests, with a higher reduction in *tet3* expression (not shown). Since TET3 is highly expressed in the adult brain and in particular in the hippocampal region, we sought to assess if the alterations

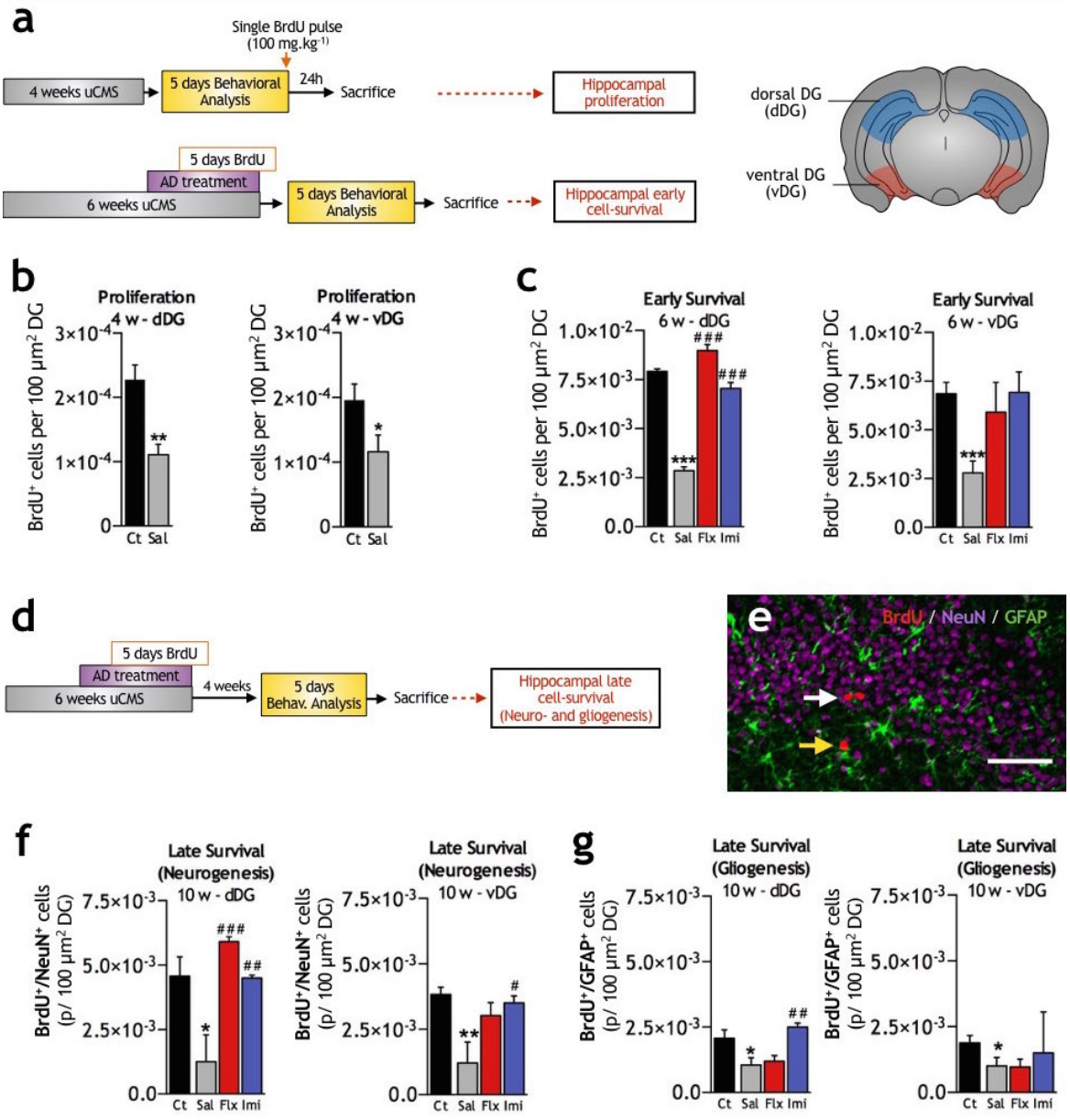


Figure 3. Hippocampal proliferation, neurogenesis and gliogenesis are decreased by stress and restored by antidepressants. a. Two independent experimental approaches, based on BrdU administration, were used to assess hippocampal cell proliferation and early (5 to 8 days) survival. **b,c.** Both measurements of cell proliferation (**b**) and early cell survival (**c**) were performed in dDG and vDG. **d-g.** A 4-5 weeks hippocampal cell survival and cell fate (**e**) analysis was additionally performed. Specifically, hippocampal neurogenesis (**f**) and gliogenesis (**g**) were assessed. The white arrow depicts a newborn neuronal (BrdU/NeuN double-positive) cell, while the yellow arrow depicts a newborn glial (BrdU/GFAP double-positive) cell. Error bars denote s.e.m. *P<0.05, **P<0.01, ***P<0.001; *denotes the effect of uCMS, #denotes antidepressant effect; n = 5 per group.

observed in TET3 expression could have repercussions on DNA 5hmC levels in this region. Hence, in the same experimental time-point (at the end of uCMS week 6), we analyzed coronal brain cryosections of both dDG and vDG stained with an anti-5hmC antibody and analyzed the signal intensity by confocal microscopy (**Fig. 4 d-f**). Interestingly, we found that signal intensity was reduced in the dDG of animals exposed to chronic stress (**Fig. 4e**). This observation was consistent among all 4 stressed animals

analyzed. In order to obtain a quantitative indicator, and to better illustrate this downregulation, we plot the intensity profile of 5hmC channel after acquiring all the images with the same confocal settings

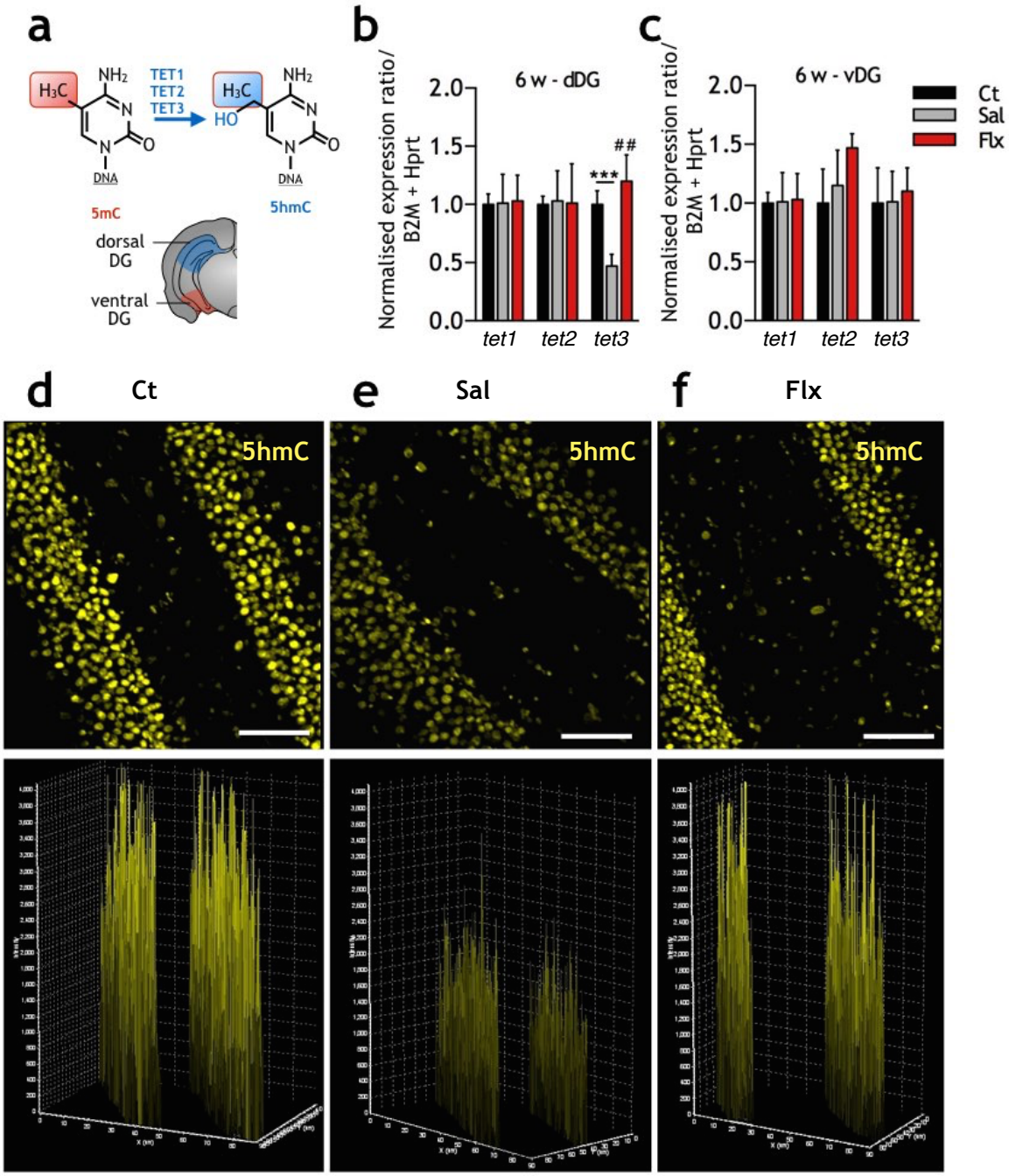


Figure 4. Stress-induced decrease of tet3 expression in the dDG, along with decreased 5hmC immunofluorescence labelling intensity are reversed by chronic fluoxetine treatment. a-c. As 5mC to 5hmC conversion is catalyzed by the TET family enzymes (**a**), we analyzed the expression of TET enzymes in dDG (**b**) and vDG (**b**). **d-f.** Representative confocal microscopy z-stacks of dDG showing 5hmC immunofluorescence labelling in control (**d**), uCMS+sal (**e**) and uCMS+Flx (**f**) animals (top panels). Lower panels depict labelling intensity plots of the images above. Error bars denote S.D.. *P<0.05, **P<0.01, ***P<0.001; *denotes the effect of uCMS, #denotes antidepressant effect; n = 3-4 per group.

(**Fig. 4 d-e,** lower panels). Here again, no alteration was found in the vDG, since signal levels for 5hmC in the vDG were similar between control and uCMS-exposed rats (**Supp. Figure 1**).

5hmC epigenetic mark is significantly lost in proliferating neural progenitor cells of the SGZ after uCMS exposure

To assess if the reduction of 5hmC in the dDG was extended to newly generated cells in the hippocampus, we performed cell counts of $BrdU^+/5hmC^+$ double-labelled cells (**Fig. 5a)**. A first analysis indicated that animals subjected to uCMS presented lower total numbers of $BrdU^+/5hmC^+$ double-labelled cells (Control= $6.6x10^{-3} \pm 1.01x10^{-3}$ positive cells per $100\mu m^2$ DG vs uCMS= $4.05x10^{-3} \pm 4.1x10^{-4}$ positive cells per $100\mu m^2$ DG; t_8= 2.329; P= 0.0241; **Fig. 5b**). However, as stress exposure is known to reduce hippocampal cell proliferation and cytogenesis, we were concerned that the observed effect was a direct consequence of the effect of stress in the reduction of the number of proliferating cells. Therefore, we analyzed the percentage of $BrdU^+$ cells in each experimental group that co-labelled with 5hmC. Data shows that within population of proliferating cells ($BrdU^+$), animals exposed to stress present a significant decrease in the DNA 5hmC mark (Control= 85.51 ± 1.14% vs uCMS= 75.61 ± 4.35%; t_8= 2.292; P= 0.0256; **Fig. 5c**). Further analysis showed that these reduction occurs at the level of the $BrdU^+/SOX2^+$ cell population (**Fig. 5 d,e**), and that this is reversed by fluoxetine

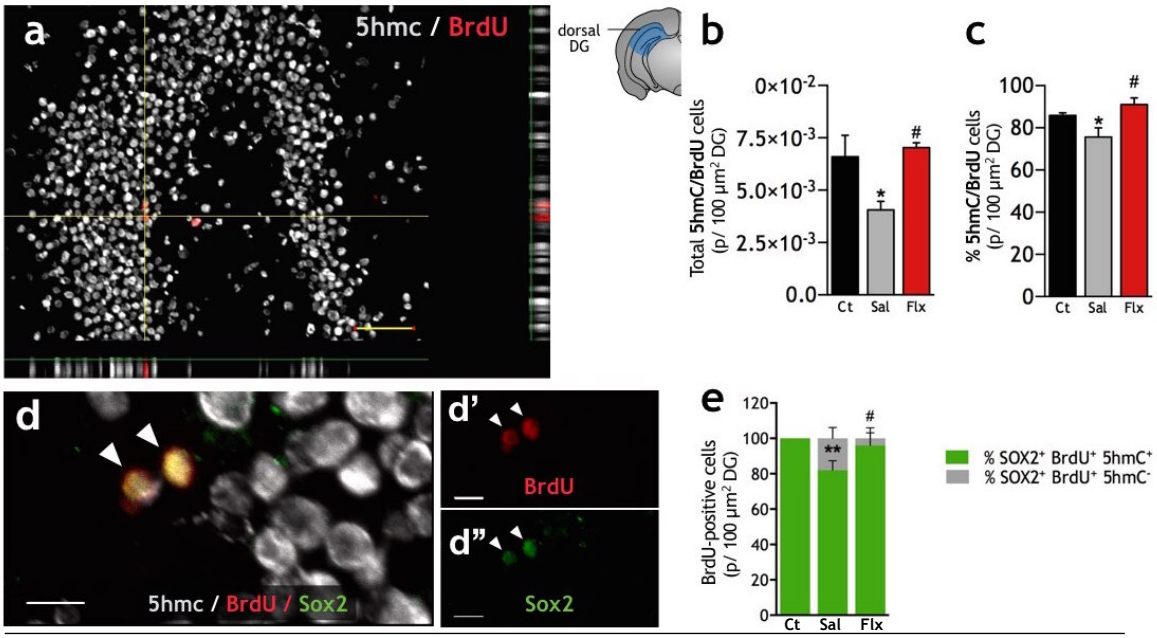


Figure 5. Loss of 5hmC in hippocampal progenitor cells. a-c. 5hmC labelling was evaluated in 5 to 8 days BrdU-retaining hippocampal cells (**a**). Total double positive 5hmC/BrdU cell density (**b**) and the percentage of BrdU positive cells co-labelled with 5hmC (**c**) was determined. **d-e.** Similar analysis was conducted in the BrdU/Sox2 double-positive progenitor cell population. Error bars denote S.D.. *P<0.05, **P<0.01, ***P<0.001; *denotes the effect of uCMS, #denotes antidepressant effect; n = 5 per group.

treatment (**Fig. 5 b,c and e**), indicating that the levels of 5hmC in hippocampal progenitor cells are significantly modulated by chronic exposure to stress and AD treatment.

Stress promotes a decrease in genome-wide levels of 5hmC specifically in the dDG

With DNA extracted from both the dDG and vDG of all animal groups, we conducted a first run of oxidative reduced representation bisulfite sequencing (oxRRBS) analysis. This recently-developed technique allows for the precise discrimination between 5hmC and 5mC. The obtained data appears to validate the previous data from our immunofluorescence analysis, showing that uCMS exposed animals present a reduction in global levels of 5hmC specifically in the dDG (**Fig. 6 a,b**). Also corroborating our previous analysis, such reduction is not present in the vDG (**Fig. 6 a,b**). Moreover, data supports the ability of fluoxetine to revert these changes, specifically in the dDG (**Fig. 6 a,b**), although by promoting hydroxymethylation in different genetic loci than those affected by stress (**Fig. 6 b**). Interestingly, our

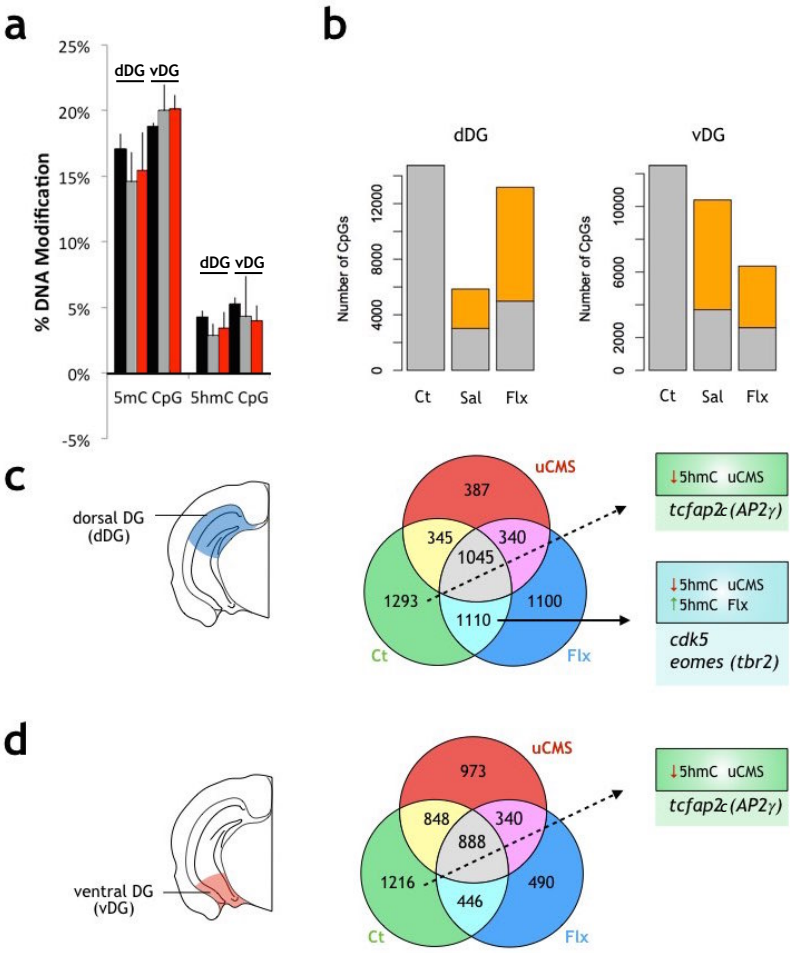


Figure 6. Genomic DNA 5hmC distribution in the dDG and vDG. a. Global levels of DNA 5mC and 5hmC were determined by oxRRBS. **a.** Number of CpG islands differentially hydroxymethylated in each experimental group; total common (grey) and unique (yellow) gene loci are displayed. **c,d.** Identified hydroxymethylated genes in the dDG (**c**) and vDG (**d**). Examples of cytogenesis regulation genes are showed (right panels). Error bars denote S.D.; n= 2-3 per group.

first screening of genes differentially hydroxymethylated between experimental groups identified transcription factors and cell cycle regulators known to promote hippocampal neurogenesis (**Fig. 6 c,d**). Curiously, the recovery of hydroxymethylation within these gene regions promoted by fluoxetine was exclusively observed in the dDG (**Fig. 6 c,d**).

DISCUSSION

In this work we addressed the question of whether chronic exposure to stress could produce significant impact on brain DNA demethylation machinery. In particular, we focused our analysis on the hippocampal region, since it is involved in the pathophysiology of many stress-related disorders, such as depression [15,16].

We were able to detect significant changes in the mRNA levels of TET3 enzyme in animals exposed to stress, that we were subsequently able to correlated with stress hallmarks, such as corticosterone levels and behavior impairments. Interestingly, stress produced alterations specifically in TET3 (and not in TET1or 2), whose expression in the brain has been shown to be the highest among TETs[11]. Moreover, it is important to note that TET3 expression was only altered in the dorsal DG. Accordingly, our first immunofluorescence analysis revealed that DNA 5hmC mark levels were globally deceased in the dorsal DG. Since we did not observe, again, the same alterations in the vDG it is likely that the reduction on 5hmC levels in the dorsal region was produced by the dowregulation of Tet3 produced by stress exposure. However, although this data suggests a rational link between the low levels of TET3 and the reduced levels of 5hmC in the dorsal hippocampus, a causative association is still to be established using alternative approaches. Hence, in the future, we will use TET3 conditional knockout mice to better dissect the putative link between stress, TET3 and hippocampal DNA 5hmC levels. It is important to note that oxRRBS technique quantitatively supported our immunohistochemical analysis by showing the reduction of 5hmC in the dDG, with no alterations in the vDG.

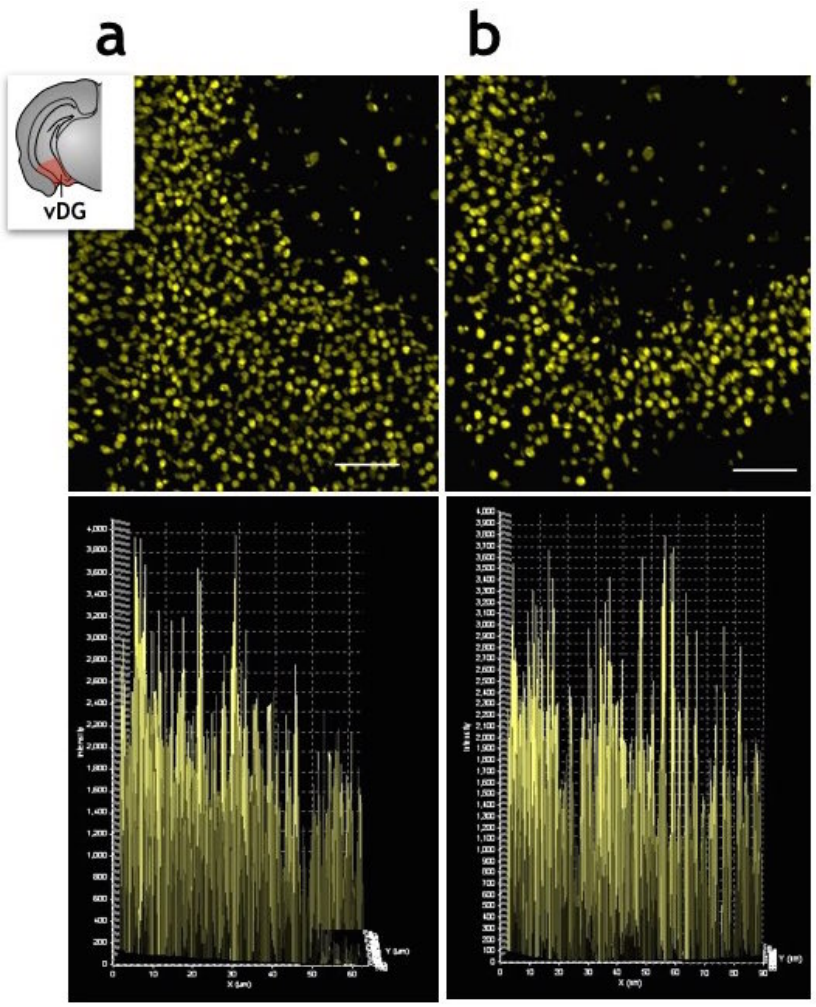
Moreover, it is well described that several behavioral dimensions are affected after chronic exposure to stress[17,18]. Some emotional and cognitive deficits emerge due to several pathological mechanisms, one of which corresponds to the deterioration of the neuroplastic capacity of the adult brain upon stress exposure. The ability to constantly generate new cells in the hippocampus, a process therefore known as hippocampal cytogenesis, is one of the neuroplastic processes that becomes compromised after stress exposure, which is believed to account for the development of

several behavioral disabilities. For such reason we investigated whether the loss of 5hmC mark in the dDG was affecting proliferating cells, or alternatively, whether it was restricted to post-mitotic cell populations. Our analysis have indeed showed that, along with post-mitotic neurons, proliferating cells were impoverished in DNA 5hmC levels. In fact, our analysis demonstrate that these proliferative cells are in fact $SOX2^{+}$ cells, again pointing for the effect of chronic stress exposure on the reduction of 5hmC levels of hippocampal neural progenitors. Importantly, data obtained from the oxRRBS analysis showed 5hmC alterations in cytogenesis regulation genes, that were only reverted by fluoxetine in the dDG. Although, this observation may correlate with the stronger pro-cytogenic action of monoaminergic ADs observed in dDG, comparatively to the vDG, additional studies are required to further dissect this link.

Finally, a brief note in respect to the functional dichotomy between the dorsal and the ventral hippocampal regions. Although it is still a matter of much controversial and debate, several authors claim that the hippocampus is not an homogeneous brain area, and a converging body of evidence indicates a functional dissociation along its septo-temporal axis, in which the dorsal part is more directly involved in learning/memory, while the ventral sub-region is linked to emotional behavior. In our uCMS model, animals acquire different deficits, both in cognitive and emotional dimensions. In a linear interpretation of our results, it is fair to hypothesize that TET3-mediated reduction of 5hmC in the dorsal hippocampus could be responsible for the emergence of cognitive disabilities; however, the lack of alterations in the ventral hippocampus are difficult to conciliate with the observed emotional deficits, such as anxiety-like behavior. Two hypothesis can be put forward: either hippocampal regional functional regionalization between the dorsal and the ventral areas is not that strict, and these two areas are quite interdependent, as many authors have also claimed; or, alternatively, DNA hydroxymethylation is primarily involved in the modulation of cognitive function, in disregard of other behavioral modalities. Considering the fact that TET3 is highly expressed in different areas of the brain and that DNA hydroxymethylation occurs in a wide range of brain regions, the last hypothesis seems quite unlikely. In the future, studying the effects of pharmacological treatments known to revert cognitive deficits caused by stress exposure, such as some anti-demential drugs (e.g. memantine), may also provide important clues in this matter.

In conclusion, this data provides important insight on the ability of chronic stress to interfere with DNA demethylation pathways in the dorsal hippocampus, resulting in significant reduction of the DNA 5hmC mark. Whether these alterations are causally linked to neuroplastic defects and to the emergence of abnormal behavior is still to be demonstrated.

SUPPLEMENTARY FIGURE



Supplementary Figure 1. No alterations in 5hmC immunofluorescence labelling intensity in the vDG, promoted by stress. a,b. Representative confocal microscopy z-stacks of vDG showing 5hmC immunofluorescence labelling in control (**a**) and uCMS+sal (**b**) (top panels). Lower panels depict labelling intensity plots of the images above.

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Chapter VI

THE SWEET DRIVE TEST: REFINING PHENOTYPIC CHARACTERIZATION OF ANHEDONIC BEHAVIOR IN RODENTS

The Sweet Drive Test: refining phenotypic characterization of anhedonic behavior in rodents

A. Mateus-Pinheiro, P. Patrício, N.D. Alves, A.R. Machado-Santos, M. Morais, J.M. Bessa, N. Sousa and L. Pinto. Frontiers in Behavioral Neuroscience, 7;8:74. (2014).

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The Sweet Drive Test: refining phenotypic characterization of anhedonic behavior in rodents

António Mateus-Pinheiro[1,2†], Patrícia Patrício[1,2†], Nuno D. Alves[1,2], Ana R. Machado-Santos[1,2], Monica Morais[1,2], João M. Bessa[1,2], Nuno Sousa[1,2*] and Luisa Pinto[1,2*]

[1] School of Health Sciences, Life and Health Sciences Research Institute (ICVS), University of Minho, Braga, Portugal
[2] ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

Edited by:
Rainer Spanagel, Central Institute of Mental Health, Germany

Reviewed by:
Christina Dalla, University of Athens, Greece
Deborah Suchecki, Universidade Federal de Sao Paulo, Brazil

***Correspondence:**
Nuno Sousa and Luisa Pinto, Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
e-mail: njcsousa@ecsaude.uminho.pt; luisapinto@ecsaude.uminho.pt

† These authors have contributed equally to this work.

Measuring anhedonic behavior in rodents is a challenging task as current methods display only moderate sensitivity to detect anhedonic phenotype and, consequently, results from different labs are frequently incongruent. Herein we present a newly-developed test, the Sweet Drive Test (SDT), which integrates food preference measurement in a non-aversive environment, with ultrasonic vocalizations (USVs) recording. Animals were placed in a soundproofed black arena, under red light illumination, and allowed to choose between regular and sweet food pellets. During the test trials, 50 KHz USVs, previously described to be associated with positive experiences, were recorded. In a first experimental approach, we demonstrate the ability of SDT to accurately characterize anhedonic behavior in animals chronically exposed to stress. In a subsequent set of experiments, we show that this paradigm has high sensitivity to detect mood-improving effects of antidepressants. The combined analysis of both food preference and the number of 50 KHz vocalizations in the SDT provides also a valuable tool to discriminate animals that responded to treatment from non-responder animals.

Keywords: depression, anhedonia, Sweet Drive Test, ultrasonic vocalizations, antidepressants, sucrose consumption test

INTRODUCTION

Animal models of depression are valuable tools to better elucidate the neuropathological basis of depressive spectrum disorders, and to provide insights into antidepressants mechanisms of action, as well as into the identification of new putative therapeutic targets (Cryan et al., 2002; Berton et al., 2012). However, it is important to consider that the translational potential of rodent models of depression relies not only on the capacity to reproduce the etiology and core pathological marks of the disease in the animals, but also in the ability to properly measure and characterize key behavioral hallmarks of depression. Anhedonia (i.e., a relative lack of pleasure in response to a formerly rewarding stimuli) is a cardinal hallmark of several forms of depression (typical and atypical major depression, dysthymic disorder or melancholic depression) (American Psychiatric Association, 2013) and it is therefore highly relevant to accurately characterize anhedonic behavior in rodent models of psychiatric disorders, such as depression. The gold-standard methods to characterize hedonic behavior involve the preference for highly palatable substances, in the usually called "sucrose (or saccharine) consumption test" (SCT); in these tests, preference for a sweetened solution, in relation to water, is assessed (Papp et al., 1991; Tye et al., 2013). However, several authors raised concerns regarding the interpretation of reduced sucrose consumption as a manifestation of anhedonia, due to confounding factors such as the long-lasting simultaneous food- and water-deprivation periods preceding the test (usually from 18 to 24 h), its moderate sensitivity do discriminate anhedonic behavior, its variability between different strains and due to the unreliability of the procedure among laboratories (Konkle et al., 2003; Anisman and Matheson, 2005; Der-Avakian and Markou, 2012; Stuart et al., 2013). Different labs have put forward alternative methods to measure anhedonic behavior in rodents (Surget et al., 2011; Stuart et al., 2013), ranging from the characterization of behavioral traits that have been arguably correlatable with the manifestation of anhedonia for instance, submissive behavior (Strekalova et al., 2004) or reduced sexual activity (Gronli et al., 2005) to more elaborate protocols involving intracranial self stimulation (ICSS) in goal-directed test paradigms (Harrison et al., 2001; Stoker and Markou, 2011).

In the attempt to refine and complement current approaches, we developed a test to characterize anhedonic behavior in rats based on the simultaneous assessment of food preference and ultrasonic vocalizations (USVs) recording- the Sweet Drive Test (SDT). Previous studies have shown that rodents USVs patterns provide important information regarding context perception and the emotional status of the animals. In particular, while USVs in the 20–22 KHz frequencies band have been associated to negative and aversive experiences, such as anxiety, fear or even pain perception, 50 KHz vocalizations have been associated to positive and pleasurable experiences. In light of these observations, we incorporated ultrasound microphones in the SDT apparatus to record USVs during test trials and to assess whether it could reinforce the assessment of hedonic behavior in rodents.

In a first set of experiments, we sought to validate SDT in an animal model of depression based in the exposure to unpredictable chronic mild stress (uCMS). In a subsequent

experimental set, we assessed SDT sensitivity to characterize the mood-improving effects of two antidepressants (fluoxetine and imipramine) and compared it to the gold-standard method, SCT.

MATERIALS AND METHODS

ANIMALS AND TREATMENTS

Two month-old male Wistar rats, weighing 200–250 g (Charles-River Laboratories) were maintained under standard laboratory conditions (12 h light: 12 h dark cycles with the dark phase beginning at 8 pm, 22°C, relative humidity of 55%, *ad libitum* access to food and water). Groups of rats ($n = 10$–18 per group) were randomly assigned to the following four experimental groups: non-stress control + saline; stress (uCMS) + saline; uCMS + fluoxetine and uCMS + imipramine. Additionally, 2 month-old Sprague Dawley male and female rats (Control + saline and uCMS + saline) were also used to assess the reproducibility and usefulness of the test in other strains and in female rodents. In the first set of experiments, an uCMS protocol was applied for 4 weeks, accordingly to what was previously validated and described (Bessa et al., 2009). In the second experimental set, animals were exposed to uCMS during 6 weeks and antidepressants (ADs) fluoxetine (10 mg.kg^{-1}; Kemprotec) and imipramine (10 mg.kg^{-1}; Sigma-Aldrich) were administered intraperitoneally (1 ml.kg^{-1}) everyday, during the 2 last weeks of the uCMS protocol. Weight gain and food intake were measured weekly during the entire protocol to monitor eventual changes induced by uCMS. All behavioral tests were conducted during the animals' nocturnal activity period. All procedures were carried out in accordance with EU Directive 2010/63/EU and NIH guidelines on animal care and experimentation.

SWEET DRIVE TEST (SDT)

SDT arena

The SDT apparatus consists in a black acrylic enclosed arena (L 82 cm × W 44 cm × H 30 cm), divided by transparent and perforated walls that defined 4 separated chambers (**Figures 1A,B** and Movie 1): a pre-chamber (PC; L 82 cm × W 12 cm × H 30 cm) in which the animal is initially placed, which is connected by an automatic trap-door to a middle chamber (MC; L 20 cm × W 30 cm × H 30 cm); once the animal enters the MC, the trap-door closes, and the animal is allowed to move freely between the right- (RC) and left-chambers (LC) (86 × 50 × 30 cm). The arena has a transparent acrylic lid used in every trial to provide noise-reduction.

Ultrasonic Vocalizations (USVs)

The RC and LC are equipped with ultrasound microphones (**Figure 1C**), so that animals' USVs can be recorded during trials. Ultrasound Microphones (CM16/CMPA, Avisoft Bioacoustics) sensitive to frequencies of 10–200 KHz were used, 20 cm above the floor, in all experiences. These were connected via an Avisoft UltrasoundGate 416H (Avisoft Biocoustics) to a personal computer. Vocalizations were recorded using the Avisoft-Recorder (version 5.1.04) with the following settings: sampling rate: 250,000; format: 16 bit. All 50 KHz vocalizations, identified by automatic data processing, were individually analyzed and validated by the experimenters. The total number of 50 KHz vocalizations emitted was assessed.

Experimental protocol

Animals were pre-habituated to sweet pellets (Cheerios®, Nestlé) in two different days, 4 and 2 weeks before the test, during the animals' activity period (60 Cheerios per cage evenly distributed among the corners of the cage); during periods of habituation to the sweet pellets, exposure to mild stressors was suspended. Furthermore, animals were firstly habituated to the SDT apparatus in the day preceding the first trial, for 6 min. In each testing day, food was removed specifically during the animals' inactive period, only 10 h before the test trial (to avoid test execution in an eventually confounding starvation state, but also to preclude odor and/or taste pre-conditioning to the usual regular food). Three SDT trials were conducted (1 trial every 48 h). Trials started at 8:30 pm (30 min after the beginning of the animals' nocturnal activity period) and were performed under red light.

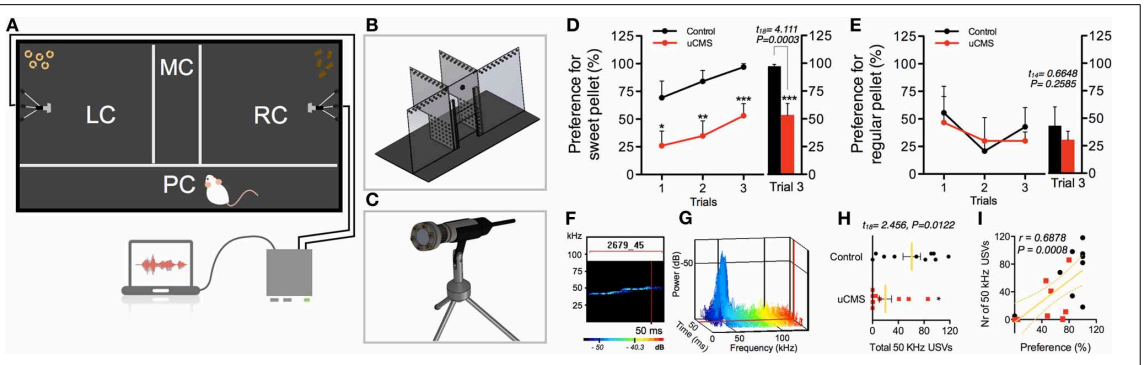

FIGURE 1 | Characterization of anhedonic behavior in rats exposed to uCMS, in the Sweet-Drive Test (SDT) paradigm. (A) Preference for sweet pellets was measured in a compartmentalized SDT apparatus **(B)**, in which ultrasonic vocalizations were recorded using ultrasound microphones **(C)**. **(D,E)** Preference values, along 3 trials, in a sweet pellet vs. regular pellet paradigm **(D)** and in a regular pellet vs. regular pellet paradigm **(E)**. **(F–I)** The total number of 50 KHz vocalizations was measured **(F,H)** and correlated with preference values **(I)**. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Error bars, s.e.m., $n = 8$–10.

Before initiating a new trial, the SDT arena was carefully cleaned with ethanol 10%. Twenty pre-weighted regular food pellets (Mucedola 4RF21-GLP; 53.3% Carbohydrates, 3700 Kcal.Kg^{-1}) were placed in the LC, and 20 pre-weighted Cheerios® (68% Carbohydrates, 3800 Kcal.Kg^{-1}) pellets were placed in the RC. After placing the animals in the PC, the transparent acrylic lid was placed. At this moment video and USVs recording were started. Once the animal crossed the trap-door and entered the MC, this door closed and the animal was allowed to freely explore the LC and the RC, for 10 min. At the end of the trial, pellets consumption was determined and preference for sweet pellets (Cheerios® pellets) was determined as follows: preference for sweet pellets (%) = Consumption of Sweet Pellets (g)/Total Food Consumption (g) × 100.

Video recording allowed to assess exploratory parameters, namely the "first choice" (first chamber in which the animal entered), "time in the pre-chamber" (latency to enter to the MC), "number of incursions" (number of entrances in each chamber) and evaluate whether these measures could differentially affect the test performance in different experimental groups. USVs analysis allowed to assess the total number of "positive" 50 KHz vocalizations during trials, and to correlate these values with the evaluated preference values.

SUCROSE CONSUMPTION TEST (SCT)

Anhedonia was assessed on weeks 4 and 6 of the uCMS protocol using the gold standard sucrose consumption test. Baseline sucrose preference values were established during a 1-week habituation period. The test consists in the presentation of two pre-weighed drinking bottles, containing water or 2% (m/v) sucrose solution for 1 h. Before each test, rats were food and water-deprived for 12 h. Sucrose preference was calculated according to the formula: sucrose preference = (sucrose intake)/(sucrose intake + water intake) × 100, as previously described (Bessa et al., 2009). SCT was performed during the nocturnal activity period of animals (starting at 8.30 p.m), 24 h after the third trial of SDT; after the SDT trial, animals were allowed to feed freely until 12 h preceding SCT.

NOVELTY SUPPRESSED FEEDING TEST (NSF)

Anxious-like behavior was assessed through the NSF test at the end of the uCMS protocol. After an 18 h food-deprivation period, animals were placed in an open-field arena, where a single food pellet was positioned in the center, as previously described (Bessa et al., 2009). After reaching the pellet, animals were individually returned to their home cage, where pre-weighed food was available, and were allowed to feed for 10 min. The latency to feed in the open-field arena was used as an anxiety-like behavior index, whereas the food consumption in the home cage provided a measure of appetite drive.

OPEN-FIELD TEST (OF)

The open field (OF) test was used as an additional measure of anxious-like behavior, as well as to evaluate locomotor performance and exploratory activity. The open field apparatus consisted of a brightly illuminated square arena of 43.2 × 43.2 cm closed by a wall of 30.5 cm high. Rats were placed individually in the center of the open field arena and their movement was traced, for 5 min, using a two 16-beam infrared system. The resulting data was analyzed using the Activity Monitor software (Med Associates, Inc.), considering two previously defined areas: a central and an outer area. Distance traveled in each of the zones was recorded and analyzed. The ratio between the distance traveled in the center and in the periphery of arena was used as indicative of anxiety-like traits.

NOVEL OBJECT RECOGNITION TEST (NOR)

Cognitive function was assessed in the NOR test. Briefly, rats were first habituated to the testing arena for 10 min. On the next day, each animal was allowed to explore two identical objects placed in the arena for 10 min. One hour later, rats explored the same arena for 5 min, with one of the familiar objects replaced by a novel object. Recognition memory was expressed by the discrimination index (D), which was defined as D = (time of exploration novel object − time of exploration familiar object)/total time of exploration.

FORCED SWIMMING TEST (FST)

Learned-helplessness was assessed using the forced swimming test. Assays were conducted 24 h after a 5-min pretest session, by placing the rats in transparent cylinders filled with water (25°C; 50 cm depth) for 5 min. Trials were video-recorded and the immobility time as well as the latency to immobility were measured using an automated video tracking system (Viewpoint). Learned-helplessness was considered as an increase in the immobility time and a decrease in the latency to immobility.

BrdU IMMUNOSTAINING

In the last day of behavioral testing, animals were given single intraperitoneal injection of Bromo-deoxyuridine (BrdU, Sigma-Aldrich, 100 mg.kg^{-1}; thymidine analog that incorporates into DNA during the S-phase of the mitotic process). Twenty-four hours after the injection, animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and were transcardially perfused with cold 4% paraformaldehyde (PFA). Brains were removed and post-fixed in 4% PFA. Serial coronal cryosections (20 μm) were cut and stained for BrdU (1:50; Dako). Secondary antibody Alexa Fluor® 488 (Molecular Probes) was used for detection. Nuclei were counterstained using DAPI. Proliferation densities were estimated in the subgranular zone (SGZ; defined as a two-cell layer-thick zone on the inner side of the granule cell layer of the dentate gyrus), using a confocal microscope (Olympus FV1000).

MORPHOLOGICAL ANALYSIS

For the three-dimensional morphometric analysis, four animals from each group were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Sanofi), transcardially perfused with 0.9% saline and processed. Briefly, brains were immersed in Golgi-Cox solution for 21 days; transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 μm) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix,

dehydrated and xylene cleared before coverslipping. Dendritic arborization was analyzed in the dentate gyrus. For each selected neuron, all branches of the dendritic tree were reconstructed at 1000x (oil) magnification using a motorized microscope (BX51, Olympus) and Neurolucida software (Microbrightfield). A three-dimensional analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). For each animal, 10 neurons were studied and total dendritic length was determined.

CORTICOSTERONE LEVELS MEASUREMENTS

Corticosterone levels were measured in blood serum, collected by tail venopuncture, using a commercially available ELISA kit (R&D Systems). Sampling was performed between 8 and 9 am (nadir) and between 8 and 9 pm (zenith; peak) at the end of the uCMS protocol. Samples were run in duplicate.

RT-PCR MEASUREMENTS

mRNA expression levels of the genes involved in the mediation of anhedonia were determined by qRT-PCR in the nucleus accumbens (NAc) and pre-frontal cortex (PFC) from six animals of each group (CT and CMS + Sal). Total RNA (500 ng) was reverse transcribed using qScript™ cDNA SuperMix (Quanta Biosciences™). Oligonucleotide primers for dopamine receptor 1 (Drd1), 2 (Drd2) and 3 (Drd3), prodynorphin (Pdyn), opioid receptor μ1 (Oprm1) and cannabinoid receptor 1 (Cnr1) were designed using Primer-BLAST software (NCBI). Sense and antisense sequences can be found in **Table 1**. Real time reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems) using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Biodyne). The housekeeping gene Beta-2-Microglobulin (B2M) was used as internal control for normalization of the target gene's expression. The relative expression was calculated using the ΔΔCt method. Results are presented as relative expression of the target gene.

STATISTICAL ANALYSIS

Statistical analyses were done using SPSS software (SPSS, Chicago, IL, USA). *t*-test was used to evaluate differences between two groups where appropriate. ADs effects on different behavioral dimensions were evaluated using One-Way analysis of variance (ANOVA). F-values and P-values derived from the between groups analysis of variance are properly indicated in the figures. Differences between groups were determined by Bonferroni's *post-hoc* multiple comparison test, and the corresponding P-values are depicted in the figures. Statistical significance was accepted for $P < 0.05$.

RESULTS

To assess the efficacy of the new behavioral paradigm described herein, we used an uCMS exposure model (Bessa et al., 2009) in Wistar-Han male rats. This protocol is known to induce core symptoms of depression in rodents, including anhedonia (Bessa et al., 2009). In a first approach, we aimed to evaluate whether SDT could discriminate the impacts of uCMS exposure in hedonic behavior of animals chronically exposed to stress during 4 weeks (**Figure 1**). In the tested paradigm, control (non-stressed) animals developed high preference for sweet pellets over the three trials, reaching over 95% of preference in trial 3; conversely, uCMS-exposed animals evidenced no significant preference for sweet food pellets over regular food pellets (preference values ≈50%) (**Figure 1D**). This group discrimination based on SDT performance was absent when using a neutral regular food vs. regular food test paradigm (**Figure 1E**).

In order to have a multi-parametric measurement of anhedonic behavior we complement the food preference analysis with simultaneous recording of 50 KHz USVs (**Figures 1F,G**). Remarkably, results showed that animals with reduced preference for sweet pellets, also presented a reduction in the number of 50 KHz "positive" vocalizations during the test (**Figure 1H**). In fact, the frequency of vocalizations correlated positively with the preference for sweet pellets (**Figure 1I**), and allowed to effectively discriminate between control and uCMS animals.

Evaluation of anhedonia with SDT was subsequently complemented with additional phenotypic characterization of animals that revealed multi-dimensional physiological and behavioral deficits normally encompassed in the symptomatic profile of depressive disorders (Bessa et al., 2009; Mateus-Pinheiro et al., 2013) (**Figure 2A**). As previously reported, uCMS exposure induced a significant reduction in total body weight gain, but no significant alterations were found in the total daily food consumption between control and uCMS-exposed animals either before, or after 4 weeks of stress exposure (**Figure 2B**). Moreover, uCMS exposure promoted the emergence of behavioral despair signs (**Figure 2C**), heightened anxiety-like behavior (**Figure 2D**) and cognitive disabilities (**Figure 2E**). These behavioral changes were accompanied with the disruption of normal corticosterone serum levels (**Figure 2F**), a decrease in overall hippocampal cell proliferation (**Figure 2G**) and dendritic atrophy of dorsal hippocampal granular neurons (**Figure 2H**).

Table 1 | Sense and antisense sequences of oligonucleotide primers used in the qRT-PCR.

Gene	Sense	Antisense	Product size (bp)
B2M	GTGCTTGCCATTCAGAAAACTCC	AGGTGGGTGGAACTGAGACA	136
Drd1	TCCTTCAAGAGGGAGACGAA	CCACAAACACATCGAAGG	168
Drd2	ATGTGCTGGTGTGCATGGCT	CACCCACCACCTCCAGGTAGAC	142
Drd3	GGGGTGACTGTCCTGGTCTA	TGGCCCTTATTGAAAACTGC	169
Pdyn	CCTGTCCTTGTGTTCCCTGT	AGAGGCAGTCAGGGTGAGAA	157
Oprm1	CAACTTGTCCCACGTTGATG	TAATGGCTGTGACCATGGAA	119
Cnr1	AGGAGCAAGGACCTGAGACA	TAACGGTGCTCTTGATGCAG	166

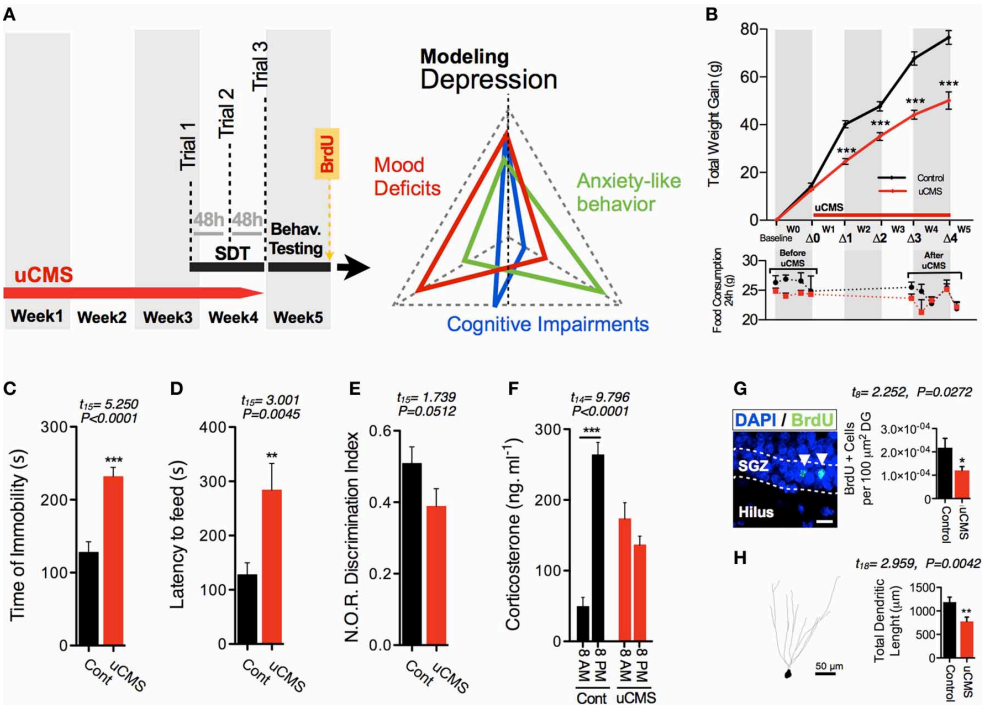

FIGURE 2 | Effects of uCMS exposure in different physiological and behavioral dimensions. (A) After 4 weeks of uCMS exposure, multi-dimensional depressive-like behavior was assessed. **(B)** Weight gain and food intake, before and after 4 weeks of uCMS. Animals exposed to uCMS presented a decreased total body weight gain during stress exposure [Week1 ($\Delta 1$): $t_{(15)} = 7.969$, $P < 0.0001$; Week2 ($\Delta 2$): $t_{(15)} = 4.948$, $P < 0.0001$; Week3 ($\Delta 3$): $t_{(15)} = 6.877$, $P < 0.0001$; Week4 ($\Delta 4$): $t_{(15)} = 5.782$, $P < 0.0001$]. Daily food consumption of uCMS animals remained unaltered after stress exposure. Animals were tested in the Forced Swimming Test **(C)**, Novelty Suppressed Feeding **(D)** and Novel Object Recognition **(E)** test paradigms. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars, s.e.m., $n = 8$–10. **(F–H)** Corticosterone serum levels **(F)**, hippocampal proliferation **(G)** and dendritic morphology of hippocampal granular neurons **(H)** were assessed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars, s.e.m., $n = 5$–10.

Importantly, we detected no significant differences between control and uCMS-exposed animals when analyzing different exploratory parameters, namely the total number of incursions to each food chamber (**Figures 3A,B**), as well as the average time animals took to leave the PC and start exploring the food chambers, and the choice of the first food chamber to explore (**Figure 3C**). Analysis of the percentage of distance spent in the center of the arena in the OF test endorsed the hyperanxious-like phenotype of uCMS-exposed animals, that was also found in NSF (**Figures 3D–F**). Together with the exploratory parameters, this data suggested an effective subtraction of any eventual anxiogenic and neophobic components of the SDT environment.

Furthermore, we analyzed the expression of molecules described to participate in the mediation of hedonic behavior in the prefrontal cortex (PFC) and in the nucleus accumbens (Nac) (Der-Avakian and Markou, 2012). From the different genes analyzed, we found that the expression of dopamine receptor D2 (Drd2) and prodynorphin (Pdyn) is decreased in the Nucleus Accumbens (Nac) of animals exposed to uCMS (**Figure 4A**). In the PFC, however, we found Drd2 mRNA levels to be increased in stressed animals, whereas uCMS exposure induced a decrease in dopamine receptor D3 (Drd3) levels in the same region (**Figure 4B**).

To further evaluate the capacity of SDT to assess anhedonic behavior, in a second approach we tested its sensitivity to detect the reestablishment of normal hedonic behavior, promoted by chronic treatment with ADs from two different classes, fluoxetine (a selective serotonin reuptake inhibitor—SSRI) and imipramine (a tricyclic agent). Moreover, we compared performance in the SDT with the performance of the same animals in the gold-standard test, SCT (**Figure 5A**). We found that both ADs were able to revert anhedonic behavior measured in the SCT and in the SDT (**Figures 5B,C**). Although group statistics allowed to discriminate the anhedonic phenotype of the stressed-untreated animal group in both tests, individual score analysis demonstrates that SDT presents a high individual discrimination accuracy (**Figure 5D**); in fact, most uCMS exposed animals were effectively discriminated based on its preference scores in SDT, with preference values outside the control group score-range (**Figure 5E**). Furthermore, simultaneous recording of 50 KHz vocalizations allowed to discriminate between uCMS animals that responded to ADs treatment (ADs R) and animals that

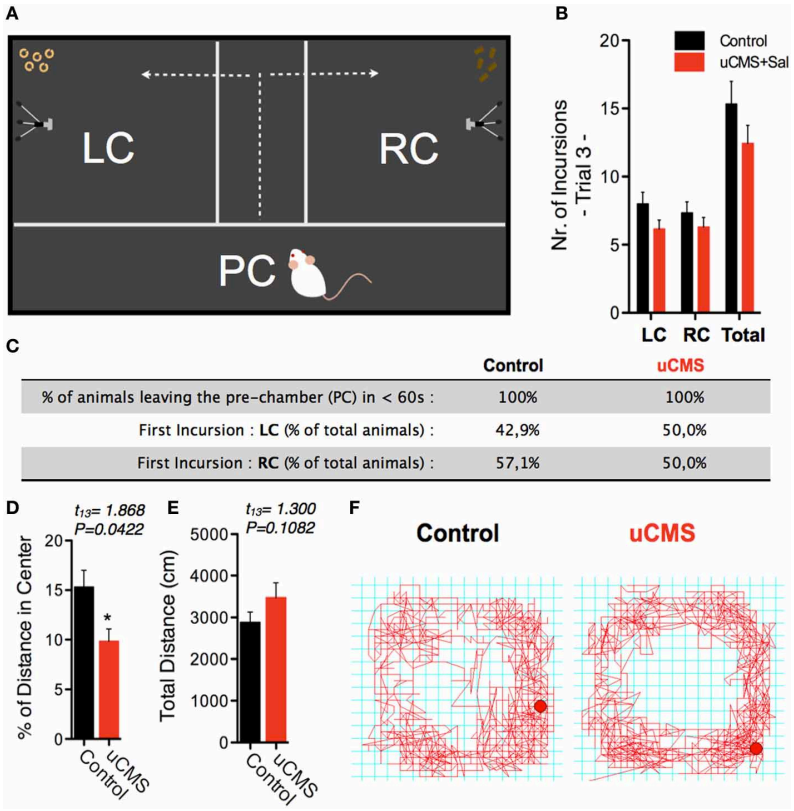

FIGURE 3 | Exploratory parameters in the SDT. (A,B) The average number of incursions into the left-chamber (LC; containing sweet pellets) and into the right-chamber (RC; containing regular pellets), during SDT was assessed and no significant differences were found. **(C)** Additional exploratory parameters were assessed in order to exclude a possible bias introduced by hyperanxious behavior of uCMS-exposed animals; the similar exploratory behavior of control and uCMS animals supports the SDT arena as a non-aversive test environment. **(D–F)** Evaluation in the open-field test, further indicates that anxious-like behavior **(D)** did not affect total exploratory activity **(E,F)**. $^*P < 0.05$ Error bars, s.e.m., $n = 7$–9.

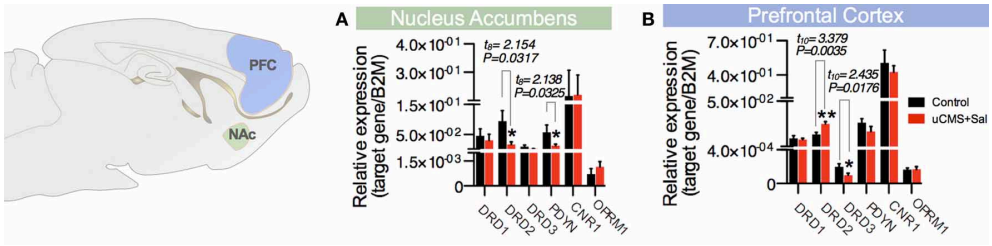

FIGURE 4 | Expression of molecular mediators of hedonia in the (A) nucleus accumbens (NAc) and (B) prefontal cortex (PFC) by RT-PCR: Dopamine Receptors D1, D2, and D3 (Drd1, Drd2, and Drd3), prodynorphin (Pdyn), Cannabinoid Receptor 1 (Cnr1) and Opioid Receptor μ1 (Oprm1). $^*P < 0.05$, $^{**}P < 0.01$. Error bars, s.e.m. *t*-test statistical measures for the analysis of uCMS vs. Control is shown on the top of the figures.

maintained the anhedonic profile and did not respond to treatment (ADs NR) (**Figure 5F**). Values of total number of 50 KHz vocalizations correlated positively with performance on SDT (**Figure 5G**). In complementary behavioral analysis, data shows that treatment with imipramine has a fast action in reverting the pro-anhedonic effects induced by stress exposure, whereas fluoxetine effects are only significant later on, namely on the third trial (**Figure 6A**). Similarly to what was found in the previous

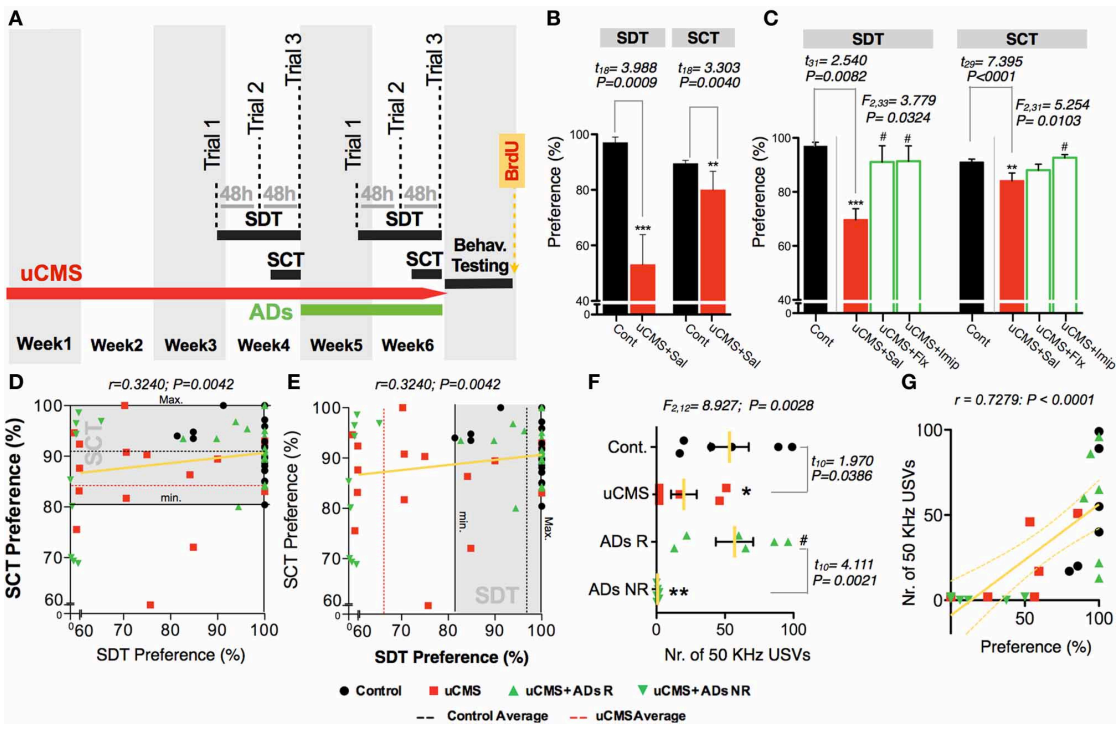

FIGURE 5 | Measuring the effects of uCMS and antidepressant treatment on anhedonic behavior with the Sweet Drive Test (SDT) and the Sucrose Consumption Test (SCT). (A–C) Anhedonic behavior was measured before **(B)** and after **(C)** 2 weeks of antidepressant treatment, in the SDT (data from the 3rd trial presented) and in the SCT (n = 10–18). **(D,E)** Performances in both tests were correlated (n = 68) and the ability of the tests to discriminate anhedonic behavior in untreated uCMS animals is showed as the density of red dots outside the performance range of control animals in each test (depicted as a shaded gray area). **(F)** Total number of 50 KHz vocalizations in control animals (Cont.), uCMS untreated (uCMS) animals and in antidepressant treatment responders (ADs R) and non-responders (ADs NR) (n = 6). **(G)** Correlation between the total number of 50 KHz vocalizations and preference values in the 3rd SDT trial (n = 24). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars, s.e.m. ANOVA statistical measures for the analysis of ADs effect is shown on the top of the figures. *Denotes the effect of uCMS exposure. #Denotes antidepressant effect.

experimental set, no differences were found in the total number of incursions that could potentially undermine SDT interpretation (**Figure 6B**). Moreover, both ADs effectively reverted the behavioral deficits induced by stress (**Figures 6C–G**).

Finally, we tested whether SDT could effectively evaluate anhedonic behavior in a different rat strain—Sprague-Dawley—both in female and male animals (**Figure 7A**). Using the same protocol of previous experiments, both female and male rats exposed to stress revealed decreased preference values in the SCT and in the SDT (**Figures 7B,C**), while the exploratory parameters in SDT were similar for all groups (**Figure 7D**).

DISCUSSION

Understanding the pathophysiology of anhedonic behavior is currently particularly relevant in different areas of neurosciences research, as it encompasses the symptomatology of different diseases with growing prevalence in modern societies. Studies aiming to characterize this pathological trait in animal models require methods with at least three fundamental characteristics, namely (i) the capacity to provide a solid measure of anhedonic behavior, with high sensitivity to both anhedonic and pro-hedonic stimuli and/or treatments, (ii) the ability to reduce or eliminate the interference of confounding factors that can undermine the interpretation of the obtained results, and (iii) the reliability of the method in different experimental contexts.

A primary concern when designing SDT was to eliminate the interference of common potential confounding factors found in animal testing. Since test performance and exploratory behavior of animals can be largely conditioned by heightened anxiety or neophobia-based test paradigms, it was our aim to subtract these factors as much as possible in the SDT. The use of perforated and transparent chamber dividers was aimed to allow the animals to acquire complete spatial and odor maps of the explorable area in any position, and to reduce neophobic behavior. This apparatus configuration, combined with the absence of white-light illumination and the sound-isolation provided by the transparent lid enclosure was intended to subtract any aversive nature of the testing context, during animal testing. Indeed, despite presenting heightened anxious-like behavior, evidenced in the NSF and OF tests, chronically stressed animals presented an

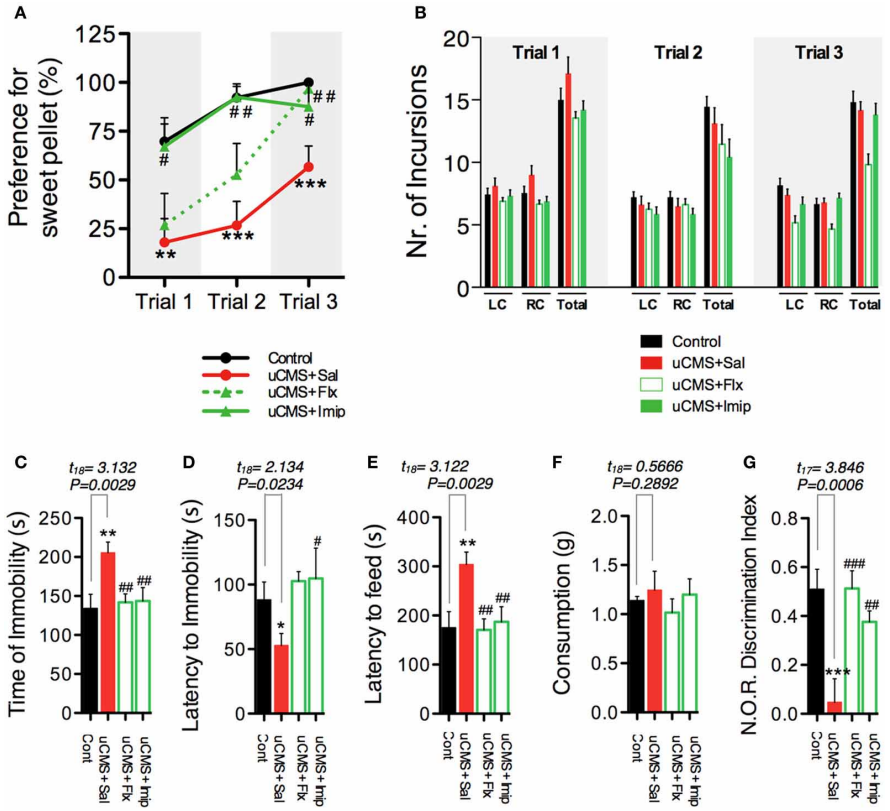

FIGURE 6 | Additional behavioral parameters determined after 6 weeks of uCMS exposure and ADs treatment. (A) uCMS exposure induced a significant decrease in preference for sweet pellets along the trials [Trial 1: $t_{(18)} = 3.011$, $P = 0.0038$; Trial 2: $t_{(18)} = 4.795$, $P < 0.0001$; Trial 3: $t_{(18)} = 4.007$, $P = 0.0004$]. This anhedonic profile was effectively reversed by imipramine in the three trials, while the improving effects of fluoxetine were mainly observed in trial 3 [Trial 1: $F_{(2,\ 27)} = 3.784$, $P = 0.0356$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} > 0.05$, $P_{uCMS\ vs.\ uCMS+Imip} < 0.05$; Trial 2: $F_{(2,\ 27)} = 7.176$, $P = 0.0032$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} > 0.05$, $P_{uCMS\ vs.\ uCMS+Imip} < 0.01$; Trial 3: $F_{(2,\ 27)} = 5.427$, $P = 0.0104$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} < 0.01$, $P_{uCMS\ vs.\ uCMS+Imip} < 0.05$]. **(B)** The average number of incursions into the left-chamber (LC; containing sweet pellets) and into the right-chamber (RC; containing regular pellets), during SDT was assessed and no significant differences were found. **(C–G)** Emotional and cognitive domains normally affected in depression were assessed in the Forced Swimming Test **(C,D)** [$ANOVA_{ADs\ effect\ on\ Time\ of\ Immobility}$: $F_{(2,\ 27)} = 4.490$, $P = 0.0050$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} < 0.01$, $P_{uCMS\ vs.\ uCMS+Imip} < 0.01$; $ANOVA_{ADs\ effect\ on\ Latency\ to\ Immobility}$: $F_{(2,\ 27)} = 3.710$, $P = 0.0377$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} > 0.05$, $P_{uCMS\ vs.\ uCMS+Imip} < 0.05$], Novelty Suppressed Feeding **(E,F)** [$ANOVA_{ADs\ effect\ on\ Latency\ to\ Feed}$: $F_{(2,\ 27)} = 7.648$, $P = 0.0023$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} < 0.01$, $P_{uCMS\ vs.\ uCMS+Imip} < 0.01$; $ANOVA_{ADs\ effect\ on\ Appetite\ Drive}$: $F_{(2,\ 27)} = 0.5522$, $P = 0.5823$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} > 0.05$, $P_{uCMS\ vs.\ uCMS+Imip} > 0.05$] and Novel Object Recognition **(G)** $ANOVA_{ADs\ effect\ on\ Exploratory\ Index}$: $F_{(2,\ 26)} = 12.45$, $P = 0.0002$, *Post-hoc*: $P_{uCMS\ vs.\ uCMS+Flx} < 0.001$, $P_{uCMS\ vs.\ uCMS+Imip} < 0.01$ test paradigms. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Error bars, s.e.m. *Denotes the effect of uCMS exposure. #Denotes antidepressant effect. Error bars, s.e.m., $n = 9$–10.

exploratory activity in the SDT that was indistinguishable from control animals, suggesting this test to be uninfluenced by this behavioral dimension. In addition, tests such as the SCT are usually conducted after long periods of simultaneous water and food deprivation (ranging from 18 to 24 h). Starvation may significantly interfere with the test outcome as animals' consumption may not reflect a "hedonistic" choice based on palatability, but rather the necessity of satiety, irrespectively of food palatability or taste. In this study, water was never removed prior testing and animals were allowed to feed freely in their active-nocturnal period (and during the first 2 h of the inactive-diurnal period). In light of the fact that in SDT trials animals had to choose between a sugared-pellet and a pellet made of the same regular food that animals were given in a daily basis, we opted to remove food from animals' cages during the remaining 10 h of the inactive period to preclude pre-conditioning to food odor or taste prior testing.

To validate the SDT, we used an uCMS-based animal model of depression which has been already pre-validated by our lab (Bessa et al., 2009; Mateus-Pinheiro et al., 2013). Animals submitted

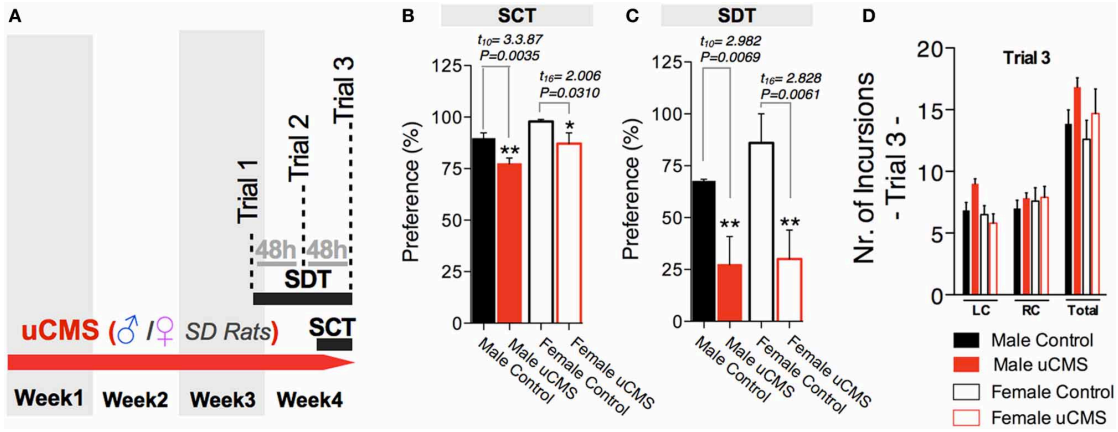

FIGURE 7 | Measuring anhedonic behavior with the Sweet Drive Test (SDT) and the Sucrose Consumption Test (SCT) in Sprague-Dawley (SD) Rats. (A) Using the same experimental approach previously used with Wistar-Han rats, male and female SD rats were tested in the SCT and the SDT. **(B)** uCMS exposure induced anhedonic behavior in both male and female animals in the SCT. **(C)** The effects of uCMS on anhedonic behavior were also detected using the SDT, evidencing a marked reduction on preference in both male and female rats. **(D)** No differences on exploratory behavior were observed between groups, during the SDT. $^{*}P < 0.05$, $^{**}P < 0.01$. Error bars, s.e.m., $n = 6$–9.

to uCMS presented several physiological and behavioral deficits encompassed in the spectrum of depressive disorders. Moreover, analysis of the expression of molecules described to participate in the mediation of hedonic behavior revealed a decrease expression of *Drd2* and *Pdyn* in the Nac. Both alterations have already been described in animals presenting increased anhedonic behavior (Blednov et al., 2006; Kruzich et al., 2006) and reinforce the idea that uCMS animals present alterations in the brain systems involved in the regulation of hedonia. Although, to the best of our knowledge, *Drd3* has not been associated with the emergence of anhedonic behavior in pre-clinical studies, we have detected a decrease of *Drd3* in the PFC; whether this finding has a causal relation with the anhedonic phenotype of stressed animals or whether it rather represents an epiphenomenon remains to be elucidated.

Using this animal model of depression, we show that SDT can effectively discriminate anhedonic behavior in stressed animals, based on preference values for sugared-pellets. In addition, we complemented food preference analysis with USVs recording. In fact, USVs have been proved to be a powerful tool to characterize rodents' behavior (Burgdorf et al., 2000, 2007). In particular, recording of 50 KHz frequency vocalizations has been shown to be associated with pleasurable and/or rewarding activities (Borges et al., 2013). Interestingly, we found that the total number of 50 KHz vocalizations emitted by uCMS exposed animals during the test is significantly lower comparatively with control animals. Moreover, the fact that the number of 50 KHz vocalizations correlated positively with preference for sugared pellets indicates that this parameter has a strong potential to complement methods currently available to characterize anhedonic behavior. The combination of these two parameters in the SDT, namely food preference and USVs quantification, offers this test the ability to provide a robust multi-parametric measure of hedonic behavior. Moreover, SDT presented high sensitivity to detect the pro-hedonic effects promoted by two different antidepressants, fluoxetine and imipramine. In fact, when comparing SDT with the *gold-standard* SCT using group statistics, results show that both tests can effectively discriminate the anhedonic behavior induced by stress exposure and the improving effects promoted by ADs. However, an individual analysis of animals' performance shows that SDT has a higher ability to discriminate uCMS-exposed animals, which presented lower preference values, prevalently outside the control group score-range. In addition, data from the second experimental set shows that USVs quantification can also be a valuable tool for the discrimination of animals that responded to treatment from non-responder animals. Once more, the quantification of 50 KHz USVs presented a positive correlation with food preference values. It is also important to mention that the longitudinal analysis of the three SDT trials, revealed that in this particular context of an uCMS-based protocol, anhedonic behavior can be detected as early as in the first trial. Although this observation validates the use of a single-trial protocol of the SDT, the longitudinal analysis of the three trials along the administration of ADs shows that different drugs differ in the time needed to elicit their improving effects, thus justifying the use of a multi-trial approach to accurately report their therapeutic effects. Therefore, a careful decision must be made regarding the use of single- or multi-trial protocol, which must be based on the main purpose and the experimental design of the study to be performed.

Finally, our results showed that SDT could effectively discriminate anhedonic behavior in two rat strains (Wistar-Han and Sprague-Dawley), as well as in male and female genders. Although these results are first indication that this test can be successfully used in different rat strains, it will be also imperative to assess its efficacy in mice models, which are currently widely used to address questions on this topic.

Overall, the present work indicates that the multi-parametric approach of SDT represents a valuable refinement of current methods to assess hedonic behavior in rodents, and can be a robust complement to the characterization of this behavioral dimension, with the potential to be implemented across labs. Improvements such as this, demonstrate that conventional paradigms are flexible to further modification, and may contribute to enhance the utility of animal models of complex neuropsychiatric disorders and to expand current knowledge on the neurobiology of mental disease.

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SUPPLEMENTARY MATERIAL

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Chapter VII

GLOBAL DISCUSSION & FUTURE PERSPECTIVES

General Discussion

Along with growing evidence unveiling the many expressions of plasticity that the mature central nervous system harbors, the relationship between this neuro-glioplasticity and the way we adapt and face an ever-changing environment is becoming increasingly comprehended. Indeed, the necessity to explore how adult neural plasticity participates in different brain functions is fundamental, not only to gain insight into the neurobiological basis of enviromentally-induced adaptations and behavioral patterns, but also to understand how our most essential neuroadaptive responses may degenerate into maladaptive pathological outcomes.

Within the work of this thesis we have focused on hippocampal adult neural plasticity, that has long been implicated in the control of different behavioral dimensions and described to be particularly vulnerable to environmental pressures. Of interest to the work here presented, the hippocampus has been purposed has the gateway of the stress response in the brain (McEwen et al., 2016). This is particular relevant since, in a likely convergence of mechanisms, chronic stress exposure has been shown to affect neural plasticity in different cortical and limbic areas, among which the hippocampus is included. The effects in brain plasticity have in turn been associated with the precipitation of multi-dimensional deficits encompassing the core symptoms and some frequent comorbid behavioral traits that relate to the clinical presentation of major depression (Bessa et al., 2009; Snyder et al., 2011; Mateus-Pinheiro et al., 2013a). Besides determining dendritic atrophy within the hippocampus, stress exposure and stress hormones were among the first known regulators of hippocampal cytogenesis (Gould et al., 1992). A pivotal question in the field lies in whether impairments in hippocampal cytogenesis are mechanistically linked to the precipitation of depressive-like behavior (Petrik et al., 2012; Tanti and Belzung, 2013), or may simply represent two coexisting epiphenomena. The second question of paramount importance is to unveil whether the capacity to generate new cells in the hippocampus is necessary to the therapeutical efficacy of antidepressant drugs (ADs).

In the first part of this thesis work we have addressed the role of hippocampal cytogenesis in the onset and recovery from depressive-like behavior, using two different cytogenesis ablation models. In a first study we have administered the anti-mitotic drug, methylazoxymethanol (MAM), to block cytogenesis in young-adult rats. Complementary, in a second study, we further dissected the effects of adult cytogenesis supression in naïve animals, using a transgenic rat model of cytogenesis ablation, the Glial Fibrillary Acidic Protein (GFAP)-Thymidine kinase (Tk) rats (GFAP-Tk rats).

7.1

Hippocampal cytogenesis as a pathophysiological trigger of depression

Decreased hippocampal cytogenesis has been consistently reported to be decreased following stress exposure (Sairanen et al., 2005; Magarinos et al., 2011) and in different animal models of depression. However, the field still struggles to conciliate into a single unifying view the apparently contradictory data regarding the putative causative role of cytogenesis in the precipitation of a multi-dimensional depressive-like phenotype.

During the last 15 years, cytogenesis ablation studies have been fundamental to study the functional correlates of adult hippocampal cytogenesis in physiological conditions, but also to explore its participation in psychopathological contexts. Three types of strategies have been adopted to abrogate post-natal cytogenesis and study its impact on brain homeostasis: **i)** the use of x-ray irradiation (Santarelli et al., 2003; Surget et al., 2011), which is a regionally restricted procedure, but that lacks cell-specificity as it affects also mature cells and elicits a moderate inflammatory response; **ii**) the use of cytostatic drugs (Bessa et al., 2009; Jayatissa et al., 2009), which are able to abrogate cytogenesis in all cytogenic niches of the brain, but that again do not spare other mitotic cell populations; **iii)** the use of transgenic mice lines (Saxe et al., 2006; Kirshenbaum et al., 2014), that allow for the specific (promoter-directed) ablation of neural stem cells, without interfering with other cell types. In a previous work from our lab, we administered MAM to naïve animals and analyzed the behavioral profile immediately after the cessation of MAM-induced cytogenesis suppression (Bessa ate al., 2009). Results showed that blocking cytogenesis did not promote the development of short-term depressive-like behavior. Indeed, the same dissociation between hippocampal cytogenesis abrogation and the precipitation of depressive-like behavior has also been documented by several authors (Holick et al., 2008; David et al., 2009). Contrastingly, some authors have challenged these results, and showed that blocking hippocampal cytogenesis produces increased immobility in the FST, as well as anhedonic behavior in the SPT (Snyder et al., 2011).

Although these results are apparently at odds with each other, we hypothesized that a likely source of discrepancy between studies was related to the experimental design and time-point of analysis. In fact, in adult rodent neurogenesis, and despite the ability of new cells to establish primordial synapses with CA3 pyramidal cells within a week, a newborn cell takes approximately 4 to 5 weeks to maturate and integrate the pre-existing circuitry (Ming and Song, 2005). Taking this temporal dynamic of the hippocampal cytogenic process, it comes to light the fact that studies analyzing the behavioral correlates of neurogenesis immediately after its abrogation are largely targeting a population of immature neurons and assessing the functional impact of these subset of cells. As a result, the full extension of the hippocampal cytogenic process has been neglected and the long-term consequences of the loss hippocampal cell turnover remain obscure.

To address this question, we have followed a similar approach as before, administering MAM to adult naïve rats, but instead of immediately analyzing the behavioral consequences of cytogenesis ablation, we only conducted behavioral tests 4 weeks post-ablation (Mateus-Pinheiro, 2013a; **Chapter 3**). Our results show that MAM administration effectively reduced the number of new neuronal ($NeuN^+$) and astroglial (GFAP) cells that succeed to differentiate and integrate into the pre-existing circuit. Regarding these ablation methods, it is important to highlight that at the time-point of our analysis, 4 week post-ablation, hippocampal proliferation was not restored to basal levels, as reflected by the reduced levels of $Ki\text{-}67^+$ cells present in MAM-treated. This aspect is critical, since it allows us to conclude that with this approach we are able to study the long-term consequences of devoiding the hippocampus of both new mature and immature cells.

Taking this into consideration, our results showed that hippocampal suppression by MAM was sufficient to induce increased time of immobility in the FST, typically interpreted as measure of depressive-like behavior in rodents (Cryan and Holmes, 2005). Moreover, the ablation of new mature cells in the adult hippocampus also induced the long-term manifestation of anhedonic behavior in the SCT. As depression has a complex and heterogeneous clinical manifestation, consisting not only in mood deficits, but also in the development of anxiety and cognitive disabilities, we explored wether hippocampal cytogenesis ablation would precipitate long-term behavioral impairments in these dimensions. Indeed, MAM-treated animals displayed heightened anxiety-like behavior manifested in two independent test paradigms use to measure this dimension (the EPM and the NSF). Moreover, and although we did not detect deficits in spatial reference memory, when measuring the escape latency, we did observe long-term deficits in working memory and behavioral flexibility, 4 weeks following ablation. Hence, our data shows that

MAM-induced hippocampal cytogenesis suppression is sufficient to elicit long-term multi-dimensional behavioral deficits commonly observed in depressed patients.

Placing this results side by side with those of Bessa et al. (2009), in which the analysis was conducted immediately after the cessation of MAM treatment, some differences are apparent. While we were able to detect long-term mood-related deficits, these were absent when the analysis was performed shortly after cytogenesis ablation (Bessa et al., 2009). However, regarding anxiety-like behavior the picture seems quite different. In fact, and similarly to our long-term analysis, Bessa et al. reported that the MAM-treatment was able to induce short-term deficits in anxiety behavior. Altogether these observations reinforce that the timeframe of the experimental design has critical influence in the results outcome. These results suggest that while mature and integrated neuronal and glial cells are likely to participate in the control of mood and hedonic behavior, immature newborn hippocampal cells seem to exert an early impact on anxiety-like behavior, which is extended to the late maturation phase. Unfortunately, a comparison with the short-term effects of cytogenesis ablation in cognitive performance is not possible, as these have not been assessed in the same study.

Although it has been shown by us and others that using MAM at a dosage bellow 10 $mg.Kg^{-1}$, does not seem to have a deleterious impact in general measures of animal well-being and inflamatory profile, as compared to untreated animals (Bessa et al., 2009; Jayatissa et al, 2009), it is difficult to control at what extent are the obtained results influenced by the cytostatic systemic effects of MAM in other mitotic cell populations. For this reason, we conducted a second study using a more directed cytogenesis ablation approach, using the GFAP-Tk rat model. With this model, we were able to selectively ablate $GFAP^+$ neural stem cells (NSCs). As the cell death mechanism underlying this approach requires the metabolization of ganciclovir (GCV) by the viral thymidine kinase, leading to the production of a cytotoxic product, it only affect dividing cells and spares $GFAP^+$ mature glial cells (Groves et al., 2013).

Using this model we conducted a subsequent study, whose design took in consideration the temporal dimension of adult cytogenesis (**Chapter 4**). For that we defined two time-points of analysis, to explore the functional consequences of ablating either immature newborn cells or fully differentiated new neuronal and glial cells. Our results show that GCV administration was able to markedly decrease the number of short-term proliferating cells ($BrdU^+$ cells) and neuroblasts (DCX^+ cells). Consequently, the same treatment also affected cell survival, showed by a significant

decrease in BrdU/NeuN and BrdU/GFAP double-positive cells, analyzed 4 weeks post-ganciclovir treatment. Making a parallelism with previous studies in which MAM was used, we started to analyze the same behavioral dimensions, immediately after ganciclovir treatment and in a second set of experiments 4-weeks post-ablation.

In our short-term analysis, we were not able to detect any effects of cytogenesis ablation in FST immobility time, or any short-term alteration of hedonic behavior in both SCT or the SDT. However, and as previously reported using MAM, GCV administration to GFAP-Tk rats elicited the short-term manifestation of anxiety-like behavior, manifested as an increased latency to feed in the NSF. Notably, these results are in line with those reported by Bessa et al., and reinforce how immature cells differentially affect mood- and anxiety-related behaviors. Furthermore, we also conducted a short-term analysis of different cognitive categories, using paradigm variation of the water maze test. Our results indicate that cytogenesis ablation did not produce short-term impairments in spatial reference memory, working memory or behavioral flexibility.

The behavioral profile of animals with cytogenesis ablation 4 weeks after GCV treatment was remarkably different. Here, the lack of new mature neuronal and glial cells produced long-term anhedonic behavior, manifested in both SCT and the SDT. However, we did not detect a significant increase in immobility in the FST. GFAP-Tk animals treated with GCV also displayed long-term anxiety-like behavior, measured in the NSF. Concerning cognitive behavioral dimensions, while we did not find any interference of cytogenesis ablation with the escape latency in a spatial reference memory task, the lack of new mature cells proved to have long-term deleterious effects in working memory and behavioral flexibility. Altogether, the results obtained using this genetic cytogenesis ablation approach largely replicate the results obtained in our previous studies with MAM (Bessa et al., 2009; Mateus-Pinheiro et al., 2013a). An exception was observed concerning the FST, where no short-term or long-term effects were observed upon genetic hippocampal cytogenesis ablation. Since this test relies in the animal motor function, it could be hypothesized that MAM could have collateral systemic effects affecting locomotion. This possibility is unlikely, since we have shown no alteration in exploratory behavior or swim velocity in our studies. A second possibility could be related with differences in the temporal persistence of the anti-cytogenic action between the two ablation methods: while in GFAP-Tk animals, normal hippocampal proliferation has been shown as early as after 8 days post-GCV treatment (Denny et al., 2012), we have shown sustained decreased of hippocampal proliferation 4 weeks post-MAM treatment. This implies that contrarily to the GFAP-Tk model, in which we are able to specifically study the

importance of new hippocampal cells at a time when they were expected to be mature cells without compromising the subsequent generation of new immature cells, with MAM , we promote a sustained abrogation of both immature and mature cells. This more severe ablation may account for the emergence of deficits in the FST. A third hypothesis, could lie on the very nature of the test itself. Although the validity of the test to measure antidepressant response is beyond contestation, the question of whether this test is actually modeling/ measuring depressive-like behavior in rodents is difficult to answer. This reinforces the need to develop more robust test paradigms to quantify expressions of emotional behavior in rodents. This is where the relevance of multi-parametric tests, such as the newly developed sweet drive test (SDT) come to light. Indeed, we have shown that the combination of quantifying preference for a sugared isocaloric food with the quantification of 50 Khz ultrasonic vocalizations presented increased sensibility to detect not only anhedonic behavior but also to distinguish antidepressant-responders, from non-responders (Mateus-Pinheiro et al., 2014; **Chapter 6**).

Fundamentally, both studies presented in this thesis support two important conclusions: first, that hippocampal cytogenesis ablation is sufficient to elicit long-term behavioral deficits in emotional and cognitive domains, known to be compromised following chronic stress exposure and in major depression; second, both studies corroborate the hypothesis that we had previously put forward concerning the importance of taking into account the full temporal extension of adult cytogenesis, as reflected by the contrasting behavioral phenotypes that emerge form the ablation of either immature or fully differentiated cells (**Figure 1**).

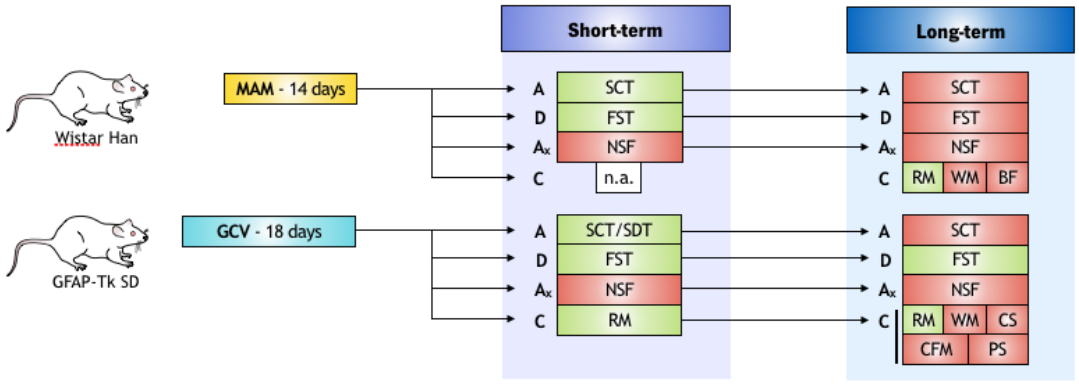

Figure 1. Results comparison between two cytogenesis ablation experiments. Both short-term and long-term behavioral profiles are shown. Green boxes depict behavioral tents in which normal performance is preserved. Red boxes depict behavioral tests, in which animals performance became affected. SCT- sucrose consumption test; SDT- sweet drive test; FST- forced swimming test; NSF- novelty suppressed feeding; RM- spatial reference memory task; WM- working memory task; BF- behavioral flexibility task; CS- cognitive strategies analysis; CFM - contextual fear memory task; PS- pattern separation task. n.a. - not assessed. A-anhedonic behavior; D-depressive-like behavior; Ax-Anxious behavior; C-Cognitive Performance

Since we observed that the ablation of newborn cells produced significant long-term cognitive deficits, we used the GFAP-Tk model to further characterize the participation of neuro- and gliogenesis in cognitive dimensions that have been previously considered as functional correlates of adult cytogenesis (**Chapter 4**). Therefore, we submitted animals to additional cognitive tasks, 4 weeks post-ablation. Curiously, although we were not able to detect effects of cytogenesis ablation on the escape latency in a spatial reference memory tasks, we report alterations at the level of cognitive strategies employed to reach the escape platform. Indeed, GFAP-Tk animals treated with GVC showed delayed transition from non-hippocampal dependent to hippocampal dependent strategies in the same test paradigm. Corroborating these results, we have reported a similar finding in animals lacking the transcription factor AP2γ, that is fundamental to preserve normal levels of adult glutamatergic hippocampal neurogenesis (**Appendix 1**). The participation of adult neurogenesis in this form of cognitive flexibility had previously been demonstrated (Gu et al., 2012; Garthe and Kempermann, 2013), and highlights how analysis refinement of test paradigms that have long been used may be fundamental to finely dissect how hippocampal cytogenesis impact on different forms of behavior. In addition, cytogenesis ablation also promoted the long-term manifestation of deficits in contextual fear memory, as well as in pattern separation, measured in an adapted version of the NOR test.

Interestingly, we also found that cytogenesis ablation promoted the development of long-term impairments in the inter-regional communication between the ventral hippocampus and the medial PFC. As both regions have been implicated in some of the studied behavioral dimensions, it is likely that the lack of new mature cells may promote profound alterations in the cortico-limbic neuro-glial networks, contributing to the observed cognitive deficits.

7.2

Hippocampal cytogenesis as a neural substrate in the spontaneous and antidepressant-mediated remission from depressive behavior

The pro-cytogenic actions of different antidepressant drugs (ADs) has been consistently reported in several works (Surget et al., 2008; Perera et al., 2011). Similarly to the conflicting reports regarding the involvement of cytogenesis in the precipitation of depressive behavior, studies addressing whether hippocampal cell turnover is essential for AD's therapeutical efficacy also report inconsistent results. In a previous work from our lab, we have provided evidence for the non-reliance of monoaminergic ADs on adult hippocampal cytogenesis; instead, short-term behavioral improving effects of these drugs seem to be attributable to rapid reversal of stress-induced neuronal dendritic atrophy in the hippocampus and prefrontal cortex (PFC). Nevertheless, and taking again into consideration the temporal progression of hippocampal cytogenesis, studies such as these have focused on the contribution of new cells to behavioral-improving actions of ADs, shortly after the conclusion of treatment, when these cells are still essentially immature.

Following the assessment of the effects of MAM administration in the long-term behavioral profile of naïve animals, we sought to explore the long-term impact of hippocampal cytogenesis to both spontaneous and ADs-induced remission from a depressive phenotype (Mateus-Pinheiro et al., 2013a; **Chapter 3**). We submitted young-adult rats to 6 weeks of an unpredictable chronic mild stress (uCMS) protocol to induce core symptoms of depressive-like behavior and analyzed the behavioral impact of MAM administration in the remission from depressive-like behavior 4 weeks later. Similarly to MAM effects on cytogenesis, uCMS exposure induced a pronounced decrease in hippocampal cyotgenesis, compromising the generation and survival of both new neuronal (BrdU/NeuN double-positive) and (BrdU/GFAP double-positive) astroglial cells. However, in spite of the fact that stressed animals without MAM treatment were able to revert hippocampal cytogenesis to basal physiological levels 4 weeks after uCMS conclusion, those receiving MAM treatment exhibited long-lasting impairments in hippocampal cytogenesis. When analyzing the implications of cytogenesis ablation to the ability of adult animals to spontaneously remit from multi-

dimensional behavioral deficits, we have been able to conclude that hippocampal cytogenesis is indeed critical for this process (**Figure 2**). In fact, MAM administration to chronically stressed animals promoted persistent and long-lasting deficits in hedonic behavior, anxiety traits and cognitive function (working memory and behavioral flexibility). Contrastingly, stressed animals in which cytogenesis was not ablated were able to, either partially or completely, recover from these deficits. Curiously, results obtained in the FST show that stressed animals are able to spontaneously recover from "depressive-like behavior" measured in this test paradigm. Considering that in the previous set of experiments MAM administration to control rats elicited long-term deficits in the FST, it becomes likely that both the mechanism and abruptness of cytogenesis ablation may account for these different results: in light of the fact that in stressed animals the progressive anti-cytogenic effect resulting from uCMS exposure precedes that of MAM administration, it is possible that these animals are able to develop compensatory mechanisms that reinforce their resilience to the subsequent anti-mitotic insult induced by this drug. In fact, unpublished data from our group shows that animals in which a stress-induced decrease in hippocampal cytogenesis had already been elicited, show increased resilience to a subsequent period of stress exposure (Alves et al., unpublished). Alternatively, and as discussed before, this apparent discrepancy may relate to the natural variability or intrinsic limitations of the FST to measure what is believed to be a proxy of depressive behavior in rodents.

Although MAM cytostatic action was not spontaneously restored, it has proved to be pharmacologically reversible by chronic administration of two monoaminergic ADs, fluoxetine and imipramine. This aspects correlates with the behavioral improving actions that we observed in treated animals. Curiously, the action of the used ADs was differentially affected by MAM co-administration. While the efficacy of fluoxetine became significantly compromised upon cytogenesis ablation, that of imipramine remained virtually unaffected. This may relate with the type of pro-cytogenic response elicited by each antidepressant, as neurogenesis had essentially a pro-neurogenic action, while imipramine promoted a strong pro-gliogenic response. Although we are unable to be sure if this relates with the different magnitude of MAM action in the efficacy of both drugs, these findings add up a new perspective into the debate of whether hippocampal cytogenesis is needed to mediate the actions of ADs. This perspective is grounded in two premisses: one relates to a "when", and the second relates to a "what". Indeed, while we have seen before that hippocampal cytogenesis was not determinant for the short-term actions of ADs, their relevance is clear in the long-term remission mediated by these drugs, highlighting how deciding *when* to experimentally approach this question dictates different outcomes. Moreover,

these results support the idea that deciding *what* to study is equally determinant. As different classes of ADs elicit different types of pro-cytogenic actions and depend at different extends from ongoing hippocampal cell genesis, this is very likely to be an important source of discrepancy among studies.

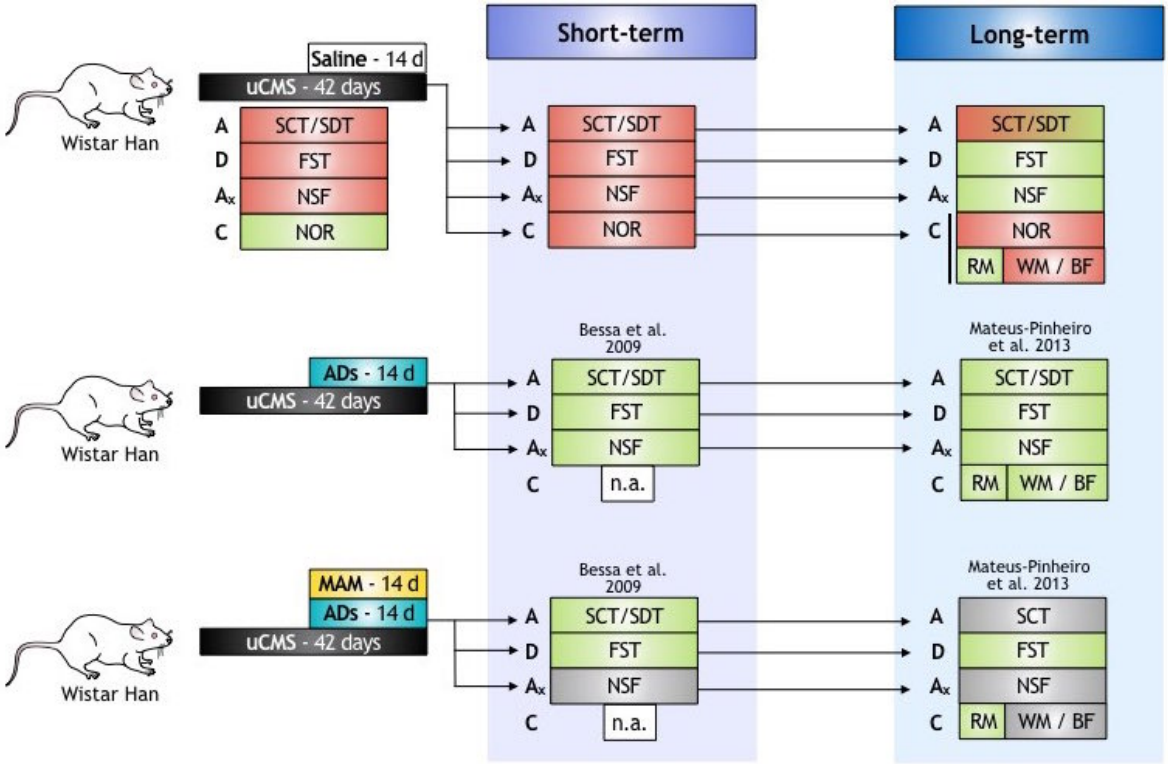

Figure 2. Spontaneous and ADs-induced remission from a multi-dimensional depressive phenotype. Both short-term and long-term behavioral profiles are shown. Green boxes depict behavioral tents in which normal performance is restored. Red boxes depict behavioral tests, in which animals performance remained affected. Gray boxes correspond to behavioral dimensions in which cytogenesis ablation promoted blunted antidepressant effects. SCT- sucrose consumption test; SDT- sweet drive test; FST- forced swimming test; NSF- novelty suppressed feeding; RM- spatial reference memory task; WM- working memory task; BF- behavioral flexibility task; CS- cognitive strategies analysis; CFM - contextual fear memory task; PS- patter separation task. n.a. - not assessed. A-anhedonic behavior; D-depressive-like behavior; Ax-Anxious behavior; C-Cognitive Performance.

7.3

The importance of neural plasticity in the adult hippocampus for the onset and remission from depression: a matter of time

Taken together, the above discussed results support the view of a time-dependent participation of hippocampal neural plasticity in both the pathological manifestation of depression-like behavior and the remission from the installed emotional and cognitive deficits.

Concerning the participation of hippocampal neural plasticity in the development of depressive traits, several works have purposed the association of hippocampal neuronal dendritic atrophy (Pittenger and Duman, 2008; Bessa et al., 2009), as well as astroglial structural remodeling (Czeh et al., 2006) with the short-term manifestation of different behavioral deficits, typically evidenced by depressed patients. In contrast, several cytogenesis ablation studies have failed to detect the short-term development of behavioral deficits in most of the dimensions that we discussed above. The exception lies on anxiety, as abrogation of hippocampal cell genesis is sufficient to induce short-term heightened anxiety signs. Contrastingly, the two ablation approaches used in this thesis work have provided consistent evidence for the participation of adult cell genesis in the long-term development of anhedonic and hyper-anxious behavior, along with different cognitive impairments. Integrating our results with those of other authors, we have purposed a bi-phasic model for the participation of hippocampal neural plasticity in the pathophysiology of depression (Mateus-Pinheiro et al., 2013b). The suggested model, has in consideration the longitudinal course of the disease, as well as the very dissimilar temporal dynamics of hippocampal neuroplastic processes (**Figure 3**). Hence, we purpose that the fast neuroplastic response at the level of neuronal synaptic dendritic remodeling and astroglial morphological changes following chronic stress exposure serve as initiators for the installment of short-term behavioral impairments. Contrarily to the rapid neuro-glial morphological changes, hippocampal neuro- and astrogliogenesis are slower processes, that take several weeks to be accomplished. Accordingly, the behavioral manifestations of anti-cytogenic insult mainly come to light approximately 4 weeks later, when cells that had been otherwise generated within the hippocampus, were expected to be fully functional and integrated in the pre-existing neuro-glial

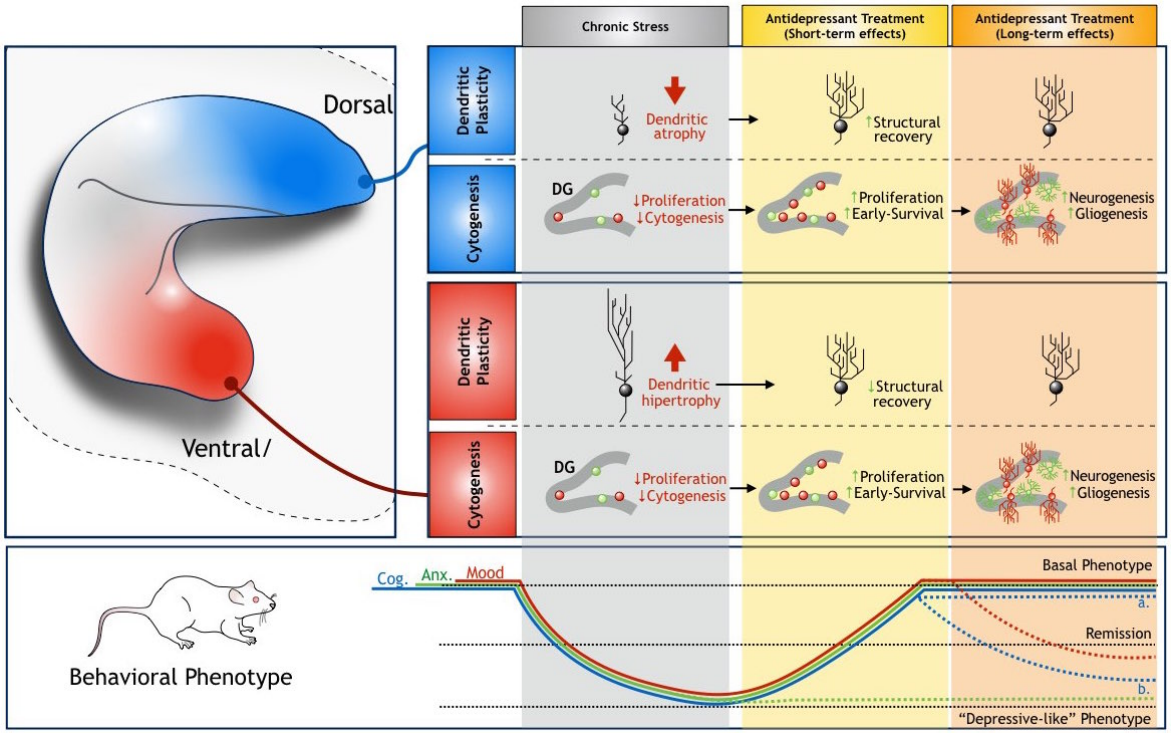

Figure 3. Hippocampal Neuroplasticity in the remission of the depressive-phenotype: the dorso-ventral dichotomy. Chronic stress exposure has opposing effects in dendritic plasticity of the dorsal (dDG) and (vDG). While promoting neuronal atrophy in granule neurons of the dDG, chronic stress promotes neuronal hypertrophy in the vDG. In both hippocampal poles, stress exerts an anti-cytogenic action. Antidepressants (ADs) revert neuroplastic deficits in both hippocampal poles and reestablish the basal phenotype in behavioral dimensions previously affected, namely mood, anxiety and cognitive performance (full lines). Dashed lines represent behavioral alterations following cytogenesis ablation, 28 days before. Cytogenesis ablation has immediate short-term detrimental effects on ADs efficacy to revert anxiety behavior (dashed green line). Contrastingly, ADs are able to promote short-term behavioral improvement on mood and cognitive performance, but fail to reveal a sustained long-term effects following cytogenesis ablation (red and blue dashed lines). While ADs present sustained effects on some cognitive dimensions (a.), cytogenesis ablation compromises remission of a subset of cognitive fundtions.

network. Interestingly, these results are in line with a previous study, reporting no alterations 1 week after cytogenesis ablation, but unveiling the development of cognitive deficits when the analysis was conducted 2 and 4 weeks post-ablation (Denny et al., 2012). In addition, a recent study has showed how optogenetically interfering with the function of 4-weeks old new neurons, but not with those of 1- or 8 weeks old, elicited the development of impairments in contextual fear memory and in cognitive strategies used in the water maze (Gu et al., 2012). Unfortunately, none of these studies addressed emotional dimensions of behavior. Nevertheless, our results further support the emerging idea of an important role of immature newly-born cells in the neurobiological mechanisms underlying anxiety behavior.

In a similar fashion, we suggest the therapeutical action of ADs to rely in a bipartite neural-plastic basis: while having a pro-cytogenic effect, the short-term action of ADs rely on their modulatory effects upon genes responsible for the observed rapid recovery from neuromorphological alterations that ensued chronic stress exposure (Pittenger and Duman, 2008; Bessa et al., 2009). The potentiation of hippocampal neuro- and gliogenesis will be fundamental latter on, once new cells attain full development and become essential to the sustained remission from depression. Moreover, differences in the potential to trigger a pro-cytogenic response by different antidepressant agents may explain, at least partially, why some antidepressant classes are more associated to relapse after initially signs of remission.

In conclusion, our results substantiate the need to consider the longitudinal course of neuro-glioplastic processes in both the development and remission from depressive behavior, in order to achieve a more comprehensive view on the participation of adult hippocampal plasticity in both contexts. Among many other influencing factors, it becomes increasingly evident that the apparent data incongruence that has marked this topic during the last years may actually relate to a matter of *time*.

7.4

Epigenetics and the stressed brain: is there a role for DNA 5hmC?

Along with the importance to study the participation of neural plasticity in physiologic and pychopathological contexts, runs the need to unveil the regulatory mechanisms behind these phenomena. Among many other forms of molecular regulation already identified to orchestrate hippocampal neural plasticity, mounting evidence support the importance of epigenetic regulation as transducer of environmental stimuli into functional and structural remodeling of the post-natal hippocampus (Sun et al., 2011).

We have conducted a longitudinal experiment, using a similar uCMS protocol as before, in which we defined three time-points of analysis, to achieve a full perspective on both short- and long-term aspects of hippocampal neural plasticity (**Chapter 6**). In brief, our longitudinal behavioral characterization allowed us to characterize how different emotional and cognitive modalities are affected across time. In our uCMS-based animal model of depression, mood related deficits seem to be transversally installed along all time-points, exhibiting signs of partial remission 4 weeks after subsiding stress exposure. In the case of anxiety behavior, deficits in this dimension are precipitated shortly following the initial weeks of stress exposure, but are readily reversed at long-term. The immediate precipitation of stress-induced anxiety signs, along with its complete reversion on latter time-points, are in agreement with the previously discussed idea of the association between this behavioral dimension and short-term neuroplastic alterations, such as rapid dendritic remodeling and the modulatory action of immature newborn hippocampal cells. Contrastingly, cognitive memory deficits are absent in earlier stages of the uCMS protocol. Instead, this deficit emerge only after 6 weeks of stress exposure and, interestingly, persists in untreated animals long after subsiding stress exposure. The late manifestation of these deficits again suggests an important association with the functional importance of new mature hippocampal cell population, that is impoverished at latter time points, following the precedent stress-induced anti-cytogenic insult.

Taking into account the neuro-anatomical and functional dichotomy existing between the dorsal and ventral hippocampus, we analyzed neural plasticity occurring in both regions independently. While we found a significant dendritic atrophy of granule cells in the dorsal DG (dDG) following stress exposure, we described a contrasting hypertrophic effect in granule cells of the ventral DG (vDG). Curiously, the vDG is anatomically and functional linked to the extended amygdala, in which neuronal hypertrophy have also been described (Vyas et al., 2004). Moreover, a similar effect was described specifically within the ventral hippocampus, in CA3 pyramidal neurons (Pinto et al., 2015). Regarding hippocampal cytogenesis, stress promoted a significant decrease in cell proliferation and long-term survival in both regions. However, as discussed in **Chapter 6** monoaminergic ADs seem to have pro-cytogenic effects that are manifested at different extents along the DG septo-temporal axis, and even differentially manifested along the transversal axis within the dDG.

When exploring wether epigenetic alterations related with DNA demethylation pathways were affected by stress and ADs treatment, the dorso-ventral dichotomy was also evident. A global decrease in DNA 5hmC levels was only detectable within the dDG, along with decreased expression specifically of TET3, suggesting perhaps a greater vulnerability of this region to environmentally-induced epigenetic alterations. Importantly, these alterations are not specific of post-mitotic cell populations, as newborn cells were also shown to be affected, supporting the possibility that DNA demethylation pathways may influence hippocampal cell genesis in stress exposure conditions. Moreover, our oxRRBS analysis revealed that, although global 5hmC variations were only detected in the dDG, both regions are differentially affected by uCMS and ADs treatment. Within the dDG, we have found decreased hydroxymethylation in genes involved in the positive regulation of hippocampal cytogenesis promoted by stress, which were reversed by fluoxetine treatment. Contrastingly, in the vDG we identified a stress-induced hydroxymethylation of GR and MR coding genes, absent in control or fluoxetine-treated animals. These results are still to be confirmed by glucMS-qPCR and to be correlated to transcriptomic data that we have recently generated using the same experimental paradigm (Patricio et al., 2015). Theoretically, if these alterations in hydroxymethylation levels are confirmed to be positively correlated with gene expression, our results may support an important role of DNA demethylation pathways in the modulation of hippocampal cytogenesis within the dDG. Interestingly, the dDG corresponds to the DG domain were we have observed a greater AD-induced cytogenesis promotion. In the same line of thought, the hyper-hydroxymethylation of genes coding corticosteroid receptors, may highlight

the already purposed pivotal importance of the ventral hippocampus in the mediation of the stress response. If confirmed, these results may pinpoint DNA demethylation mechanisms as determinant for the role of the hippocampus as a gateway of the systemic stress response into the CNS. Interestingly, we have recently identified the presence of glucocorticoid response elements (GRE) within the promoter region of *tet3* (*unpublished data*), further suggesting a functional crosstalk between stress response and the DNA demethylation pathways.

Besides the already mentioned confirmatory analysis, this study would benefit from the following subsequent steps:

- To assess wether chronic administration of corticosterone to naïve animals can reproduce our findings, further endorsing the relation between stress hormones and the observed alterations;

- To study alterations in 5hmC levels and *tet* genes expression in both undifferentiated and differentiated neurosphere cultures, supplemented with corticosterone and monoaminergic neurotrasnmitters, to pinpoint the epigenetic alterations that are specifically promoted in either pos-mitotic or progenitor cell populations.

- To characterize hippocampal neuroplastic and behavioral alterations found in a conditional forebrain tet3 knockout model, to further dissect the link between tet enzymes and the observed neuro-behavioral phenotype.

7.5

Concluding Remarks & Future Perspectives

In conclusion, through complementary experimental approaches, we have provided evidence for the participation of different forms of hippocampal neural plasticity in several cognitive dimensions and emotion-related behaviors, both in the healthy and stressed/depressed brain. Moreover, the results of this thesis contribute to a better comprehension on how adult hippocampal cytogenesis modulate different modalities in a time-dependent manner, emphasizing the importance of considering the longitudinal course of neural plasticity processes in the evaluation of their impact in both basal and psychopathological conditions.

Further insight on this field will come with the development of strategies to selectively ablate either neuro- or gliogenesis independently. Such approach will allow to precisely underpin the individual participation of new neuronal and glial cells to different brain functions, as well as their role in the onset and remission from stress-related disorders, such as depression. Moreover, viral tracing techniques may be used to better elucidate how new cells promote the reconfiguration of cortico-limbic circuits. In the context of depression, this would allow to characterize a possible underlying “deconectome” or “reconectome”, and how inter-regional connections are modulated by different antidepressant pharmacological classes. Although we normally associate typical antidepressant treatment with the potentiation of the endogenous brain plasticity, additional therapeutical advances may stem from exploring the potential of alternative approaches seeking to extend the cytogenic potential to other fundamental brain regions, such as the prefrontal cortex (PFC). These approaches, although challenging in many aspects, may contribute to reinforce cortico-limbic connections, known to become impoverished in depression, such as the ventral hippocampal-PFC projections. Finally, progress may come from the effort to develop non-invansive neuroimaging techniques to monitor forms of neuroplasticity in the human brain. Even if presently this may configure an utopian idea, to embrace this challenge is also to bravely acknowledge that this field is arriving to a developmental plateau from which progression is strikingly limited due to the lack of translational validation, that would ideally come from human data.

What remains indisputable, in virtue of its participation in a broad range of physiological and functional brain processes, is the need to continue to unveil the many facets of adult neural plasticity. Indeed, in a broader perspective, understanding how neural plasticity remodels our brain is to seek comprehension on how it reshapes the way we behave and, ultimately, how it defines the very essence of what we are.

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Chapter VIII

APPENDIX

AP2γ regulates adult glutamatergic neurogenesis and modulates cognitive function

A. Mateus-Pinheiro*, N.D. Alves*, P. Patrício, A.R. Machado-Santos, E.L. Campos, J. Silva, V.M.Sardinha, J. Reis, H. Schorle, J.F. Oliveira, J. Ninkovic, N. Sousa, L. Pinto

(*submitted*)

AP2γ regulates adult glutamatergic neurogenesis and modulates cognitive function

António Mateus-Pinheiro[1,2*], Nuno Dinis Alves[1,2*], Patrícia Patrício[1,2], Ana Rita Machado-Santos[1,2], Eduardo Loureiro Campos[1,2], Joana Silva[1,2], Vanessa Morais Sardinha[1,2], Joana Reis[1,2], Hubert Schorle[3], João Filipe Oliveira[1,2], Jovica Ninkovic[4,5], Nuno Sousa[1,2], Luisa Pinto[1,2]

[1]Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal

[2]ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

[3]Department of Developmental Pathology, Institute for Pathology, University of Bonn Medical School, Bonn, Germany

[4]Institute for Stem Cell Research, Helmholtz Centre Munich German Research Center for Environmental Health (GmbH), 85764 Neuherberg, Germany

[5]Physiological Genomics, Medical Faculty, University of Munich, Schillerstrasse 46, 80633 Munich, Germany

* These authors contributed equally to this work

Correspondence to: luisapinto@ecsaude.uminho.pt

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Abstract

Adult neurogenesis represents one form of non-synaptic neuroplasticity that is critical both for brain homeostasis and for adaptations to the ever-changing environments; however, there is still much to disclose regarding the functional role of transcription factors that regulate adult neurogenesis. Here, we identified AP2γ as transcription factor regulating the generation and maturation of new glutamatergic neurons in the adult brain. In addition, we demonstrate the relevance of AP2γ in hippocampal and prefrontal cortex activity and its implications to distinct emotional and cognitive functions.

Introduction

Although this notion is very likely to be revisited in the near future, there are two brain regions consensually accepted as neurogenic niches in the adult brain, where new cells are persistently generated throughout life: while cells born in the subgranular zone (SGZ) become mostly glutamatergic neurons of the hippocampal dentate gyrus, those originated in the subependymal zone (SEZ) become largely GABAergic interneurons of the olfactory bulb[]. Research in recent years has provided insights on the transcriptional network involved in the regulation of neurogenesis, both in early developmental stages and during adulthood[3,6-11]. During cortical development the regulation of glutamatergic neurogenesis is controlled by a set of transcription factors including Pax6, Tbr2, NeuroD and Tbr1, with implications on proliferation, cell cycle kinetics, lineage and fate specification, regional identity, axonal growth and cell adhesion processes[]. Interestingly, the transcriptional sequence (Pax6→Tbr2→NeuroD→Tbr1) regulating cortical glutamatergic neurogenesis during development is recapitulated in the adult hippocampus and, with some variations, also participates in cell commitment to glutamatergic lineages in the SEZ[]. Activating Protein 2 gamma (AP2γ, also known as Tcfap2c or Tfap2c) is a transcription factor that integrates the transcriptional network of

glutamatergic neurogenesis during early developmental stages[], as it directly regulates the basal progenitor fate determinants Math3 and Tbr2[]. Deletion of AP2γ in the developing cortex results in a specific reduction of upper layer neurons in the occipital cerebral cortex, while its overexpression potentiates region- and time-specific generation of cortical layers II/III. Yet, the function of AP2γ in the adult neurogenic niches remains to be disclosed. Herein, we addressed the question of whether AP2γ is an active determinant of adult glutamatergic neurogenesis and whether its function is relevant for the homeostasis of different emotional dimensions, as well as for the integrity of cognitive function. Indeed, we report here an important role of AP2γ in the regulation of adult glutamatergic neurogenesis, both in the adult hippocampal dentate gyrus (DG) and in the SEZ, as well as in the preservation of emotional and cognitive behaviors.

Results

AP2γ expression in the adult brain

As a first step to characterize AP2γ function in the adult CNS, we used an in situ hybridization approach to identify the regional expression patterns of the gene. AP2γ mRNA expression was exclusively observed in two adult neurogenic niches, the subependymal zone (SEZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) (**Fig. 1a**). Specifically, in the DG AP2γ-mRNA positive cells were predominantly localized in the SGZ, although positive cells were also found more distally in the granular region (**Fig. 1a**). In addition, AP2γ-mRNA positive cells were present along the SEZ, including the dorsal wall (**Fig. 1a**), as well as in the rostral migratory stream (RMS) and the molecular cell layer (MCL) and glomerular layer (GL) of the olfactory bulbs (OB) (**Supplementary Fig. 1a**). Both in the dorsal wall of the SEZ and SGZ of the DG, AP2γ-mRNA positive cells were co-labeled with BrdU-label retaining progenitor cells (neural stem cells labeled

after an 8-week chase period), and with Tbr2 positive cells (**Fig.1b**). Furthermore, in the OB, AP2γ-mRNA positive cells were also co-labeled with BrdU-positive newly generated neurons and with Tbr2-positive cells both in the MCL and GL (**Supplementary Fig. 1b-d**). Immunohistochemistry analysis revealed that most AP2γ positive cells in the SEZ and in the SGZ were also positive for doublecortin (DCX) (61.5% ±2.7 and 53.6% ±3.6 in the DG and SEZ, respectively; **Fig. 1c-e**), while a smaller subset of these cells expressed Tbr2 (21.3% ±4.1 and 10.9% ±7.9 in the DG and SEZ, respectively; **Fig. 1c, d**), indicating a predominant commitment of AP2γ positive cells to the glutamatergic neuronal lineage. AP2γ positive cells were also positive for BrdU-label retaining progenitor cells (after an 8-week chase period), both in the SEZ and DG (13.9% ±3.5 and 7.7% ± 2.4 in the DG and SEZ, respectively; **Fig. 1c, d**), showing that a small portion of AP2γ positive cells are slow dividing progenitor cells. Importantly, we did not find any co-localization between AP2γ positive cells and astroglial cells ($GFAP^+$ cells) or mature neurons ($NeuN^+$ cells) in both neurogenic niches, while most were co-localizing with $Ki67^+$ proliferating cells (**Supplementary Fig. 2**). Thus, this data reveals that AP2γ (both mRNA and protein) expression persists in the adult brain, being present in the neurogenic niches and most predominantly expressed in cells of the glutamatergic lineage.

AP2γ regulates adult neurogenesis

After identifying the presence of AP2γ in progenitors and neuroblasts of the adult neurogenic niches, we sought to understand its role in the neuronal fate specification of Neural Stem Cells (NSCs) primary cultures, derived from both the adult SEZ and DG[21]. For that we used Moloney murine leukemia virus–based retroviral vectors containing Cre-IRES-GFP to infect cultured NSCs from controls and mice containing AP2γ flanked by loxP sites (henceforth referred to as AP2γfl/fl) to delete AP2γ (**Fig. 2**). In the SEZ, viral-mediated deletion of AP2γ reduced the percentage of mixed clones (clones containing both neuronal (Tuj1-positive) and non-neuronal cells; IRES-GFP: 59% ± 3.7,

Cre-IRES-GFP: 27.3% ± 3.1, t_{18} =6.567, P <0.0001) at the expense of a significant increase in the generation of non-neuronal clones (exclusively formed by non-neuronal (TuJ1-negative) cells; IRES-GFP: 27.4% ± 7.1, Cre-IRES-GFP: 60.6.3% ± 6.2, t_{18} =3.522, P =0.0012) (**Fig. 2a-b**). Similarly, AP2γ deletion in the NSCs from the DG also produced a decrease in the generation of mixed clones (IRES-GFP: 49% ± 2.0, Cre-IRES-GFP: 14% ± 5.8, t18 =5.705, P <0.0001), counterbalanced by a marked increase in the formation of non-neuronal clones (IRES-GFP: 51% ± 3.4, Cre-IRES-GFP: 86% ± 3.5, t_{18} =7.173, P <0.0001), supporting the role of AP2γ in commitment and differentiation into the neuronal lineage (**Fig. 2d**). We did not observe a significant difference in the clone size of control and AP2γfl/fl infected cells from the SEZ and DG (**Fig. 2c,e**).

To verify if the effects observed in vitro upon deletion of AP2γ were present in the adult mouse brain, we used tamoxifen-inducible GLAST:CreErt2/Z/EG mice and crossed with AP2γfl/fl mice (henceforth referred to as AP2γ$^{-/-}$, **Fig. 2f**). This allowed us to promote tamoxifen inducible excision of AP2γ in the progeny, and assess how the lack of this transcription factor affects hippocampal neurogenesis 1 week after induction (cells with AP2γ deletion become detectable by immunohistochemistry as GFP positive cells). In the AP2γ$^{-/-}$ mice we observed a significant decrease in the percentage of GFP/DCX double-positive cells both in the SEZ and in the DG in comparison to wild-type (Wt) mice (in the SEZ, Wt: 58% ± 12.3, AP2γ$^{-/-}$: 30% ± 1.8, t_{18} =2.252, P =0.0185; in the DG, Wt: 64% ± 2.8, AP2γ$^{-/-}$: 45% ± 3.5, t_{18} =4.239, P =0.0002) (**Fig. 2g-h**). In both regions, the decrease of AP2γ negative neuroblasts was accompanied with a marked increase in GFP/GFAP double-positive cells (in the SEZ, Wt: 10% ± 0.7, AP2γ$^{-/-}$: 36% ± 7.7, t_{18} =3.361, P =0.0017; in the hippocampal DG, Wt: 8.4% ± 0.5, AP2γ$^{-/-}$: 41% ± 7.8, t_{18} =4.171, P =0.0003; **Fig. 2g,h**). This increase in GFAP positive cells likely represents an increase in the GFAP-expressing progenitors pool or in the number of newly formed glial cells, as a result of a defect at the level of lineage commitment and subsequent differentiation into glutamatergic neurons. To complement this data,

an additional experiment in which we promoted the deletion of AP2γ in the forebrain, by crossing Emx1-Cre with AP2γfl/fl mice (henceforth referred to as AP2γfl/fl::Emx1-Cre) was performed; In fact we observed a decrease in DCX-positive neuroblasts in the DG (**Supplementary Fig. 3**). Effects in the neuroblast populations of the SEZ were not pronounced in AP2γfl/fl::Emx1-Cre mice (**Supplementary Fig. 4a,b**); however, AP2γfl/fl::Emx1-Cre mice show a decrease in Tbr2 positive cells in the granular cell layer (GCL) of the olfactory bulbs, as well as a decrease in Tbr1 positive cells in the molecular cell layer (MCL) of the same region, indicating an impairment in the progression to the glutamatergic neuronal lineage throughout development (**Supplementary Fig. 4c,d**).

In vivo AP2γ overexpression promotes neuronal differentiation in the adult brain

To assess whether increased levels of AP2γ in the adult neurogenic niches could modulate the generation of new neurons, we used retroviral vectors containing either IRES-GFP as control or AP2γ-IRES-GFP for AP2γ overexpression, and injected both in the DG and SEZ of adult mice (from now on designated as AP2γ^{OE}) (**Fig. 3a,b**). In this way, proliferative cells were stably transduced by the viral vectors, resulting in the overexpression of AP2γ and in the co-expression of GFP. Analysis performed at 1 week post-injection showed an increase in SEZ neuroblasts, reflected by an increase in GFP/DCX double-positive cells (Wt= 64% ± 2.9, AP2γ^{OE}= 71% ± 2.8, t_{18} =1.749; P =0.0487, **Fig. 3c,d**). In the hippocampal DG, the large proportion of GFP positive cells corresponded to the neuroblasts population (DCX$^+$ cells). We found a reduction in the percentage of neuroblasts in AP2γ^{OE} animals in relation to control animals in the DG 1 week post-injection (Wt= 66% ± 0.7, AP2γ^{OE}= 55% ± 3.5; t_{18} =3.082; P <0.0032) (**Fig. 3c,e**); moreover, this reduction in neuroblasts was accompanied by a significant increase detected in mature granular neurons (NeuN$^+$ cells) in AP2γ^{OE} mice (Wt= 12% ± 3.4 and AP2γ^{OE}= 30% ± 1.3, t_{18} =4.945; P <0.0001) (**Fig. 3c,e**), indicating the promotion of

neurogenesis and the acceleration of the differentiation process in AP2γ^{OE} mice. A reduction was also detected in the GFAP-positive proliferating cell population of AP2γ^{OE} mice (Wt= 5.8% ± 0.34 and AP2γ^{OE}= 2.0% ± 1.3, t_{18} =2.828; P =0.0056) (**Fig. 3e**). Furthermore, in animals sacrificed 1 month post-injection, most GFP$^+$ cells were now mature neurons (NeuN$^+$ cells), whereas the GFP$^+$/ DCX$^+$ cell population was scarce (**Fig. 3f**). At this time-point, the increase in the differentiation of neuronal cells in the DG was maintained in AP2γ^{OE} mice that presented a significant increase in the percentage of GFP$^+$/ NeuN$^+$ cells (Wt= 69% ± 1.9 and AP2γ^{OE}= 95 %± 3.5, t_{18} =6.529; P <0.0001) (**Fig. 3f**). Few GFP$^+$ cells co-localized with GFAP$^+$ cells in the DG and no significant variation was observed in the percentage of GFP$^+$/ GFAP$^+$ cells (**Fig. 3f and Supplementary Figure 5**). Together, these data support the participation of AP2γ in the regulation of adult neurogenesis.

AP2γ integrates the transcriptional network regulating glutamatergic neurogenesis

To better understand how AP2γ participates on the regulation of adult glutamatergic neurogenesis modulators, we transfected mouse embryonic carcinoma P19 cells with a pGL3 luciferase vector containing either NeuroD or Tbr2 promoters (which contain AP2γ binding sites) and with a control, Sox2, Pax6 or AP2γ cDNA expression vector. Transfection with AP2γ led to a significant activation of both promoters (Tbr2, P <0.001; NeuroD, P =0.031; **Fig. 4a**) as previously described[]. We also observed significant activation of these promoters by Sox2 (Tbr2, P =0.001; NeuroD, P =0.001; **Fig. 4a**) and Pax6 (Tbr2, P =0.026; NeuroD, P =0.022; **Fig. 4a**), but no cooperative effects of Pax6 with AP2γ. However, simultaneous transfection with Sox2 and AP2γ potentiated the activation of the Tbr2 promoter (Tbr2, P =0.026; NeuroD, P =0.022; **Fig. 4a**). To uncover the upstream regulators of AP2γ, we transfected P19 cells with a pGL3 luciferase vector containing the AP2γ promoter and with a control, Pax6, Neurogenin 1 (Ngn1), Neurogenin 2 (Ngn2), Neurogenin 3 (Ngn3), Mash1, Sox2 or AP2γ cDNA expression vector (**Fig. 4b**). We observed that Pax6 and Sox2 activate the

AP2γ promoter while Mash1 and Ngn1 inhibit this promoter (**Fig. 4b**). AP2γ was also able to promote its self-activation, as previously described().

To further clarify the transcriptional network involving AP2γ in vivo, we crossed AP2γ$^{fl/fl}$mice with Glast:CreEtrt2 mice and obtained tamoxifen inducible AP2γ conditional knockout mice - AP2γfl/fl// Glast::CreErt2 (henceforth referred as AP2γ cKO). Here, we focused our analysis on the hippocampal region, since we were interested in characterizing the importance of AP2γ regulation to behavioral dimensions depending on hippocampal function. Twenty-one days after tamoxifen treatment of adult mice (2 month-old), we studied the impact of AP2γ deficiency on the above-mentioned proteins (**Fig. 4c**), in the hippocampus of both heterozygous (AP2γ$^{+/-}$ cKO) and homozygous conditional knockout (AP2γ$^{-/-}$ cKO) mice. In fact, AP2γ deficiency triggered decreased levels of Pax6 and Tbr2 protein, but not Sox2, in the adult dorsal and ventral DG (**Fig. 4d)**. Together, both in vitro and in vivo results are consistent with the view of AP2γ as a regulator of its targets Pax6 and Tbr2 also in the adult hippocampus (**Fig. 4e**).

AP2γ cKO adult mice display hippocampal neurogenesis impairments but no alterations in neuronal morphology

After using complementary approaches to manipulate AP2γ levels both in vitro and in vivo, we have further used the AP2γ cKO mouse model to better dissect the functional implications of AP2γ deficiency in the adult brain.

We have previously confirmed by western blot, that 2 month-old AP2γ$^{-/-}$ cKO animals present significantly lower levels of this transcription factor in the ventral and dorsal DGs, as well as in other

brain regions, such as the prefrontal cortex (PFC) and the SEZ (**Supplementary Fig. 6**), 21 days after tamoxifen administration (**Fig. 4c**). Analysis of proliferation and neurogenesis in the adult hippocampal DG (**Fig. 5a-c**) showed that AP2γ$^{-/-}$ cKO animals present deficits in hippocampal proliferation (decrease in BrdU-positive cells), an effect that is more pronounced in homozygous mice (dorsal DG: Wt= 3.89 ± 0.62 x10^{-3} cells/100µm^2, AP2γ$^{-/-}$ cKO= 1.04 ± 0.28 x10^{-3} cells/100µm^2, $F_{3\text{-}37}$ =11.97, P <0.0001; Post-hoc: P <0.001; ventral DG: Wt= 3.13 ± 0.8 x10^{-3} cells/100µm^2, AP2γ$^{-/-}$ cKO = 0.55 ± 0.1 x10^{-3} cells/100µm^2, $F_{3\text{-}37}$ =8.596, P <0.0001; Post-hoc: P <0.001, **Fig. 5b**) as well as in hippocampal neurogenesis (detected as lower number of BrdU/DCX double-positive cells; dorsal DG: Wt= 2.79 ± 0.48 x10^{-3} cells/100µm^2, AP2γ$^{-/-}$ cKO = 0.29 ± 0.09 x10^{-3} cells/100µm^2, $F_{3\text{-}37}$ =19.87, P <0.0001; Post-hoc: P <0.001; ventral DG: Wt= 1.75 ± 0.66 x10^{-3} cells/100µm^2, AP2γ$^{-/-}$ cKO = 0.16 ± 0.09 x10^{-3} cells/100µm^2, $F_{3\text{-}37}$ =4.678, P = 0.0154; Post-hoc: P <0.01, **Fig. 5c**).

To explore whether AP2γ deficiency could affect additional neuroplastic processes in the adult DG, such as dendrite morphogenesis, we analyzed the dendritic morphology of DG granular neurons using 3D morphometric reconstruction (**Fig. 5d, e**), and spines's densities and morphology (**Fig. 5f**). Neither of these parameters was affected in both AP2γ$^{+/-}$ cKO or AP2γ$^{-/-}$ cKO animals.

AP2γ deficiency induces deficits in specific cognitive modalities

Given the role of AP2γ in adult hippocampal neurogenesis we tested AP2γ cKO mice in different behavioral paradigms to assess its relevance in several emotional and cognitive domains (**Fig. 6**). We used two behavioral tests to detect anxious-like behavior, namely the open field (OF) test and the elevated plus maze (EPM). In the OF test (**Fig. 6a**), although not statistically significant, both AP2γ$^{+/-}$ cKO or AP2γ$^{-/-}$ cKO mice displayed a decrease in the total distance travelled in the center of the arena (Wt= 168.25 ± 55.7 cm vs AP2γ$^{+/-}$ cKO= 73.57 ± 45.19 cm and AP2γ$^{-/-}$ cKO= 88.08 ± 20.23

cm, F_{3-27} =1.405, P =0.263), while average velocity was identical, (**Supplementary Fig. 7**). In accordance, AP2γ cKO animals displayed a decreased exploration of open arms in the EPM (**Fig. 6b**), (Wt= 21.0 ± 7 % vs AP2$\gamma^{+/-}$ cKO= 12.12 ± 4.87 % and AP2$\gamma^{-/-}$ cKO= 7.86 ± 3.23 cm, F_{3-27} =1.623, P =0.216). Both trends point for a possible effect of the reduced levels of AP2γ levels in anxiety-like behavior, although results in both tests failed to reach statistical significance. In the forced swimming test (FST) that assesses behavioral despair, AP2γ cKO mice displayed mobility levels similar to control animals, discarding the contribution of AP2γ to this behavioral domain, at least in basal conditions (**Fig. 6c**). Next, we tested the animals in a contextual fear conditioning (CFC) task, previously described to be sensitive to neurogenesis impairments[22]. In the CFC task (**Fig. 6d**), animals were submitted to a context probe (on day 2), aimed to test hippocampal-dependent memory, and a light-cued probe (on day 3), aimed to assess the integrity of extra-hippocampal memory circuits[22]. All groups presented similar average freezing percentages after the conditioning trials (**Fig. 6e**). In the context probe, AP2$\gamma^{-/-}$ cKO presented a reduction in the percentage of freezing, while Wt animals presented higher freezing percentages when exposed to a familiar context (Wt= 77.37 ± 4.67% vs AP2$\gamma^{-/-}$ cKO = 60.47 ± 7.24%, F_{3-15} =3.767, P =0.047; Post-hoc: P <0.05 **Fig. 6f**). Switching to a new environment, promoted a decrease in freezing in both groups (Wt= 30.29 ± 4.65% vs AP2$\gamma^{-/-}$ cKO = 36.69 ± 3.18%, **Fig. 6g**). Curiously, this impairment in contextual memory was not observed in AP2$\gamma^{+/-}$ cKO animals. In the light probe, both animal groups presented high percentages of freezing after exposure to the light cue (Wt= 58.61 ± 5.74% vs AP2$\gamma^{-/-}$ cKO = 65.46 ± 5.53%, t_{14} =0.7959, P =0.219**, Fig. 6h**). Overall, CFC results show that AP2$\gamma^{-/-}$ cKO display specific deficits in contextual hippocampal-associated memory, while AP2γ deletion leaves associative non-hippocampal dependent memory intact.

We proceeded with the cognitive characterization of AP2γ cKO using different test paradigms based on the Morris Water Maze (as previously described[0]) (**Fig. 7**). In a reference memory task, which

relies at great extent on hippocampal function[], Wt and AP2γ$^{-/-}$ cKO mice presented similar learning curves, without significant differences in the overall distances swum along the four trials days (**Fig. 7a**). However, analysis of the strategies adopted to reach the escape platform[] showed that AP2γ$^{-/-}$ cKO mice are able to maintain test performances comparable to Wt animals by delaying the switch from non-hippocampal dependent strategies ("Block 1") to hippocampal dependent strategies ("Block 2") (**Fig. 7b-h**). In fact, the majority of AP2γ$^{-/-}$ cKO animals initiate Block 2 strategies by test days 3 and 4, while presenting an increased mean duration of Block 1 in relation to Wt mice (**Block1:** Wt = 5.2 ± 1.1 vs AP2γ$^{-/-}$ cKO = 9.0 ± 1.58; t_{18} = 1.966; P = 0.0324; **Block2:** Wt = 10.0 ± 1.2 vs AP2γ$^{-/-}$ cKO = 4.8 ± 1.5; t_{18} = 2.690; P = 0.0075**, Fig. 7e-h**). Furthermore, in a working memory test paradigm, both AP2γ$^{-/-}$ cKO and Wt mice presented similar performances along all the trials (**Fig. 7i**), showing that post-natal deletion of AP2γ does not interfere with this cognitive domain in basal conditions. In terms of behavioral flexibility, AP2γ$^{-/-}$ cKO displayed increased time spent on the "older quadrant", in relation to Wt mice (t_{18} =4.806, P <0.0001, **Fig. 7j**). Interestingly, these results were only found in homozygous mice, as AP2γ$^{+/-}$ cKO present test performances similar to Wt animals (**Supplementary Fig. 8**).

The hippocampal-to-PFC link is impaired in AP2γ$^{-/-}$ cKO mice

In order to better understand the translation of AP2γ deficiency into poor reference memory and behavior flexibility performances, we sought for a functional characterization of the hippocampus-PFC network by analyzing electrophysiological features of local field potentials (LFPs) recorded in these areas (**Fig. 8a**). Interestingly, the temporal structure of the LFPs recorded from AP2γ cKO animals was affected. Specifically, in AP2$^{-/-}$ cKO coherence measurements between simultaneously recorded LFPs[] of the medial PFC and the ventral hippocampus are significantly decreased in all bands when compared to similar recordings obtained from Wt animals (theta: Wt= 0.71 ± 0.06,

AP2γ$^{-/-}$ cKO= 0.44 ± 0.03, $F_{3\text{-}12}$ =6.788, P =0.011, Post-hoc: P <0.01; beta: Wt= 0.79 ± 0.03, AP2γ$^{-/-}$ cKO= 0.40 ± 0.05, $F_{3\text{-}12}$ =28.72, P <0.001, Post-hoc: P <0.001; low gamma: Wt= 0.48 ± 0.03, AP2γ$^{-/-}$ cKO= 0.21 ± 0.04, $F_{3\text{-}12}$ =19.77, P <0.001, Post-hoc: P <0.001; **Fig. 8b**), thus showing a compromised connection between these two brain regions. Power spectra densities (PSD) translate the amplitude of the signals recorded in a brain region across the frequency domain and are important read-outs of the function of that region[]. AP2γ depletion did not exert an effect in PSD of the ventral hippocampus (**Fig. 8c**) but promoted a reduction in PSD of the PFC, namely in the beta and low gamma frequency bands (beta: Wt= 67.6 ± 0.58μV^2/Hz, AP2γ$^{-/-}$ cKO= 62.1 ± 0.68μV^2/Hz, $F_{3\text{-}12}$ =11.94, P =0.0014, Post-hoc: P <0.01; low gamma: Wt= 59.0 ± 0.71μV^2/Hz, AP2γ$^{-/-}$ cKO= 54.9 ± 0.86μV^2/Hz, $F_{3\text{-}12}$ =11.03, P =0.0038, Post-hoc: P <0.01; **Fig. 8d**).

Discussion

This study identifies, for the first time, AP2γ as a molecular regulator that integrates the transcriptional network responsible for the generation of new glutamatergic neurons in the post-natal brain, and reveals its modulatory function in different cognitive modalities.

The function and the underlying mechanisms of AP2γ in adult neurogenesis

The functional importance of transcription factors in adult neurogenesis is now becoming increasingly comprehended. On the premise that adult hippocampal neurogenesis may recapitulate embryonic cortical neurogenesis in its regulatory mechanisms[], we investigated if the function of AP2γ in the developmental brain was preserved in the adult brain. Indeed, we found that AP2γ is present in the two main post-natal neurogenic niches, the SEZ and the SGZ. Previous studies have shown that the adult hippocampus NSCs are mainly specified towards the glutamatergic lineage[]. Based on these observations, the presence of AP2γ, a molecular partner in the glutamatergic

neurogenesis is not unexpected in the SGZ, neither in the SEZ. In fact, despite the predominant GABAergic nature of the SEZ neurogenic niche, our results show that a proportion of AP2γ positive cells are also Tbr2 positive, suggesting that AP2γ may regulate the specification of a subpopulation of neuronal progenitors towards the glutamatergic lineage in this brain region. This finding is in line with the observation that SEZ progenitors also give rise to a subpopulation of adult-born glutamatergic juxtaglomerular neurons of the olfactory bulb().

After the demonstration of the presence of AP2γ in the two neurogenic niches, we explored the molecular mechanisms regulating its action in adult neurogenesis. Our in vitro approaches, suggest that AP2γ acts as an effector of Sox2 and Pax6 in the promotion of Tbr2 expression. Furthermore, the fact that AP2γ can bind to its own promoter, and positively regulate its expression, suggests a dose-dependent mechanism whose exact importance is yet to be fully elucidated. It is well established that Pax6 continues to be expressed in the adult SGZ; in fact Pax6-positive cells co-express GFAP and Nestin, both markers of type I progenitors(). Pax6 expression appears to persist to some extent in Type-2 progenitors co-expressing DCX, but is not present in postmitotic neuroblasts(). It is therefore comprehensible that AP2γ, as a Pax6-downstream target, preserves its regulatory function on glutamatergic neurogenesis in the adult brain, as demonstrated herein. In fact, our results demonstrate that AP2γ is a positive regulator of adult neurogenesis in the hippocampal DG and in the SEZ as its overexpression increments the generation of new neurons in these two regions. Accordingly, our experiments of AP2γ deletion, both in vitro and in vivo, confirm that the absence of this transcription factor produces a marked decrease in the neuroblast populations both in the SGZ and SEZ.

Importantly, the present data points out that AP2γ expression alterations produce a net effect in the $Tbr2^{+}$ cells (either an increment in overexpression experiments, or a decrease when using retroviral-

mediated deletion of AP2γ). Therefore, this transcription factor regulates post-natal glutamatergic neurogenesis by mobilizing transient amplifying progenitors (TAPs), rather than interfering with the NSCs pool. The presence of an alternative regulatory pathway using AP2γ as an intermediate transcriptional regulator, in parallel with the direct regulation of Tbr2 by Pax6, suggests that AP2γ function may allow a fine-tuning of the neurogenic process, by either rapidly expand or restrict the TAPs pool[34,35].

AP2γ modulates cognitive function

In this work, we aimed not only to identify the molecular/cellular consequences of AP2γ function, but also to explore how this transcriptional modulation could impact on different behavioral dimensions. Since impairments in hippocampal neurogenesis have been associated with the emergence of depressive-like behavior[0], we analyzed the performance of AP2γ cKO mice (with significant reduction in AP2γ protein levels in Glast positive cells) in the FST, but no significant impact in behavioral despair was detected in animals with reduced levels of AP2γ. Interestingly, AP2γ cKO mice performance in the open-field test and elevated-plus maze tests suggests an hyper-anxious state of these animals. Such effect of AP2γ depletion would be consistent with previous works suggesting a link between anxiety and decreased neurogenesis[23]. However, since we were unable to find statistical significant differences in this domain, further studies are needed to clarify the role of AP2γ in the control of anxiety behavior.

Furthermore, impairments in the generation of new hippocampal neurons have also been associated with cognitive deficits[0]. Indeed, spatial memory assessment revealed that AP2γ regulation of the population of hippocampal TAPs seems essential to the preservation of hippocampal-dependent cognitive tasks, such as strategies on spatial memory tasks. In addition, cognitive dimensions that

are based in the interaction of the hippocampal formation and prefrontal cortical areas, such as spatial behavioral flexibility were also found to be impaired in AP2γ$^{-/-}$ cKO animals. Such phenotype suggests that AP2γ deficiency also impacts on PFC function. Strikingly, our electrophysiological studies revealed that AP2γ deficiency in the adult brain led to a significant decrease of coherence between the ventral hippocampus and the PFC, indicating a decrease in the ability of these regions to functionally interact, including the theta and beta frequencies, previously shown to be critically related with behavior outputs dependent on the cortico-limbic networks[29,()]. Altogether, the data presented here suggest that this impairment of neuronal activity and interregional communication occurs as a consequence of glutamatergic network malfunction triggered by the lack of AP2γ regulation in adult neurogenesis. This may likely underlie the poor cognitive performance observed in animals with depleted levels of AP2γ in the CNS.

In conclusion, the present study identifies for the first time AP2γ as a key regulator of adult neurogenesis and demonstrates its modulatory role in hippocampal and PFC activity. In light of the role of these brain regions in emotional and cognitive behaviors and of the behavioral and functional data presented here, AP2γ has a potential relevance to normal cortico-limbic functions.

Online Methods

Animals

AP2γ$^{loxP/loxP}$(AP2γfl/fl) (Kindly provided by Dr. Hubert Schorle), Emx1-cre, Glast:CreErt2[44] and Z/EG[45] mice were maintained on a C57Bl/6J background (also used as wild type) and identified the genotypes by PCR of genomic DNA[46]. loxP sites flanked exon 5, whose deletion caused a loss of the helix-span-helix domain near the protein carboxy terminus. AP2γfl/fl mice were crossed with Emx1-cre or Glast:CreEtrt2 and Z/EG mice and considered the day of vaginal plug as E0 and the day of birth as P0. Animals were housed in polypropylene cages. All procedures were carried out in accordance with EU Directive 2010/63/EU and NIH guidelines on animal care and experimentation and were approved by the Portuguese Government/Direção Geral de Alimentação e Veterinária (DGAV) with the project reference 0420/000/000/2011 (DGAV 4542).

Tamoxifen administration

Tamoxifen (Sigma, St. Louis, MO; T-5648) was dissolved in corn oil (Sigma; C-8267) at 20 mg/ml. For the deletion experiments with the AP2γfl/fl//Glast::CreErt2//Z/EG mice, 1 mg was injected intraperitoneally (i.p.) twice a day for 5 consecutive days[44]. Animals were sacrificed 1 week after the end of tamoxifen administration. For the deletion experiments with the AP2γfl/fl//Glast::CreErt2$^{+/-}$ mice, 2 month-old animals were i.p. injected with 1 mg twice a day for 5 consecutive days, with 7 days break followed by injections for 5 additional consecutive days. Animals were subjected to behavioral testing 21 days after the end of the injections (n=6-10).

Viral Constructs, Virus Production, and Stereotactic Injections

We cloned the full-length cDNA of mouse AP2γ (1,816 bp, EMBL accession number X94694, kindly provided by M. Moser, Max Planck Institute of Biochemistry) into the EcoRI unique restriction site of the retroviral vector pMXIG between the upstream long terminal repeat and the IRES sequence as previously described[20]. Retrovirus was produced in a packaging cell line (GPG-293) after transient transfection with the retroviral expression plasmid. Estimation of the viral titer was performed as described[0] and titered to $5x10^7$ colony-forming units (cfu) ml^{-1} . For all overexpression experiments, 8-week-old males were stereotactically injected with 1 ml of either CAG-IRES-GFP (IRES-GFP) or CAG-IRES-AP2γ (AP2γ-IRES-GFP) retroviruses into the left and the right SEZ or DG (coordinates relative to the bregma were (in mm) for the SEZ: 0.75 anterior/posterior, 1.2 medial/lateral, and -1.7 dorsal/ventral from the dura; for the DG: -2.0 anterior/posterior, 1.6 medial/lateral, and -1.9 dorsal/ventral from the dura). For the deletion experiments, 2-month-old AP2γfl/fl males were injected with 1 ml of either IRES-GFP or Cre-IRES-GFP retroviruses. Analyses were performed 1 week or 1 month post-injection (n=5 mice per group for each experimental condition).

BrdU labelling

Mice used for cell type analyses with ISH and immunofluorescence co-labeling analyses were given BrdU in drinking water (1 mg/ml, Sigma Aldrich) for 2 weeks, and sacrificed 8 weeks after. For the remaining experiments, mice were injected once with BrdU (100mg/Kg, i.p.), 24 hours before sacrifice.

Primary SEZ and DG cultures

For primary subependymal and dentate gyrus cultures, six mice (AP2γfl/fl mice, 2 months-old) were used. Appropriate dissection was confirmed by qRT-PCR analysis using genes that are specific for these regions. Cells were directly plated after preparation on six coverslips coated with poly-D-lysine in DMEM/F12-supplemented medium (without any addition of EGF and FGF2) and were transduced

with a retroviral vector IRES-GFP or CRE-IRES-GFP 2h later[()]. After 7d in culture, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature and processed for antibody staining.

Histology and Immunostaining

Animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and were transcardially perfused with cold 4% paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde. Coronal vibratome sections (40 mm) were obtained and immunostainings were performed as previously described[11]. Antibodies used were: Beta3Tubulin (Tuj1, Sigma, Mouse IgG2b, 1:2000), Doublecortin (DCX, Chemicon, Guinea pig, 1:1000), GFAP (SIGMA, IgG1, 1:100), GFP (Chemicon, rabbit, 1:5000 or Aves, chicken 1:500), NeuN (Chemicon, IgG1, 1:75), Pax6 (Chemicon, rabbit, 1:700), TH (Chemicon, chick, 1:200), BrdU (rat, 1:200, Abcam), Ki67 (DAKO, rat, 1:50), Tbr2 (Abcam, rabbit, 1:100), Tbr1 (Abcam, rabbit, 1:100), AP2γ (specific to an N-terminal epitope, Santa Cruz, rabbit, 1:100; Abcam, mouse IgG1, 1:100), beta3-tubulin (Sigma, mouse IgG2b, 1:100), PSANCAM (Millipore, Marseille, mouse IgGM, 1:500). For AP2γ immunostaining, brain sections were first incubated for 20 min with citrate buffer (10mM) at the microwave (400 Watts) for antigen retrieval. After cooling for 15 min, sections were washed in phosphate-buffered saline (PBS) and incubated in hydrogen chloride (HCl) solution for 15 min at room temperature. Primary antibodies were incubated overnight at 4°C. The subclass-specific antibodies Alexa Fluor 488 and Alexa Fluor 594 (Molecular Probes) were used for detection. Nuclei were counterstained using DAPI. Analyses were performed in a confocal microscope (Olympus FV1000).

In Situ Hybridization

Digoxigenin-labeled RNA probes for AP2γ (1,816 bp, EMBL accession number X94694) (kindly provided by M. Moser) were used as described previously[11].

3D morphological analysis

To assess the 3D dendritic morphology of hippocampal neurons we performed Golgi-Cox impregnation technique. Briefly, brains were immersed in Golgi-Cox solution for 21 days and then transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Rochester, NY, USA), fixed in Kodak Rapid Fix, dehydrated and xylene cleared. Dendritic arborization and spine numbers/ density were analyzed in the dentate gyrus of control and $AP2^{-/-}$ cKO mice as previously described (10-15 neurons for each animal; n=4 per group)[23,24]. Dendritic trees were reconstructed at 1000x (oil) magnification using a motorized microscope (Axioplan 2, Carl Zeiss, Germany) and Neurolucida/NeuroExplorer software (Microbrightfield, U.S.A.).

Western Blot

Hippocampus, SEZ and PFC of AP2γ cKO mice and Wt littermates were carefully dissected out after decapitation. Tissue was weighted and homogenized in RIPA buffer [containing 50 mM Tris HCl, 2 mM EDTA, 250 mM NaCl, 10 % glycerol, 1 mM PMSF protease inhibitors (Roche)] and then sonicated for 2 min. The samples were centrifuged for 25 min at 10.000 rpm at 4°C. The protein concentration of the supernatant was determined using Bradford assay. Samples with equal amounts of protein, 30 μg, were analyzed using the following primary antibodies: mouse anti-tubulin (1:200, DSHB), mouse anti-actin (1:250, Abcam) goat anti-AP2γ (1:250, Abcam), rabbit anti-PAX6 (1:2000, Chemicon), rabbit anti-SOX2 (1:500, Chemicon) and rabbit anti-TBR2 (1:500, Abcam). Secondary antibodies were purchased from BioRad (anti-mouse, 1:10.000; anti-rabbit, 1:10.000)

and Santa-Cruz Biotechnologies (anti-goat, 1:5000). Membranes were developed using ECL Clarity reagent (BioRad) an developed in ChemiDoc XRS System from BioRad. After developing, images were quantified using ImageLabTM Software, BioRad. ".

Luciferase Assay

Expression plasmids for luciferase reporter assays were constructed using full-length cDNA of mouse Sox2 clone into the pCAG vector, Pax6 cloned into the pMXIG vector and the full-length cDNA of Mash1, Ngn1, Ngn2 and Ngn3 cloned into the pcDNA expression vector. The promoters of Tbr2, NeuroD and AP2γ were cloned into the pGL3 vector (Promega) as previously described[20]. We used mouse embryonic carcinoma P19 cells ($0.5x10^5$ cells/cm^2) and assays were performed according to the manufacturer's instructions (Promega).

Electrophysiological studies

Electrophysiological recordings were obtained from anesthetized Wt, AP2$\gamma^{+/-}$ cKO and AP2$\gamma^{-/-}$ cKO mice (sevoflurane 2.5%; 800 mL/min). Mice rectal temperature was maintained at 37°C by means of a homoeothermic blanket (Stoelting, Ireland). A surgical procedure was used to carefully insert platinum/iridium concentric electrodes (Science Products, Germany) in the target positions as described previously[29] following the mouse brain atlas (from Paxinos): medial pre-frontal cortex (mPFC, prelimbic region; coordinates, 1.22 mm anterior to bregma, 0.4 mm lateral to the midline, 1.7 mm below bregma); ventral hippocampus (vHIP; coordinates, 3.2 mm posterior to bregma, 3.1 mm lateral to the midline, 2.5 mm below bregma).

LFP signals obtained from the mPFC and vHIP were amplified, filtered (0.1–3000 Hz, LP511 Grass Amplifier, Astro-Med, Germany), acquired (Micro 1401 mkII, CED, UK) and recorded running Signal Software (CED, UK). Local field activity was recorded at the sampling rate of 1000 Hz during 100s.

At the end of the electrophysiological protocols, a biphasic 1 mA stimulus was delivered to electrodes in order to mark the local of recording. Afterwards, mice were deeply anesthetized with sodium pentobarbital, brains carefully removed, immersed in paraformaldehyde (PFA) 4% for 48h and sectioned (50 μm) in the vibratome apparatus. Next, brain slices containing the mPFC and vHIP were staining with Cresyl Violet for determination of the recording site. Animals with recording positions outside at least in one of the two regions under study were excluded from analysis (n= 5 animals per group for each experimental condition).

Coherence analysis was based on multi-taper Fourier analysis. Coherence was calculated by custom-written MATLAB scripts, using the MATLAB toolbox Chronux (http://www.chronux.org)[47]. Coherence was calculated for each 1 s long segments and their mean was evaluated for all frequencies from 1 to 90 Hz. The power spectral density (PSD) of each channel was calculated through the 10 × log of the multiplication between the complex Fourier transform of each 1s long data segment and its complex conjugate. The mean PSD of each channel was evaluated for all frequencies from 1 to 90 Hz[29]. Both coherence and PSD measurements were assessed in the following frequencies: theta (4–12Hz); beta (12–20Hz); low gamma (20–40Hz).

Behavioral analysis

Elevated-Plus Maze (EPM)

Anxiety-like behavior was examined through the EPM test, in a 5 min. session, as previously described[48]. The percentage of time spent in the open-arm was used as an index of anxiety-like

behavior and the number of entries in the closed-arms was taken as an indicator for locomotor activity. Trials were video-recorded and analyzed using ethovision software (Noldus).

Open field test (OF)

The open field (OF) test was used to evaluate locomotor performance and exploratory activity. The open field apparatus consisted of a brightly illuminated square arena of 43.2 X 43.2 cm closed by a wall of 30.5 cm high. Mice were placed individually in the center of the open field arena and their movement was traced, for 5 minutes, using a two 16-beam infrared system. The resulting data was analyzed using the Activity Monitor software (Med Associates, Inc.), considering two previously defined areas: a central and an outer area. Due to the thigmotaxic exploratory activity of rodents, the ratio between the time spent in the center and in the periphery of the open field arena can also be indicative of anxiety-like traits[49]. Therefore, both distance travelled and time spent in each of the zones were recorded and analyzed. Average velocity was also determined for each group.

Forced Swimming Test (FST)

Learned-helplessness was assessed through the FST. Assays were conducted in a single trial, by placing the mice in transparent cylinders filled with water (25°C; 50 cm of depth) during 5 min. According to the methodology already described[50], the first three minutes were considered as an habituation period and the last two minutes as the test period. Therefore, only the last two minutes were considered for behavioral analysis. Trials were video-recorded and the immobility time was measured through the Etholog (vs.2.2) software.

Water Maze Tasks

Water maze tests were used to test the performance in spatial reference and behavioral flexibility tasks as described previously[48]. Briefly, these tests were conducted in a circular black pool (170 cm diameter) filled with water at 22°C to a depth of 34 cm in a room with extrinsic clues (triangle, square, cross and horizontal stripes) and dim light. An invisible platform was placed in one of four quadrants. In the reference memory task the platform position was maintained in the same quadrant for 4 days. At the fifth day, the behavioral flexibility performance of animals was tested by positioning the platform in a new (opposite) quadrant. Animals were tested in four trials. Besides the time of escape latency, the time spent in both new and old quadrants were recorded.

To confirm that differences observed in the escape latency were not due to distinct locomotor performance, we measured the average swimming velocities during trials.

Data collection and analyses of Morris Water Maze spatial reference trials were performed using a video tracking system (Viewpoint, Champagne au mont d'or, France). Search strategies were defined as previously described[26,28]. Quantitative analyses and strategy classification was performed with data collected by the Viewpoint software, using an algorithm that we developed for automatic strategy attribution based on the following main parameters: (i) thigmotaxis (Tt): >70% of swim distance within the outer ring area (8 cm from the pool border); (ii) random swim (RS): >80% of swim distance within the inner circular area; balanced quadrant exploration (all quadrants explored with a percentage of swim distance not >50% for none of the quadrants); non-circular trajectory; (iii) scanning (Sc): >80% of swim distance within the inner circular area; balanced quadrant exploration (all quadrants explored with a percentage of swim distance not >50% for none of the quadrants); non-circular trajectory; percentage of swim distance in the platform corridor area (funnel shaped area centered along the imaginary axis connecting the start position and platform position) ≤60%; (iv) chaining (Ch): >80% of swim distance within the inner circular area; balanced quadrant exploration (all quadrants explored with a percentage of swim distance not >50% for none of the quadrants); percentage of swim distance in the platform corridor area ≤60%; circular trajectory (v)

directed search (DS): >80% of swim distance within the inner circular area; percentage of swim distance in the platform corridor area >60% with shifts in the trajectory direction (vi) focal search (FS): percentage of swim distance in the perimeter of the escape platform (30 cm perimeter) > 50%; (vii) direct swim (DSw): >80% of swim distance within the inner circular area; percentage of swim distance in the platform corridor area >90% with no shifts in the trajectory direction ("direct trajectory").

For strategy blocks analysis, we defined two blocks of strategies: Block1, comprising the "non-hippocampal dependent strategies" (Thigmotaxis, Random Swim and Scanning) and Block2, comprising the "hippocampal dependent strategies" (Directed Search, Focal Search and Direct Swim). Strategy blocks were defined as a sequence of at least three trials with the strategies from the same class. For block lengths, a maximum of two-trial interruptions were tolerated but not counted: for example, in the following strategies sequence "DS-DS-FS-DS-Sc-DS-FS-FS", the block length for Block2 was scored 8, despite the presence of one trial in which the attributed strategy belongs to Block1 (Sc). Independent analysis was performed for both Wt, AP2$\gamma^{+/-}$ and AP2$\gamma^{-/-}$ cKO mice

Contextual Fear Conditioning (CFC)

Contextual Fear Conditioning was conducted in white acrylic chambers with internal dimensions of 20 cm wide, 16 cm deep and 20.5 cm high (Med Associates), with an embedded light bulb mounted directly above the chamber to provide illumination. Each chamber contained a stainless steel shock grid floor inside a clear acrylic cylinder, where animals were placed. Animals were subjected to two probes, a **context probe** and a **cue (light) probe**. The CFC procedure was conducted over 3 days (see **Fig. 5**), as follows:

Day 1. Mice were placed in the conditioning white chamber (Context A) and received 3 pairings between a light (20 s) and a coterminating shock (1 s, ≈0.5 mA). The interval between pairing was set as 180 s, and the first tone presentation commenced 180 s after the mouse was placed into the chamber. After three light shock pairings, freezing behavior was measured during 3 minutes, in order to determine whether Wt and AP2γ cKO had similar baseline freezing values during the conditioning trials. Freezing was defined as the complete absence of motion, including motion of the vibrissae, for a minimum of 1s. At the end of the three pairings, mice remained in the chamber for a further 30 s before being returned to their home cage. The chambers were cleaned with 10% ethanol solution between each trial.

Day 2. For the context probe, animals were placed in the white chamber (Context A), where they were originally shocked, 24h after the light-shock pairings. Freezing behavior was measured during 3 minutes. Two hours later, animals were put in a modified version of the chamber (Context B) that was sheeted with a black plasticized cover that was previously sprayed with vanilla oil, in order to alter both spatial and odor references; the ventilation fan was not operated; the experimenter wore a different style of gloves and lab coat. Again, freezing was measured during 3 minutes.

Day 3. For the cue probe, test settings for Context B were maintained and animals were placed inside the chamber 24h after the context probe. After 3 minutes, a light was turned on during 20 s and freezing behavior was subsequently measured during 1 minute.

Data analysis and Statistics

Statistical analyses were done using SPSS software. After confirmation of homogeneity, data was subjected to appropriate statistical tests. ANOVA repeated measures was used to analyze cognitive-learning tasks performance. One-way ANOVA was used to evaluate the remaining behavioral and molecular results. F-values and P-values derived from the between groups ANOVA analyses are

properly indicated along the text. Differences between groups were determined by Bonferroni's post-hoc multiple comparison test, and the corresponding P-values are indicated in the figures. A t-test was used two evaluate differences between two groups where appropriate. Statistical significance was accepted for $P<0.05$.

Power Spectrum Density (PSD) of the mPFC and vHIP as well as the mPFC-vHIP coherences were obtained by the recording of LFP signals during 100 s. Each measure was applied on 1 s long segments and the average of all segments was considered for statistical group analysis. Values were obtained using custom-written MATLAB-based programs (MathWorks, Natick, MA).

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None of the authors have any conflicts of interest to declare.

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MAIN FIGURES

FIGURE 1

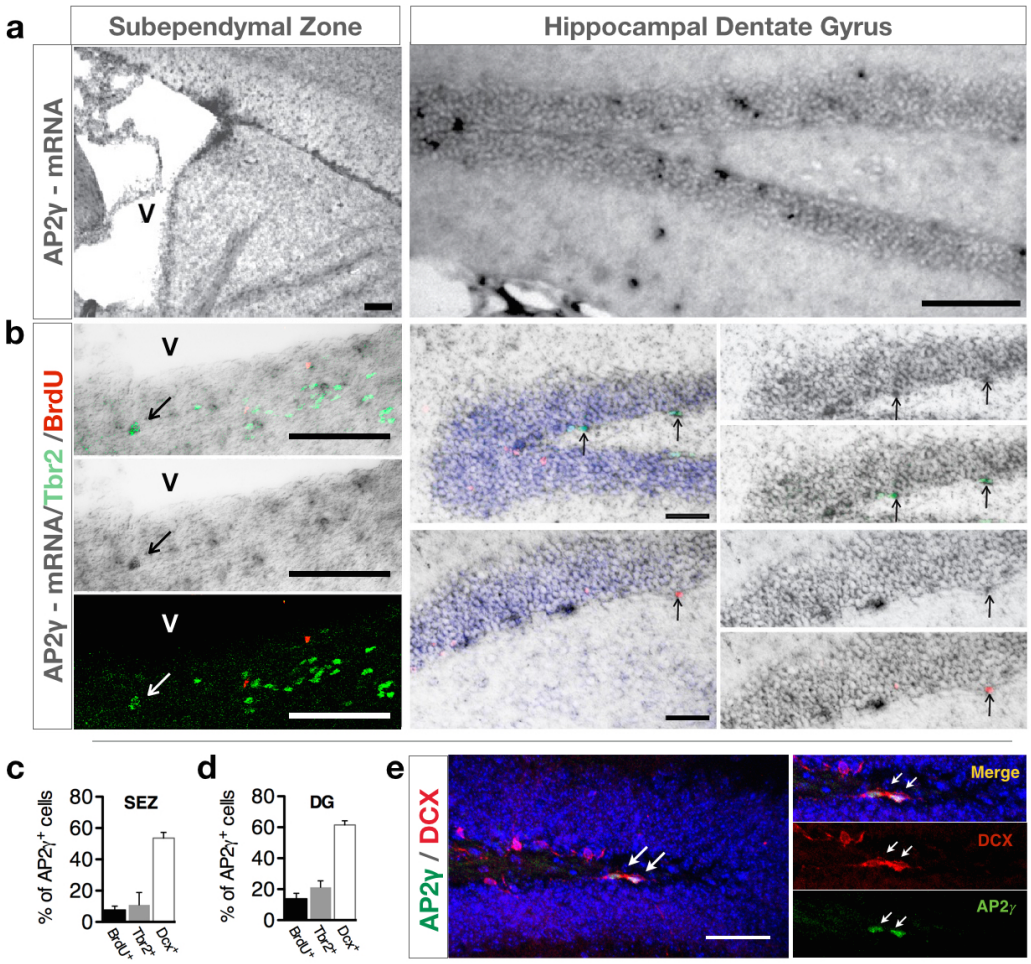

Figure 1. AP2γ expression in the adult mouse brain. (**a**) *In situ* hybrization (ISH) of AP2γ in the adult subependymal zone (SEZ) (left panel) and hippocampal dentate gyrus (DG) (right panel). (**b**) The left panel shows the combination of ISH of AP2γ with immunolabelled BrdU+ cells in the SEZ. The right panel shows ISH of AP2γ in the DG, with immunolabelled BrdU+ (in red) and Tbr2+ cells (in green). (**c-e**) Immunohistochemical quantification of the percentage of AP2γ+ cells co-labelled with BrdU, Tbr2 or DCX in the SEZ (**c**) and the DG (**d,e**). Error bars represent s.e.m. Scale

FIGURE 2

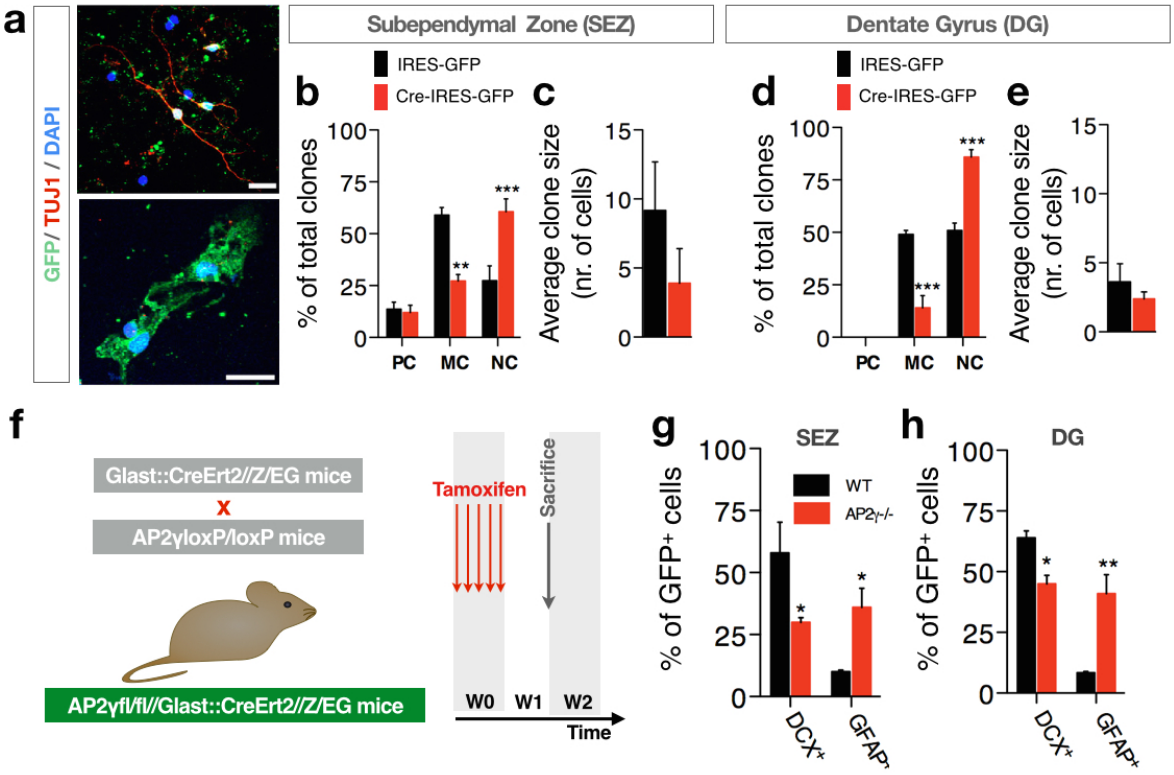

Figure 2. *In vitro* and *in vivo* deletion of AP2γ. (**a-e**) *In vitro* viral-mediated deletion of AP2γ in NSCs primary cultures (**a**) with quantification of the percentage of pure neuronal (PC), mixed neuronal and non-neuronal (MC) and non-neuronal clones (NC) (**b**) and average clone size (**c**) in adult SEZ NSCs primary cultures. Similar analysis of clone type (**d**) and average size (**e**) was performed in adult DG NSCs primary cultures, n=10, Student's t test, ** $P \leq 0.01$,*** $P \leq 0.001$. Error bars represent s.e.m. (**f-h**) Tamoxifen was administered to adult mice obtained after crossing Glast::CreErt2//Z/EG mice with mice containing AP2γ flanked by loxP sites (AP2γfl/fl), in order to promote AP2γ deletion in Glast expressing cells, and animals were sacrificed after 1 week (**f**). Quantification of the percentage of GFP-positive cells co-labelled with DCX or GFAP in the SEZ (**g**), and in the hippocampal DG (**h**) (n=6). Student's t test, * $P \leq 0.05$,** $P \leq 0.01$. Error bars represent s.e.m. Scale bars represent 20µm.

FIGURE 3

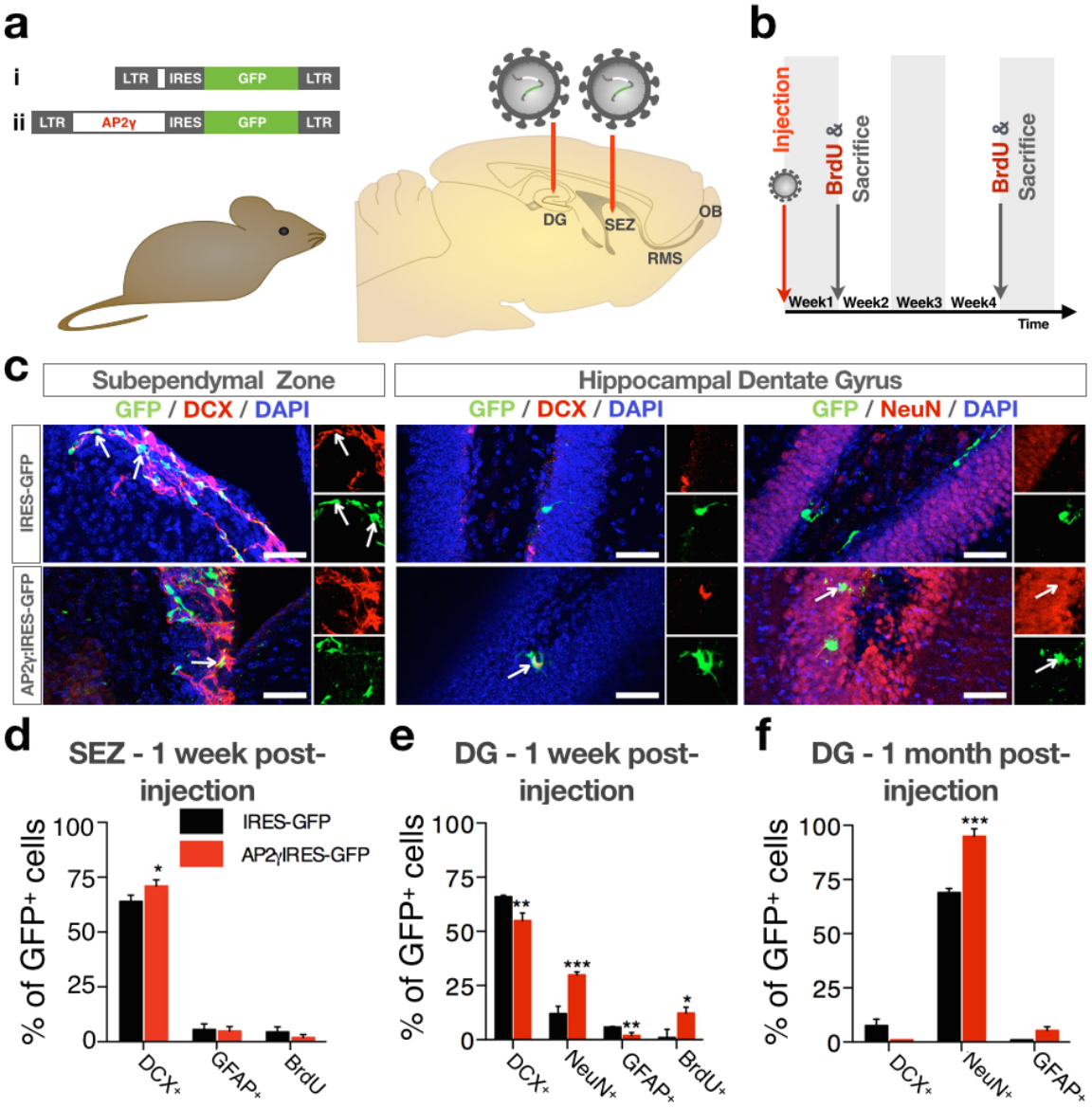

Figure 3. Viral-mediated overexpression of AP2γ in the post-natal neurogenic niches. (**a-b**) Adult mice were injected with retrovirus containing AP2γIRES-GFP or simply IRES-GFP as an experimental control, either in the dorsal hippocampus or in the subependymal zone (SEZ) (**a**) and sacrificed either 1 week post-injection or 4 weeks post-injection. (**c**) High magnification images showing GFP-positive transfected cells in the SEZ (left panels) and in the hippocampal dentate gyrus (DG, right panels) in sections immunolabelled to doublecortin (DCX, in red, left and mid panels) or NeuN (in red, right panels). (**d-f**) Quantification of the percentage of GFP-positive cells co-labelled with NeuN, DCX, GFAP or BrdU in the SEZ, 1 week post-injection (**d**), and in the hippocampal DG, 1 week (**e**) and 1 month post-injection (**f**). n=10, Student's t test, * $P \leq 0.05$,** $P \leq 0.01$. Error bars represent s.e.m. Scale bars represent 50µm.

FIGURE 4

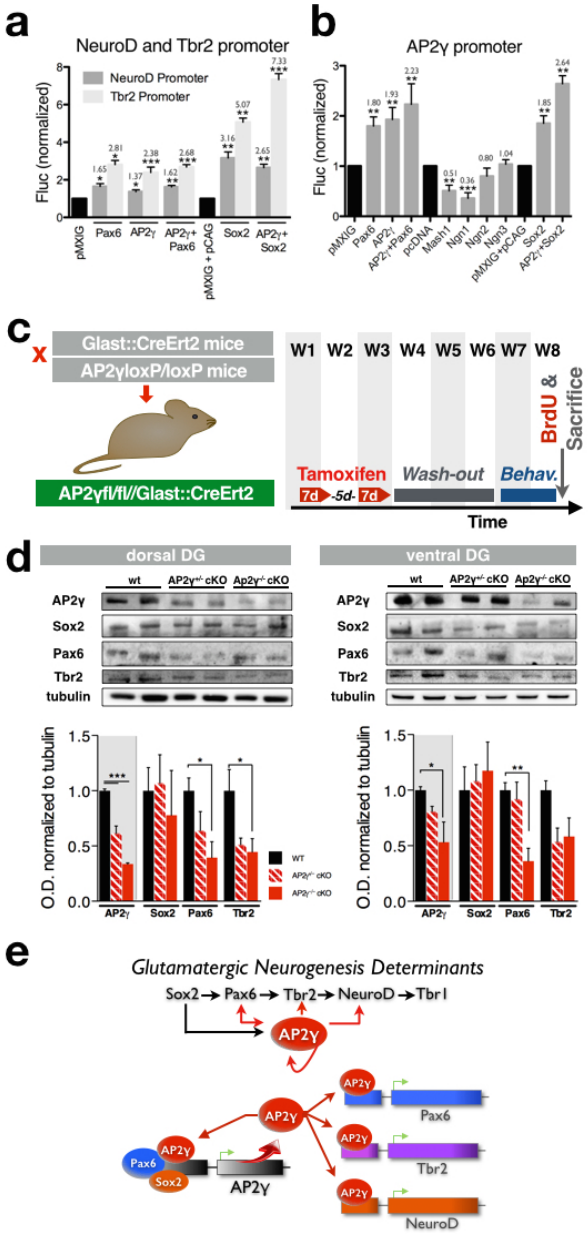

Figure 4. Regulation of glutamatergic determinants by AP2γ. (**a-b**) Histograms depicting the luciferase luminescence intensity normalized to Renilla intensity from embryonic carcinoma P19 cells transduced with the firefly or Renilla luciferase constructs (Fluc or Rluc, respectively) using either AP2γ (**a**) or NeuroD and Tbr2 promoters (**b**). Values were normalized to the pMXIG empty vector containing only GFP (4 independent experiments; Student's t test, * $P \leq 0.05$,** $P \leq 0.01$, ***$P \leq 0.001$). (**c**) For western-blot analysis, 2month-old AP2γ cKO (AP2γfl/fl//Glast::CreErt2) animals were injected with tamoxifen, tested 21 days after and subsequently sacrificed; (**d**) Western-blot analysis of AP2γ, Sox2, Pax6 and Tbr2 in adult hippocampal protein extracts from Wt, AP2$\gamma^{+/-}$ cKO and AP2$\gamma^{-/-}$ cKO mice (n=5-6; Student's t test, * $P \leq 0.05$,** $P \leq 0.01$). (**e**) Illustration depicting different regulatory functions of AP2γ in the adult hippocampus; both upstream transcription factors, Sox2 and Pax6 induce the expression of AP2γ, whereas the last presents self-regulatory activity and regulates the expression levels of Pax6, Tbr2 and NeuroD. Error bars represent s.e.m.

FIGURE 5

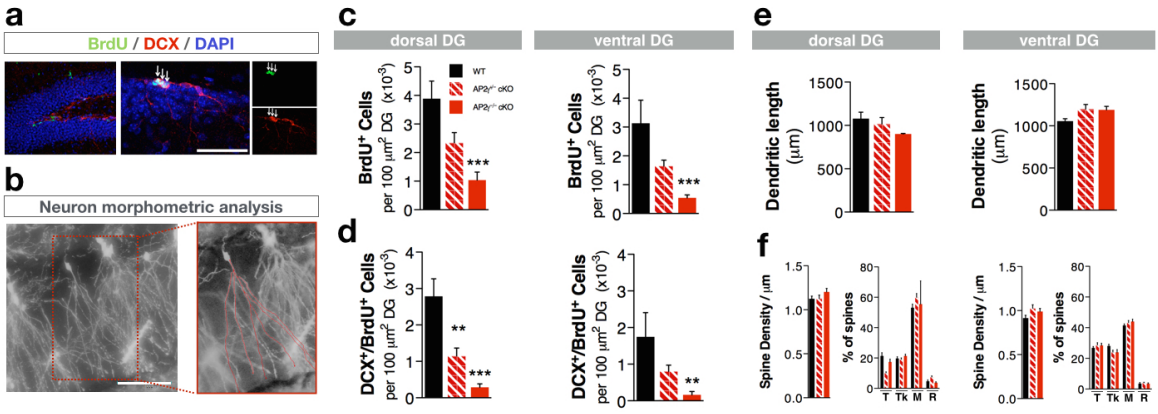

Figure 5. Hippocampal neurogenesis and dendritic morphology in adult AP2γ cKO (AP2γfl/fl//Glast::CreErt2) mice. (**a**) Dorsal hippocampal coronal section stained for BrdU (in green) and DCX (in red). Double-stained BrdU and DCX are indicated by white arrows. (**b**) Representative 3D morphometric reconstruction of a DG granular neuron. **(c,d)** Cell counts of BrdU-positive cells (**c**) and BrdU/DCX double-positive cells (**d**) in the hippocampal DG, n=6, Student's t test, * $P \leq 0.05$,** $P \leq 0.01$. (**e,f**) Dendritic length **(e)** and spines density and morphology of hippocampal granular neurons **(f)**, n=10, Student's t test, * $P \leq 0.05$,** $P \leq 0.01$. Error bars represent s.e.m. Scale bars represent 50 µm. Abbreviations: T, Thin Spines; Tk, Thick Spines; M, Mushroom Spines; R, Ramified Spines.

FIGURE 6

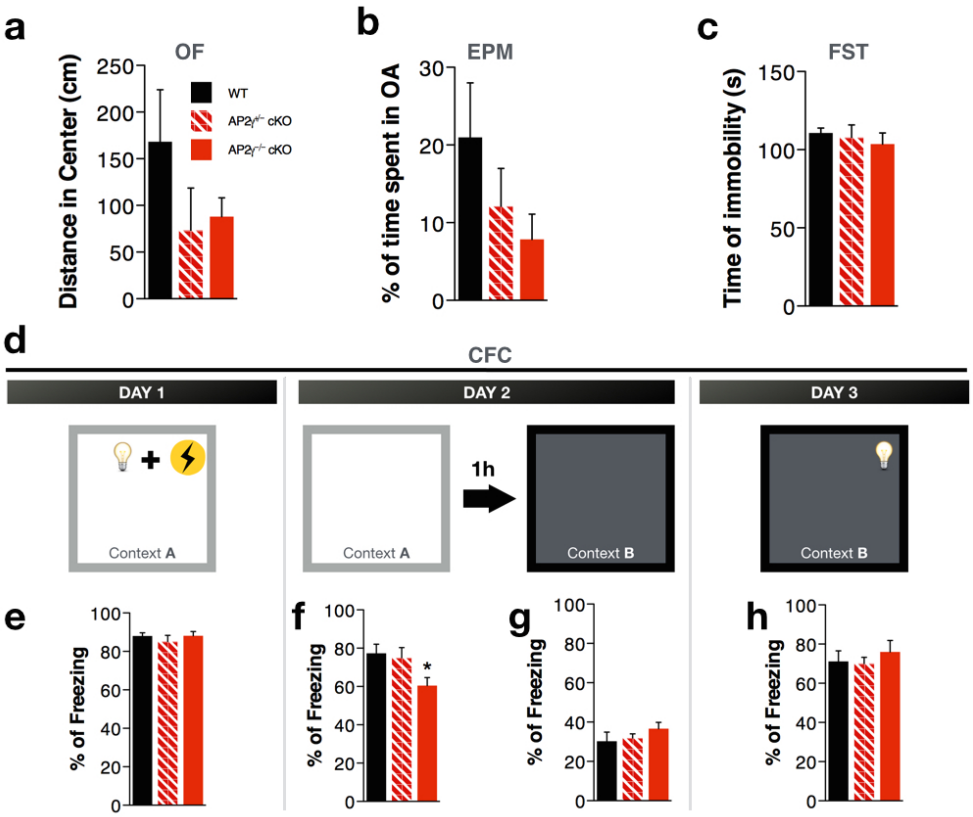

Figure 6. Behavioral analyses of AP2γ cKO mice (**a-e**) AP2γ cKO animals were tested in different behavioral paradigms. (**a,b**) Anxious-like behavior was tested both in the open-field test (**a**) and in the elevated plus maze (**b**). **(c)** The presence of depressive-like behavior was assessed in the Forced Swimming Test . **(d-h)** In addition, animals were tested in a contextual fear conditioning paradigm, n=10, Student's t test, * $P \leq 0.05$,** $P \leq 0.01$. Abbreviations: OF, open field; EPM, elevated plus maze; OA, open arms; FST, forced swimming; CFC, contextual fear conditioning; error bars represent s.e.m.

FIGURE 7

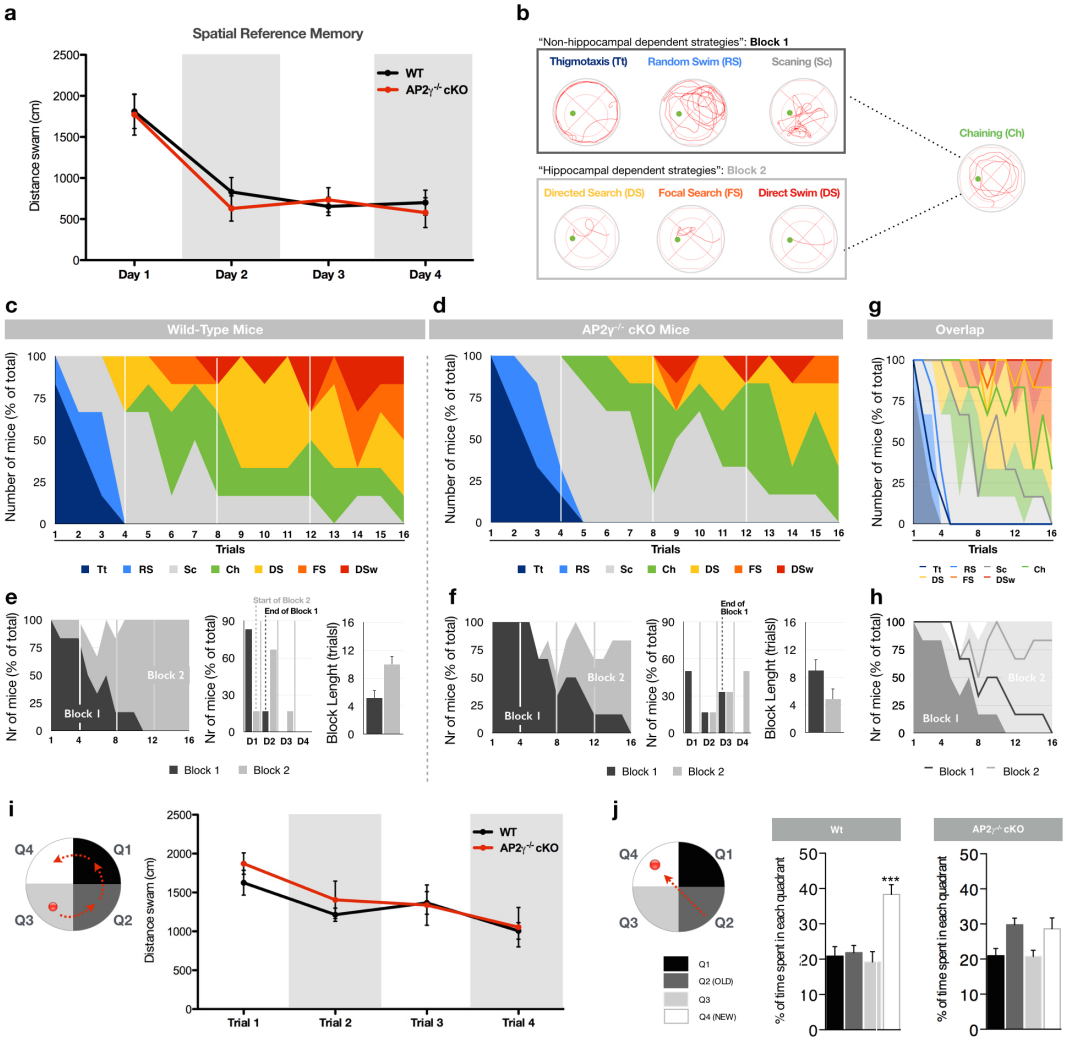

Figure 7. Cognitive strategies during water maze learning in AP2γ$^{-/-}$ cKO mice. (**a-h**) Spatial reference memory was evaluated as the average escape latency in each test day. A schematic representation and color code for each strategy (**b**) and the average prevalence of each strategy by trial number are shown both for Wt (**c**) and for AP2γ$^{-/-}$ cKO animals (**d**). The prevalence of each strategy-block along trials (Block 1: "Non-hippocampal dependent strategies"; Block 2: "Hippocampal dependent strategies"), the distribution of strategies-block boundaries and overall block length is shown for Wt (**e**) and AP2γ$^{-/-}$ cKO animals (**f**); graphical comparison of these parameters is shown in **(g)** and **(h). (i,j)** Furthermore, animals were tested in a working memory task **(i)** and in reversal learning task **(j).** n=10, Student's t test, * $P \leq 0.05$,** $P \leq 0.01$; error bars represent s.e.m.

FIGURE 8

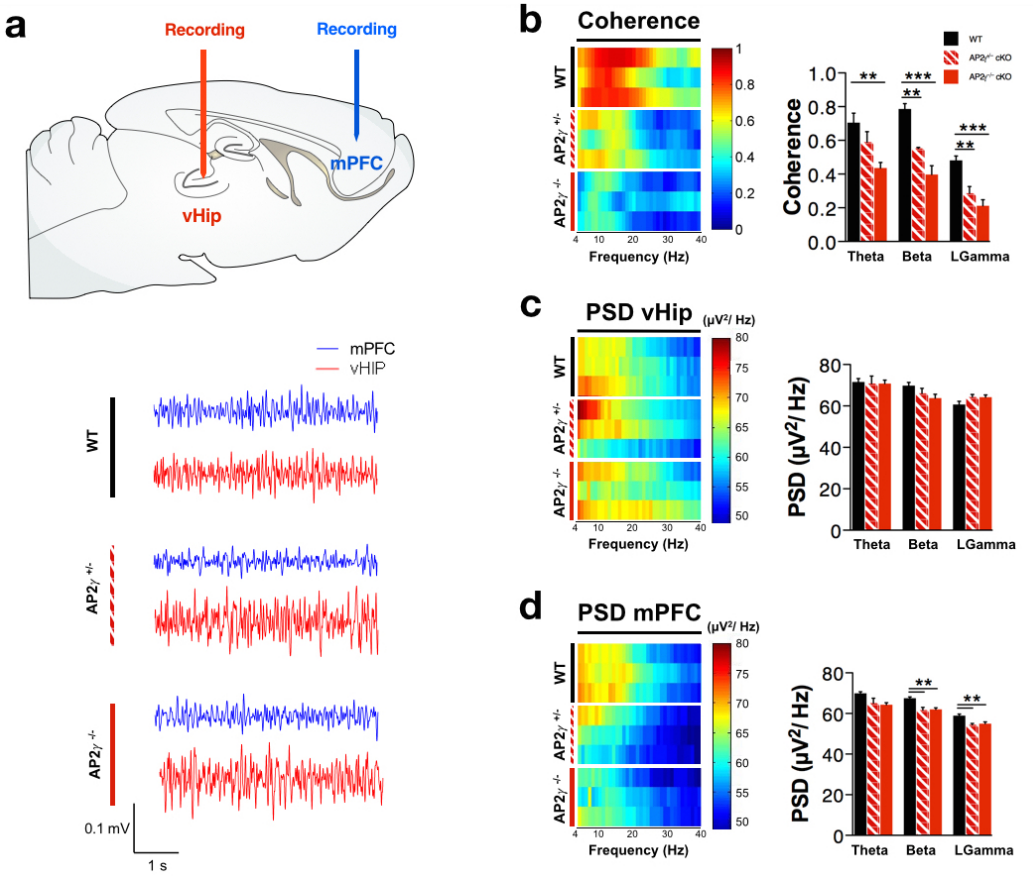

Figure 8. AP2γ deficiency decreases spectral coherence between the ventral hippocampus (vHIP) and the medial prefrontal cortex (mPFC) and neuronal activity within each region. (**a**) Upper panel depicting LFP recording sites and depiction of the electrode positions; lower panel shows representative LFP signals. (**b**) Spectral coherence between vHIP and mPFC of Wt and AP2γ cKO mice (left panel); Group comparison of the coherence values for each frequency band (right panel). (**c**) Power spectral density (PSD) measured in the vHIP (left panel); group comparison of the PSD values for each frequency band (right panel). **(d**) PSD measured in the mPFC (left panel); group comparison of the PSD values for each frequency band (right panel). In the spectrograms each horizontal line in the Y-axis represents the spectrogram of an individual mouse (3 representative mice from each group are shown). ; n=5, One-way ANOVA, * $P \leq 0.05$,** $P \leq 0.01$.; error bars represent s.e.m.