Universidade do Minho
Escola de Ciências da Saúde

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Molecular regulation of the hippocampal neurogenic niche in depression and by antidepressants: Insights from an unpredictable Chronic Mild Stress rat model

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

fevereiro 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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ABSTRACT

Molecular regulation of the hippocampal neurogenic niche in depression and by antidepressants*: Insights from an unpredictable Chronic Mild Stress rat model.*

ABSTRACT

Depression is a complex multidimensional disorder affecting around 20% of the world population. Nevertheless, its pathophysiology is still incompletely understood. Currently available antidepressant treatments are ineffective in a significant proportion of the depressed patients and relapse rates are high, emphasizing the need for better therapeutic strategies. Depression is thought to result from the interplay between genetic predisposition and environmental factors, such as stress, a major precipitating factor for depression. Depressed individuals and chronic stress animal models of depression present impairments in several systems, including neurotransmitters, neuroimmune, endocrine systems and neurotrophic factors.

Adult brain retains the capacity to adapt and re-organize neural networks in response to stimuli. This ability to change occurs in several brain regions and is called neural plasticity. Neural plasticity phenomena include synapto-dendritic changes and the generation of new cells. Neural plasticity impairments in specific brain regions are involved in the pathophysiology of depression. One of these regions is the hippocampus, which is involved in several forms of memory and in stress response. Moreover, the hippocampus comprises one of the few brain regions in the adult brain where neurogenesis occurs, the dentate gyrus (DG). Previous works showed that decreased cell genesis and dendritic morphology alterations are detected in the hippocampus of depressed individuals and animal models of depression, whereas antidepressant treatment prevents these changes.

Here, we used a validated rat model of depression, the unpredictable chronic mild stress (uCMS), to explore the molecular underpinnings of neural plasticity in the hippocampal DG (hDG), in the context of depression and antidepressant treatment. For that, in a first study we performed a transcriptome analysis, in the hDG of untreated depressive-like animals and after chronic treatment with either fluoxetine, imipramine, tianeptine or agomelatine. Behavioral characterization and neuromorphological analysis in the DG revealed that all antidepressants, except agomelatine, were able to reverse the depressive-like profile and the dendritic plasticity impairments induced by uCMS. UCMS-exposure produced molecular changes that were partly reversed by antidepressants. Interestingly, agomelatine reversed less uCMS-induced changes compared to other treatments. In general, fluoxetine impacted more on neurons to reduce the expression of pro-inflammatory

response genes, promoting the expression of cell metabolism-related molecules. Imipramine and tianeptine presented similar molecular profiles, promoting pathways related to cellular protection. Agomelatine produced changes in several pathways, namely on Rho-GTPase-related pathways, impacting on neurons and oligodendrocytes. In summary, antidepressants belonging to different pharmacological classes generated distinct molecular profiles that may be useful in different clinical contexts.

In a second study, we aimed to assess the cell cycle mechanisms regulating hippocampal cell proliferation in the context of depression and antidepressant treatment. For that we used both *in vivo* - uCMS rat model -and *in vitro* - rat hippocampal-derived neurospheres - approaches. UCMS-exposed animals presented decreased proliferation and generation of newborn neurons that were reversed by fluoxetine. In our *in vivo* model, results suggest that uCMS-exposure produces a cell cycle arrest in the hDG progenitor cells that is accompanied by decreased levels of cyclins D1, E and A expression. Chronic fluoxetine treatment reversed the G1 phase arrest and increased cyclin E expression. Dexamethasone (DEX), used to mimic the elevation of glucocorticoids after chronic stress, decreased proliferation *in vitro*, whereas the administration of serotonin (5-HT), a neurotransmitter involved in the actions of fluoxetine, partly reversed this decrease. DEX also induced a G1-phase arrest, decreasing the expression of cyclins D1, cyclin D2 and Cdk6, while increasing p27 expression. 5-HT treatment partly reversed the G1-phase arrest and restored the expression of cyclin D1 and Cdk6. These results suggest that the anti-proliferative actions of chronic stress in the adult hDG result from a glucocorticoid-mediated G1-phase arrest in the progenitor cells that may be overcome by antidepressant treatment.

Together, these findings contribute to the identification of novel molecular players and targets underlying neural plasticity phenomena in the hDG in the context of depression, unravelling unique properties of different classes of antidepressant. This knowledge may help paving the way for the implementation of more personalized treatments and open new avenues for the development of novel antidepressant strategies. Future studies will further disclose the molecular changes in this and other brain regions implicated in depression and hopefully provide clues for forthcoming translational approaches in depressive patients.

RESUMO

Regulação molecular do nicho neurogénico hippocampal na depressão e pela ação de antidepressivos: *Pistas de um modelo animal de stress crónico moderado.*

RESUMO

A depressão é uma doença complexa que afeta cerca de 20% da população mundial. No entanto, a patofisiologia da doença é ainda pouco compreendida. Uma parte significativa dos doentes não responde aos antidepressivos existentes e os índices de recidiva são elevados, sublinhando a necessidade de melhores estratégias terapêuticas. Pensa-se que a depressão resulte da interação entre predisposição genética e fatores ambientais, como por exemplo o stress, um dos principais fatores precipitantes da depressão. Indivíduos com depressão e modelos animais de stress crónico apresentam alterações em vários sistemas, incluindo sistemas de neurotransmissores, neuroimunológicos e endócrinos e fatores neurotróficos.

O cérebro adulto mantém a capacidade de se adaptar e reorganizar as redes neurais em resposta a estímulos. Estas respostas, que incluem alterações sinapto-dendríticas e geração de novas células, ocorrem em várias regiões do cérebro e designam-se globalmente por plasticidade neural. Modificações na plasticidade neural em regiões específicas estão envolvidas na patofisiologia da depressão. Uma destas regiões é o hipocampo, região responsável por várias formas de memória e envolvida na resposta ao stress. É também no hipocampo que se encontra a circunvolução dentada (DG), uma das regiões neurogénicas do cérebro adulto. Em doentes com depressão e em modelos animais verifica-se uma diminuição da geração de novas células e alterações na morfologia dendrítica no hipocampo; estas alterações são prevenidas pela administração de antidepressivos.

Neste trabalho, usou-se um modelo animal de depressão, o modelo de exposição a stress crónico moderado e imprevisível (uCMS), para identificar as bases moleculares da plasticidade neural no DG hipocampal (hDG), no contexto da depressão e do tratamento com antidepressivos. Num primeiro estudo, procedeu-se à análise do transcritoma no hDG de ratos com fenótipo do tipo depressivo e após tratamento com fluoxetina, imipramina, tianeptina ou agomelatina. A caracterização comportamental e a análise neuromorfológica no hDG revelaram que todos os antidepressivos, exceto a agomelatina, revertem o perfil do tipo depressivo e os défices de plasticidade dendrítica produzidos pelo uCMS. A exposição ao uCMS promoveu alterações moleculares que foram parcialmente revertidas pelo tratamento com antidepressivos. Curiosamente, a agomelatina reverteu um menor número de alterações quando comparada com os

outros antidepressivos. Em geral, a fluoxetina teve um impacto significativo em neurónios, reduzindo a expressão de genes de resposta inflamatória e promovendo a expressão de moléculas relacionadas com metabolismo celular. A imipramina e a tianeptina apresentaram um perfil molecular semelhante, promovendo vias de sinalização relacionadas com a proteção celular. A agomelatina produziu alterações em diversas vias, nomeadamente em vias relacionadas com as Rho-GTPases, em neurónios e oligodendrócitos. Em suma, antidepressivos de diferentes classes farmacológicas induzem perfis moleculares distintos que podem ser úteis em diferentes contextos clínicos.

Num segundo estudo, avaliaram-se os mecanismos de ciclo celular que regulam a proliferação hipocampal no contexto da depressão e do tratamento com antidepressivos. Usaram-se duas abordagens complementares: *in vivo* – o modelo animal uCMS – e *in vitro* – neuroesferas derivadas do hipocampo de rato. Os animais uCMS apresentaram uma diminuição da proliferação e da geração de novos neurónios que foram revertidas pela fluoxetina. A exposição ao uCMS produziu uma ligeira repressão da progressão do ciclo celular na fase G1, nas células progenitoras do hDG, que foi acompanhada por uma diminuição dos níveis de expressão das ciclinas D1, E e A. O tratamento crónico com fluoxetina reverteu esta repressão promovendo a expressão da ciclina E. *In vitro*, o tratamento com dexametasona (DEX), para mimetizar a elevação dos glucocorticóides (GCs) induzida pela exposição prolongada a stress, promoveu uma diminuição da proliferação. Por sua vez, a administração de serotonina (5-HT), neurotransmissor envolvido na ação da fluoxetina, reverteu parcialmente estas alterações. A DEX também induziu repressão do ciclo celular na fase G1, diminuindo a expressão das ciclinas D1, D2 e da Cdk6, e aumentando a expressão do p27. O tratamento com 5-HT reverteu parcialmente esta repressão e repôs a expressão da ciclina D1 e da Cdk6. Estes resultados sugerem que as ações anti-proliferativas do stress crónico no hDG adulto resultam de uma repressão na fase G1 em células progenitoras, mediada por GCs, que pode ser revertida pelo tratamento com antidepressivos.

Em conjunto, estes resultados contribuem para a identificação de novos intervenientes na regulação dos fenómenos de plasticidade neural no hDG, na depressão, revelando ainda propriedades particulares de cada antidepressivo. Este conhecimento poderá auxiliar na implementação de tratamentos mais personalizados e encetar oportunidades para o desenvolvimento de novas estratégias terapêuticas. Estudos futuros permitirão desvendar as alterações moleculares nesta e outras regiões do cérebro envolvidas na depressão, esperando-se que estas possam fornecer pistas importantes que promovam o estudo da depressão em pacientes.

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ABBREVIATIONS

ABBREVIATIONS

5-HT – serotonin

A

ACTH – adrenocorticotropic hormone

AD – antidepressant

AGOM – agomelatine

AR – adrenergic receptor

AVP – arginine-vasopressin

B

BDNF – brain-derived neurotrophic factor

BMP – bone morphogenetic protein

BrdU – bromodeoxyuridine

C

CA – cornu ammonis

CDK – cyclin-dependent kinase

CDKi – cyclin-dependent kinase inhibitor

CIP – CDK inhibitory protein

CNS – central nervous system

CORT – corticosterone

CREB – cAMP-response element binding protein

CRF – corticotropin releasing factor

CUS – chronic unpredictable stress

D

DA – dopamine

DALY's – disability adjusted life years

DBS - deep brain stimulation

DCX - doublecortin

DEX – dexamethasone

DG – dentate gyrus

DRN – dorsal raphe nuclei

DSM – Diagnostic and Statistical Manual of Mental Disorders

E

EC – entorhinal cortex

ECT – electroconvulsive therapy

F

FLX – fluoxetine

FST – forced swim test

G

GABA - gamma-aminobutyric acid

GC – glucocorticoid

GCL – granule cell layer

GFAP – glial fibrillary acidic protein

GR – glucocorticoid receptor

GWAS – genome-wide association studies

H

HPA – hypothalamic-pituitary-adrenal

I

IL-6 – interleukin 6

IMIP – imipramine

IRS – inflammatory response system

K

KIP – kinase inhibitory protein

L

LC – locus coeruleus

LTD – long-term depression

LTP – long-term potentiation

M

MAO – monoamine oxidase

MAOi – monoamine oxidase inhibitor

MFB – medial forebrain bundle

mGluR – metabotropic glutamate receptor

MR – mineralocorticoid receptor

MRS – magnetic resonance spectroscopy

N

NAc – nucleus accumbens

NE - norepinephrine

NET – norepinephrine transporter

NIMH – National Institute of Mental Health

NMDA – N-methyl-D-aspartate

NSC – neural stem cell

NSF – novelty suppressed feeding

NPC – neural progenitor cell

P

PAG – periaqueductal gray area

PFC – prefrontal cortex

POMC – pro-opiomelanocortin

PSA-NCAM - polysialylated-neural cell adhesion molecule

PTSD – Posttraumatic stress disorder

PVN – paraventricular nucleus

S

SDT – sweet drive test

SERT – serotonin transporter

SEZ – subependymal zone

SGZ – subgranular zone

SNRI – serotonin norepinephrine reuptake inhibitor

SPT – sucrose preference test

SSRI – selective serotonin reuptake inhibitor

STAR*D – Sequenced Treatment Alternatives to Relieve Depression

T

tANP – transit amplifying neural progenitor

TCA – tricyclic agent

TIAN – tianeptine

TMS – transcranial magnetic stimulation

TNF-α – tumor necrosis factor alfa

U

uCMS – unpredictable Chronic Mild Stress

V

VEGF – vascular endothelial growth factor

VTA – ventral tegmental area

W

WHO – World Health Organization

THESIS LAYOUT

THESIS LAYOUT

Chapter 1 presents a general introduction on depression epidemiology, treatment and the major neurobiological mechanisms involved in its pathophysiology, with a particular emphasis on the role of stress and neural plasticity in the hippocampus.

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

Chapter 3 comprises the research work on the molecular mechanisms regulating neural plasticity in the dentate gyrus (DG) of an animal model of depression and after the treatment with different classes of antidepressants. This chapter is presented as a final original paper published in Neuropsychopharmacology in 2015 (Patrício *et al.* 2015).

Chapter 4 displays the research work on the cell cycle regulation in proliferative cells of the adult hippocampal neurogenic niche, in an animal model of depression and after fluoxetine treatment.

Chapter 5 encompasses a general discussion, in which the major findings of the research work are debated in light of the current relevant literature, as well as its limitations. Future perspectives on how the field may evolve are also discussed.

Appendices

I) Review paper on the cell cycle mechanisms regulating adult hippocampal proliferation and neurogenesis in the context of depression and antidepressant treatment, published during my PhD (Patrício *et al.* Mol Neurobiol, 2013).

II) Research paper on the development of a new behavioral paradigm to assess anhedonia in rodents, published during the period of my PhD (Mateus-Pinheiro*; Patrício*, *et al.* Front Behav Neurosci, 2014, *authors contributed equally).

III) Preliminary data - Relevance of miRNA-411-5p for hippocampal neuroplasticity in the uCMS rat model of depression and after fluoxetine treatment.

IV) Published abstract: Poster from the European College on Neuropsychopharmacology (ECNP) Workshop 2015 (P.1.018, Patrício *et al.* Eur. Neuropsychopharmacol., 2015).

V) Published abstract: Oral communication from the 28th ECNP Congress 2015 (S.15.06, Patrício *et al.* Eur. Neuropsychopharmacol., 2015).

1. INTRODUCTION

1. INTRODUCTION

1.1 DEPRESSION: A GLOBAL SOCIOECONOMIC BURDEN

1.1.1 EPIDEMIOLOGY OF DEPRESSION

Major Depression is among the most prevalent psychiatric disorders and is estimated to affect 350 million people worldwide (WHO, 2012). This disorder affects all communities across the world and is more prevalent in women than in men (1.5 to 3-fold higher rates). Major Depression may appear at any age but it often starts early in life, with the likelihood of onset increasing markedly with puberty (American Psychiatric Association, 2013). Moreover, depression is a chronic disorder, presenting high rates of recurrence, with at least 50% of patients who had a first episode of depression having one or more episodes during their lifetime (Burcusa and Iacono, 2007). Depression has also been associated with the development of other medical conditions, including cardiovascular disorders (Van der Kooy et al., 2007), stroke (Pan et al., 2011), Alzheimer's disease (Green et al., 2003, Ownby et al., 2006), type 2 diabetes (Knol et al., 2006, Nouwen et al., 2010) and cancer (Gross et al., 2010, Currier and Nemeroff, 2014). A growing body of evidence suggests a bidirectional link between depression and other medical illnesses, with each condition having a negative impact on the onset, progression, prognosis and treatment of the other (Katon, 2003, Ramasubbu et al., 2012). This puts depression as the second leading cause of disability worldwide, accounting for 65.6 million disability adjusted life years (DALYs) (Collins et al., 2011, Kessler and Bromet, 2013).

1.1.2 SYMPTOMS OF DEPRESSION

Major Depression is a complex and heterogeneous clinical entity poorly characterized in terms of cellular and molecular pathophysiological factors. In this way, the diagnosis of depression is clinical. The clinical classification of depression has been structured in diagnostic tools such as the Diagnostic and Statistical Manual of Mental Disorders (DSM). According to DSM-V, Major Depression symptoms include depressed mood and anhedonia, i.e. diminished interest or pleasure in all, or almost all activities, which are core features of depression (Nelson and Charney, 1981), neurovegetative signs, including changes in sleeping patterns and appetite, fatigue or loss of energy,

feelings of worthlessness or guilt, diminished ability to concentrate and suicidal ideation (Hasler et al., 2004, American Psychiatric Association, 2013). The diagnosis of a major depressive episode must meet at least five of these symptoms during a 2-week period, being one of the symptoms depressed mood or anhedonia. Additionally, the symptoms must cause clinically significant distress or impairments in relevant areas of functioning, such as social, professional or occupational (American Psychiatric Association, 2013).

Depression symptomatology is often accompanied by anxiety and cognitive dysfunction. Anxiety and depression are highly co-morbid, suggesting a common pathophysiology for both conditions (Hettema, 2008). Changes in executive function, attention deficits, and short-term, long-term and working memory impairments have been found in depressed patients (Castaneda et al., 2008, Gonda et al., 2015) and may play a crucial role in therapeutic response and risk of relapse (Gonda et al., 2015).

1.2 TREATMENT IN DEPRESSION

1.2.1 ANTIDEPRESSANT DRUGS

Antidepressants are the first line treatment for depression. Despite the significant advances in the pharmacotherapy of depression during the last decades, our current knowledge on the mechanisms of action of this broad class of drugs is still scarce. An important study, completed in 2006, the National Institute of Mental Health (NIMH)-funded Sequenced Treatment Alternatives to Relieve Depression (STAR*D) trial, provided important data on the effectiveness of antidepressant drugs treatment in major depressive disorder patients. This trial was the largest and longest study ever conducted to evaluate depression treatment and enrolled 4,041 outpatients with non-psychotic depression, though from these only 2,876 met the study requirements and proceeded to evaluation (Deakin and O'Loughlin, 2009, Sinyor et al., 2010). Results from this trial showed that only one third of the patients remitted after first antidepressant treatment. Over the course of the study, i.e. after one year and four treatment levels, almost 70 percent of those who did not withdraw from the study, remitted. Overall, STAR*D data showed that some patients may need to try several treatment strategies in order to remit from depression, though the probability of treatment decreased with every additional treatment strategy needed (NIMH, 2006). Most strikingly, it called the attention for the fact that a substantial proportion of patients do not achieve remission with the currently available pharmacological treatments (Pigott, 2015). Together, this emphasizes the need for a better understanding on the mechanisms of action of antidepressants and on how this relates to people's response to different treatments. The expectations for the development of novel, more effective antidepressants are now higher, first because there are more people suffering from this disorder and not benefiting from the available drugs, and second, because treatments are expected not only to promote symptoms relief but also to allow good performances at both social and professional levels.

Most antidepressants have been found by serendipity, which partly explains the frankly limited knowledge about their mechanisms of action. Given that and their complex pharmacology there is no uniform classification system. In general, these are classified according to their primary pharmacological targets, while others take their names from their chemical structure. The following subsection does not aim at an exhaustive description of all antidepressants and their mechanisms of action. Its purpose instead, is to provide a general overview on the major classes of antidepressants

and their major pharmacological mechanisms, and to give a chronological perspective on how the antidepressant research field has evolved along the years (**Figure 1**).

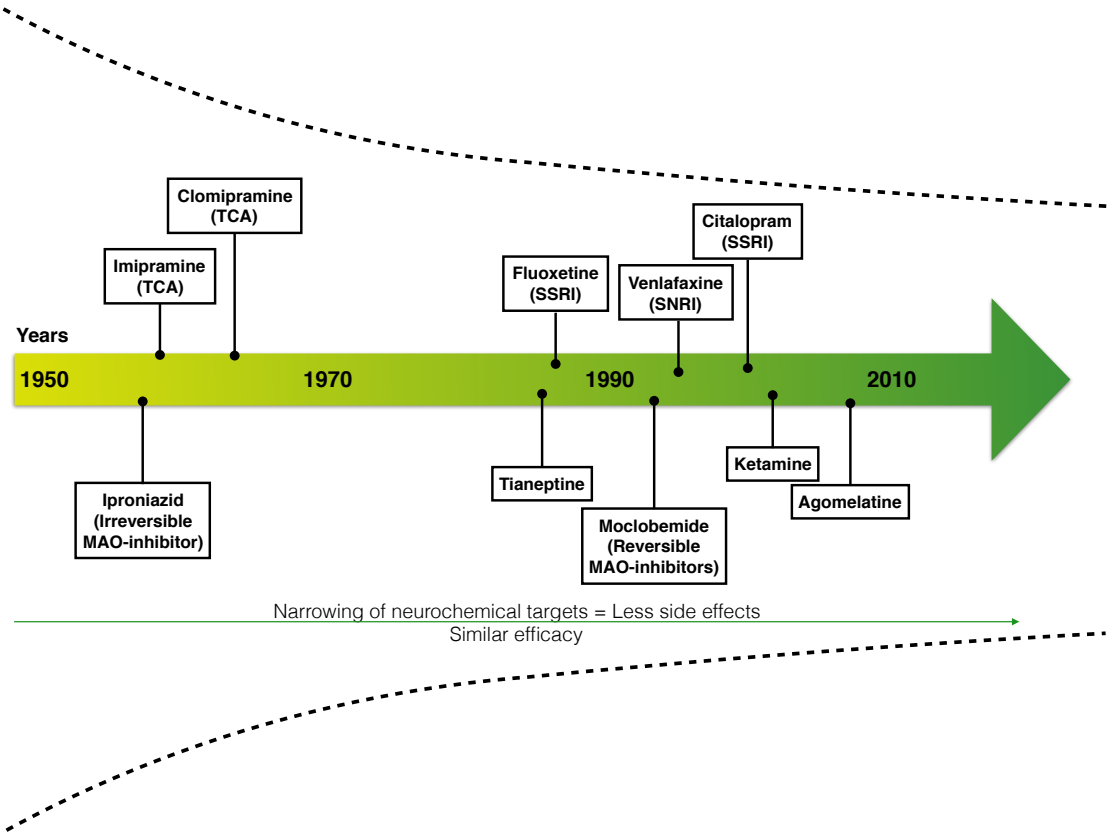


Figure 1: Schematic representation of the chronology for introduction of drugs for the treatment of depression. The generic names of each class of drugs or individual drug are presented. Not all drugs are presented, only those mentioned in the text. Abbreviations: TCA – tricyclic; MAO – monoamine oxidase; SSRI – selective serotonin reuptake inhibitor; SNRI – serotonin norepinephrine reuptake inhibitor. *Adapted from Millan et al. 2015.*

Monoamine oxidase inhibitors (MAOIs)

The first therapeutically used antidepressants were inhibitors of the enzyme monoamine oxidase (MAO) (Stahl, 2013). MAOs are a family of enzymes that catalyse the oxidation of monoamines. There are two types of MAO in mammals: MAO-A, an enzyme that preferentially metabolizes serotonin, and MAO-B, that mainly metabolizes phenethylamines. In most species, both enzymes are able to oxidize dopamine, norepinephrine, epinephrine, tryptamine and tyramine (Youdim et al.,

2006). Drugs from this class of antidepressants interfere with the catabolism of monoamines, thus increasing the availability of these neurotransmitters. These antidepressants emerged in the 1950's by serendipity when treating tuberculosis patients with isoniazid. Iproniazid, a related compound, was the prototype of this class of antidepressants and presented powerful antidepressant effects, but had to be withdrawn due to toxicity and serious side-effects (Millan et al., 2015). Since then, the research on MAOIs has been further driven to develop newer, safer drugs, namely reversible inhibitors of MAO and MAO-A specific inhibitors (Millan et al., 2015). This class includes the antidepressants tranylcypromine, phenelzine and moclobemide.

Tricyclics (TCAs)

TCA antidepressants are a group of structurally similar compounds also developed in the late 1950's (Millan et al., 2015). TCAs inhibit the reuptake of both norepinephrine and serotonin, by blocking the norepinephrine transporter (NET) and the serotonin transporter (SERT), respectively (Nojimoto et al., 2010, Stahl, 2013). Interestingly, different tricyclic antidepressants have been shown to present distinct selectivity for each transporter. Also contributing for their therapeutic profile is the 5-HT2A and 5-HT2C receptors antagonistic actions observed for some TCAs (e.g. amitriptyline and clomipramine)(Stahl, 2013). Adding to these beneficial antidepressant properties, TCAs present several other pharmacologic targets that are responsible for relevant side effects of these drugs. These include antagonism of muscarinic cholinergic receptors, H_1-histaminic receptors, α_1-adrenergic receptors, and voltage-sensitive sodium channels (**Table 1**)(Richelson and Nelson, 1984, Nojimoto et al., 2010). This class includes amitriptyline, imipramine, clomipramine and desipramine.

Selective Serotonin Reuptake Inhibitors (SSRIs)

SSRIs were firstly introduced in the 1980s and are now the predominant first-line treatment for major depression. These drugs are as effective, but better tolerated and safer than TCAs. SSRIs major pharmacological mechanism is the blockade of serotonin reuptake through inhibition of SERT on presynaptic neurons (Kennedy and Rizvi, 2014). As a consequence, serotonin accumulates in the synapse and promotes long-term desinhibition of serotonergic neurotransmission (Stahl, 1998,

Stahl, 2013). There are six drugs included in this class - fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram and escitalopram – all of them sharing the same major pharmacological mechanism. However, each of these drugs has secondary pharmacological targets (Stahl, 2013, Kennedy and Rizvi, 2014) that may account for their individual differences in efficacy.

Serotonin and Norepinephrine reuptake Inhibitors (SNRIs)

Introduced in the early 1990's, this class of antidepressants is also currently used as first-line treatment for depression (Block and Nemeroff, 2014). As the name suggests these are inhibitors of serotonin and norepinephrine reuptake, thus increasing the levels of these neurotransmitters in the synaptic cleft. Additionally, NET inhibition in the prefrontal cortex (PFC) promotes dopamine increases in this brain region (Stahl, 2013). This class includes the antidepressants venlafaxine and duloxetine.

Other antidepressant drugs

Next I will describe some antidepressant drugs that are not included in any of the above-mentioned classes.

Tianeptine

Tianeptine was introduced in the 1980's in Europe, to treat depression (Millan et al., 2015). Although it is chemically classified as a TCA, tianeptine is usually not included in this class of antidepressants, due to its distinct pharmacological properties (McEwen et al., 2010). Contrary to other TCAs, the mechanism of action of this antidepressant does not involve modulation of the monoaminergic neurotransmission. Instead, preclinical data suggest, it acts as a modulator of the glutamatergic neurotransmission in particular limbic brain regions, including the hippocampus, amygdala and PFC (Kasper and McEwen, 2008).

Agomelatine

Agomelatine was first approved for the treatment of depression in Europe, in 2009. It is a melatonergic agonist of MT1 and MT2 receptors, and an antagonist of 5-HT2c receptors. The melatonergic actions of this antidepressant appear to promote resynchronization of circadian rhythms and reverse the phase delay of depression (Stahl, 2013). However, melatonergic actions alone are not sufficient to promote its antidepressant effects, as melatonin or selective MT agonists do not show antidepressant effects (Hickie and Rogers, 2011). The antagonist effects on 5-HT2c have been shown to prevent inhibition of norepinephrine and dopamine release in the PFC (Stahl, 2013).

Ketamine

Ketamine was introduced more than 40 years ago as an anesthetic drug used in surgical and diagnosis procedures (Haas and Harper, 1992). Pharmacologically, it is a high affinity non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist (Naughton et al., 2014). In 2000, Berman et al. showed for the first time that a subanesthetic dose of ketamine reduced depression symptoms (Berman et al., 2000). Some years later another study corroborated these results by showing the rapid effects of ketamine in reversing depressive symptoms in treatment resistant patients (Zarate et al., 2006). Since then, many studies have addressed the mechanisms of action and efficacy of ketamine, although different rates of response have been found (Naughton et al., 2014). Given the insufficiency of comprehensive clinical trials showing its efficacy and few data on long-term effects, ketamine is still not routinely recommended (Naughton et al., 2014).

Table 1: Summary of representative antidepressant agents and their major pharmacological targets (adapted from Millan et al., 2015).

Representative antidepressant	Class name	Major pharmacological mechanism
Phenelzine	MAO inhibitor	Irreversible MAO inhibition.
Moclobemide	MAO inhibitor	Reversible MAO-A inhibition.
Imipramine	TCA	5-HT/NE reuptake inhibition. α1-AR, histamine H1 and muscarinic receptors antagonism.
Amitriptyline	TCA	5-HT/NE reuptake inhibition. α1-AR, histamine H1, 5-HT2A/2C and muscarinic receptors antagonism. NA+/Ca+ ion channels blocking.
Clomipramine	TCA	5-HT/NE reuptake inhibition. α1-AR, histamine H1, 5-HT2A/2C and muscarinic receptors antagonism.
Fluoxetine	SSRI	5-HT reuptake inhibition.
Venlafaxine	SNRI	Serotonin-norepinephrine reuptake inhibition.
Tianeptine	-	Glutamatergic neurotransmission modulation.
Agomelatine	-	MT1 and MT2 receptor agonism. 5-HT2C antagonism.
Ketamine	-	NMDA receptor antagonism

Abbreviations: MAO – Monoamine oxidase; TCA – Tricyclic agent; SSRI – Selective serotonin reuptake inhibitor; 5-HT – serotonin; NE – norepinephrine; AR – adrenergic receptor; MT – Melatonin; NMDA – N-methyl-D-aspartate.

1.2.2 Combination/augmentation strategies and somatic treatments for depression

In cases in which standard antidepressant treatment fails or patients respond insufficiently to monotherapy, alternative interventions may be considered to treat depression. These include changing antidepressant treatment, combining antidepressants from the same of from different pharmacological classes, or augmentation strategies that consist in the addition of a non-antidepressant treatment (e.g. lithium, atypical antipsychotics). Non-pharmacological, non-psychotherapy treatments may also be used alone or in combination with pharmacological treatments. These include vagus nerve stimulation (VNS), electroconvulsive therapy (ECT), transcranial magnetic stimulation (TMS) and deep brain stimulation (DBS).

VNS involves implantation of a device connected to electrodes that deliver low-frequency pulses to the vagus nerve. This therapy has been shown to increase activation of neurons in the locus

coeruleus (LC) and dorsal raphe nuclei (DRN) (Kennedy and Giacobbe, 2007), and consequently produce changes in the levels of monoamines, including serotonin, norepinephrine, GABA and glutamate, in several brain regions (Akhtar et al., 2016). ECT consists in the administration of repeated generalized electrically induced seizures. This is one of the most effective treatments for depression, however it is usually only applied in severely ill patients or patients that do not respond to antidepressant drug treatment (Berton and Nestler, 2006). The major drawback of this treatment is the potential side effect on memory (Block and Nemeroff, 2014). TMS is the least invasive of these brain stimulation techniques and is used in patients that did not respond to one antidepressant treatment trial. It consists in the production of a magnetic field around the brain and in the delivery of an electrical current most frequently targeting the dorsolateral PFC (Block and Nemeroff, 2014). DBS involves implantation of electrodes in the brain promoting neuromodulatory effects on specific circuits. Although still not an approved treatment for depression, several clinical trials have reported the efficacy of DBS for treating refractory depression (Mayberg et al., 2005, Schlaepfer et al., 2013). Most trials have targeted brain regions involved in the reward response, such as the subgenual cingulate, nucleus accumbens (NAc) and medial forebrain bundle (MFB) (Schlaepfer et al., 2014). Interestingly, this technique has helped to improve the knowledge on the neuronal circuitry involved in depression.

1.3 AETIOLOGY OF DEPRESSION: INTERPLAY BETWEEN GENETICS AND ENVIRONMENTAL FACTORS

Depression is a complex heterogeneous clinical entity that is thought to result from the interplay between genetic predisposition and environmental factors (Sullivan et al., 2000). Genetic factors may play a significant role in the development of this disorder. However, notwithstanding the considerable evidence supporting a genetic contribution to disease predisposition, genome-wide association studies (GWAS) and meta-analyses have failed to find robust susceptibility genes associated with depression (Flint and Kendler, 2014). Epidemiologic studies show that depression is only moderately heritable (around 40%) and cannot be considered a genetic disorder (Levinson, 2006), with around 60% of the factors involved in its aetiology not being explained by genetic variability (Flint and Kendler, 2014). Environmental factors have been shown to play a prominent role, increasing the risk for the development of depression and include early life trauma, drugs, medical illness and chronic stress (Wong and Licinio, 2001, Saveanu and Nemeroff, 2012).

1.3.1 THE ROLE OF STRESS

Stress is one of the most widely recognized precipitating factors of depression. Hans Selye coined the term “stress” in 1936, in a Letter to Nature. At that time, he described the General Adaptation Syndrome, as a set of physiological changes occurring in response to adverse stimuli (Selye, 1936). In other words, stress can be defined as any event interpreted as a threat to the physiological or psychological integrity of an individual that produces physiological and/or behavioral responses (McEwen, 2000). These responses are expected to allow the individual to cope with the situation. However, stress is not a single entity and several types of stress can be defined; stress may be acute or chronic, may occur only once or be repetitive in time, can be predictable or unpredictable, and mild or severe (Lucassen et al., 2014). Indeed, not all types of stress are detrimental or have been linked with development of depression. In fact, stress has an important adaptive function with beneficial effects to the organism, when there is reinstatement of homeostasis (allostasis)(McEwen and Gianaros, 2011).

1.3.1.1 The Hypothalamic-Pituitary-Adrenal (HPA) axis in the stress response

Stress elicits a complex response that encompasses autonomic, physical, behavioral and neuroendocrine changes (Fulford and Harbuz, 2005). The brain is central in this process, as it perceives both sensory inputs from the external environment and internal inputs from the body. During this processing, it coordinates and adjusts physiological and behavioral responses to fit the imposed challenges (McEwen and Gianaros, 2011).

The HPA axis is one of the biological systems involved in the stress response. The activated HPA axis regulates peripheral functions, including metabolic and immune functions, but also impacts on the central nervous system (CNS) (Pariante and Lightman, 2008). During stress response, activation of the paraventricular nucleus (PVN) in the hypothalamus results in the secretion of corticotropin-releasing factor (CRF) and arginine-vasopressin (AVP), which in turn promotes the secretion of adrenocorticotropic hormone (ACTH) from the pituitary into the blood stream. This hormone stimulates glucocorticoids (GCs; cortisol in humans and corticosterone in rodents) release from the adrenal cortex. GCs will then promote the regulation of both brain and peripheral function by interacting with their receptors in several target tissues (Fulford and Harbuz, 2005, Stahl, 2013). As important as to initiate this response is to ensure that it is terminated; auto-regulation of the HPA axis is achieved by both rapid and delayed negative feedback at several sites. The rapid feedback occurs immediately after the increase in circulating GCs and lasts 5 to 15 min. This rapid feedback is probably a consequence of non-genomic actions of GCs and is responsible for the inhibition of ACTH synthesis and release at the pituitary level. ACTH levels decrease as a consequence of down-regulated CRF and AVP expression in the hypothalamus. On the other hand, the delayed feedback occurs 1 to 2 h after the rise of GCs and may continue from 4 up to 24 h, if there is a prolonged activation of the axis. This delayed profile suggests that the response depends on genomic actions of GCs and manifests as a decreased mRNA expression of the ACTH precursor protein, the pro-opiomelanocortin (POMC), in the hypothalamus and pituitary. Forebrain limbic structures are involved in the regulation of autonomic and HPA axis stress response, and may serve as links between the stress response and the development of neuropsychiatric disorders, such as depression (Smith and Vale, 2006). The hippocampus is highly involved in the termination of stress response, since the stimulation of hippocampal neurons decreases the neuronal activity in the PVN inhibiting GCs secretion. Moreover, the hippocampus encodes sensorial information from the environment associated with the stressor (Herman et al., 2005). The PFC also negatively regulates the HPA axis

in response to stress, by attenuating ACTH and GCs secretion. Moreover, medial PFC neurons are activated and promote catecholamines release in response to stress. On the other hand, the amygdala positively regulates the HPA axis, promoting the release of GCs (Smith and Vale, 2006). The articulation of these responses is crucial to ensure the termination of the stress response and to reinstate homeostasis (Fulford and Harbuz, 2005). Two receptors are primarily involved in the actions of GCs: the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). GR is a low affinity GC selective receptor, whereas MR is a high affinity receptor with equal affinity for aldosterone. These receptors are located in the cytoplasm; once steroids diffuse across the plasma membrane and bind to the receptor, the receptor-ligand complex translocates to the nucleus and acts as a transcription factor, by interacting with DNA-responsive elements (Fulford and Harbuz, 2005). MRs are activated during basal physiological conditions, at low corticosterone concentrations and have been described to play a role in stress assessment, response initiation and maintenance. On the other hand, GRs are activated at higher corticosterone concentrations (e.g. circadian peak or stress) and are responsible for stress response termination and network normalization (Reul and de Kloet, 1985, Sousa et al., 2008). The distribution of both receptors in the CNS is distinct, although some overlap may be found in particular brain regions. Whereas, MRs have a limited distribution, being predominantly found in hippocampal and septal neurons, but also in the amygdala, PVN and LC neurons, GRs are ubiquitously found in neurons and astrocytes, but enriched in the hippocampus, PVN and the lateral septum (reviewed in Fulford and Harbuz, 2005, Sousa et al., 2008, Joels and Baram, 2009). **Figure 2** shows a schematic representation of the HPA axis in humans and rodents under a physiological stress response.

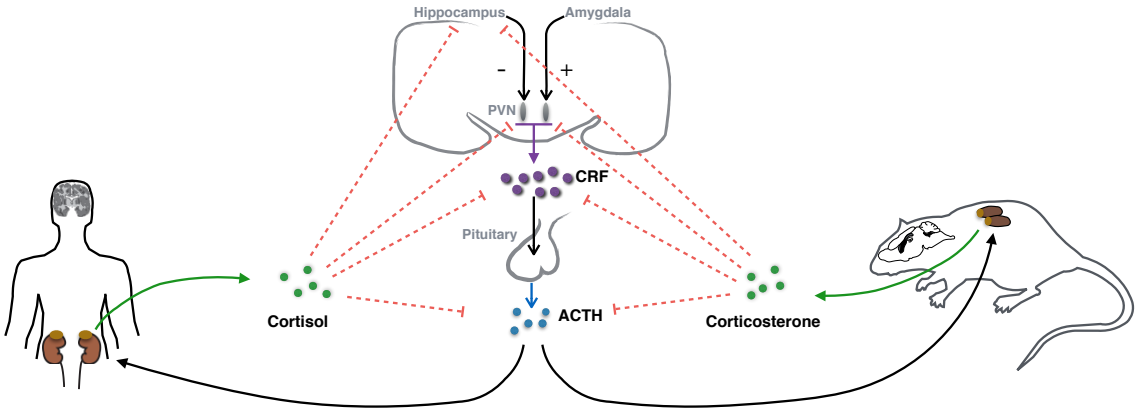


Figure 2: Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis regulation in humans and rodents. PVN – paraventricular nucleus; CRF - corticotropin releasing factor; ACTH - adrenocorticotropic hormone.

1.3.1.2 Chronic stress and depression

Decades of research on stress have demonstrated that the stress response and its consequences depend on numerous factors including, timing of the stressor, inherent characteristics of the stressor (intensity, duration and nature of the stressor), predictability and individual susceptibility (Sousa, 2016). In this sense, stressors occurring in specific neurodevelopmental stages or exceeding a certain intensity or duration, may elicit a dysregulated maladaptive response (Sousa and Almeida, 2012) and trigger the development of neuropathological scenarios, including anxiety-related disorders, post-traumatic stress disorder (PTSD), and depression. Furthermore, the perception of an event as stressful, and consequently the stress response, are dramatically variable among individuals. Thus, variations in the way distinct individuals cope with stress may be determinant in the development of stress-related neuropathology (Sapolsky, 2015).

Chronic stress is indeed one of the most potent precipitating factors of depression. As mentioned earlier, the HPA axis is central to the stress response, as it rapidly incites the secretion of GCs to promote alertness, vigilance, attention and acquisition through activation of both brain and peripheral functions. When the stress stimulus is over, these reactions are blunted in order to re-establish normalization, promoting adaptation and consolidation. Prolonged exposure to stress promotes continuous activation of this response producing changes in several neurobiological

systems involved in the pathophysiology of depression (Herbert et al., 2006, Pariante and Lightman, 2008). In animal models, exposure to chronic stress or GCs leads to a decreased expression of GR in the hippocampus, which will consequently lead to disinhibition of the HPA axis and to a further increase in GCs secretion (Willner et al., 2013, Wang and Harris, 2015). Importantly, around 50% of depressed patients present hyperactivation of the HPA axis, which results in increased cortisol levels (Anacker et al. 2011). Deficient GC-mediated feedback inhibition may explain this impairment. In these patients, successful antidepressant treatment re-establishes the negative feedback on the HPA axis by GCs, thus being a significant predictor of long-term clinical response (de Kloet et al., 2005, Pariante and Lightman, 2008).

How the transition from acute to chronic stress translates in the brain is still under investigation, but data suggests that during chronic stress there is an involvement of additional neuronal projections that result in a distinct network activation state when compared to acute stress (Sousa, 2016). Ultimately, further investigating this transition from a beneficial moderated stress response to a chronic maladaptive one may be of relevance to understand how stress increases vulnerability to several neuropsychiatric disorders, including depression. The consequences of chronic stress and their role in depression in particular brain regions, namely the hippocampus, will be addressed more in-depth later in this chapter.

1.4 ANIMAL MODELS OF DEPRESSION: TOOLS TO STUDY DISEASE PATHOPHYSIOLOGY

One of the main challenges when defining the etiopathogenesis of depression is the necessary difficulty in studying this disorder in humans. On the other hand, the fact that depression is a complex, multidimensional disorder, affecting higher cognitive processes, such as motivation and self-esteem, makes it difficult to approach in a clinical setting and also to model in animals (Wong and Licinio, 2001). Nonetheless, the knowledge on the neurobiological mechanisms underlying depression has progressed substantially in the last years, partly due to preclinical studies (Song and Leonard, 2005, Krishnan and Nestler, 2008, Cryan and Holmes, 2005).

Animal models of depression are usually evaluated for their face validity, etiological validity and pharmacological validity (Belzung and Lemoine, 2011, Krishnan and Nestler, 2011). Face validity is the ability to induce behavioral and neurobiological signs in animals that are similar to the symptoms of the human disease. Etiological validity refers to the similarity between the precipitating factors used to induced these signs in animals and those assumed to precipitate the human disease (Berton and Nestler, 2006). Pharmacological, or predictive, validity refers to the ability of effective antidepressant treatments to reverse the depressive-like signs (Bessa et al., 2009b, Krishnan and Nestler, 2011).

Most likely, animal models will never be able to fully recapitulate the complexity and heterogeneity of the human disease, especially because some of the symptoms are presumably limited to humans (e.g. feelings of worthlessness, guilt and suicidal thoughts). Even so, many features present in depressive patients, including anhedonia, changes in neurovegetative functions (appetite and sleep patterns) and cognitive changes have been replicated in preclinical models (Berton and Nestler, 2006, Bessa et al., 2009b, Krishnan and Nestler, 2011). These animal are thus potentially useful and robust approaches to study the neurobiology of depression and explore novel therapeutic opportunities (Belzung and Lemoine, 2011), when used with an understanding of its limitations.

Animal models of depression include lesion animal models (e.g. olfactory bulbectomy model (Song and Leonard, 2005)), immune stimulation models (e.g. endotoxin; proinflammatory cytokines (De La Garza, 2005)), and stress models (Berton and Nestler, 2006). Animal models that use stress as a precipitating factor include: acute stressor models (e.g. novel environment, forced swimming, tail suspension, inescapable foot shock (Nestler et al., 2002)), and prenatal, maternal separation and chronic stressors models (e.g. chronic mild stress, chronic unpredictable stress, sustained social stress (Berton and Nestler, 2006)). For some of these models, the previously referred validity criteria

are not fulfilled, first, because some use etiological factors which are not usual in human depression, and second, because they do not cover the multidimensional nature of depressive behavior. Stress models, including prenatal, maternal separation and chronic stressors models are apparently more closely related to the human disease, as they are based on clinical evidence that chronic stressful life events or during specific developmental stages significantly increase the risk for depression (Berton and Nestler, 2006). As mentioned earlier in this chapter, stress exposure is one of the most consensual precipitating factors for depression. Noteworthy, depression may develop in the absence of noticeable stress, and individuals experiencing chronic stress exposure do not necessarily develop depression, further underscoring the multidimensional etiopathogenesis of human depression and the difficulty in developing flawless animal models (Kendler et al., 1999, Feder et al., 2009).

1.4.1 The unpredictable chronic mild stress model (uCMS)

Originally developed by Willner and colleagues in the 1980's, the uCMS model is a widely used rodent model of depression, consisting of continuous exposure to a set of mild stressors in an unpredictable fashion, over a relatively prolonged period of time (Willner et al., 1987). 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; habituation 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. Because an unexpected stressor cannot be prevented or controlled it produces more severe consequences. Conversely, stimuli that vary in a predictable manner, such as seasonal periods, homeostatically increase the stress response (Franklin et al., 2012).

The development of this model was based on observations that rats reduced their sucrose preference – assumed as a reflection of anhedonia, and a core symptom of human depression – and presented changes in locomotor activity, when exposed to relatively severe stressors for a prolonged period (Katz, 1981). The alternative proposed by Willner, incorporated two main changes: first, a reduction in the severity/intensity of the stimuli, to more realistically resemble the human daily life stressors; and second, reactivity towards reward was taken as the most relevant behavioral endpoint, instead of locomotor activity (Willner, 2005).

More than 25 years after the development of this paradigm, laboratories from all over the world have explored other features and endpoints of this animal model (Willner, 2005, Hill et al., 2012). Indeed, animals subjected to the uCMS protocol present a set of behavioral changes with strong face validity to human depression, that include not only anhedonia, but also anxiety and cognitive impairments in spatial memory and object recognition tasks (Bessa et al., 2009a, Bessa et al., 2009b). In addition, this protocol produces a variety of neurobiological alterations akin to human depression, such as neuroplastic alterations in the hippocampus, amygdala and PFC, changes in neurotransmitters systems and hyperactivation of the HPA axis (Willner, 2005, Hill et al., 2012). Importantly, most of these changes are reversed by chronic, but not acute, treatment with clinically relevant antidepressant drugs from different pharmacological classes (Willner, 2005), thus supporting the predictive validity of the model.

The relevance of this animal model to study depression and antidepressants actions was recently addressed in a very interesting and comprehensive review (Hill et al., 2012). The authors summarized and collected literature data from the neurobiological alterations induced by uCMS exposure, and subsequently examined which changes could be comparable to those seen in human depression (Hill et al., 2012). Their review suggests that the uCMS is a useful model to study the neurobiology of the human disease and endorses the use of particular neurobiological variables, alone or in conjunction with behavioral endpoints, to assess antidepressants efficacy and the antidepressant potential of novel therapeutic agents (Hill et al., 2012).

1.5 PATHOPHYSIOLOGY OF DEPRESSION

Notwithstanding its high prevalence and significant clinical and social burden, depression's pathophysiology is still poorly understood compared to other disorders with equivalent impact. Nonetheless, during the last decades, the efforts of many researchers resulted in the development of several hypotheses to explain the neurobiology of depression. Current views, integrating biochemical, cellular and neuroimaging data from both clinical and preclinical settings, suggest that depression is a systems disorder affecting several pathways and mediators.

In this section, I will briefly address some of the major findings that led to the proposal of key theories to explain the pathophysiology of depression. Later in this chapter, a specific section will be dedicated to exploring the neural plasticity theory of depression, as a downstream target for dysregulation in depression. Emphasis will be put in hippocampal neuroplasticity and its molecular regulation, as that is the main focus of the experimental work of this thesis.

1.5.1 SYSTEMS DYSREGULATION IN DEPRESSION: ONE DISORDER, MANY TARGETS

1.5.1.1 Neural circuitry

Despite the progress made in the understanding of depression's neurobiology, the details on the disorder's neural circuitry are still emerging. The convergence of data from neuroimaging, post-mortem histopathological studies and DBS in humans, as well as data from preclinical models, has greatly contributed to unveil the complex neural circuitry of depression. Human functional neuroimaging studies have most consistently demonstrated that depression is associated with dysregulated activity in the PFC and subgenual cingulate cortex (Cg 25), probably involved in emotion experience and processing, and in the hippocampus and amygdala, which are involved in emotional memory formation and retrieval (Ressler and Mayberg, 2007, Schlaepfer et al., 2014). Additionally, these studies show that the Cg25 and amygdala are hyperactive in depressive patients returning to normal functioning after effective antidepressant treatment (Krishnan and Nestler, 2008). The brain's reward circuitry, namely the ventral tegmental area (VTA), NAc and related pathways, has also been extensively implicated in depression (Russo and Nestler, 2013). Dysfunction in these brain regions may be responsible for the hedonic deficits and decreased motivation observed in most depressed individuals (Berton and Nestler, 2006). Targeting these brain

regions using DBS has produced robust antidepressant effects in treatment-resistant depressive patients (Ressler and Mayberg, 2007, Schlaepfer et al., 2014). These various forebrain regions receive monoaminergic projections: dopamine from the VTA, serotonin from the dorsal raphe in the periaqueductal grey area (PAG), and norepinephrine from the LC.

In summary, several brain regions and neural pathways responsible for controlling emotion, reward response and executive function have been described as dysregulated in depression, including the limbic-cortical, amygdalar, striatal, dorsal raphe, hippocampal and mesolimbic dopamine circuits (reviewed in (Drevets et al., 2008, Price and Drevets, 2012, Tye and Deisseroth, 2012).

1.5.1.2 Monoaminergic neurotransmission

A role for monoaminergic neurotransmission has long been implicated in the development and treatment of depression (Berton and Nestler, 2006). Indeed, one of the first hypotheses of the neurobiology of depression was based on observations that antidepressants, namely MAOis and TCAs, were able to increase the levels of serotonin and norepinephrine (Millan et al., 2015). This finding led to the suggestion that depression was a monoamine deficiency disorder. Paradoxically, these antidepressants were later found to produce immediate increases in the levels of monoamines, while their beneficial effects are only seen around two to three weeks after the beginning of the treatment (Krishnan and Nestler, 2008). Moreover, serotonin and norepinephrine depletion does not induce depressive symptoms in healthy individuals, suggesting that depression does not directly result from a monoaminergic deficiency (Ruhe et al., 2007). Changes in the monoaminergic neurotransmission may marginally contribute to the genetic susceptibility for depression (Lopez-Leon et al., 2008). However, current data suggests that additional downstream effects are most likely on the origin of antidepressants actions, thus reconciling previous findings with more recent knowledge on the neurobiology of depression. For example, chronic administration of monoaminergic antidepressants upregulates cyclic-AMP-response-element binding protein (CREB) in the hippocampus, a transcription factor that regulates expression of several genes involved in neuroplasticity, cell survival, and cognition (Tardito et al., 2006). These changes in neuroplasticity may partly explain the observed antidepressants effects, as will be discussed later in this chapter.

1.5.1.3 Glutamatergic neurotransmission

Glutamate is the major excitatory neurotransmitter in the brain. Moreover, glutamatergic neurotransmission is widely connected with γ-amino butyric acid (GABA)ergic and monoaminergic systems. Mounting evidence suggests a role for glutamatergic neurotransmission in the pathophysiology and treatment of depression. Indeed, *in vivo* and postmortem studies have revealed increased levels of glutamate measured in the plasma and PFC of depressive patients, while antidepressants could restore the plasma levels (Hashimoto et al., 2007, Kucukibrahimoglu et al., 2009). Since postmortem interval impacts on glutamate measurements and the origins of plasma glutamate are undetermined, *in vivo* magnetic resonance spectroscopy (MRS) analysis has been more recently performed in the brains of depressed individuals. Using this technique, glutamate and its metabolites (also referred to as Glx) are measured both intra and extracellularly; though, it should be noted that only a small fraction of the measured metabolites corresponds to synaptic glutamate. Despite some conflicting results, the majority of the studies reports reduced levels of Glx in the frontal cortex and cingulate regions of depressed patients (reviewed by (Yuksel and Ongur, 2010)). Notwithstanding the limitations of this technique and the seemingly contradictory changes in the levels of these metabolites, evidence suggests an aberrant glutamatergic function in the brains of depressed individuals. Glutamate is also an important mediator of structural and functional changes in the brain and may be involved in the deleterious downstream effects of increased GCs observed in depressed patients and animal models of chronic stress (McEwen et al., 2015). Preclinical studies using animal models of chronic stress exposure, support the participation of the glutamatergic system in the pathophysiology of depression and in the actions of antidepressants (Pittenger and Duman, 2008). Antidepressants have been shown to affect several aspects related to glutamatergic neurotransmission and dysfunction, namely by interfering with the function of NMDA and metabotropic (mGluR) glutamate receptors, and by preventing the stress-induced increases in glutamate release (Sanacora et al., 2012). The fast acting antidepressant properties of ketamine, an NMDA receptor antagonist, further reinforce the involvement of glutamatergic neurotransmission in the pathophysiology of depression and open new avenues for the development of novel antidepressant strategies (Naughton et al., 2014).

1.5.1.4 Neurotrophins

Neurotrophins constitute a family of secreted growth factors that bind to specific membrane receptors to activate downstream intracellular signalling cascades involved in cell growth, proliferation and differentiation. Decreased levels of neurotrophins have been found in particular brain regions, such as the hippocampus and PFC, of preclinical chronic stress models of depression and in depressed individuals (Duman, 2004, Duman and Monteggia, 2006). Moreover, neurotrophins, namely brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF), have been implicated in the actions of antidepressants (Warner-Schmidt and Duman, 2007). This has led to the proposal of a neurotrophic hypothesis of depression. However, other studies in rodents have failed to observe such changes, thus generating controversy around this hypothesis (Groves, 2007). Additional findings have questioned this hypothesis by exploring the role of BDNF in other brain regions such as the ventral tegmental area (VTA) and NAc. Strikingly, in these regions, BDNF was found to produce a pro-depressive-like action (Berton et al., 2006). Together, these results suggest that the neurotrophin-mediated responses are region-specific and may be partly determined by genetic and environmental factors, such as stress. Interestingly, neurotrophins have been suggested to mediate the neuroplastic changes in neuronal pathways underlying the behavioral responses in depression (Castren et al., 2007).

1.5.1.5 Neuroimmune system

During the early 1990's works by Maes *et al.* and by Smith *et al.* were the first to suggest the role of inflammation and cell-mediated immune activation for the etiopathogenesis of depression (reviewed by Maes, 1995, 2011). During the last decades, both clinical and preclinical studies have further demonstrated that depression is accompanied by immune dysregulation, namely through activation of the inflammatory response system (IRS), thus suggesting a neuroimmune hypothesis for depression. Supporting these findings, a recent meta-analysis reported increased levels of pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor alfa (TNF-α) in the serum of depressed patients (Dowlati et al., 2010). Shelton *et al.* also reported increased levels of pro-inflammatory cytokines in the frontal cortex of depressed individuals (Shelton et al., 2011), suggesting that previously demonstrated increases in peripheral cytokines are reflected in the brain. Several antidepressants suppress pro-inflammatory cytokine production, while promoting the

secretion of endogenous anti-inflammatory cytokines (reviewed by Walker, 2013). Moreover, clinical investigations have examined the effects of anti-inflammatory drugs on depression, with some promising results (Hodes et al., 2015). Together, these data shows that at least some depressed patients present alterations in inflammation, which has the potential to be used has a treatment target. Apparently, neuroinflammation can either trigger downstream changes on systems involved in depression neuropathology, by eliciting endocrine (Silverman and Sternberg, 2012) and neuroplastic effects (Wager-Smith and Markou, 2011) or occur as a result of these changes (Kubera et al., 2011), in a bidirectional framework that is not yet fully uncovered (Leonard, 2010).

1.5.2 Neural plasticity as a target for disruption in depression

As discussed in the previous section, several systems have been described as dysregulated in the context of depression. The articulation of seemingly unrelated hypotheses may be challenging. However, in recent years, keystone data on the involvement of neural plasticity events in the development and remission from depression have helped to reconcile these findings. This hypothesis represents a possible framework in which all the above-mentioned changes seem to converge.

In the next section, I will first describe the different forms of neural plasticity, namely in the hippocampus, and then review the literature on their relevance for depression and antidepressant effects.

1.5.2.1 Different forms of neural plasticity in the adult brain: from synapses to the generation of new cells

Previously regarded as stable and unchanging, the adult brain is now recognized as endowed with a considerable capacity to change structurally and functionally in response to intrinsic and extrinsic signals; this ability to "change itself" is also called neural plasticity. Neural plasticity encompasses a series of physiological changes that include: re-wiring of neural circuits, remodelling of dendrites and synapses, and the generation of new cells. These phenomena produce functional alterations and are accompanied by rapid gene expression changes responsible for the responsiveness of the system to environmental stimuli. Several brain regions undergo different forms of plasticity, including the

hippocampus, amygdala and PFC. Changes in these brain regions have been reported to be involved in perception, emotional processing, memory and cognition. Importantly, histological and functional neuroimaging studies have disclosed morphological changes in these brain regions in depressed individuals and animal models of depression (McKinnon et al., 2009, Sacher et al., 2012).

Synaptic and dendritic plasticity

Dendritic and synaptic plasticity allow the adult brain to rapidly respond and adapt to both intrinsic and extrinsic cues. These changes have either been interpreted as an attempt to maintain functional homeostasis, in order to cope with environmental challenges and achieve adaptation, or as a protective mechanism, as for example the case of hibernating animals (Tavosanis, 2012). Structural changes occurring in neural cells include pre- and post-synaptic formation, loss or rearrangement, spatial re-orientation of dendrites and axons, and alterations in branching patterns (Caroni et al., 2012, Tavosanis, 2012). Adding to these changes, two forms of functional neuronal plasticity can be defined: long-term potentiation (LTP) and long-term depression (LTD).

Excitatory synapses are mainly located on dendritic spines. Dendritic spines are highly dynamic specialized subcellular compartments that function as chemically and electrically separated structures (Lai and Ip, 2013, Bosch et al., 2014). These exhibit structural plasticity, including changes in shape and size, which may occur as a consequence of LTP or LTD (Bosch et al., 2014, Meyer et al., 2014). In this way, spine formation and changes in spine morphology represent fast adaptive responses that may be modulated by environmental cues in the form of synaptic activity (Caroni et al., 2012, Lai and Ip, 2013). Adding another degree of complexity to this system, recent data has challenged the classical view of dendrites as solely responsible for collecting information from all connected input cells and transmitting it to the axon. Current views support that dendrites may function as individual signalling units by locally computing incoming inputs (reviewed by (Branco and Hausser, 2010)). This puts forward the hypothesis that dendritic structural changes induce alterations in neuronal connectivity but also in input processing (Tavosanis, 2012).

Neurogenesis in the adult brain

Neurogenesis is the process by which new neurons are generated from precursor cells. Traditionally, this phenomenon was assumed to be limited to the embryonic and early postnatal stages; however,

over the years this view has changed and it is now firmly established that neurogenesis occurs in the adult mammalian brain. In the 1960's, Altman's studies showed for the first time that new cells could be generated in the adult brain of rats (Altman, 1963, Altman and Das, 1965). Several works, by Kaplan and Nottebohm, that followed corroborated this idea of new neurons being generated and integrated in the adult brain of several species, from birds to primates (Kaplan and Hinds, 1977, Kaplan, 1981, Kaplan and Bell, 1984, Paton and Nottebohm, 1984, Burd and Nottebohm, 1985, Gould et al., 1999a). Adult neurogenesis is thus the process by which neural progenitors divide mitotically to produce new neurons in the adult brain, in a process that involves neuronal commitment, migration and maturation of the cells. It is now widely accepted that adult neurogenesis occurs at least in two regions of the adult mammalian brain — the subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus and in the subependymal zone (SEZ). Apart from these two regions, a growing number of studies supports the generation of new neurons from progenitor cells in the neocortex (Altman, 1963, Gould et al., 2001, Dayer et al., 2005), striatum (Gould et al., 1999b, Bernier et al., 2002, Dayer et al., 2005, Ernst et al., 2014), amygdala (Bernier et al., 2002, Fowler et al., 2002) and hypothalamus (Huang et al., 1998, Fowler et al., 2002, Kokoeva et al., 2005). Still, these data are controversial, largely due to the existence of contradictory reports (Kornack and Rakic, 2001, Koketsu et al., 2003, Bhardwaj et al., 2006), and require definite confirmation.

Despite most of the research in the context of adult brain has focused on the generation of new neurons from neural stem cells (NSCs), it is important to highlight that new glial cells are also generated in the adult brain, not only in the neurogenic niches, but in a more wide-spread manner (Cameron et al., 1993, Suh et al., 2007, Encinas et al., 2013, Peretto and Bonfanti, 2015). Only recently, more attention has been given to the gliogenic process in the adult neurogenic niches; hence, it is still a matter of debate how these two cell lineages are related.

1.5.2.2 Hippocampal neural plasticity

The hippocampus

The hippocampus is a medial temporal lobe brain region with a heterogeneous organization and function. It is part of a brain system called the hippocampal formation that includes the cornu ammonis (CA) fields, or hippocampus proper, the dentate gyrus (DG), the subiculum and the

entorhinal cortex (EC) (Amaral and Lavenex, 2007). The hippocampus proper can be differentiated into three main subdivisions in rodents, the CA1, CA2 and CA3, and in an additional CA4 in humans. These fields are distinct in a number of ways, including in their connectivity (Treves, 2004, Strange et al., 2014).

The intrinsic hippocampal circuit is mostly unidirectional. The EC is the major source of input information to the hippocampus as most of the neocortical multimodal sensory information reaches the hippocampus through this structure. Neurons in layer II of the EC convey the information to the dendrites of granule cells in the DG via the perforant path. The CA3 and CA1 subregions of the hippocampus also receive inputs from the EC: layer II EC cells innervate CA3 pyramidal neurons (perforant path to CA3), whereas layer III cells project to the CA1 region (temporoammonic pathway). Granule cells in the DG then project their axons (mossy fibers) to the proximal apical dendrites of CA3 neurons that will, in turn, establish connections with the ipsilateral CA1 pyramidal neurons, through the Schaffer collaterals. Projections to the contralateral CA1 and CA3 regions are achieved through commissural connections (Neves et al., 2008). CA1 pyramidal cells project to the subiculum and to the deep layers of the EC (Amaral et al., 2007), closing the hippocampal-processing loop (**Figure 3**).

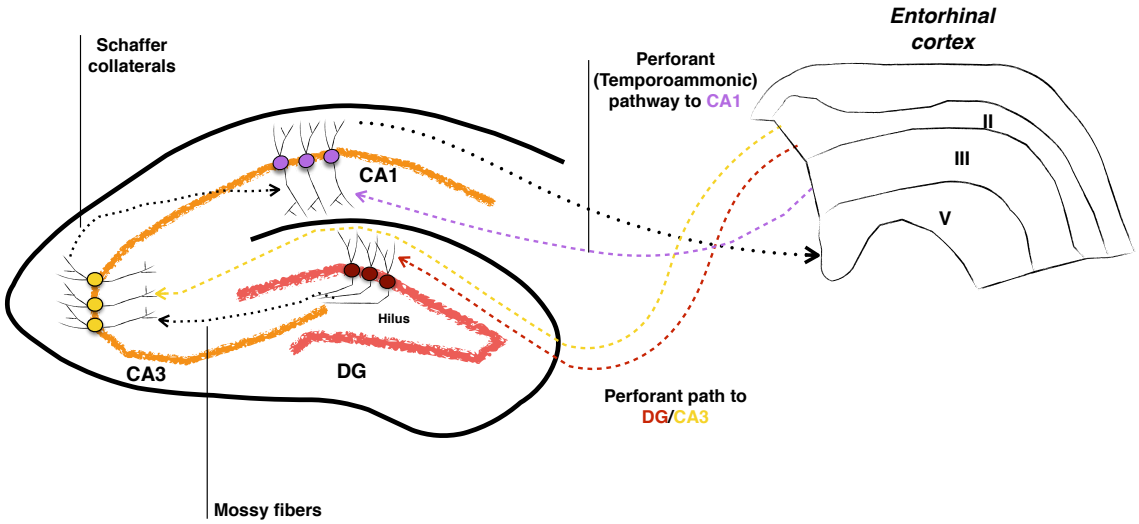


Figure 3: Schematic representation of the hippocampal intrinsic circuitry. Dashed arrows depict the connections between the different hippocampal subregions. DG – dentate gyrus; CA – cornu ammonis.

The hippocampus extends along a dorsal (septal) to ventral (temporal) axis in rodents, that corresponds to a posterior-anterior axis in humans (Strange et al., 2014). Although the basic intrinsic structure and circuitry is maintained along this axis, a clear functional dissociation is now supported by solid evidence (reviewed in Strange et al., 2014). Accounting for the functional dissociation are the distinct connectivities with cortical and subcortical areas in the dorsal versus the ventral hippocampus. The most dorsal parts of the hippocampus establish connections with retrosplenial and anterior cingulate cortices, mediating more cognitive-related processes, such as learning, memory, navigation and exploration (Moser et al., 1995, Fanselow and Dong, 2010). The connectivity of most ventral parts of the hippocampus is more dense with brain regions related to emotional processing and general affective states regulation, including the medial PFC, the amygdala, the NAc and the hypothalamic endocrine and autonomic nuclei (Fanselow and Dong, 2010, Strange et al., 2014). Noteworthy, connectivity and gene expression data further sustain the hypothesis of a functional gradient rather than a simplistic dorsal-ventral dichotomy (Kjelstrup et al., 2008, Thompson et al., 2008, Strange et al., 2014).

The hippocampus plays an essential role in both cognitive function and emotional processing (Fanselow and Dong, 2010, Strange et al., 2014). It is also one of the key brain structures involved in the regulation of the stress response; first, because it encodes contextual information from the environment that is associated with the perceived threat (Herman et al., 2005), and second because it is implicated in the termination of the HPA axis response upon stress, via a GR-dependent mechanism (Hariri and Holmes, 2015). The hippocampus was the first non-hypothalamic structure recognized to have GC receptors (McEwen et al., 1968), with both subtypes of these receptors being highly expressed there, a feature that renders it particularly sensitive to the effects of GCs. When stimulated, hippocampal neurons promote a decrease in the neuronal activity of the PVN inhibiting GCs secretion. Lesion experiments in the hippocampus or interrupting the signals between the hippocampus and the PVN produce an elevation of GC levels in basal conditions (Smith and Vale, 2006), highlighting the relevance of this structure to the feedback regulation of the HPA axis.

Neurogenesis in the adult hippocampus

The hippocampus is one of the few brain regions that retains the ability to generate new neurons in the adult brain. In the adult hippocampus, the generation of new neurons and glial cells occurs from NSCs located in the SGZ, a narrow layer of cells in the DG between the granule cell layer (GCL) and the hilus (Ehninger and Kempermann, 2008, Ming and Song, 2011, Sun et al., 2015). There, multipotent precursors expressing glial fibrillary acidic protein (GFAP), Nestin and Sox2 represent the first step of the cell genesis process, thus being named Type-1 cells. These cells can be divided in radial and non-radial (horizontal), according to their morphology and properties; Radial Type-1 cells are typically quiescent, slow-dividing and possess a single radial process that crosses the GCL of the DG, whereas horizontal Type-1 cells divide faster than radial cells and present short, horizontal processes extending through the SGZ (Kriegstein and Alvarez-Buylla, 2009, Hodge et al., 2012). Current theories suggest that one or both of these subtypes divide asymmetrically, and horizontally to the SGZ, generating one daughter cell and one NSC. The daughter cells are Type-2, transit-amplifying neural progenitors (tANPs) or neural progenitor cells (NPCs) that divide actively and lack GFAP expression. These cells divide approximately 2.5 times before exiting the cell cycle (Encinas et al., 2011, Encinas et al., 2013) and are thus greatly responsible for the expansion of the pool of newly born cells (Ehninger and Kempermann, 2008). They can be segregated in the developmentally younger subtype Type-2a Nestin-expressing cells, and Type-2b cells, which start to express markers of neuronal committed cells, namely Tbr2 followed by the expression of NeuroD and Prox1. Remarkably, a specific marker for lineage restricted astrocyte precursors has not yet been fully established (Encinas et al., 2013). Finally, Type-2 cells give rise to neuroblasts, or Type-3 cells; these are slowly proliferating cells that are exiting the cell cycle to produce a post-mitotic immature cell (Ehninger and Kempermann, 2008). They express markers of the neuronal lineage, including doublecortin (DCX), polysialylated-neural cell adhesion molecule (PSA-NCAM) and calretinin. Increased expression of CREB promotes survival and maturation of the newborn neurons, mediating dendritic branching and growth (Jagasia et al., 2009). At this transitional point, cells start receiving GABAergic inputs that promote neuronal differentiation (Tozuka et al., 2005). Strikingly, around two thirds of these immature cells will be eliminated by apoptosis and cleared by phagocytosis (Kempermann et al., 2004, Sierra et al., 2010, Lu et al., 2011). The precise mechanism by which this occurs is still debatable (Sierra et al., 2010, Lu et al., 2011), but activation of NMDA receptors may be a positive regulatory mechanism (Tashiro et al., 2006). These

newly born neurons will then fully maturate into calbindin/neuronal nuclei (NeuN)-expressing granule neurons, elongating their mossy fiber axons towards the hilar and CA3 region of the hippocampus. During the first week after birth, these cells receive GABAergic, but not glutamatergic, inputs (Esposito et al., 2005, Overstreet Wadiche et al., 2005). Neurons aged from 14 to 21 days start developing spines and dendrites (Zhao et al., 2006). At that stage they also start receiving glutamatergic synaptic input (Aimone et al., 2010) and making synapses with CA3 neurons (Faulkner et al., 2008, Toni et al., 2008). After a maturation phase, around 4 to 8 weeks after birth, adult-born neurons present electrophysiological properties comparable to mature neurons (reviewed by Aimone et al., 2010). Though the great majority of new cells generated in the adult hippocampus from NSCs are neurons, there is also a proportion (around 15%) of new glial cells being generated there. Although the regulation of their differentiation process is largely undetermined (Rajkowska and Miguel-Hidalgo, 2007), some authors suggest the existence of distinct subsets of progenitors that may be committed to a glial lineage (Encinas et al., 2011; Encinas et al. 2013). **Figure 4** shows the steps involved in the generation of new neurons in the adult hippocampal neurogenic niche.

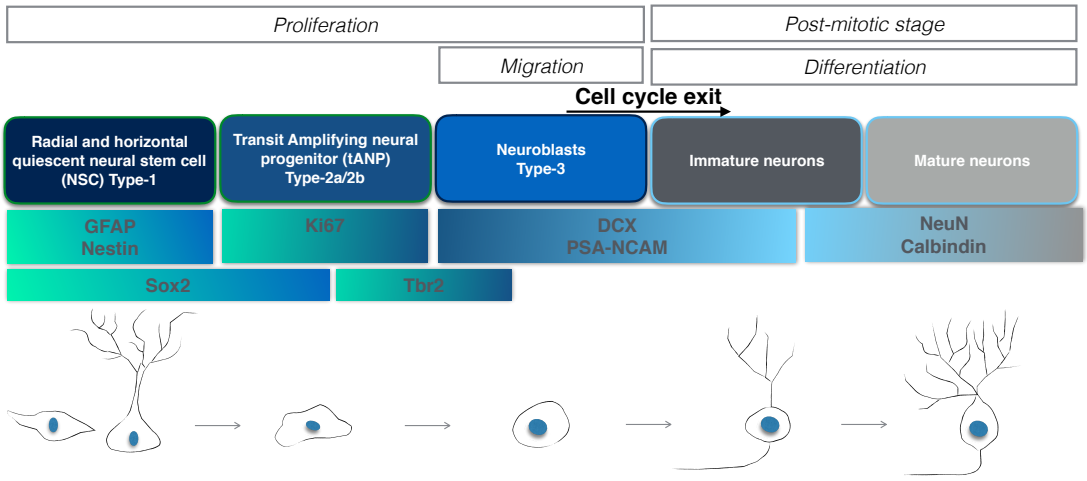


Figure 4: Schematic representation of the adult hippocampal neurogenic process. Summary of the neurogenesis developmental stages and the corresponding stage-specific markers. GFAP - glial fibrillary acidic protein; Sox2 – SRY box 2; Tbr2 –T box brain protein 2; PSA-NCAM – Polysialylated-neural cell adhesion molecule; DCX - doublecortin; NeuN - neuronal nuclei.

Cell cycle control in the adult hippocampal neurogenic niche

Proliferation of cells is determined by both intrinsic and extrinsic factors. These growth promoting or repressing signals converge onto the cell cycle machinery determining whether cells enter the cell cycle or are maintained in a quiescent state. The regulation of the cell cycle in the adult hippocampus relies on a precise control over the mechanisms of cell cycle entry by NSCs, cell cycle progression or arrest, cell cycle length and cell cycle exit. These events will determine the timing of proliferation and the net result of each cell cycle round.

The cell cycle comprises a series of events that lead to cell division. In eukaryotes, the cell cycle is divided in 4 major phases: the first gap (G1) phase, during which the cell prepares for a period of DNA replication; the synthetic (S) phase, in which the cell duplicates its DNA content; a second gap (G2) phase during which proteins are synthesized in preparation for mitosis while cell growth continues, and finally mitosis (M), where cell division occurs. After termination of division, cells may be recruited to a new round of division or enter a quiescent phase (G0). The progression through each of these phases is regulated by a specific set of molecules, including cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. CDKs are serine/threonine kinases that form catalytic heterodimers by associating with cyclins, their regulatory subunits. Together, these are responsible for cell cycle progression. Whereas CDKs expression is relatively stable throughout the cell cycle, the levels of cyclins vary during the cell cycle progression. Each CDK associates with distinct cyclins that will further determine the protein targets to be phosphorylated by the complex. There are several types of cyclins in mammals; generally, D-type cyclins are expressed during G_1 phase in response to mitogens, and bind to Cdk4 or Cdk6. These complexes phosphorylate the members of the retinoblastoma protein (Rb) family and inhibit their activity. This, in turn promotes the release of E2F proteins stimulating the transcription of genes necessary for S-phase entry. These include E-type cyclins, which bind to Cdk2, leading to irreversible inactivation of Rb proteins and consequent G1/S transition. The Cdk2-cyclin A complex is responsible for S-phase progression, and finally Cdk1-cyclin B and A complexes promote G2/M transition (Galderisi et al., 2003, Beukelaers et al., 2012). CDK inhibitors are negative regulators of the cell cycle progression. These molecules can be divided in two families - the Inhibitor of kinase 4/Alternative reading frame (Ink4/ARF) family and the CDK inhibitory protein/Kinase inhibitory protein (Cip/Kip) family – and act at a post-translational level. The members of INK4 family include p16INK4a, p15INK4b, p18INK4c, and p19INK4d and bind to

CDK4 and CDK6 to inhibit their association with D-type Cyclins. The Cip/kip gene family includes p21Cip1, p27Kip1 and p57Kip2. These bind to both cyclins, including D-, E-, A-, and B-type cyclins, and CDKs, modulating their activities (Sherr and Roberts, 1999, Besson et al., 2008). **Figure 5** depicts the general cell cycle phases in mammalians and their regulators.

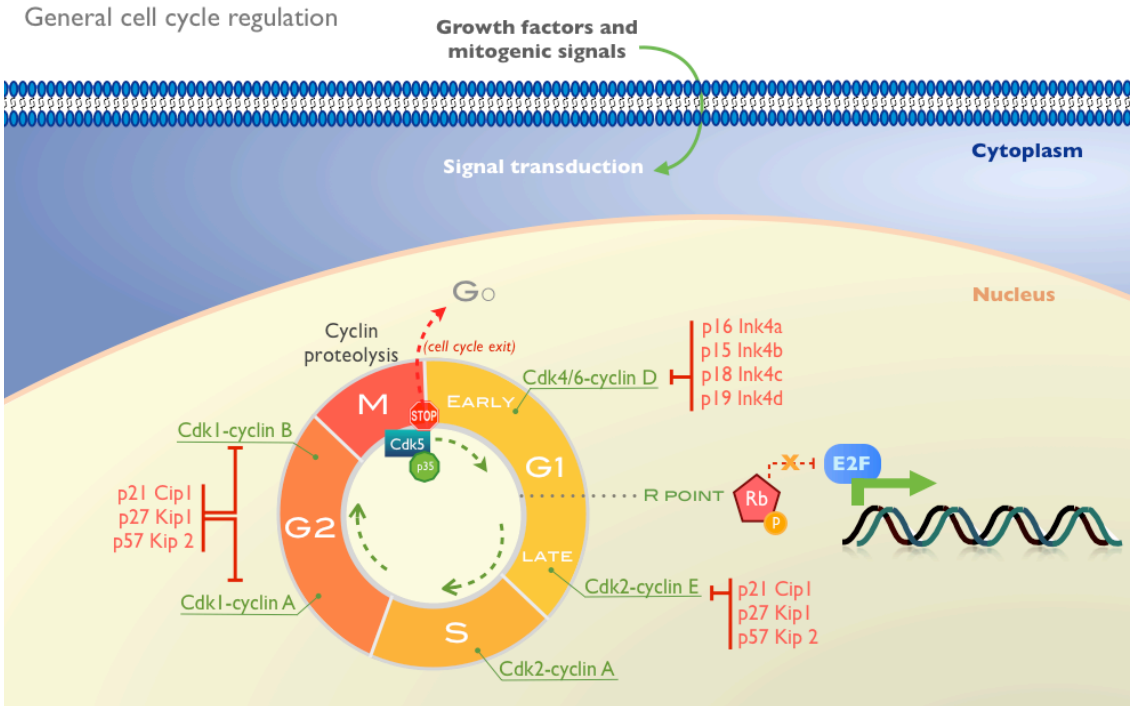


Figure 5: The general cell cycle phases and regulators in mammalian cells. Abbreviations: G0 – quiescence; G1 – gap phase 1; S – synthesis phase; G2 – gap phase 2; M – mitosis; Cdk – Cyclin-dependent kinase. *Adapted from Patrício et al. 2013.*

Several cell cycle markers have been described in the context of adult hippocampal neurogenesis. Regarding the role of D-type cyclins, it has been suggested that only cyclin D2 is expressed in dividing cells derived from neuronal precursors (Kowalczyk et al., 2004). Others have shown that cyclin D1 is expressed in both mitotic and post mitotic neurons and may be involved in other functions rather than in cell cycle regulation (Glickstein et al., 2007). Cyclin E is traditionally associated with the late stage of G1 and G1/S transition. In the adult hippocampus, cyclin E is highly expressed in the vertical processes of Nestin and GFAP-expressing NSCs, but only faintly expressed in mitotically active cells (Ikeda and Ikeda, 2015). As previously reported during mouse development (Geng et al., 2003) and in hematopoietic cells (Campaner et al., 2013), cyclin E may be mediating cell cycle entry from quiescence (Ikeda and Ikeda, 2015). Cyclin E is also expressed in

post mitotic neurons where it is suggested to perform functions related to synapse formation (Odajima et al., 2011).

Cdk6 has been implicated in the switch between proliferation and differentiation by regulating the G1 phase duration, supporting the previously described "cell-cycle length hypothesis". This model predicts that shorter G1 phases are characteristic of proliferative divisions, whereas longer G1 phases are needed for cell fate determinants to induce a cellular response, and thus to promote differentiation (Salomoni and Calegari, 2010, Beukelaers et al., 2011). Cdk6 was reported to promote specific and cell-autonomous expansion of Type-1 NSCs and Type-2 NPCs pool at the expense of neurogenesis, when inducibly expressed in conjunction with cyclin D1 (Artegiani et al., 2011). CDK2 was found to be dispensable not only for proliferation control in the adult hippocampal neurogenic niche but also for differentiation and survival of adult-born DG neurons (Vandenbosch et al., 2007). One major limitation of these studies is the use of constitutive KO models to explore the function of molecules in the adult context. It is not possible to exclude that compensatory effects, as a result of functional redundancy among molecules, may affect the outcomes and lead to misinterpretations in the field.

CDK inhibitors have also been described to play a role in the context of adult hippocampal neurogenesis. P27Kip1 is expressed in quiescent NSCs (Andreu et al., 2015) and in dividing NPCs that co-express DCX, in the SGZ (Qiu et al., 2009). P27Kip1 KO mice present increased BrdU positive cells in the SGZ and in the SEZ compared to WT, suggesting a role in constraining NPCs proliferation (Qiu et al., 2009). More recently, p27Kip1 was also described as a regulator of stem cell quiescence (Andreu et al., 2015). This study suggested that P27Kip1 might be one of the effector molecules downstream of bone morphogenetic protein (BMP) signalling pathway (Andreu et al., 2015), a pathway involved in stem cell maintenance in the adult hippocampal DG (Mira et al., 2010). Moreover, p27Kip1 was shown to promote cell cycle exit in immature neurons (Andreu et al., 2015). Together, these data indicate that this protein can act at different levels during the neurogenic process. P57Kip2 expression was also found in quiescent radial NSCs in the adult hippocampus, but not in fast dividing progenitor cells. Furthermore, conditional deletion of this molecule revealed that p57Kip2 is also involved in stem cell quiescence maintenance (Furutachi et al., 2013).

P21Cip1 expression was found in tANPs and in neuronal committed early developing cells. Moreover, its expression was negatively correlated with the degree of cells differentiation (Pechnick

et al., 2008, Pechnick et al., 2011). Whereas two studies by the same group showed that p21Cip1 depletion promotes increased proliferation in the SGZ (Pechnick et al., 2008, Pechnick et al., 2011), others have failed to disclose changes in proliferation in the DG of p21-/- mice (Qiu et al., 2009). Importantly, two different mouse strains and distinct BrdU protocols were used to assess proliferation levels (Qiu et al., 2009, Pechnick et al., 2011), probably accounting for these conflicting results. BrdU is a thymidine analogue that incorporates into the DNA of dividing cells during the S-phase of the cell cycle and is widely used for birthdating of cells and to assess cell proliferation. However, careful must be taken when interpreting these data as it does not discriminate among other effects that may cause an increased labelling of S-phase, including G1/G2 shortening, lengthening of S phase and increase in the growth fraction (Salomoni and Calegari, 2010). Additionally, distinct labelling paradigms may be used according to the purpose of the study; this is done by varying the number of BrdU pulses and by varying the period between each BrdU pulse, or between the BrdU pulse(s) and the time of sacrifice (Taupin, 2007). As such, different experimental BrdU paradigms may result in different scientific conclusions. This further encourages the use of the appropriate controls and additional endogenous markers of proliferation to ensure comparable and replicable results.

1.5.2.3 Hippocampal neural plasticity in depression

Changes in structural plasticity

Neural plasticity in the adult hippocampus is critical to promote adaptation and a target for dysregulation in several neuropathologies, namely depression. As previously discussed herein, chronic stress exposure is a key factor contributing for the development of depression. The effects of chronic stress encompass not only hyperactivation of the HPA axis with increased secretion of GCs, but also dysregulation of glutamatergic neurotransmission (Popoli et al., 2012) and neuroimmune function (Hodes et al., 2015), and changes in the levels of neurotrophic factors (McEwen et al., 2015). Importantly, these changes are thought to, at least partially, mediate the neuroplasticity impairments observed in the hippocampus of depressed patients and animal models of depression (Pittenger and Duman, 2008).

Meta-analyses studies have revealed that depressed individuals present hippocampal atrophy (Campbell et al., 2004, Videbech and Ravnkilde, 2004) making it one of the most consistently

observed changes in depression. Still, it is not clear if the decreased hippocampal volume observed in depressed individuals is a cause rather than a consequence of depression. Arguments disfavouring this hypothesis include the fact that duration and number of the depressive episodes have been suggested to negatively correlate with the size of the hippocampus (MacQueen and Frodl, 2011, Cobb et al., 2013); one study in particular detected these differences only in patients with a disease course of more than 2 years and that had more than one depressive episode (McKinnon et al., 2009), thus supporting hippocampal atrophy as a consequence of depression. Moreover, hippocampal atrophy is observed in numerous other neuropsychiatric and neurological disorders, including schizophrenia (Adriano et al., 2012), Parkinson's (Junque et al., 2005) and Alzheimer's disease (Vijayakumar and Vijayakumar, 2013) and posttraumatic stress disorder (Villarreal et al., 2002), suggesting common pathophysiological mechanisms between these and depression. The precise cellular correlates underlying the hippocampal shrinkage in major depression are still debatable. Postmortem studies have shown that depressed patients present decreased neuropil, including dendritic atrophy, decreased dendritic spine complexity, and glial processes, but no changes in neuronal density (Stockmeier et al., 2004, Cobb et al., 2013). Additional evidence on this topic comes from preclinical studies using mostly chronic stress animal models of depression. A large body of evidence shows that chronic stress impairs LTP in the DG and in the CA1 region of the hippocampus. Conversely, LTD is enhanced in these same regions, supporting decreased functional plasticity in the hippocampus of these animals (Kim and Diamond, 2002). Likewise, animal studies have consistently demonstrated that chronic stress exposure produces reductions in spine density (Ota and Duman, 2013) and atrophy of hippocampal neurons (Pittenger and Duman, 2008). Antidepressant treatment was shown to reverse these stress-induced deficits in dendritic length (McEwen and Olie, 2005, Bessa et al., 2009a). Interestingly, more recent data suggest that chronic stress exposure impacts differently along the longitudinal axis of the hippocampus (Pinto et al., 2015). This is in line with the recently disclosed anatomical and functional dissociation between the dorsal and ventral hippocampus (Strange et al., 2014). Animal studies have also provided evidence for the involvement of glial cells in the pathophysiology of depression. Glial cells in the hippocampus of animal models of depression present morphological and molecular changes that are reversed by antidepressant treatment (Czeh et al., 2006, Czeh et al., 2013).

ADULT HIPPOCAMPAL NEUROGENESIS: IMPLICATIONS FOR BEHAVIOR AND DEPRESSION

It is currently well established, using various experimental approaches, that adult-born neurons in the hippocampal DG of mammals are functional and able to integrate the pre-existing neuronal network (reviewed in Aguilar-Arredondo et al., 2015). These adult-born neurons respond from early stages and display different functions throughout their maturation process (Aimone et al., 2010, Aguilar-Arredondo et al., 2015). Adult-born neurons synaptically connected and at specific stages of development are highly plastic and display hyperexcitability (Ming and Song, 2011, Gu et al., 2012). These features suggest that these neurons may provide unique contributions to information processing in the hippocampus (Ming and Song, 2011). Adding to this, seminal work by Spalding et al. has recently provided strong evidence for extensive neurogenesis (around 700 new neurons per day) in the human hippocampus throughout life, representing an annual turnover of DG neurons of approximately 1.75%. A slight decline during aging was observed, comparable to the levels of neurogenesis occurring in middle aged mouse (Spalding et al., 2013). This and other studies reveal that the hippocampus is a highly dynamic structure that must continuously adapt to the process of new neurons being generated and integrated, and supports a role for adult hippocampal neurogenesis in human brain function.

Though there is increasing evidence supporting a role for hippocampal neurogenesis in brain function, its precise function is still debatable. Most of the data on the function of adult-born neurons in the hippocampus comes from ablation studies in rodents. The heterogeneity of the experimental models, timeframes and methodological approaches used to suppress cell genesis have yielded conflicting results regarding the impacts of the newborn cells on behavior. These approaches include the use of cytostatic agents (e.g. Methylazoxymethanol), focal irradiation, transgenic models, and more recently optogenetic control; these methods vary in specificity and efficacy. Adding to this, studies have been performed in different species and strains creating another source of variability on experimental results (Snyder et al., 2009). Converging data has supported a role for adult hippocampal neurogenesis in certain types of hippocampal-dependent learning and memory, including long-term spatial memory, cognitive flexibility and pattern separation (Ming and Song, 2011, Miller and Hen, 2015). Interestingly, stimuli that enhance memory, such as learning, running and environmental enrichment, are pro-neurogenic, whereas during aging, stress and neurodegenerative disorders there is a decrease in neurogenesis and memory impairments (Aimone et al., 2010). New neurons in the adult hippocampus have also been suggested to play a role in

memory acquisition and retrieval. Specifically silencing 4 weeks old functional and integrated adult born neurons that were shown to be recruited during memory acquisition, impairs memory retrieval (Gu et al., 2012). Moreover, by examining the retrograde impact of neurogenesis manipulation it was found that high levels of neurogenesis disturb established hippocampal-dependent memories. In line with previous hypotheses on the balance between plasticity and stability for memory persistence, these results support a role for new neurons in memory clearance (Akers et al., 2014).

Given the involvement of adult neurogenesis in such complex behaviors it is plausible to anticipate that its disruption may impact on the neuronal circuitry and ultimately be implicated in the development of psychiatric and neurodegenerative disorders. As such, adding to its cognitive- and memory-related functions, adult hippocampal neurogenesis has been extensively studied in the context of depression and antidepressant treatment (Miller and Hen, 2015). Furthermore, memory-related functions, such as pattern separation were suggested to participate in mood regulation (Kheirbek et al., 2012), revealing the complexity and intertwined function of newborn neurons in neural networks.

Initial findings that decreased hippocampal proliferation and neurogenesis occur in chronic stress animal models of depression and that conversely, antidepressant treatment and stimuli can increase these levels, have led to the suggestion that adult hippocampal neurogenesis is involved in the pathophysiology and treatment of depression (Kempermann and Kronenberg, 2003). This theory is further supported by the fact that most antidepressants take 3 to 4 weeks to exert their beneficial effects in patients and animal models, a period that corresponds to the period of maturation of adult born neurons (Balu and Lucki, 2009). Moreover, depressed individuals may display cognitive impairments (Gonda et al., 2015) and patients undergoing treatment for cancer with cytostatic agents, often display cognitive impairments and depressive symptoms (Pereira Dias et al., 2014). Several studies have addressed this hypothesis, but the existence of a causal relationship between neurogenesis and the development of depression/therapeutic action of antidepressants is still not consensual. Moreover, whereas most studies refer to neurogenesis, the majority of methods used actually suppress cell genesis in general, as the generation of glial cells is also repressed.

The relevance of hippocampal cell genesis in the context of depressive-like behaviors has been widely investigated in preclinical models. While some studies have shown that blocking/decreasing cell genesis in naive rodents, either by using a cytostatic agent (Bessa et al., 2009a, Mateus-Pinheiro et al., 2013), focal irradiation (Fuss et al., 2010), or using transgenic models (Revest et al.,

2009, Snyder et al., 2011, Yun et al., 2016), produces depressive- and/or anxiety-like signs in rodents, others have failed to observe these changes (Santarelli et al., 2003, Surget et al., 2008, Jayatissa et al., 2009, Surget et al., 2011, Deng and Gage, 2015). Interestingly, it has been suggested that new neurons may be relevant for buffering the stress effects thus maybe playing a role on depression vulnerability (Snyder et al., 2011). Regarding the relevance of adult cell genesis for the actions of antidepressants in animal models of depression, robust evidence has shown that at least some of the behavioral effects of antidepressants rely on active neurogenesis (Santarelli et al., 2003, Perera et al., 2007, Surget et al., 2008, Bessa et al., 2009a, David et al., 2009, Mateus-Pinheiro et al., 2013). Again, these apparently conflicting results have been suggested to encompass differences in experimental layout, including the stress state of the animals, the behavioral tests used, the species and strains of the animals, the mode of cell genesis ablation employed and the time-point of ablation (Petrik et al., 2012, Miller and Hen, 2015). One of the key points that have been discussed as relevant to interpret the observed effects on behavior is the maturation stage of the new neurons (Mateus-Pinheiro et al., 2013, Tanti and Belzung, 2013). As previously referred, the adult hippocampal neurogenic process takes approximately 4 to 6 weeks to be completed, the time needed for new neurons to be integrated in the pre-existing neural network. It is thus expectable that the time and duration of ablation protocols, as well as the time behavioral analysis takes place will influence the behavioral outcomes and thus the interpretation of the results. In line with this, the behavioral effects of monoaminergic antidepressants were shown to be independent of active cell genesis when analysis is performed two weeks after ablation (Bessa et al., 2009a); however, when this assessment is made 4 to 6 weeks after ablation, an impact in both the HPA axis function and behavior was observed (Surget et al., 2011, Mateus-Pinheiro et al., 2013). Likewise, a recent report showed that specifically ablating 4-weeks old neurons produces impacts in memory (Gu et al., 2012). Unfortunately, no other behavioral dimensions were assessed in that study, to further disclose the relevance of these neurons for other behavioral dimensions. Conversely, other studies, using transgenic rodent models, have suggested that immature adult-born neurons are relevant for several aspects of cognitive and emotional behavior (Deng et al., 2009, Deng and Gage, 2015, Yun et al. 2016). The evident complexity of this system urges the need for more detailed studies specifically inhibiting the generation of new neurons or glial cells, and targeting distinct maturation stages of adult born cells to disclose the relevance of the hippocampal cell genesis dynamics in the context of depression and antidepressants treatment.

A few recent reports have assessed the levels of proliferating and progenitor cells in the human brain

of both antidepressant-treated and untreated depressive patients (Reif et al., 2006, Boldrini et al., 2009, Lucassen et al., 2010b, Boldrini et al., 2012). In one of the first works, the authors reported no differences in the number of proliferative cells in the hippocampal DG of depressed patients compared to healthy controls (Reif et al., 2006); of notice, as the number of individuals in this study was too small, treated and untreated patients were not divided, which could have accounted for the negative results (Reif et al., 2006). More recent studies have assessed these differences in larger cohorts of depressive patients (Boldrini et al., 2009, Boldrini et al., 2012). Increased levels of proliferating neural progenitor cells were specifically disclosed in the anterior part of the DG of antidepressant treated patients compared to untreated patients (Boldrini et al., 2009, Boldrini et al., 2012). Although differences did not reach statistical significance, untreated depressive patients presented half the number of cells compared to healthy individuals (Boldrini et al., 2009), maybe reflecting the relatively small number of samples in this study. Others have found decreased levels of proliferating cells in the hippocampus of elderly depressed patients but no effects of antidepressants (Lucassen et al., 2010b), suggesting that proliferation in response to antidepressants is age-dependent. In summary, human studies analysing proliferation and neurogenesis in this context are still scarce, and only partly replicate the findings from animal models. Still, more studies are needed for a comprehensive understanding of the relationship between the generation of new cells in the hippocampus and depression.

Molecular regulation of adult hippocampal neurogenesis in the context of depression

Few studies have addressed the molecular regulation of proliferation in the adult hippocampus in the context of depression and antidepressants. Notch and BMP signalling, two pathways implicated in stem and progenitor cells pool maintenance (Breunig et al., 2007, Ehm et al., 2010, Mira et al., 2010), have been described to participate in the pro-neurogenic actions of physical exercise (Gobeske et al., 2009, Brandt et al., 2010), a stimulus with suggested antidepressant effects (Ernst et al., 2006, Lucassen et al., 2010a). Adding to its previously described functions, Notch1 was shown to mediate the running-induced survival and cell cycle exit of Type-3 cells in the SGZ (Brandt et al., 2010). On the other hand, elective running in mice promoted inhibition of BMP signalling and consequent increase in hippocampal proliferation (Gobeske et al., 2009).

Regarding the cell cycle machinery, one study examined the role of cell cycle regulators in the SGZ

of chronically stressed rats. These depressive-like animals presented decreased numbers of proliferating cells (Ki67 positive) in this region that were accompanied by increased expression of p27Kip1 (Heine et al., 2004). Together, this data suggests that p27Kip1 inhibits the cell cycle in the SGZ after chronic stress exposure. In line with this, chronic treatment with different classes of antidepressants (fluoxetine, imipramine and desipramine) with recognized pro-neurogenic actions was found to down-regulate p21Cip1 but not p27Kip1 expression in the SGZ, when compared to vehicle animals, an effect that was linked to increased neurogenesis (Pechnick et al., 2008, Pechnick et al., 2011). Regrettably, the involvement of this CDK inhibitor in the pro-neurogenic effects of antidepressants was not evaluated in the context of a depressive-like phenotype, thus limiting the translational potential of these findings.

Table 2: Summary of the molecular signalling and cell cycle alterations regulating proliferation and neurogenesis in animal models of depression and by antidepressant drugs/stimuli. *Adapted from Patrício et al., 2013*.

Experimental approach	Proliferation/ neurogenesis DG	Molecular changes	Refs.
Signalling pathways			
Voluntary exercise in mice (antidepressant stimulus)	⇧	Inhibition of BMP signalling (⇩ BMP4; ⇧ noggin)	(Gobeske et al., 2009)
		⇧ Notch1 activity in DCX+ cells (cell cycle exit promotion)	(Brandt et al., 2010)
Cell cycle regulators			
Chronic stress exposed rats	⇩	⇧ p27Kip1 expression in the SGZ of the DG	(Heine et al., 2004)
Naïve mice chronically treated with fluoxetine, imipramine or desipramine	⇧	⇩ p21Cip1 expression in the SGZ of the DG	(Pechnick et al., 2011)

Abbreviations: BMP – Bone morphogenetic protein; SGZ – Subgranular zone; DG- dentate gyrus; DCX – doublecortin

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2. BACKGROUND AND AIMS

2. BACKGROUND AND AIMS

Despite the great advances in the past few decades, our understanding of the pathophysiology of depression and of the action of antidepressants is rather limited. Therefore, the concerted actions of researchers must be driven forward in this field.

In this thesis we focused our analysis in a specific brain region widely reported to be involved in the pathophysiology of depression – the hippocampus. Specifically in the dentate gyrus (DG), a sub-region of the hippocampus, changes in two major forms of neural plasticity have been identified and associated with depression: synapto-dendritic plasticity and cell genesis. In order to better understand the molecular mechanisms regulating these neural plasticity phenomena in this brain region, in depression and after antidepressants treatment, we used a robust and validated rat model of depression, the unpredictable chronic mild stress model (uCMS) and *in vitro* approaches.

In this context, two major aims were established:

1. Perform a comprehensive characterization of the hippocampal DG transcriptome in depressive-like animals and after the treatment with four antidepressants from different pharmacological classes (fluoxetine, imipramine, tianeptine and agomelatine), and correlate this with the behavioral and neuroplasticity status (Chapter 3).

2. Characterize the cell cycle regulatory mechanisms in the hippocampal progenitor cells, in an animal model of depression and after the antidepressant treatment, the SSRI fluoxetine (Chapter 4).

3. Differential and Converging Molecular Mechanisms of Antidepressants' Action in the Hippocampal Dentate Gyrus

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Differential and Converging Molecular Mechanisms of Antidepressants' Action in the Hippocampal Dentate Gyrus

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Major depression is a highly prevalent, multidimensional disorder. Although several classes of antidepressants (ADs) are currently available, treatment efficacy is limited, and relapse rates are high; thus, there is a need to find better therapeutic strategies. Neuroplastic changes in brain regions such as the hippocampal dentate gyrus (DG) accompany depression and its amelioration with ADs. In this study, the unpredictable chronic mild stress (uCMS) rat model of depression was used to determine the molecular mediators of chronic stress and the targets of four ADs with different pharmacological profiles (fluoxetine, imipramine, tianeptine, and agomelatine) in the hippocampal DG. All ADs, except agomelatine, reversed the depression-like behavior and neuroplastic changes produced by uCMS. Chronic stress induced significant molecular changes that were generally reversed by fluoxetine, imipramine, and tianeptine. Fluoxetine primarily acted on neurons to reduce the expression of pro-inflammatory response genes and increased a set of genes involved in cell metabolism. Similarities were found between the molecular actions and targets of imipramine and tianeptine that activated pathways related to cellular protection. Agomelatine presented a unique profile, with pronounced effects on genes related to Rho-GTPase-related pathways in oligodendrocytes and neurons. These differential molecular signatures of ADs studied contribute to our understanding of the processes implicated in the onset and treatment of depression-like symptoms.
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INTRODUCTION

Major depression is one of the most prevalent neuropsychiatric disorder and is characterized by a multifaceted profile of behavioral deficits, such as depressed mood, anxiety, and cognitive impairments (Bessa *et al*, 2009b; Villanueva, 2013). Strikingly, a high percentage of patients treated with the currently available therapies do not show full remission (Lang and Borgwardt, 2013) and present treatment resistance (Blier and Blondeau, 2011). Although the pathophysiology of depression is still incompletely understood, dysregulation of monoaminergic systems, neuroplasticity, and immunological responses (Villanueva, 2013; Willner *et al*, 2013) are considered to contribute to the disease. In addition, alterations in dendritic plasticity and cytogenesis in the hippocampal dentate gyrus (DG) are observed in the brains of animal models of depression and depressed patients (Lucassen *et al*, 2014; Pittenger and Duman, 2008). Importantly, these changes have been implicated in the onset of depressive-like symptoms and in the actions of antidepressants (ADs) in animal models of depression (Bessa *et al*, 2009a; Mateus-Pinheiro *et al*, 2013a, b; Surget *et al*, 2011).

ADs are generally classified according to their primary pharmacological targets. The first developed ADs were monoamine oxidase inhibitors (MAOi) and tricyclic (TCA) and tetracyclic agents (Li *et al*, 2012). Second-generation ADs include the selective serotonin reuptake inhibitors (SSRIs), still the most prescribed ADs worldwide, and norepinephrine and serotonin reuptake inhibitors (NSRIs). Atypical ADs include tianeptine whose mechanisms of action are poorly understood but which exert potent neuroplastic effects (McEwen *et al*, 2010), and agomelatine, an agonist of melatonin receptors (MT1 and MT2) and an antagonist of the $5HT_{2c}$ receptor that was recently introduced as an AD (Tardito *et al*, 2012). Despite their diverse pharmacological profiles, all of these drugs result in similar behavioral outcomes, suggesting overlapping mechanisms of action. Insights into their potentially common molecular

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targets and divergent mechanisms may help develop new treatment strategies that exploit specific properties of each individual drug.

Application of genome-wide analyses of specific brain regions is a potentially valuable approach to study drug-specific molecular targets and to generate information that may facilitate the development of more efficacious treatments. Such approaches have been previously used to investigate the molecular alterations induced by ADs in the hippocampus and other brain regions of naive animals (Conti *et al*, 2007; Gaska *et al*, 2012; Korostynski *et al*, 2013; Landgrebe *et al*, 2002; Lee *et al*, 2010; Sillaber *et al*, 2008; Surget *et al*, 2009; Takahashi *et al*, 2006); interpretation of these studies is, however, limited by the fact that treatment of individuals who do not display signs of depressive-like behavior is not comparable to a pathological context (Cryan and Slattery, 2007). Previous comparable studies relied on measures of a single behavioral index measurement (Drigues *et al*, 2003; Nakatani *et al*, 2004), while others using multidimensional animal models of depression focused on a single class of AD (Andrus *et al*, 2012; Datson *et al*, 2012; Lisowski *et al*, 2013; Surget *et al*, 2009) or two monoaminergic ADs (Malki *et al*, 2012).

Given the complex nature of depression and the heterogeneous pharmacological profiles of available ADs, we considered it important to undertake a comprehensive comparison of the molecular effects of commonly used ADs, focusing on the effects elicited in the DG of rats exposed to a well-characterized multidimensional animal model of depression, the unpredictable chronic mild stress (uCMS) (Bessa *et al*, 2009b; Hill *et al*, 2012). Exposure to uCMS results in animals with behavioral deficits and biometric and neuroplastic changes that match many of those found in patients with major depression (Bessa *et al*, 2013, 2009b; Hill *et al*, 2012; Lucassen *et al*, 2014). Following induction of disease-like symptoms, animals were treated with either fluoxetine, imipramine, tianeptine, or agomelatine, and transcriptome analysis was subsequently performed on their DGs.

MATERIALS AND METHODS

Animals and Treatments

Male Wistar rats (200–250 g, 2-month old; Charles River Laboratories) were maintained under standard laboratory conditions (lights on: 0800–2000 hours; 22 °C, relative humidity of 55%, *ad libitum* access to food and water). Rats ($n = 8$–12/ group for behavioral analysis, of which 3 were considered for microarray analysis and 5/6 for morphological analysis) were randomly assigned to one of the eight groups: non-stressed control (CT) + vehicle; stress-exposed (uCMS) + vehicle; and uCMS + fluoxetine (FLX)/ imipramine (IMIP)/tianeptine (TIAN)/agomelatine (AGOM). A validated uCMS protocol was applied for 6 weeks, as previously described (Bessa *et al*, 2009b). During the last 2 weeks of the uCMS, animals were injected intraperitoneally daily with one of the four ADs: fluoxetine (10 mg/kg in ultra-pure water; Kemprotec, Middlesborough, UK); imipramine (10 mg/kg in 0.9% saline solution; Sigma-Aldrich, St Louis, MO, USA); tianeptine (10 mg/kg in 0.9% saline solution; Kemprotec); and agomelatine (40 mg/kg^1 in 0.5% hydroxyethylcellulose; Kemprotec). The doses were chosen based on previous studies (Banasr *et al*, 2006; Bessa *et al*, 2009a; McEwen *et al*, 2010). Body weight was monitored weekly (Supplementary Figure S1a). All procedures were carried out in accordance with EU Directive 2010/63/EU and NIH guidelines on animal care and experimentation.

Behavioral Analysis

Sucrose consumption test (SCT). Anhedonia was assessed for all animals by the SCT 4 and 6 weeks into the uCMS protocol (Figure 1a). Baseline sucrose preference (SP) was determined immediately before the start of the uCMS protocol (three independent trials). Before each trial, rats were food and water deprived for 12 h. For testing, animals were presented with two preweighed bottles, containing water or 2% (m/v) sucrose solution for 1 h. SP was calculated as previously described (Bessa *et al*, 2009b).

Sweet Drive Test (SDT). The SDT test was used as an additional measure of anhedonic behavior for all animals, as previously described (Mateus-Pinheiro *et al*, 2014). Briefly, each animal was allowed to explore the SDT box for 10 min where sweet (Cheerios, Nestlé) or regular pellets (Mucedola 4RF21-GLP) were available. After each trial, preference for sweet pellets was calculated as follows: Preference for sweet pellets (%) = sweet pellets consumed (g)/total pellets consumed (g) × 100. Three SDT trials were conducted during the last week of the uCMS protocol (1 trial every 48 h; Figure 1a). The number of entries into each chamber was used as a measure of exploratory behavior (Supplementary Figure S1b).

Novelty suppressed feeding (NSF) test. Anxiety-like behavior was assessed using the NSF test at the end of the uCMS protocol. Food-deprived (18 h) animals were placed in an open-field arena for a maximum of 10 min, where a single food pellet was positioned in the center, as previously described (Bessa *et al*, 2009b). After reaching the pellet, animals were individually returned to their home cage and were allowed to feed for 10 min. The latency to feed in the open-field arena was used as an index of anxiety-like behavior, whereas the food consumption in the home cage provided a measure of appetite drive (Supplementary Figure S1c).

Forced swim test (FST). The FST was performed at the end of the uCMS protocol. Assays were conducted 24 h after a 5-min pretest session by placing the rats in glass cylinders filled with water (23 °C; 50 cm deep) for 5 min. Trials were video-recorded and an increase in immobility time was considered to be a higher degree of depressive-like behavior.

Corticosterone Levels Measurement

For all animals, corticosterone levels were measured in blood serum using a [^{125}I] radioimmunoassay kit (MP Biomedicals, Costa Mesa, CA), according to the manufacturer's instructions. Blood sampling (tail venipuncture) was performed during the diurnal nadir (N, 0800–0900 hours) and diurnal zenith (Z, 2000–2100 hours) in the fourth (start

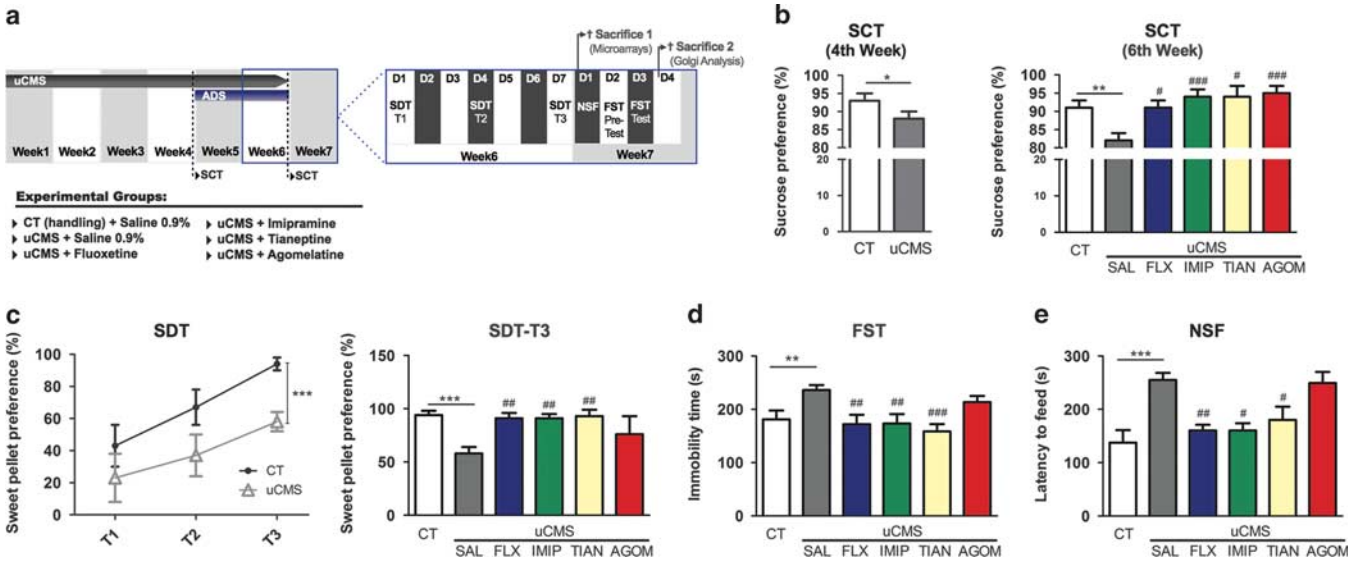


Figure 1 Multi-dimensional behavioral characterization of the animal model of depression used (unpredictable chronic mild stress—uCMS) before and after treatment with four different ADs. (a) uCMS protocol was applied to the animals for 6 weeks; four different ADs (FLX, fluoxetine; IMIP, imipramine; TIAN, tianeptine; AGOM, agomelatine) were administered in the last 2 weeks of the uCMS protocol. Behavioral profiling was performed using a battery of tests to assess mood and anxiety-like behavior ($n = 8$–12). Animals used for microarray analysis ($n = 3$) were killed immediately after the end of uCMS protocol/AD-treatment (Sacrifice 1). Animals used for morphological analysis ($n = 5$/6) were killed on week 7, after performing all behavioral tests (Sacrifice 2). (b) Sucrose consumption test (SCT) was performed at the fourth and sixth weeks of the uCMS protocol to evaluate anhedonia. uCMS induced an anhedonic profile in untreated rats (SAL), but all ADs reversed this phenotype. (c) Sweet Drive Test (SDT) was used as an additional measure of anhedonia. uCMS proved to induce anhedonia as observed in Trial 3 (T3; Trial 1 (T1) and Trial 2 (T2)—data not shown) that was reversed by FLX, IMIP, and TIAN but not AGOM. (d) In the Forced Swim Test (FST), uCMS induced increased immobility that was reversed by administration of FLX, IMIP, and TIAN but not AGOM. (e) uCMS exposure produced anxious-like behavior, as assessed in the Novelty Suppressed Feeding (NSF) test. All ADs, except AGOM reversed this phenotype. Error bars denote SEM. *Denotes the effect of uCMS-exposure; #Denotes the effect of ADs compared with untreated uCMS-exposed animals. $^{*/\#}p < 0.05$; $^{**/\#\#}p < 0.01$; $^{***/\#\#\#}p < 0.001$. $n = 8$–12 animals per group.

of ADs treatment) and sixth (end of ADs treatment) weeks of the uCMS protocol.

Morphological Analysis

Three-dimensional morphometric analysis was performed on Golgi-Cox stained material obtained from rats (5-6/group) that had been transcardially perfused with 0.9% saline and further processed, as previously described (Bessa *et al*, 2009a). For each animal, at least eight neurons fulfilling previously described criteria (Pinto *et al*, 2014) were analyzed in the dorsal and ventral hippocampal DG. For each selected neuron, dendritic branches were reconstructed at $\times 1000$ (oil) magnification using a motorized microscope (Axioplan 2; Carl Zeiss, LLC, United States) and the Neurolucida software (MBF Bioscience, Williston, VT). Three-dimensional analysis of the reconstructed neurons was performed using the NeuroExplorer software (MBF Bioscience). Measurements from individual neurons from each animal were averaged. Total dendritic length was compared among the experimental groups. Branching of the neurons was evaluated using 3D Sholl analysis; for this, the number of dendritic intersections with concentric circles positioned at radial intervals of 20 μm was determined.

Statistical Analysis of Behavioral Data and Biometric Parameters

Statistical analysis was performed using the SPSS software (SPSS, Chicago, IL, USA). The underlying assumptions of all statistical procedures were assessed. The normal distribution was tested using the Kolmogorov–Smirnov test. Student's *t*-test was used to assess differences between Control and uCMS groups and one-way ANOVA followed by Bonferroni *post-hoc* test was performed to determine the differences between AD-treated groups and uCMS-exposed untreated group. Repeated-measures ANOVA was used to assess differences in the body weight changes and in the Sholl analysis of the 3D morphometric analysis. Homogeneity of variances was assessed with Levene's test when different groups were compared and with Mauchly's Test of Sphericity when repeated measures were compared. Data transformations were tested when the described assumptions were violated. As these transformations did not prove to be useful to accomplish normality or homogeneity of variances, the alternative non-parametric tests were applied (Mann–Whitney, Kruskal–Wallis, and Friedman tests). As all the significant results remained the same, the results for the parametric statistical tests were presented. Test statistics and *p*-values are shown for each test. Significance was set at $p < 0.05$. Effect size, Cohen's *d* for *t*-test (*d*), and eta-squared for ANOVA (η^2), is presented whenever statistical significance is reached.

Transcriptome Analysis

DG macrodissection. Whole DG was collected 24 h after the last stressor/AD injection ($n = 3$ biological replicates/group; these animals did not perform NSF and FST tests; Figure 1a). Animals were first anesthetized with pentobarbital and

transcardially perfused with 0.9% saline. Immediately after dissection, tissues were frozen and stored at −80 °C until further analysis.

To avoid experimenter-dependent bias, brains were macrodissected by a single investigator. Moreover, to confirm the accuracy of the macrodissections, total RNA was isolated from both the DG and the remainder hippocampus of CT animals, and gene expression analysis of genes predominantly expressed in the DG (*Prox1*, *Dsp*, and *NeuroD1*; Supplementary Table S1) compared with the remainder hippocampus was performed by qRT-PCR.

RNA isolation. Total RNA was isolated from the macrodissected DGs using the miRNeasy Mini kit (Qiagen, Germantown, MD, USA), according to the manufacturer's instructions. The Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was used to assess RNA quality. Only high-quality RNA (RIN > 7) was used for microarray analysis.

Transcriptome analysis. Total RNA (200 ng) was amplified using the Ambion WT Expression Kit and the WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). In all, 2.75 μg of amplified cDNA was hybridized on Affymetrix Rat Gene 1.0 ST arrays containing about 28 000 probe sets. Staining (Fluidics script FS450_0007) and scanning was done according to the Affymetrix expression protocol.

Expression console (v.1.2, Affymetrix) was used for quality control and to obtain annotated normalized RMA gene-level data (standard settings, including median polish and sketch-quantile normalization). Statistical analyses were performed by utilizing the statistical programming environment R (R_Development_Core_Team, 2013) implemented in CARMAweb (Rainer *et al*, 2006). Genewise testing for differential expression was done by employing the limma *t*-test and Benjamini–Hochberg multiple testing correction (FDR < 10%). Regulated gene sets were defined by $p < 0.01$ (limma *t*-test), fold-change > 1.2X, and an average expression > 100 in at least one group in each pairwise comparison. Heatmaps were generated with the CARMAweb. Canonical pathway and Function and Disease enrichment analyses were done using the Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, CA, USA) software. Array data are publically accessible from NCBI/GEO (GSE56028).

Cell-type enrichment and mRNA half-life were assessed using previously published data sets (Cahoy *et al*, 2008; Sharova *et al*, 2009) and analyzed using the online resource Genes2mind (www.genes2mind.org) (Korostynski *et al*, 2013). Enrichment was calculated as fold difference between the level of gene expression in a particular cell type and median value of gene expression in all three cell types. Mean enrichment of gene expression was calculated as the mean value of enrichment for each set of drug-regulated genes (Cahoy *et al*, 2008).

cDNA synthesis and real-time PCR analysis. Total RNA (500 ng) from the samples used for microarray analysis (DG) and from those used for determination of the macrodissections' accuracy (remainder hippocampus) was reverse-transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA); for microRNAs analysis we used the qScript microRNA cDNA Synthesis Kit (qScript microRNA Quantification System, Quanta Biosciences).

For real-time RT-PCR, oligonucleotide primers for selected genes of interest for microarrays confirmation and macrodissections' accuracy determination were designed (NCBI Primer-BLAST software) (Supplementary Table S1). Reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, LLC, CA, USA) using 5X HOT FIREPol EvaGreen qPCR Mix Plus, ROX (Solis Biodyne, Tartu, Estonia), or PerfeCTa SYBR Green SuperMix, Low ROX (Quanta Biosciences) for microRNA confirmation analysis. Target gene expression levels were normalized against the housekeeping gene Beta-2-Microglobulin (B2M) or RNA-U6 small nuclear 2 (RNU6) for microRNA expression analysis. The relative expression was calculated using the ΔΔCt method. Results are presented as fold-change of mRNA levels between the respective experimental groups after normalization to B2M or RNU6 levels.

RESULTS

Multi-Dimensional Behavioral Profile

In order to assess the common beneficial effects of four ADs belonging to different classes—fluoxetine, imipramine, tianeptine, and agomelatine, we used a well-established animal model of depression, the uCMS protocol (Figure 1a). uCMS induced anhedonia, as demonstrated by the reduced preference for a sucrose solution in the SCT, both after 4 ($t_{22} = 1.768$, $p = 0.045$, $d = 0.12$) and 6 weeks of uCMS exposure ($t_{22} = 3.182$, $p = 0.0043$, $d = 0.3152$; Figure 1b). The four ADs reversed the uCMS-induced deficits in the SCT ($F_{(4,50)} = 6.468$, $p = 0.0003$, $\eta^2 = 0.3410$; Figure 1b). Also in the SDT, reduced preference for sweet pellets was observed throughout a three-trial testing paradigm, compared with control (CT) animals ($t_{19} = 4.887$, $p = 0.0001$, $d = 0.1884$; Figure 1c). All ADs except agomelatine reversed the uCMS anhedonic effects ($F_{(4,43)} = 5.134$, $p = 0.0018$, $\eta^2 = 0.3232$; Figure 1c and Supplementary Figure S1b). uCMS also induced increased immobility in the FST ($t_{18} = 3.109$, $p = 0.006$, $d = 0.3493$; Figure 1d), a measure of behavioral despair, which is another hallmark symptom of depressive-like behavior. Again, all ADs except agomelatine reversed this phenotype ($F_{(4,39)} = 6.197$, $p = 0.0006$, $\eta^2 = 0.3886$; Figure 1d).

Anxious-like behavior was assessed in the NSF test (Figure 1e and Supplementary Figure S1c). As expected, uCMS-exposed animals presented higher latency to feed in the NSF compared with CT ($t_{22} = 4.303$, $p = 0.0003$, $d = 0.4570$; Figure 1e), indicating increased anxiety-like phenotype. All ADs except agomelatine reversed this phenotype ($F_{(4,55)} = 7.335$, $p < 0.0001$, $\eta^2 = 0.3479$; Figure 1e).

As an additional measure of face and predictive validity, corticosterone levels were measured in the serum (Supplementary Figure S2). Disruption of the diurnal pattern of corticosterone production in uCMS-exposed animals and reversion by ADs was observed (Supplementary Figure S2).

Neuroplastic Changes in the Hippocampal DG

Neuroplastic changes were assessed in the dorsal (dDG) and ventral (vDG) hippocampal DG. As previously described (Bessa *et al*, 2009a; Pinto *et al*, 2014), uCMS-exposed animals presented significantly shorter granule neurons in the dDG compared with CT animals ($t_8 = 4.560$, $p = 0.0019$, $d = 0.7221$; Figure 2a). All ADs, excluding agomelatine, reversed this structural change up to the levels of CT ($F_{(4,21)} = 5.344$, $p = 0.0040$, $\eta^2 = 0.5054$; Figure 2a). Sholl analysis revealed less complex granule neurons in uCMS-exposed animals compared with CT and a reversion by tianeptine and imipramine (Supplementary Figure S3a).

Concerning the vDG, no statistically significant differences were observed in the dendritic length between the experimental groups (CT *vs* uCMS: $t_{11} = 0.7387$, $p = 0.4756$; uCMS_ADs *vs* uCMS: $F_{(4,21)} = 1.910$, $p = 0.1463$; Figure 2b). Interestingly, however, the Sholl analysis disclosed significantly more complex neurons in the uCMS animals compared with fluoxetine and agomelatine-treated animals (Supplementary Figure S3b).

Microarray Analysis of the DG of uCMS-Exposed and AD-Treated Rats

To unravel the molecular regulation in the hippocampal DG after uCMS exposure and treatment with each of the four ADs, microarray analysis of macrodissected DG was performed. To confirm the specificity of the macrodissections, gene expression analysis was performed in the DG of CT animals and compared with the remainder tissue of the hippocampus. The accuracy of the dissections was validated by an enrichment of genes predominantly expressed in the DG (Neurogenic differentiation 1 (*NeuroD1*) Desmoplakin, (*Dsp*) and Prospero homeobox 1 (*Prox1*)) compared with the remaining hippocampus (Supplementary Figure S4). A heatmap and a cluster dendrogram were generated according to the expression profile of 1311 significantly regulated ($p < 0.01$) probe sets (Figure 2c). Mild gene expression changes were observed, with most transcripts presenting fold changes between 1.2 and 1.5 (Figure 2d). This genome-wide analysis revealed both upregulation and downregulation of a large number of molecules, mainly in

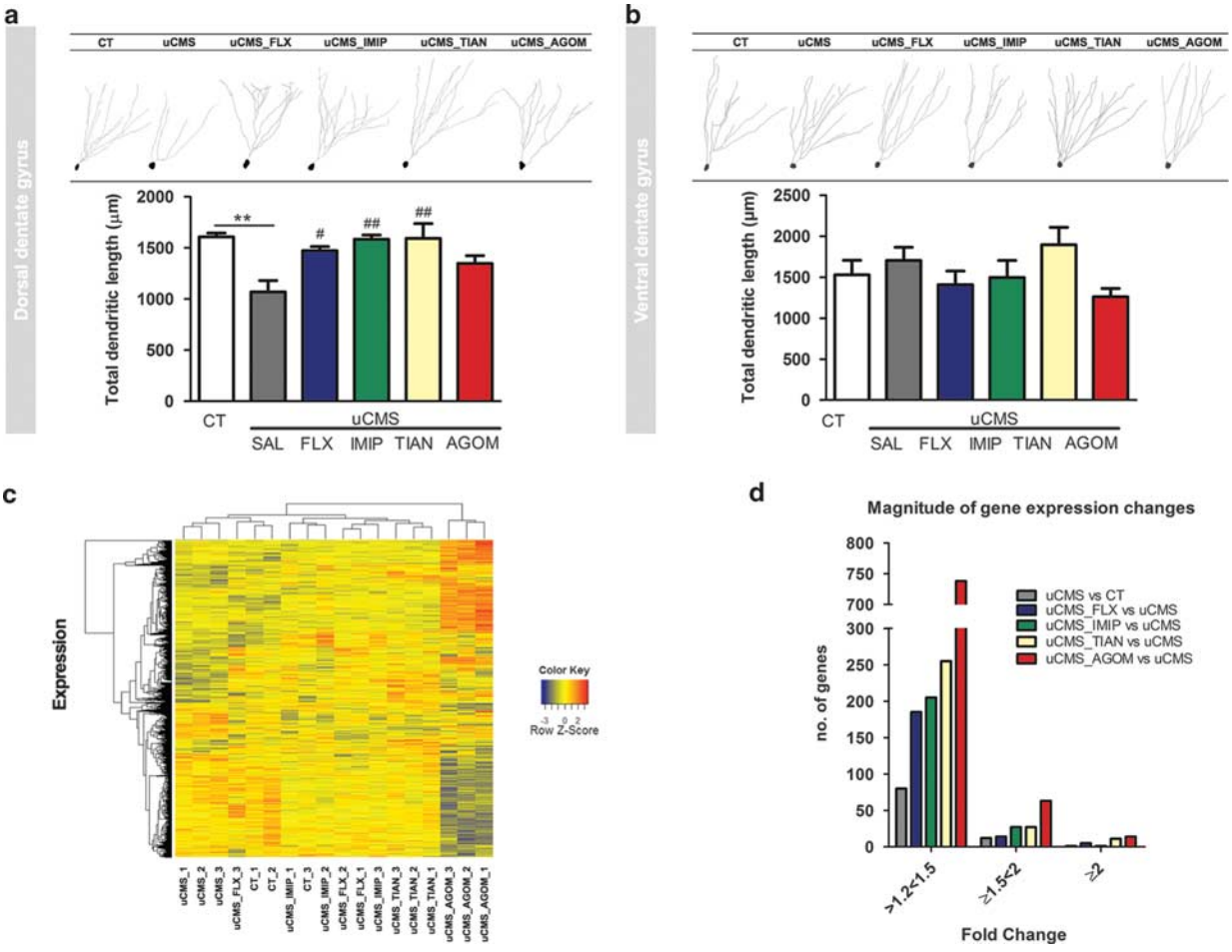


Figure 2 Neuronal morphology and gene expression analysis of the hippocampal DG. (a) Representative pictures of reconstructed Golgi-impregnated granule neurons of the dorsal DG and average total dendritic length. Untreated uCMS-exposed animals (SAL) show a decrease in the total dendritic length that is recovered by fluoxetine (FLX), imipramine (IMIP), and tianeptine (TIAN) administration. (b) Representative pictures of reconstructed Golgi-impregnated granule neurons of the ventral DG and total dendritic length. No statistically significant differences were observed between groups. Error bars denote SEM. *Denotes the effect of uCMS-exposure; #Denotes the effect of antidepressants compared with untreated uCMS-exposed animals. $^{*/\#}p < 0.05$; $^{**/\#\#}p < 0.01$; $^{***/\#\#\#}p < 0.001$. $n = 5$ or 6 animals per group. (c) Heatmap and cluster dendrogram obtained from the microarray analysis of all samples generated according to the expression profile of 1311 significantly regulated ($p < 0.01$) probe sets. (d) Magnitude of the transcriptional response to chronic stress exposure (uCMS) and ADs' treatment in the DG; the majority of fold changes are between 1.2- and 1.5-fold.

AD-treated animals (Figure 3a). The ratio between upregulated and downregulated genes was positive for all ADs, except agomelatine (FLX: 2.2; IMIP: 1.8; TIAN: 5; AGOM: 0.89).

Transcriptional Regulation Induced by uCMS Exposure and AD Treatment

A total of 93 transcripts were altered in uCMS, when compared with CTs (Figure 3a). The top 10 regulated functional terms associated with these probe sets were identified (Figure 3b). Functions related to the categories Cancer, Inflammatory Disease, and Metabolic Disease showed the highest number of altered transcripts.

Regarding the effects of AD administration, a total of 209 transcripts in fluoxetine-treated animals, 293 in tianeptine-treated animals, 233 in imipramine-treated animals, and 815 in agomelatine-treated animals were regulated compared with uCMS animals (Figure 3a and c and Supplementary Table S2). Despite the higher number of regulated transcripts in agomelatine-treated animals, fluoxetine, imipramine, and tianeptine reversed more uCMS-induced changes, thus normalizing the levels of these molecules to the levels of CT (25, 22, and 32% of reversion, respectively, compared with 12% in agomelatine-treated animals; Table 1 and Supplementary Table S3). Of notice, the list of genes whose expression was reversed after AD treatment included several microRNAs (eg, miR-409, miR-411), dual specificity protein phosphatase 1 (*Dusp1*), and Metallothionein 4 (*Mt4*) (Table 1).

ADs Commonly Regulated Genes

Independently of the AD treatment, 11 transcripts were commonly regulated (Figure 3c). The majority of them are novel transcripts or coding for uncharacterized predicted proteins (Supplementary Table S4). As most of the behavioral effects were observed after treatment with fluoxetine, imipramine, or tianeptine, and because these three ADs could reverse more transcriptional alterations induced by uCMS, we decided to further explore their common effects. The analysis of commonly regulated genes revealed 33 probe sets, including several small nucleolar RNAs (SnoRNA) and microRNAs (eg, miR-409, miR-411, miR-412), tissue plasminogen activator (*Plat*), *Mt4*, and *Dusp1* (Supplementary Tables S4 and S5). A predicted network where these genes interact to induce the cellular and behavioral beneficial effects observed is depicted in Supplementary Figure S5. The IPA software functions associated to this network are related to tissue morphology and connective tissue development and function.

To confirm the differential expression of genes identified by microarray analysis, real-time RT-PCR of the selected genes for each relevant comparison was performed (Figure 3d–h). These genes were chosen based on their neuroplasticity-related function; those without neuroplasticity-related function were randomly selected from the list of significantly altered genes to avoid bias in the microarray confirmation. Moreover, genes with annotated function whose expression was reversed by at least three of the

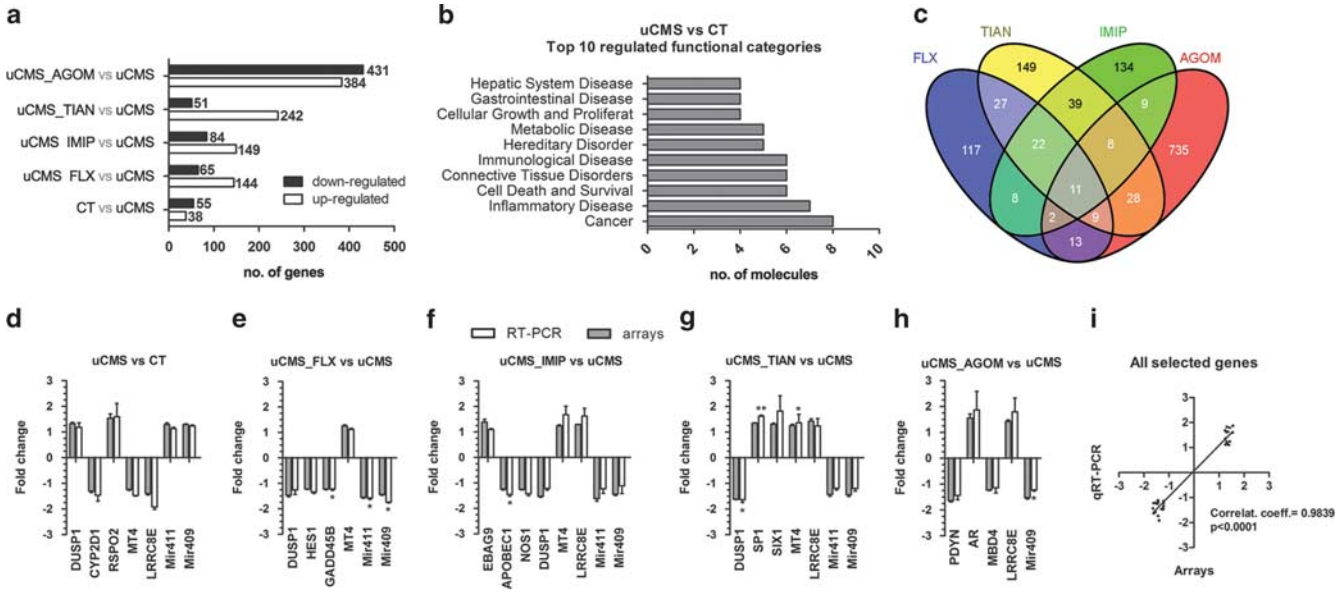


Figure 3 Microarray analysis of the DG of Control, uCMS-exposed and antidepressant-treated rats. (a) The number of upregulated and downregulated genes in uCMS *vs* CT animals and in AD-treated *vs* uCMS animals. (b) Top 10 significantly enriched functional categories identified with Ingenuity Pathway Analysis (IPA) Software in uCMS-exposed *vs* Control animals. Shown are the terms with the highest number of genes, as indicated by the bars. (c) Venn diagram depicting the number of significantly regulated transcripts ($p<0.01$, fold change (FC) $>1.2\times$, average expression >100) after 2 weeks of AD treatment and overlap between treatments. (d–h) Confirmation of the microarrays data was performed by qRT-PCR of selected genes, for each relevant comparison. White bars depict the linear FC of mRNA levels between the respective experimental groups after normalization to B2M/RNU6 (microRNAs) mRNA levels; Grey bars depict the observed linear FC in the Affymetrix microarrays. Error bars denote SEM. *Denotes statistical differences between groups for each relevant comparison after normalization to B2M/RNU6 mRNA levels; *$p<0.05$; $n=3$ per group. (i) Linear regression analysis of FC from microarrays and qRT-PCR of all selected genes for confirmation. Correlation coefficient and *p*-value are indicated in the graph. AGOM, agomelatine; CT, control; FLX, fluoxetine; IMIP, imipramine; TIAN, tianeptine; uCMS, unpredictable chronic mild stress.

Table 1 List of uCMS-Induced Transcriptional Alterations Reversed by Antidepressants (ADs) Treatment

Probe set	Gene symbol or ID	Fold change				
		uCMS-exposed	**FLX-treated**	**IMIP-treated**	**TIAN-treated**	**AGOM-treated**
10764367	*Mir181b-1*	1.49	—	−1.62	−1.52	—
10887050	*Mir411*	1.29	−1.55	−1.61	−1.43	—
10887104	*Mir409*	1.29	−1.44	−1.44	−1.44	−1.52
10816485	*Mir9-1*	1.29	—	−1.27	—	—
10887110	*Mir410*	1.23	−1.36	—	−1.24	—
10887048	*Mir379*	1.21	−1.47	−1.34	—	—
10863679	*Cml5*	1.35	—	—	−1.41	−1.76
10732652	*Dusp1*	1.32	−1.47	−1.53	−1.61	—
10725778	*Nupr1*	1.31	—	—	−1.31	—
10799977	*Enkur*	1.23	−1.23	—	−1.34	—
10719648	*Zfp61*	1.23	—	—	—	−1.28
10864918	*Ret*	1.21	—	—	—	−1.31
10828841	*Rab44*	−1.22	—	—	1.24	—
10809406	*Mt4*	−1.23	1.25	1.24	1.25	—
10712196	*Pkp3*	−1.24	—	—	1.21	—
10722273	*Fancf*	−1.27	1.22	—	1.22	—
10747214	*Krt31*	−1.29	—	—	1.28	1.27
10759553	*Lrrc8e*	−1.40	—	1.29	1.43	1.42
10731444	*Rsl1d1*	−1.71	—	—	—	1.41
10788070	*ENSRNOT00000059482*	−1.47	1.59	1.61	1.54	1.48
10806413	*ENSRNOT00000060679*	−1.34	—	1.44	1.49	—
10767075	*ENSRNOT00000053847*	−1.38	1.52	—	1.48	—
10722578	*ENSRNOT00000055917*	−1.38	—	1.39	1.42	—
10752628	*ENSRNOT00000063656*	−1.42	1.41	—	—	—
10775226	*ENSRNOT00000047758*	−1.43	1.42	1.50	—	—
10755670	*ENSRNOT00000053925*	−1.57	—	1.94	2.15	—
10749818	*ENSRNOT00000053950*	−1.66	2.04	1.73	1.85	—
10785624	*ENSRNOT00000057823*	−1.22	1.23	—	—	—
10763318	*ENSRNOT00000049616*	−1.24	1.28	1.27	—	—
10744141	*ENSRNOT00000054292*	−1.25	—	—	1.24	—
10780922	*ENSRNOT00000032631*	−1.25	1.26	—	1.38	1.34
10857470	*ENSRNOT00000056864*	−1.26	—	1.25	—	—
10702579	*ENSRNOT00000060826*	−1.27	1.32	1.30	1.36	—
10724150	*ENSRNOT00000031043*	−1.28	—	—	1.22	—
10707740	*ENSRNOT00000055911*	−1.29	1.30	1.34	1.34	—
10873723	*LOC100360708*	1.28	—	−1.38	−1.43	—
10744306	*LOC497940*	1.24	−1.26	—	—	—
10926285	*LOC680955*	1.21	—	—	−1.28	—
10805591	*LOC689153*	−1.26	1.22	—	1.35	1.44
10937658	*LOC686031*	−1.58	1.58	1.48	1.48	1.56
10783022	*RGD1310110*	1.25	—	—	—	—
10726241	*RGD1560958*	−1.29	1.22	—	1.22	—
10817543	*FQ225205*	−1.87	2.10	—	—	—
Percentage of reversed alterations		—	25	22	33	12

Last row shows the percentage of molecules whose expression was reversed by each of the ADs.

ADs were analyzed (Supplementary Table S1). For all the selected genes, the differential expression observed by Affymetrix arrays (Figure 3d–h) was confirmed. Moreover, a strong correlation between the microarrays and the qRT-PCR fold changes was found (correlation coefficient 0.9839, $p<0.0001$; Figure 3i).

Canonical Pathways Analysis

PA software was used to identify pathways sensitive to stress and to AD treatment. A list of significantly enriched canonical pathways and the corresponding genes is provided for each relevant comparison (Figure 4a–d and Supplementary Table S6). Chronic stress exposure induced the upregulation of only one gene related to the glutathione redox reactions pathway. Fluoxetine treatment promoted, among others, the downregulation of genes involved in pro-inflammatory response pathways (eg, IL-6 signaling, NF-$\kappa\beta$ signaling, acute phase response signaling; Figure 4a) and the upregulation of genes from metabolic pathways (eg, pentose phosphate pathway and PPAR signaling; Figure 4a). Imipramine treatment induced the downregulation of several genes involved in drug metabolism pathways and the upregulation of DNA-damage and oxidative stress response pathways-related genes (eg, DNA double-strand break repair by non-homologous end joining, NRF2-mediated oxidative stress response; Figure 4b). Tianeptine treatment also promoted the downregulation of genes from drug metabolism pathways and the upregulation of biosynthetic and DNA-damage response pathways (Figure 4c). Finally, agomelatine treatment induced the downregulation of genes involved in Cdk5 Signaling, Netrin signaling, and synaptic long-term depression and the upregulation of genes involved in Rho-GTPases-related pathways (Figure 4d and Supplementary Table S6).

Nervous Tissue Cell-Type Enrichment and Transcript Stability Analysis

To understand the cellular impact of each AD, we analyzed which cells types were expressing the genes significantly altered after uCMS exposure and AD treatment. For that, publicly available data representing cellular enrichment of individual transcripts in neurons, astrocytes, or oligodendrocytes were used (Cahoy *et al*, 2008; Korostynski *et al*, 2013; Figure 4e; and Supplementary Figure S6). Fluoxetine treatment was characterized by an enrichment of genes expressed in neurons, whereas agomelatine treatment showed an enrichment of genes expressed both in oligodendrocytes and neurons, in comparison to astrocytes. Imipramine and

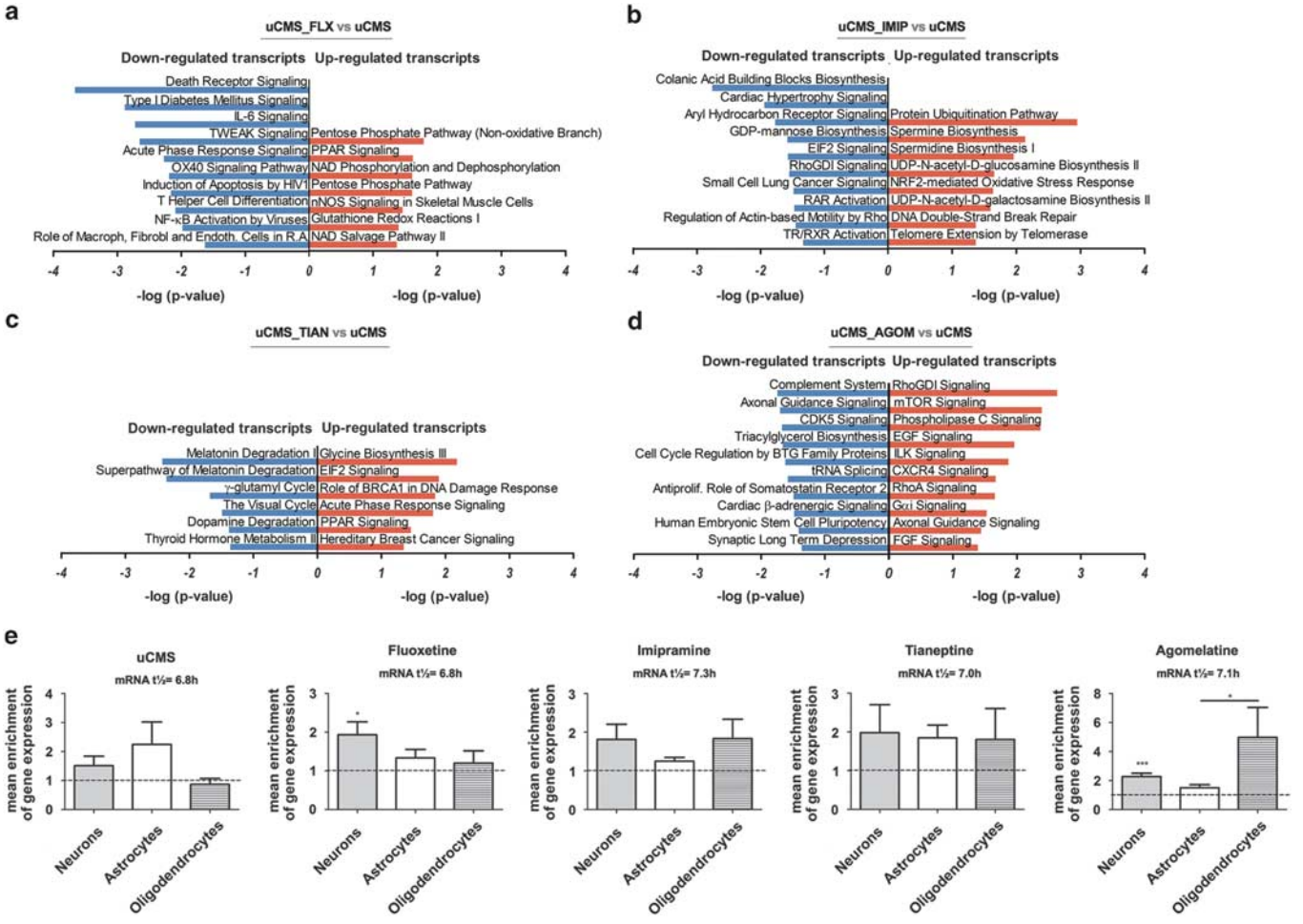


Figure 4 Characterization of ADs' effects and cellular targets. (a–d) Top 10 significantly enriched canonical pathways identified with Ingenuity Pathway Analysis (IPA) Software in untreated uCMS rats *vs* FLX-treated (a), IMIP-treated (b), TIAN-treated (c), and AGOM-treated (d) animals. Pathways presenting the higher number of regulated transcripts were selected. (e) Predicted cell type enrichment and median mRNA half-life (mRNA t½) of uCMS- and ADs-responsive genes. Median mRNA t½ for whole transcriptome was 7.1 h. Error bars denote SEM. *$p<0.05$; ***$p<0.001$. AGOM, agomelatine; CT, control; FLX, fluoxetine; IMIP, imipramine; TIAN, tianeptine; uCMS, unpredictable chronic mild stress.

tianeptine treatments did not reveal enrichment in any particular cell type. Although not reaching statistical significance, uCMS exposure showed a trend for enrichment in transcripts from astrocytes and neurons compared with oligodendrocytes (Figure 4e and Supplementary Figure S6).

To further dissect the function of the differentially regulated genes, we analyzed their transcript stability (Schwanhausser *et al*, 2011). Genes with low mRNA stability are frequently involved in the regulation of intracellular signaling, whereas long-lived transcripts have a role in cell metabolism (Korostynski *et al*, 2013; Schwanhausser *et al*, 2011). Similar median mRNA half-life for each of the treatments was found (Figure 4e). Moreover, these values were close to the whole genome median mRNA half-life (7.1 h).

DISCUSSION

Although most ADs produce similar behavioral and neuroplastic effects, each AD has a characteristic pharmacological and molecular signature, the full exploitation of which could be helpful in designing treatments that capture the various pathological facets presented by individual depressed patients. To this end, we here sought to identify common and divergent molecular targets and pathways of four distinct classes of ADs, represented by fluoxetine, imipramine, tianeptine, and agomelatine. Our genome-wide analysis focused on the hippocampal DG—one of the most studied neural targets of stress and ADs (Lucassen *et al*, 2014; Wainwright and Galea, 2013)—from animals displaying behavioral and endocrine impairments akin to depression. These behavioral anomalies were reversed after 2 weeks of treatment with fluoxetine, imipramine, and tianeptine; agomelatine resulted in only partial behavioral recovery although it re-synchronized the diurnal pattern of corticosterone secretion. Notably, fluoxetine, imipramine, and tianeptine, but not agomelatine, also restored dendritic arborization of the dorsal dentate granule cells of the hippocampus to prestress levels. No differences were disclosed in neuronal dendritic length in the vDG. Given the evidence for a heterogeneous structure and function along the septo-temporal axis of the DG (Kheirbek *et al*, 2013; Tanti and Belzung, 2013), with the dDG contributing mainly to functions related to learning and memory, whereas the vDG is more related to anxiety and emotional regulation (Tanti and Belzung 2013), we would expect also an impact on the vDG. Moreover, while previous reports have shown that the effects of some AD treatments on adult hippocampal neurogenesis are region specific along the septo-temporal axis of the hippocampus, the implication of morphological changes in granule neurons has not been fully elucidated (Felice *et al*, 2012; O'Leary *et al*, 2012), even though a contrasting gradient of stress-induced morphological and physiological changes along the septo-temporal axis of the hippocampus has been shown (Pinto *et al*, 2014).

Transcriptional Changes Induced by uCMS and Common Reversal Mechanisms by AD Treatment

Depression is associated with risk for other pathologies, including cancer and cardiometabolic disease (Lang and Borgwardt, 2013), which themselves are associated with stress. The present analysis of uCMS-exposed animals revealed upregulation and downregulation of genes related to these disorders.

Dusp1, a key negative regulator of the MAP kinase signaling pathway (Huang and Tan, 2012), previously related to the pathophysiology of depression in humans and animal models of depression (Duric *et al*, 2010) was upregulated by uCMS exposure. Importantly, MAP kinase pathway has been previously implicated in synaptic plasticity (Duric *et al*, 2010; Sweatt, 2004) and may partly explain the altered neuroplasticity observed in the dDG of uCMS-exposed animals. Consistent with their behavior- and neuroplastic-improving actions, all ADs except agomelatine, reversed uCMS-upregulated *Dusp1* expression. Interestingly, at least two other studies have shown the involvement of MAP kinase pathway-related genes in the actions of monoaminergic ADs (Malki *et al*, 2012; Surget *et al*, 2009), further endorsing the role of this pathway as a common strategy used by different AD treatments to reverse the depressive-like behaviors. We also observed that a significant number of miRNA precursors were upregulated and downregulated by uCMS and ADs, respectively. Importantly, among the predicted targets of these miRNAs are genes of the MAP kinase pathway (eg, *Map2k1*; *Map3k1*), calcium-signaling-related genes (eg, *Calml4*, *Camkk2*), and Rho-signaling-related genes (eg, *RhoGef*, *Rnd2*) (Lewis *et al*, 2005). This finding is interesting in light of the role of miRNAs in neuronal development and neuroplasticity (McClung and Nestler, 2008) and their potential for serving as new therapeutic targets (Hansen and Obrietan, 2013). Indeed, several recent studies have demonstrated that miRNAs are both targets not only for disruption in mental illness (Kohen *et al*, 2014) but also for AD treatment action (Baudry *et al*, 2010; O'Connor *et al*, 2013).

Noticeably, and given the role of the hippocampal DG cell genesis changes in the onset of depressive-like behavior and in the actions of ADs, only minor contribution of key neurogenesis-related genes emerged from the comparisons between experimental groups (eg, *Sox11*, *Hes1*). We assume that this under-representation may be due to the small proportion of progenitor cells in the tissue (representative of the SGZ) when compared with the remainder mature cells. However, an enrichment of genes related to particular aspects of neurogenesis, such as neuronal migration/axonogenesis (eg, *RhoC*, *Slit1*, *Epha6*, *Rasa1*, *Mapk3*, *Cxcl12*) and neuronal fate determination and plasticity (eg, *Dusp1*, *Cdc20*), was found.

AD-specific Transcriptional Changes

The present transcriptome analysis showed that not only each class of AD left its own molecular signature on the DG but also that they triggered common regulatory effects on a number of gene families and pathways.

Depressed patients and animal models of depression frequently display a deregulated neuroinflammatory response (Shelton *et al*, 2011; Sukoff Rizzo *et al*, 2012), resulting in increased production of pro-inflammatory cytokines that alter neurotransmitter metabolism and neural plasticity (Willner *et al*, 2013). It was interesting to observe here that fluoxetine reduced the expression of IL6-signaling and of

TNF signaling-related molecules. These pathways have been implicated in the development of depressive-like behaviors (Manosso *et al*, 2013) and were shown to be over-represented in this and other brain regions in response to chronic stress (Datson *et al*, 2012; Sukoff Rizzo *et al*, 2012). In addition, fluoxetine treatment in uCMS rats activated pathways related to cellular respiration and metabolism, a finding in line with the presence of long-lived transcripts (Korostynski *et al*, 2013; Schwanhausser *et al*, 2011). Further, consistent with the findings of other studies (Encinas *et al*, 2006; Mateus-Pinheiro *et al*, 2013b; Surget *et al*, 2011), the actions of fluoxetine were more pronounced in neurons than in astrocytes and oligodendrocytes.

Interestingly, imipramine and tianeptine were found to act similarly; the mechanism of action of tianeptine, a drug structurally similar to tricyclic agents, is largely unknown (McEwen *et al*, 2010). However, we recently predicted that the transcriptional effects of tianeptine, like those of imipramine, may involve the modulation of norepinephrine, serotonin, and dopamine systems in the striatum of naive mice (Korostynski *et al*, 2013). In the present study, imipramine and tianeptine co-regulated a large number of genes; notably, those were implicated in pathways related to drug metabolism, biosynthesis, and DNA-damage response; this finding suggests that the mechanisms of action of the two drugs may involve neuroprotection against the neurotoxic effects of stress. Moreover, as both neuronal and non-neuronal cells were predicted to respond to these drugs, it is likely that they exert broad actions that ultimately converge to reverse the harmful structural and molecular effects of uCMS.

In contrast to imipramine, fluoxetine, and tianeptine, agomelatine acts on two pharmacological substrates: melatonin and $5HT_{2c}$ receptors. Thus, it was not surprising that its application produced a behavioral and molecular therapeutic profile that was distinct from that of the other ADs tested. Remarkably, agomelatine produced the highest number of transcriptional changes but reversed only 12% of the transcriptional changes induced by uCMS. Rho-GTPase-signaling related genes were among those showing the highest upregulation by agomelatine. Although this signaling pathway is an important regulator of morphological neuroplasticity (Negishi and Katoh, 2005), agomelatine was strikingly poor in reversing the maladaptive structural (and behavioral) alterations induced by uCMS. *In silico* analysis predicted that agomelatine acts on neurons and oligodendrocytes, matching previous observations that melatonin promotes oligodendroglial maturation (Olivier *et al*, 2009).

This study identifies new molecular correlates of chronic stress that are subject to differential regulation by different classes of ADs and which may therefore underlie their different efficacies in reversing the maladaptive neurostructural and behavioral changes observed in the DG of animal models of depression. Interestingly, and according to previous studies (Datson *et al*, 2012), little overlap was found between the AD-regulated genes and pathways in the context of this paradigm of induced depressive-like behavior (uCMS) and those regulated in naive AD-treated animals (Gaska *et al*, 2012; Landgrebe *et al*, 2002; Sillaber *et al*, 2008). This further emphasizes the relevance of using animal models of depression to explore the molecular mechanisms of depressive-like phenotype reversion in the brain. As a result, the new information gained may allow exploitation of the unique properties of each AD in the search for the next generation of ADs; to further pursue this aim, molecular profiling of other brain regions affected in depression will be necessary. The data reported here may also serve to guide drug choice in managing symptoms in individual patients with specific genetic variants.

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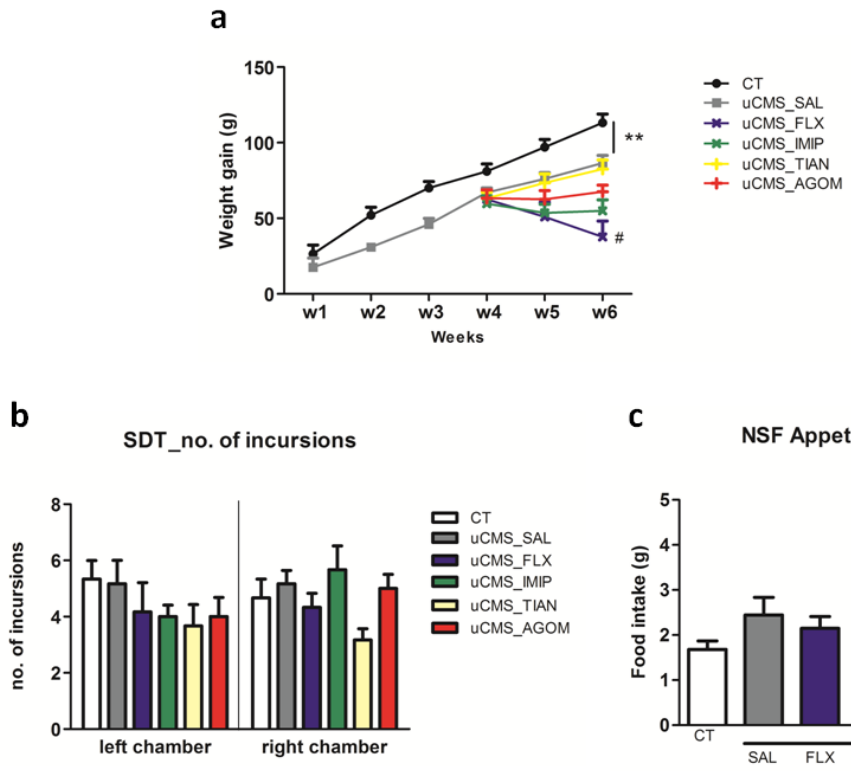
Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

3.1. Supplementary Information

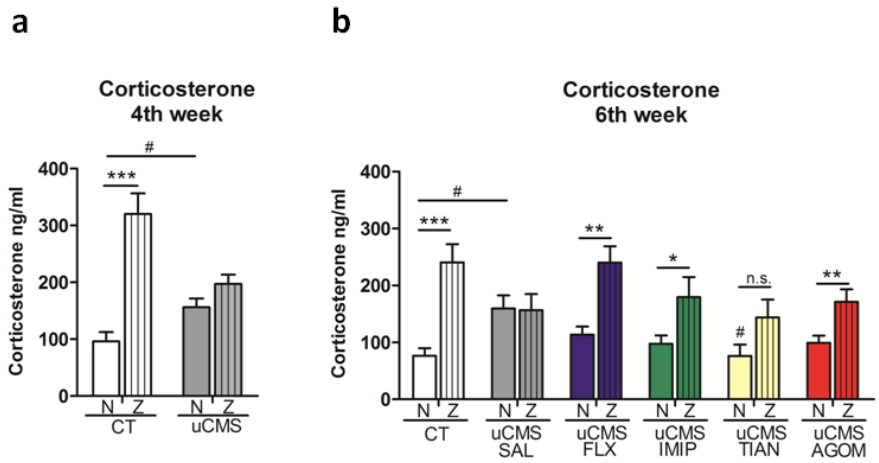
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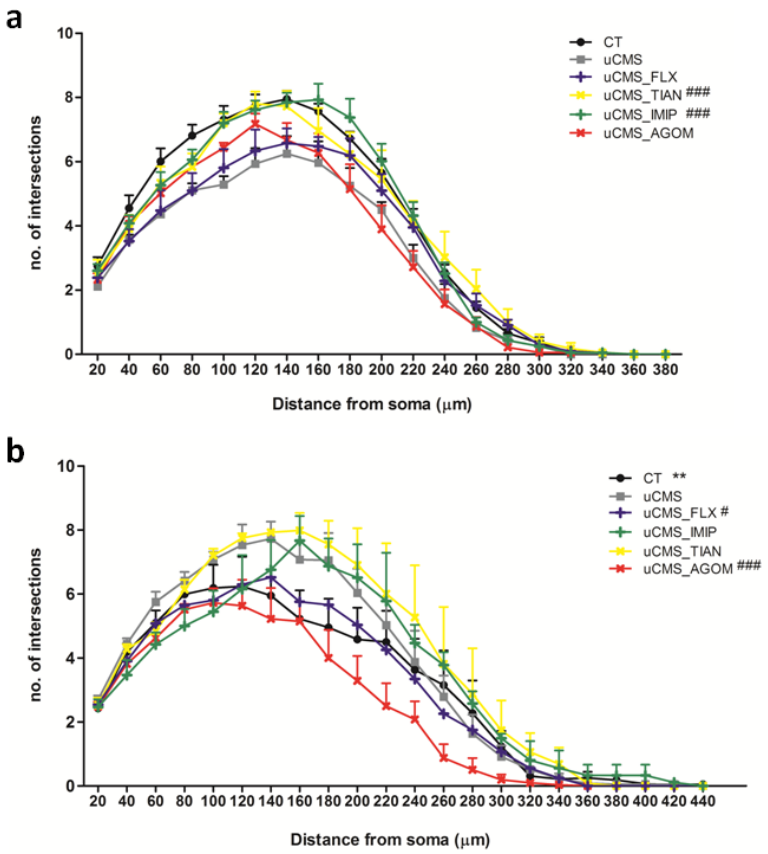
SUPPLEMENTARY FIGURES



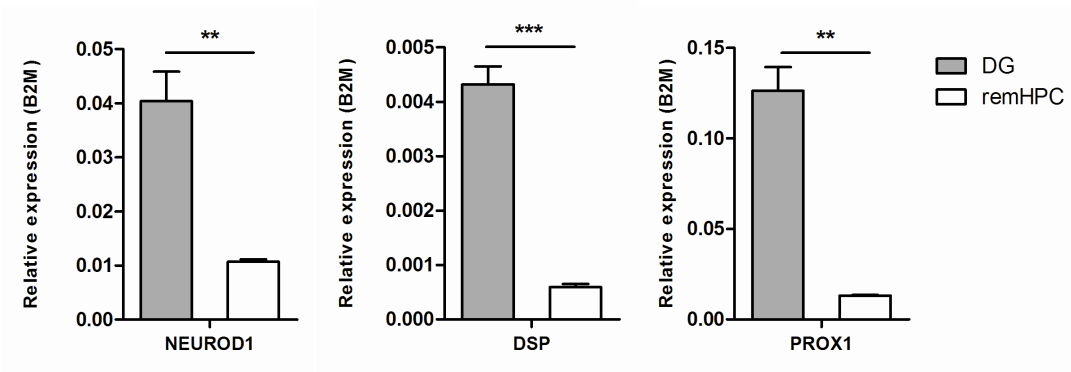
Supplementary Figure S1: Additional behavioral parameters assessed at the end of the uCMS protocol. (**a**) Weight gain variation along the 6 weeks of uCMS exposure. uCMS-exposed animals gained significantly less weight than control animals ($F_{(1,16)}$=13.566, p=0.002, η2=0.459). Not surprisingly, uCMS_FLX animals gained significantly less weight than uCMS ($F_{(4,40)}$=3.961, p=0.008, η2=0.284) . (**b**) The SDT apparatus consisted in a black arena divided by transparent perforated walls that define 4 separated chambers: a pre-chamber, which is connected by a trap-door to a middle chamber that leads to a right- (RC) and a left-chamber (LC). Sweet pellets (Cheerios®, Nestlé) were placed in the LC, and regular food pellets (Mucedola 4RF21-GLP) were placed in the RC. For the test, each animal was allowed to move freely during 10 min to reach and eat the pellets. Average number of incursions in the left and right chambers of the Sweet Drive Test (SDT) box was assessed during the test and no differences were observed among experimental groups (Left chamber: $F_{(5,42)}$=0.8258, p=0.5385; Right chamber: $F_{(5,42)}$=2.179, p=0.0746). (**c**) Food consumption in a 10 min period assessed immediately after Novelty Suppressed Feeding (NSF) test; average food intake did not differ among experimental groups ($F_{(5,54)}$=1.557, p=0.1880). Abbreviations: Control = CT; unpredictable chronic mild stress = uCMS; uCMS rats treated with fluoxetine = uCMS_FLX; uCMS rats treated with imipramine = uCMS_IMIP; uCMS rats treated with tianeptine = uCMS_TIAN; uCMS rats treated with agomelatine = uCMS_AGOM.



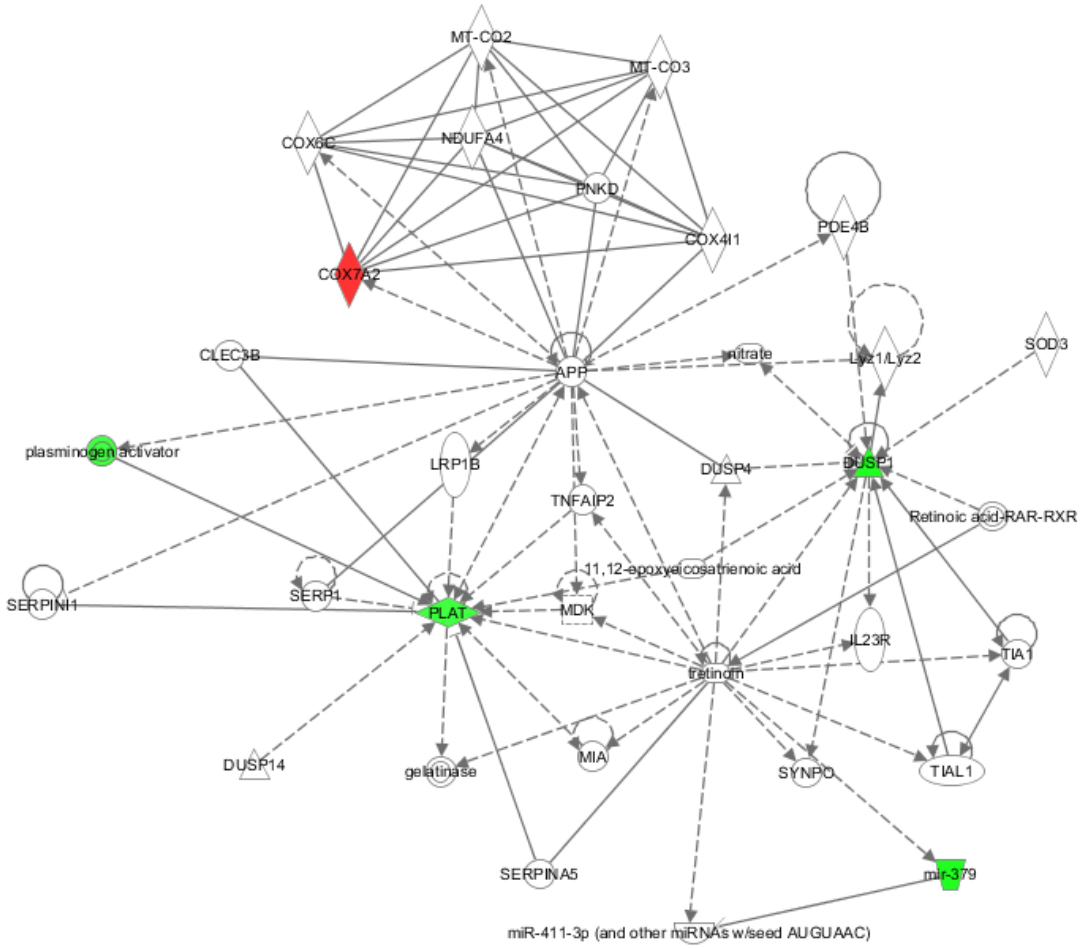
Supplementary Figure S2: Corticosterone levels measured in the serum of rats between 8:00 and 9:00 (basal levels; nadir, N) and between 20:00 and 21:00 (peak levels; zenith, Z). (**a**) At the 4th week of the uCMS protocol, control (CT) animals presented a functional circadian regulation of the corticosterone production (nadir vs zenith: t_{22}=5.628, p<0.0001, η2=0.59019) whereas uCMS-exposed animals presented significantly higher basal (nadir, N) levels of corticosterone than CTs (t_{68}=1.766, p=0.0409, d=0.04387), and no circadian regulation (nadir vs zenith: t_{114}=1.847, p=0.0673). (**b**) At the 6th week, the CT (nadir vs zenith: t_{22}=4.724, p<0.0001, d=0.5035) and uCMS-exposed (nadir vs zenith: t_{22}=0.08231, p=0.9351) animals presented the same profile as at the 4th week (CT_{nadir} vs $uCMS_{nadir}$: t_{22}=3.132, p=0.0048, d=0.3084). All ADs except tianeptine, could revert the uCMS effects, presenting a circadian regulation of the corticosterone secretion (nadir vs zenith: FLX: t_{16}=3.980, p=0.0011, d=0.4975; IMIP: t_{22}=2.148, p=0.0430, d=0.1734; AGOM: t_{22}=2.853, p=0.0093, d=0.2701). Although not statistically significant, tianeptine showed a strong trend to induce a reestablishment of the circadian corticosterone secretion pattern (t_{18}=1.821, p=0.0853). Error bars denote SEM. * Denotes differences between nadir and zenith measurements. # Denotes differences between basal levels (Nadir, N) of corticosterone, after ADs treatment comparing to uCMS untreated animals. n.s.-not statistically significant. */# p<0.05; **/## p<0.01; ***/### p<0.001. n=8-12 animals per group. Abbreviations: N= Nadir; Z= Zenith; Control = CT; unpredictable chronic mild stress = uCMS; uCMS rats treated with fluoxetine = uCMS_FLX; uCMS rats treated with imipramine = uCMS_IMIP; uCMS rats treated with tianeptine = uCMS_TIAN; uCMS rats treated with agomelatine = uCMS_AGOM.



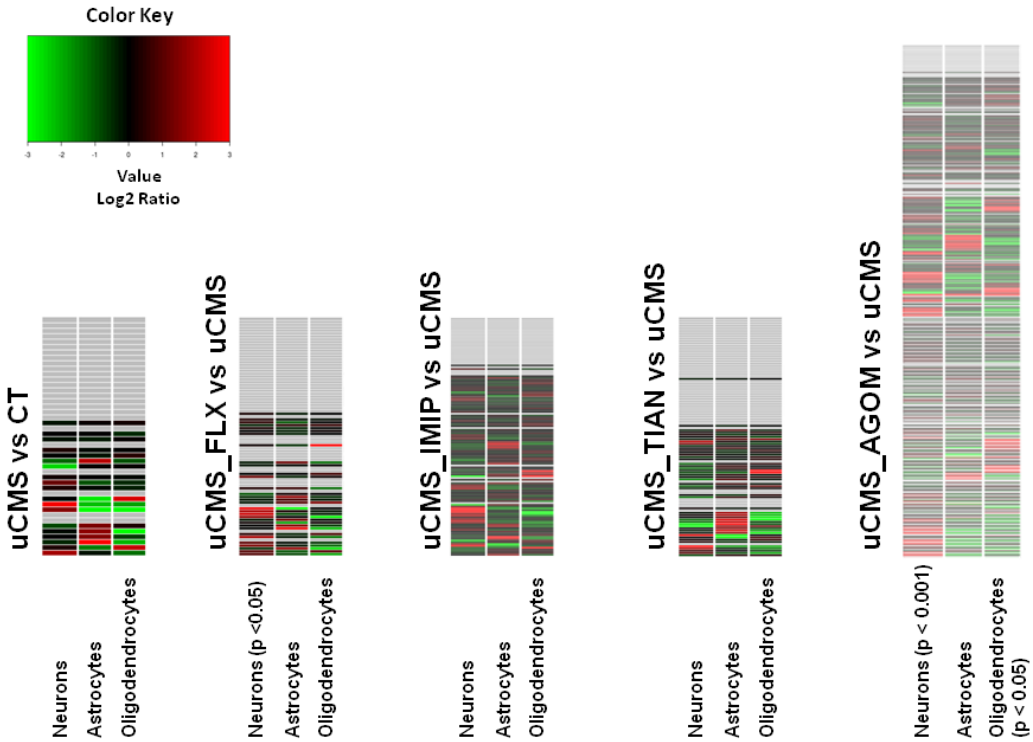
Supplementary Figure S3: Additional morphological data on the hippocampal DG granule cells. (**a**) Sholl analysis of reconstructed granule neurons of the dorsal DG shows higher complexity of neurons from control (uCMS vs CT: $F_{(1,7)}$= 16.264, p=0.005, η2=0.699), imipramine-treated and tianeptine-treated animals compared to uCMS-exposed ($F_{(4,18)}$=15.03, p<0.0001, η2=0.4550, post-hoc uCMS_IMIP/TIAN vs uCMS: p<0.0001). (**b**) Sholl analysis of reconstructed granule neurons of the ventral DG. Control animals, and animals treated with fluoxetine or agomelatine present significantly less complex granule neurons than uCMS-exposed animals ($F_{(4,21)}$=18.63, p<0.0001, η2=0.4701; post-hoc FLX: p<0.05; post-hoc AGOM: p<0.0001). Error bars denote SEM. * Denotes the effect of uCMS-exposure; # Denotes the effect of antidepressants comparing to untreated uCMS-exposed animals. */# p<0.05; **/## p<0.01; ***/### p<0.001. n=5/6 animals per group. Abbreviations: Control = CT; unpredictable chronic mild stress = uCMS; uCMS rats treated with fluoxetine = uCMS_FLX; uCMS rats treated with imipramine = uCMS_IMIP; uCMS rats treated with tianeptine = uCMS_TIAN; uCMS rats treated with agomelatine = uCMS_AGOM.



Supplementary Figure S4: Confirmation of the macrodissections' accuracy. Gene expression analysis of three genes that are predominantly expressed in the DG of CT animals compared to the remainder tissue of the hippocampus: *NeuroD1*, *DSP* and *Prox1*. This specificity was confirmed by an enrichment in the expression of these three genes in the DG comparing to the remaining hippocampus (*NeuroD1*: t_4=5.436, p=0.0056, d=0.8808; *DSP*: t_4=11.12, p=0.0004, d=0.9687; *Prox1*: t_4=8.583, p=0.001, d=0.9485). Bars represent the relative expression of each target gene after normalization to *B2M* mRNA levels; error bars denote SEM. *Denotes statistical differences between groups after normalization to *B2M* mRNA levels; **p<0.01; ***p<0.001; n=3 per group.



Supplementary Figure S5: Common transcriptional regulation of fluoxetine, imipramine and tianeptine. Predicted gene network (IPA software) including some of the co-regulated genes between fluoxetine, imipramine and tianeptine. The functions associated to this network are tissue morphology and connective tissue development and function. Green symbols: significantly down-regulated genes; red symbols: significantly up-regulated genes. Genes in white color were not significantly regulated in the analysis. Significance was set at $p<0.01$.



Supplementary Figure S6: Neural cell type enrichment heatmaps obtained from Genes2mind online resource (genes2mind.org), using as input the significantly regulated ($p<0.01$) transcripts in each comparison. Grey color indicates genes for which there is no available data.

SUPPLEMENTARY TABLES

Supplementary Table S1: Sense and antisense sequences of oligonucleotide primers used in the quantitative real time polymerase chain reaction (qRT-PCR), the corresponding product size and function of the selected genes. For microarrays confirmations, most genes were chosen based on their neuroplasticity-related function; those without neuroplasticity-related function were randomly selected from the list of significantly altered genes to avoid bias in the microarrays confirmation. Moreover, genes with annotated function which expression was reversed by at least three of the ADs were analyzed. For macrodissections' accuracy determination, genes predominantly expressed in the DG comparing to the CA regions were chosen.

Gene symbol	Gene name	Selected functions/features	Sense	Antisense	Product size (bp)
Microarrays confirmation					
HES1	Hairy and enhancer of split-1	Repressor of neuronal commitment in stem cells (Nakamura *et al*, 2000).	TGTCAACACGACACCGGACAAAC	TGGAATGCCGGGAGCTATCTTT	161
GADD45B	Growth arrest and DNA-damage-inducible, beta	Role in region-specific DNA demethylation to control key aspects of activity-dependent adult neurogenesis (Ma *et al*, 2009).	GCTGCGACAATGACATTGAC	GTATGACAGTTCGTGACCAG	136
APOBEC1	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	Involved in neuronal activity-induced active DNA demethylation and subsequent gene expression in the dentate gyrus of the adult mouse brain *in vivo* (Guo *et al*, 2011).	ACATAAGCTCCCGAGGAAGG	GGCTCAATTCTTCTCCTCAGAGT	102
NOS1	Nitric oxide synthase 1	Role in the manifestation of depression-like behavior (Yazir *et al*, 2012)	GACAACGTTCCTGTGGTCCT	GAAGAGCTGGTCCTTTGTGC	276
AR	Androgen receptor	Role of androgens in synaptic plasticity (Ooishi *et al*, 2012)	GGGTGACTTCTCTGCCTCTG	CCACAGATCAGGCAGGTCTT	210
PDYN	Prodynorphin	Involvement in neuroplasticity and depression-like behavior (Schwarzer, 2009)	CCTGTCCTTGTGTTCCCTGT	AGAGGCAGTCAGGGTGAGAA	157
CYP2D1	Cytochrome P450 2D1	Role in response to centrally acting drugs (Miksys and Tyndale, 2004)	GTGCAGCGCAGAGTCCAACAA	ATGGAAGCGGTGGGGCTTCT	270
EBAG9	Estrogen Receptor Binding Site Associated, Antigen, 9	Role of estrogen in synaptic plasticity (Ooishi *et al*, 2012)	TAAGCAGACAGACGTGGAGGA	TGCTACCATCTGGGACACCAA	218

SP1	Specificity protein 1	Role in neuronal plasticity by activation of synapsin I gene (*SYN1*) (Paonessa *et al*, 2013)	CTGCCACCATGAGCGACCAAGAT	CCCTGGGAGTTGTTGCTGTTCTC	259
DUSP1	Dual specificity protein phosphatase 1	Reversed by at least three of the ADs. Negative regulator of MAP kinase and involvement in the development of depressive-like behavior (Duric *et al*, 2010)	CTCCAAGGAGGATATGAAGCGTTTT	TGGTAAGCACTGCCCAGGTACA	203
MT4	Metallothionein 4	Reversed by at least three of the ADs.	CACGTGTATGTCTGGAGGGATCT	GCACTTGTCTGAGCCCCCTTTG	157
LRRC8E	Leucine rich repeat containing 8 family, member E	Reversed by at least three of the ADs.	CCCAGAGCAACAGGATGATCCCAG	TGCACCCGAACACCCCAATCA	144
MIR409-5P	microRNA 409, 5p	Reversed by all the ADs.	AGTGTCTCTCGACAGGCACAGGCT	PerfeCTa Universal PCR Primer (Quanta Biosciences*)	-
MIR411-5P	microRNA 411, 5p	Reversed by at least three of the ADs.	AAAGAGTCTAAACAGGATGAGTCC	PerfeCTa Universal PCR Primer (Quanta Biosciences*)	-
RNU6	RNA-U6 small nuclear 2	Reference gene for microRNAs expression analysis	TGGGACAGGAAGTGATCTTGC	PerfeCTa Universal PCR Primer (Quanta Biosciences*)	-
SIX1	Sine Oculis Homeobox Homolog	-	CAAGGAAAGGGAGAACACCGAAAA	CCGTTAGGCCCGGAAGAGAA	206
RSPO2	R-spondin 2	-	GGAGACGCAGTAAGCGAGCTAGT	TGGGGCTCGGTGTCCATAGT	188
MBD4	Methyl-CpG-binding domain protein 4	-	CTCAATCGGACCTCAGGCAA	AGTTCTGACACGTCCCTCCA	110
B2M	Beta-2-microglobulin	Reference gene.	GTGCTTGCCATTCAGAAAACTCC	AGGTGGGTGGAACTGAGACA	136
Macrodissections' accuracy confirmation					
NEUROD1	Neurogenic differentiation 1	DG-specific (Pleasure *et al*, 2000)	ACAACAGGAAGTGGAAACATGAC	ACTGAGGCACTCGTCTGTCC	102
DSP	Desmoplakin	DG-specific (Lein *et al*, 2004)	CCAGCACCAGACAGTCACCAAAA	GCAGCCACGTCTCCTGTTTGT	116
PROX1	Prospero-related homeobox 1	DG-specific (Iwano *et al*, 2012)	CGGGAAGCGCAATGAAGGGCTAT	GATGGCTTGGCGCGCATACTT	211

* MicroRNAs expression analysis was assessed using the qScript™ microRNA Quantification System (Quanta Biosciences™, Gaithersburg, MD, USA).

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Supplementary Table S2
Xls document.

Please go to:
http://www.nature.com/npp/journal/v40/n2/suppinfo/npp2014176s1.html

Supplementary Table S3: List of uCMS-induced transcriptional alterations and reversion by antidepressants (ADs) treatment. Last row shows the number of molecules altered by uCMS-exposure and those reversed by each AD-treatment. Genes highlighted in bold showed reversion of the uCMS-induced expression changes by at least one antidepressant. Datasets were defined as described in the paper ($p<0.01$, fold-change>1.2x, average expression >100).

Probeset	Gene symbol or ID	Fold Change				
		uCMS-exposed	FLX-treated	IMIP-treated	TIAN-treated	AGOM-treated
10764367	***Mir181b-1***	1.49	•	-1.62	-1.52	•
10887050	***Mir411***	1.29	-1.55	-1.61	-1.43	•
10887104	***Mir409***	1.29	-1.44	-1.44	-1.44	-1.52
10816485	***Mir9-1***	1.29	•	-1.27	•	•
10887110	***Mir410***	1.23	-1.36	•	-1.24	•
10887048	***Mir379***	1.21	-1.47	-1.34	-	•
10863679	***Cml5***	1.35	•	•	-1.41	-1.76
10732652	***Dusp1***	1.32	-1.47	-1.53	-1.61	-
10725778	***Nupr1***	1.31	•	•	-1.31	•
10799977	***Enkur***	1.23	-1.23	•	-1.34	•
10719648	***Zfp61***	1.23	•	•	•	-1.28
10864918	***Ret***	1.21	•	•	•	-1.31
10828841	***Rab44***	-1.22	•	•	1.24	•
10809406	***Mt4***	-1.23	1.25	1.24	1.25	•
10712196	***Pkp3***	-1.24	•	•	1.21	•
10722273	***Fancf***	-1.27	1.22	•	1.22	•
10747214	***Krt31***	-1.29	•	•	1.28	1.27
10759553	***Lrrc8e***	-1.40	•	1.29	1.43	1.42
10731444	***Rsl1d1***	-1.71	•	•	•	1.41
10903538	*Rspo2*	1.54	•	•	•	•
10827592	*Clic2*	1.36	•	•	•	•
10718666	*Apeg3*	1.32	•	•	•	•
10790446	*Gprin2*	1.25	•	•	•	•
10791522	*Hpgd*	1.24	•	•	•	•
10755728	*Aifm3*	1.24	•	•	•	-
10894221	*Zfp472*	1.23	•	•	•	•
10801859	*Prrc1*	1.22	•	•	•	•
10860815	*Samd9l*	1.22	•	•	•	•
10802593	*Mro*	1.22	•	•	•	•
10930749	*Sept10*	1.21	•	•	•	•
10722845	*Agbl1*	-1.21	•	•	•	•
10709473	*Olr143*	-1.21	•	•	•	•
10742919	*Olr1414*	-1.21	•	•	•	•
10855096	*Olr811*	-1.22	•	•	•	•
10851667	*Neurl2*	-1.21	•	•	•	•
10921457	*Guca1a*	-1.22	•	•	•	•

10777229	*Apex2*	-1.22	•	•	•	•
10831236	*Ly6g6c*	-1.23	•	•	•	•
10744947	*Serpinf2*	-1.27	•	•	•	•
10905738	*Cyp2d1*	-1.30	•	•	•	•
10905732	*Cyp2d5*	-1.30	•	•	•	•
10910678	*Itga11*	-1.36	•	•	•	•
10813949	*Fam134b*	-1.39	•	•	•	•
10846740	*Frzb*	-1.54	•	•	•	•
10806413	***ENSRNOT00000060679***	-1.34	•	1.44	1.49	•
10767075	***ENSRNOT00000053847***	-1.38	1.52	•	1.48	•
10722578	***ENSRNOT00000055917***	-1.38	•	1.39	1.42	•
10752628	***ENSRNOT00000063656***	-1.42	1.41	•	•	•
10775226	***ENSRNOT00000047758***	-1.43	1.42	1.50	•	•
10788070	***ENSRNOT00000059482***	-1.47	1.59	1.61	1.54	1.48
10755670	***ENSRNOT00000053925***	-1.57	•	1.94	2.15	•
10749818	***ENSRNOT00000053950***	-1.66	2.04	1.73	1.85	•
10785624	***ENSRNOT00000057823***	-1.22	1.23	•	•	•
10763318	***ENSRNOT00000049616***	-1.24	1.28	1.27	•	•
10744141	***ENSRNOT00000054292***	-1.25	•	•	1.24	•
10780922	***ENSRNOT00000032631***	-1.25	1.26	•	1.38	1.34
10857470	***ENSRNOT00000056864***	-1.26	•	1.25	•	•
10702579	***ENSRNOT00000060826***	-1.27	1.32	1.30	1.36	•
10724150	***ENSRNOT00000031043***	-1.28	•	•	1.22	•
10707740	***ENSRNOT00000055911***	-1.29	1.30	1.34	1.34	•
10740320	*ENSRNOT00000061717*	1.72	•	•	•	•
10731408	*ENSRNOT00000061717*	1.72	•	•	•	•
10795400	*ENSRNOT00000035939*	1.26	•	•	•	•
10827367	*ENSRNOT00000036927*	1.23	•	•	•	•
10894216	*ENSRNOT00000037985*	1.21	•	•	•	•
10710087	*ENSRNOT00000054414*	-1.22	•	•	•	•
10867488	*ENSRNOT00000041141*	-1.22	•	•	•	•
10928544	*ENSRNOT00000054361*	-1.22	•	•	•	•
10743964	*ENSRNOT00000057054*	-1.23	•	•	•	•
10756127	*ENSRNOT00000033247*	-1.23	•	•	•	•
10820122	*ENSRNOT00000061985*	-1.23	•	•	•	•
10894854	*ENSRNOT00000049028*	-1.26	•	•	•	•
10873723	***LOC100360708***	1.28	•	-1.38	-1.43	•
10744306	***LOC497940***	1.24	-1.26	•	•	•
10926285	***LOC680955***	1.21	•	•	-1.28	•
10805591	***LOC689153***	-1.26	1.22	•	1.35	1.44
10937658	***LOC686031***	-1.58	1.58	1.48	1.48	1.56
10771492	*LOC100361238*	1.40	•	•	•	•
10785174	*LOC100359986*	1.30	•	•	•	•
10712047	*LOC680907*	-1.23	•	•	•	•

10796555	*LOC100365771*	-1.25	•	•	•	•
10917992	*LOC100361110*	-1.26	•	•	•	•
10783022	***RGD1310110***	1.25	•	•	•	•
10726241	***RGD1560958***	-1.29	1.22	•	1.22	•
10740325	*RGD1565158*	1.89	•	•	•	•
10761736	*RGD1563482*	1.28	•	•	•	•
10798130	*RGD1562844*	1.27	•	•	•	•
10803895	*RGD1310251*	-1.22	•	•	•	•
10755281	*RGD1560559*	-1.23	•	•	•	•
10808094	*RGD1311422*	-1.69	•	•	•	•
10817543	***FQ225205***	-1.87	2.10	•	•	•
10930616	*FQ214145*	7.80	•	•	•	•
Total # of molecules		**93**	**23**	**20**	**30**	**11**

Supplementary Table S4: Genes commonly regulated by the 4 antidepressants, Fluoxetine (FLX), Imipramine (IMIP), Tianeptine (TIAN) and Agomelatine (AGOM), and by 3 antidepressants (FLX, IMIP, and TIAN). Regulated genes as shown in Figure 3i were used for the analysis ($p<0.01$, fold change > 1.3x, and average expression >100). Two probe sets corresponding to the same gene were removed.

Treatments	Probe set	Gene	Fold change			
			FLX	IMIP	TIAN	AGOM
FLX/IMIP/TIAN/ AGOM	10937658	LOC686031	1.58	1.48	1.48	1.56
	10722423	ENSRNOT00000053799	1.48	1.54	2.10	1.35
	10887106	Mir412	-1.44	-1.38	-1.47	-1.52
	10722465	ENSRNOT00000053934	1.63	1.75	2.08	1.47
	10722425	ENSRNOT00000052934	1.60	1.55	1.72	1.35
	10722471	ENSRNOT00000052507	1.46	1.47	1.64	1.37
	10722481	ENSRNOT00000053855	1.37	1.53	1.79	1.39
	10722459	ENSRNOT00000053880	1.25	1.76	1.45	2.16
	10788070	ENSRNOT00000059482	1.25	1.76	1.45	2.16
	10894159	ENSRNOT00000031816	1.38	1.28	1.23	1.44
	10887104	Mir409	-1.52	-1.44	-1.44	-1.44
FLX/IMIP/TIAN	10937658	LOC686031	1.58	1.48	1.48	1.56
	10722423	ENSRNOT00000053799	1.48	1.54	2.10	1.35
	10887106	Mir412	-1.44	-1.38	-1.47	-1.52
	10722465	ENSRNOT00000053934	1.63	1.75	2.08	1.47
	10722425	ENSRNOT00000052934	1.60	1.55	1.72	1.35
	10722471	ENSRNOT00000052507	1.46	1.47	1.64	1.37
	10722481	ENSRNOT00000053855	1.37	1.53	1.79	1.39
	10722459	ENSRNOT00000053880	1.25	1.76	1.45	2.16
	10788070	ENSRNOT00000059482	1.25	1.76	1.45	2.16
	10894159	ENSRNOT00000031816	1.38	1.28	1.23	1.44
	10887104	Mir409	-1.52	-1.44	-1.44	-1.44
	10930569	NC_001665	1.85	1.96	2.30	-
	10930299	ENSRNOT00000054648	1.31	1.26	1.41	-
	10732652	Dusp1	-1.47	-1.53	-1.61	-
	10707740	ENSRNOT00000055911	1.30	1.34	1.34	-
	10915103	ENSRNOT00000054091	1.35	1.45	1.51	-
	10722449	ENSRNOT00000053529	1.57	1.36	2.04	-
	10702579	ENSRNOT00000060826	1.32	1.30	1.36	-
	10826416	ENSRNOT00000053097	1.32	1.31	1.35	-
	10756530	ENSRNOT00000061078	1.52	1.30	1.29	-
	10722451	ENSRNOT00000053019	1.41	1.48	1.76	-
	10722429	ENSRNOT00000053184	1.49	1.50	1.68	-
	10722433	ENSRNOT00000053117	1.32	1.40	1.52	-
	10887050	Mir411	-1.55	-1.61	-1.43	-
	10792421	Plat	-1.26	-1.34	-1.31	-
	10770892	Vom2r65	1.27	1.24	1.31	-
	10722431	ENSRNOT00000052564	1.23	1.27	1.34	-
	10766882	Mir29c	-1.57	-1.71	-1.60	-
	10749818	ENSRNOT00000053950	2.04	1.73	1.85	-
	10722441	ENSRNOT00000052661	1.28	1.28	1.42	-
	10809406	Mt4	1.25	1.24	1.25	-

Supplementary Table S5: Twenty commonly regulated genes by 3 (FLX, IMIP, and TIAN) of the 4 antidepressants. Regulated genes as shown in Figure 3c were used for the analysis ($p<0.01$, fold change > 1.3x, and average expression >100). Two probe sets corresponding to the same gene were removed.

Probe set	Gene	Fold Change		
		FLX	IMIP	TIAN
10930569	NC_001665	1.85	1.96	2.30
10930299	ENSRNOT00000054648	1.31	1.26	1.41
10732652	Dusp1	-1.47	-1.53	-1.61
10707740	ENSRNOT00000055911	1.30	1.34	1.34
10915103	ENSRNOT00000054091	1.35	1.45	1.51
10722449	ENSRNOT00000053529	1.57	1.36	2.04
10702579	ENSRNOT00000060826	1.32	1.30	1.36
10826416	ENSRNOT00000053097	1.32	1.31	1.35
10756530	ENSRNOT00000061078	1.52	1.30	1.29
10722451	ENSRNOT00000053019	1.41	1.48	1.76
10722429	ENSRNOT00000053184	1.49	1.50	1.68
10722433	ENSRNOT00000053117	1.32	1.40	1.52
10887050	Mir411	-1.55	-1.61	-1.43
10792421	Plat	-1.26	-1.34	-1.31
10770892	Vom2r65	1.27	1.24	1.31
10722431	ENSRNOT00000052564	1.23	1.27	1.34
10766882	Mir29c	-1.57	-1.71	-1.60
10749818	ENSRNOT00000053950	2.04	1.73	1.85
10722441	ENSRNOT00000052661	1.28	1.28	1.42
10809406	Mt4	1.25	1.24	1.25

Supplementary Table S6: Significantly ($p<0.05$) enriched canonical pathways in uCMS-exposed vs CT animals and after ADs (fluoxetine, FLX; imipramine, IMIP; tianeptine, TIAN; agomelatine, AGOM) treatment. Regulated gene sets as shown in Figure 3a were analyzed separately for up- and down-regulation. Ratio is defined as the number of regulated genes in the array data divided by the number of all genes in the pathway. Pathways in each comparison are ordered by increasing p-values.

Canonical pathway (Ingenuity)	No of genes	List of genes	Ratio	-log (p-value)
uCMS vs CT: upregulated				
Glutathione Redox Reactions I	1	CLIC2	4.17E-02	1.46
uCMS_FLX vs uCMS: downregulated				
Death Receptor Signaling	3	NFKBIA,TNFRSF25,TNFSF10	4.41E-02	3.66
Type I Diabetes Mellitus Signaling	3	NFKBIA,GZMB,TNFRSF11B	2.48E-02	2.88
IL-6 Signaling	3	NFKBIA,IL6R,TNFRSF11B	2.42E-02	2.72
TWEAK Signaling	2	NFKBIA,TNFRSF25	5.13E-02	2.65
Acute Phase Response Signaling	3	NFKBIA,IL6R,TNFRSF11B	1.66E-02	2.27
OX40 Signaling Pathway	2	NFKBIA,CD4	2.06E-02	2.19
Induction of Apoptosis by HIV1	2	NFKBIA,TNFRSF11B	2.99E-02	2.16
ATM Signaling	2	GADD45B,NFKBIA	3.03E-02	2.16
T Helper Cell Differentiation	2	IL6R,TNFRSF11B	2.78E-02	2.09
NF-κB Activation by Viruses	2	NFKBIA,CD4	2.41E-02	1.98
HMGB1 Signaling	2	TNFRSF11B,PLAT	1.83E-02	1.80
PPAR Signaling	2	NFKBIA,TNFRSF11B	1.87E-02	1.77
T Cell Receptor Signaling	2	NFKBIA,CD4	1.83E-02	1.73
iCOS-iCOSL Signaling in T Helper Cells	2	NFKBIA,CD4	1.59E-02	1.72
CD28 Signaling in T Helper Cells	2	NFKBIA,CD4	1.47E-02	1.65
PKCθ Signaling in T Lymphocytes	2	NFKBIA,CD4	1.39E-02	1.63
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	3	NFKBIA,IL6R,TNFRSF11B	8.77E-03	1.63
Type II Diabetes Mellitus Signaling	2	NFKBIA,TNFRSF11B	1.17E-02	1.59
Granzyme B Signaling	1	GZMB	5.56E-02	1.53
Hepatic Cholestasis	2	NFKBIA,TNFRSF11B	1.09E-02	1.49
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2	IL6R,TNFRSF11B	1.29E-02	1.48
GADD45 Signaling	1	GADD45B	4.17E-02	1.45
Tec Kinase Signaling	2	TNFRSF25,TNFSF10	1.09E-02	1.41
Dendritic Cell Maturation	2	NFKBIA,TNFRSF11B	9.48E-03	1.40
NF-κB Signaling	2	NFKBIA,TNFRSF11B	1.15E-02	1.39
Systemic Lupus Erythematosus Signaling	2	LSM6,IL6R	7.81E-03	1.35
Role of NFAT in Regulation of the Immune Response	2	NFKBIA,CD4	1.00E-02	1.35
Tumoricidal Function of Hepatic Natural Killer Cells	1	GZMB	3.70E-02	1.31
uCMS_FLX vs uCMS: upregulated				
Pentose Phosphate Pathway (Non-oxidative Branch)	1	TKTL2	7.69E-02	1.79
PPAR Signaling	2	INS,SCAND1	1.87E-02	1.62
NAD Phosphorylation and Dephosphorylation	1	ACPT	5.26E-02	1.61
Pentose Phosphate Pathway	1	TKTL2	4.35E-02	1.61
nNOS Signaling in Skeletal Muscle Cells	1	CHRNA1	3.23E-02	1.46
Glutathione Redox Reactions I	1	MGST3	4.17E-02	1.40
NAD Salvage Pathway II	1	ACPT	3.12E-02	1.37

uCMS_IMIP vs uCMS: downregulated				
Colanic Acid Building Blocks Biosynthesis	2	TSTA3,PMM1	5.56E-02	2.76
GDP-L-fucose Biosynthesis I (from GDP-D-mannose)	1	TSTA3	1.43E-01	2.05
Cardiac Hypertrophy Signaling	4	MYL6,RHOC,ADRA2C,EIF2B2	1.61E-02	1.94
Coenzyme A Biosynthesis	1	PPCS	7.14E-02	1.87
Aryl Hydrocarbon Receptor Signaling	3	GSTM3,RXRA,AIP	1.75E-02	1.77
Heme Biosynthesis from Uroporphyrinogen-III I	1	UROD	9.09E-02	1.75
Citrulline-Nitric Oxide Cycle	1	NOS1	6.25E-02	1.65
GDP-mannose Biosynthesis	1	PMM1	7.69E-02	1.58
EIF2 Signaling	3	EIF2B2,RPL7,RPSA	1.49E-02	1.57
RhoGDI Signaling	3	CDH7,MYL6,RHOC	1.49E-02	1.55
Small Cell Lung Cancer Signaling	2	NOS1,RXRA	2.13E-02	1.48
RAR Activation	3	PRMT2,DUSP1,RXRA	1.57E-02	1.47
Regulation of Actin-based Motility by Rho	2	MYL6,RHOC	2.20E-02	1.45
Heme Biosynthesis II	1	UROD	4.17E-02	1.45
TR/RXR Activation	2	RXRA,THRSP	1.83E-02	1.33
uCMS_IMIP vs uCMS: upregulated				
Protein Ubiquitination Pathway	5	DN1AJC24,USP14,USP15,CDC20,DNAJB6	1.85E-02	2.95
Spermine Biosynthesis	1	SMS	1.43E-01	2.14
Spermidine Biosynthesis I	1	SMS	1.25E-01	1.96
UDP-N-acetyl-D-glucosamine Biosynthesis II	1	GNPNAT1	5.88E-02	1.66
NRF2-mediated Oxidative Stress Response	3	USP14,DNAJA2,DNAJB6	1.54E-02	1.64
UDP-N-acetyl-D-galactosamine Biosynthesis II	1	GNPNAT1	4.00E-02	1.60
DNA Double-Strand Break Repair by Non-Homologous End Joining	1	NBN	5.00E-02	1.37
Telomere Extension by Telomerase	1	NBN	5.56E-02	1.37
uCMS_TIAN vs uCMS: downregulated				
Melatonin Degradation I	2	Sult1d1,CYP1B1	3.03E-02	2.42
Superpathway of Melatonin Degradation	2	Sult1d1,CYP1B1	2.47E-02	2.36
γ-glutamyl Cycle	1	GGACT	3.45E-02	1.68
The Visual Cycle	1	DHRS3	3.57E-02	1.49
Dopamine Degradation	1	Sult1d1	2.63E-02	1.38
Thyroid Hormone Metabolism II (via Conjugation and/or Degradation)	1	Sult1d1	1.89E-02	1.36
uCMS_TIAN vs uCMS: upregulated				
Glycine Biosynthesis III	1	AGXT	1.67E-01	2.18
EIF2 Signaling	3	INS,RPL32,RPL13	1.49E-02	1.90
Role of BRCA1 in DNA Damage Response	2	BARD1,FANCF	2.82E-02	1.84
Acute Phase Response Signaling	3	FTL,C3,OSMR	1.66E-02	1.81
PPAR Signaling	2	INS,SCAND1	1.87E-02	1.46
Hereditary Breast Cancer Signaling	2	BARD1,FANCF	1.49E-02	1.35
uCMS_AGOM vs uCMS: downregulated				
Complement System	3	CD55,C4BPB,C4BPA	8.57E-02	1.74
Axonal Guidance Signaling	14	EPHA8,PLXNA3,SLIT1,MAPK3,BAIAP2,ABLIM3,SMO,UNC5D,WNT4,WNT16,SEMA6B,ABLIM2,EFNB3,GNG7	2.90E-02	1.70

L-glutamine Biosynthesis II (tRNA-dependent)	1	QRSL1	9.09E-02	1.70
Sulfite Oxidation IV	1	SUOX	2.50E-01	1.70
CDK5 Signaling	5	PPP2R4,MAPK3,CAPN1,PPM1L,ADCY1	5.15E-02	1.67
Triacylglycerol Biosynthesis	3	PPAPDC1A,PPAP2A,DGAT2	6.52E-02	1.66
Cell Cycle Regulation by BTG Family Proteins	3	PPP2R4,PPM1L,BTG1	7.69E-02	1.62
tRNA Splicing	3	TSEN54,PDE1B,TRPT1	6.52E-02	1.58
Antiproliferative Role of Somatostatin Receptor 2	4	SSTR2,MAPK3,NPR2,GNG7	5.56E-02	1.49
Cardiac β-adrenergic Signaling	6	PPP2R4,PPM1L,ADCY1,PDE1B,PKIA,GNG7	3.80E-02	1.48
Netrin Signaling	3	ABLIM3,UNC5D,ABLIM2	5.17E-02	1.45
Human Embryonic Stem Cell Pluripotency	6	FGFR4,SMAD3,SMO,WNT16,WNT4,SMAD5	3.73E-02	1.41
Alanine Degradation III	1	GPT	1.67E-01	1.40
Alanine Biosynthesis II	1	GPT	1.67E-01	1.40
Uridine-5'-phosphate Biosynthesis	1	CAD	5.00E-02	1.40
Synaptic Long Term Depression	6	PLA2G2D,PPP2R4,GRM1,MAPK3,PPM1L,NPR2	3.73E-02	1.36
uCMS_AGOM vs uCMS: upregulated				
EIF2 Signaling	4	MAP2K4,ITPR1,RASA1,PRKCA	4.48E-02	3.31
RhoGDI Signaling	9	PDE8A,ENPP6,LPAR1,HTR1F,CREB5,RAP1A,RASA1,CHRM2,PRKCA	3.98E-02	2.63
mTOR Signaling	7	MAP2K4,RHOQ,ARHGEF6,PPP2R2B,TMSB10/TMSB4X,CREB5,FNBP1	3.76E-02	2.39
Phospholipase C Signaling	7	MAP2K4,RAP1B,RHOQ,PPP2R2B,RAP1A,FNBP1,PRKCA	3.40E-02	2.37
Corticotropin Releasing Hormone Signaling	5	MAP2K4,RHOQ,ITPR1,FNBP1,PRKCA	4.14E-02	2.24
Cdc42 Signaling	5	MAP2K4,RAP1B,HGF,RAP1A,PRKCA	3.23E-02	2.04
EGF Signaling	7	MAP2K4,RAP1B,RHOQ,RALA,TSPAN2,RAP1A,FNBP1	6.25E-02	1.96
G-Protein Coupled Receptor Signaling	2	HIST1H1A,HMGB2	3.26E-02	1.88
ILK Signaling	10	MAP2K4,RAP1B,RHOQ,RALA,ARHGEF6,RAP1A,RASA1,XIAP,FNBP1,PRKCA	3.41E-02	1.87
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	6	MAP2K4,RHOQ,ITPR1,ELMO1,FNBP1,PRKCA	3.30E-02	1.85
Cholecystokinin/Gastrin-mediated Signaling	5	LPAR1,RDX,ARHGAP12,SEPT4,PIP4K2A	4.72E-02	1.78
HGF Signaling	5	RALA,LPAR1,HTR1F,RAP1A,CHRM2	4.50E-02	1.74
Integrin Signaling	1	MPST	3.37E-02	1.71
Granzyme A Signaling	2	CMPK1,AK7	1.00E-01	1.7
Molecular Mechanisms of Cancer	5	RAP1B,ITPR1,CREB5,RAP1A,PRKCA	2.58E-02	1.68
CXCR4 Signaling	11	RAP1B,EPHA6,MAG,SRGAP1,ARHGEF6,HHIP,RAP1A,RASA1,NTN1,PRKCA,ADAMTS4	3.45E-02	1.67
RhoA Signaling	7	MAP2K4,SP1,SGK1,ITPR1,CREB5,RASA1,PRKCA	4.10E-02	1.66
Gαi Signaling	7	MAP2K4,RHOQ,ARHGEF6,R	3.70E-02	1.53

		DX,SEPT4,PIP4K2A,FNBP1		
L-cysteine Degradation III	7	PDE8A,ENPP6,LPAR1,HTR1F,CREB5,RAP1A,CHRM2	1.11E-01	1.5
Pyrimidine Ribonucleotides Interconversion	2	CMPK1,AK7	5.88E-02	1.49
Synaptic Long Term Potentiation	4	HGF,ITPR1,CREB5,PRKCA	3.85E-02	1.49
Axonal Guidance Signaling	10	PDE8A,ENPP6,RAP1B,DUSP5,HIST1H1A,ITPR1,CREB5,RAP1A,NTN1,PRKCA	2.28E-02	1.44
Huntington's Disease Signaling	5	EIF4G2,PPP2R2B,RPS19,EIF3A,EIF3J	2.78E-02	1.42
Signaling by Rho Family GTPases	4	MAP2K4,RHOQ,SP1,FNBP1	2.67E-02	1.42
cAMP-mediated signaling	5	PDE8A,ENPP6,RAP1B,GUCY1A3,RAP1A	3.10E-02	1.42
Pyrimidine Ribonucleotides De Novo Biosynthesis	4	MAP2K4,ITPR1,RASA1,PRKCA	4.17E-02	1.41
FGF Signaling	9	PDE8A,ENPP6,LPAR1,HTR1F,CREB5,RAP1A,RASA1,CHRM2,PRKCA	4.26E-02	1.39
Protein Kinase A Signaling	7	MAP2K4,RHOQ,ARHGEF6,PPP2R2B,TMSB10/TMSB4X,CREB5,FNBP1	2.46E-02	1.39
Regulation of eIF4 and p70S6K Signaling	7	MAP2K4,RAP1B,RHOQ,PPP2R2B,RAP1A,FNBP1,PRKCA	2.86E-02	1.37
HMGB1 Signaling	5	MAP2K4,RHOQ,ITPR1,FNBP1,PRKCA	3.67E-02	1.34
Relaxin Signaling	5	MAP2K4,RAP1B,HGF,RAP1A,PRKCA	3.05E-02	1.33

4. Cell cycle regulation of the hippocampal progenitor cells in a rat model of depression and after fluoxetine treatment

Manuscript to be submitted

Cell cycle regulation of the hippocampal progenitor cells in a rat model of depression and after fluoxetine treatment

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ABSTRACT

Changes in adult hippocampal cell proliferation and genesis have been largely reported in the context of depression and antidepressants action. Adult hippocampal cell genesis encompasses several steps including cell division, differentiation and integration of cells in the pre-existing network. Surprisingly, the cell cycle regulatory mechanisms implicated in the regulation of proliferation and cell genesis in the context of depression and antidepressant treatment are largely undisclosed. Using both *in vivo* - unpredictable chronic mild stress (uCMS) rat model of depression -and *in vitro* - rat hippocampal-derived neurospheres culture - approaches, we aimed to unravel the cell cycle mechanisms regulating hippocampal cell proliferation and genesis in the context of depression and antidepressant treatment. Here we show that uCMS-exposed animals presented decreased proliferation (BrdU+ and Ki67+ cells) and generation of newborn neurons (BrdU+DCX+) that was reversed by fluoxetine treatment. Moreover, the hippocampal dentate gyrus of uCMS animals presents a slightly increased percentage of cells in the G1 phase of the cell cycle and a decreased proportion in the G2/M phase, suggesting a G1 phase arrest. These cells presented decreased levels of cyclin D1, E and A expression, compared to control animals. Chronic fluoxetine treatment (2 weeks) reversed the G1 phase arrest and promoted an up-regulation of cyclin E expression. Dexamethasone (DEX) decreased proliferation of progenitor cells *in vitro*, whereas the administration of serotonin (5-HT), a neurotransmitter involved in the actions of SSRIs, could partly reverse this decrease. Moreover, DEX also induced a G1-phase arrest and decreased the expression of cyclin D1, cyclin D2 and Cdk6, while increasing p27Kip1 expression. 5-HT treatment could only partly reverse the G1-phase arrest and restored the expression of cyclin D1 and Cdk6. Together, these results suggest that the anti-proliferative actions of chronic stress in the adult hipocampal DG may result from a glucocorticoid-mediated G1-phase arrest in the progenitor cells that may be overcome by antidepressant treatment. This G1 arrest seems to be at least partly mediated by decreased cyclin D1 expression. Interestingly, we found a putative glucocorticoid responsive element (GRE) in the promoter region of cyclin D1. Complementary experimental approaches may be useful to further understand the molecular mechanisms underlying these changes.

Keywords: depression, antidepressants, hippocampal neurogenesis, cell cycle.

INTRODUCTION

Although evidence suggests that new cells can be produced in several brain regions during adulthood (Gould, 2007), cell genesis from neural progenitors is generally accepted to occur mainly in two regions of the mammalian brain: the subgranular zone (SGZ) of the hippocampal dentate gyrus (hDG) and in the subependymal zone (SEZ) of the lateral ventricles (Aimone et al., 2010). Particularly, cells generated in the adult hDG have an important role in the regulation and integration of information, with major repercussions for many behavioral manifestations (Aimone et al., 2010, Glasper et al., 2012); Changes in the number of newly generated cells in this brain region impact on cognitive performance, memory, anxiety behavior, stress response and psychotropic drugs effects (Schloesser et al., 2010, O'Leary et al., 2012, Mateus-Pinheiro et al., 2013b, Egeland et al., 2015).

One of the most striking hypotheses in this field was the involvement of hippocampal cell genesis in the pathophysiology of depression and in the efficacy of antidepressants (ADs) (Malberg et al., 2000, Santarelli et al., 2003, Perera et al., 2007, Snyder et al., 2011, Mateus-Pinheiro et al., 2013b, Hill et al., 2015). However, the understanding of this phenomenon and the molecular cues guiding its course are far from being completely understood. The generation of new cells from a neural progenitor involves several steps, namely cell division, cell fate commitment, migration and maturation, and the establishment of proper synaptic connections that culminate with a full integration of cells in the pre-existing network (Kempermann et al., 2004, Patricio et al., 2013). As in other tissues, the cell cycle of adult hippocampal progenitor cells is governed by cyclins and cyclin-dependent kinases (Cdk) that promote cell cycle progression, and Cdk inhibitors that restrain its progression, promoting cell cycle exit and post-mitotic states maintenance. However, the cell cycle changes implicated in the decreased cell genesis occuring in the hippocampus of depressed individuals and animal models of depression and the pro-neurogenic/gliogenic effects produced by ADs are poorly understood. Previous works have briefly suggested the implication of Cdk inhibitors in the decreased proliferative capacity of progenitor cells in the SGZ of animal models of depression (Heine et al., 2004, Chesnokova and Pechnick, 2008). Nevertheless, no studies have looked at this phenomenon in an integrated multimodal fashion.

To understand the cell cycle mechanisms regulating hippocampal cell proliferation and cell genesis in the context of depression and ADs treatment, we used two complementary approaches: an *in vivo* multidimensional rat model of depression – the unpredictable Chronic Mild Stress (uCMS), and *in*

vitro tools – rat hippocampal-derived neurospheres culture. Indeed, chronic stress exposure is a major precipitating factor of depression; both in humans and animal models, chronic stress promotes a disruption of the HPA axis resulting in increased glucocorticoids (GCs) secretion, which ultimately leads to loss of corticosteroid feedback inhibition. Glucocorticoid receptors (GRs) are largely expressed in the hippocampus, thus rendering this region particularly susceptible to the effects of stress. Indeed, excessive GCs are also known to increase Ca2+ influx, thus making neurons more vulnerable to excitotoxicity and oxidative stress (Stein-Behrens et al., 1994, Behl et al., 1997, Sousa and Almeida, 2012). These molecules have potent growth-inhibitory effects in several peripheral tissues and also in the hippocampus, thus inhibiting proliferation and neurogenesis (Mirescu and Gould, 2006, Schoenfeld and Gould, 2013). In this study, the cell cycle of the hippocampal progenitor cells in depressive-like and fluoxetine-treated animals was characterized. To mimic the *in vivo* elevation of GCs after chronic stress exposure, rat hippocampal neurospheres cultures were stimulated with dexamethasone 1 μM (DEX), an agonist of GR. Cells were then treated with serotonin (5-HT), one of the major neurotransmitters implicated in fluoxetine treatment. Using this multidimensional approach, we could further elucidate the molecular players and mechanisms regulating progenitor cells proliferation in the hippocampal neurogenic niche after chronic stress exposure and ADs treatment.

MATERIALS AND METHODS

Animals and treatments

Male Wistar Han rats (200–250g, 2-month old; Charles River Laboratories) were maintained under standard laboratory conditions (lights on: 08:00–20:00 hours; 22° C, relative humidity of 55%, *ad libitum* access to food and water). Rats were randomly assigned to one of the following groups (n=10/group): non-stressed control (CT) vehicle; stress-exposed (uCMS) vehicle; and uCMS treated with fluoxetine (uCMS+FLX). A validated uCMS protocol was applied for 6 weeks, as previously described (Bessa et al, 2009b). During the last 2 weeks of the uCMS, animals were injected daily with fluoxetine (intraperitoneal; 10 mg/kg in ultra-pure water; Kemprotec). The dose was chosen based on previous studies (Bessa et al., 2009a, Mateus-Pinheiro et al., 2013a, Patricio et al.,

2015). All procedures were carried out in accordance with EU Directive 2010/63/EU on animal care and experimentation.

Behavioral analysis

Sucrose consumption test (SCT): Anhedonia was assessed during the last week of uCMS using the SCT. Briefly, animals were allowed to habituate to the sucrose solution in the week before the beginning of the uCMS protocol to establish baseline preference levels. To test sucrose preference, animals were food and water deprived for 12 h and then presented with two pre-weighed bottles containing 2% sucrose solution or tap water for a period of 1 h (starting at the beginning of the dark period). Sucrose preference was calculated as previously described (Bessa et al., 2009b, Patricio et al., 2015).

Novelty suppressed feeding (NSF): Anxiety-like behavior was assessed through the NSF test at the end of the uCMS protocol. Animals were food-deprived for 18h and then placed in an open-field arena for a maximum of 10 min, where a single food pellet was positioned in the center, as previously described (Bessa et al., 2009b). After reaching the pellet, animals were returned to their home cage where they were allowed to feed for 10 min. The latency to feed in the open-field arena was used as an anxiety-like behavior index; food consumption in the home cage provided a measure of appetite drive.

Forced swim test (FST): Depressive-like behavior was assessed at the end of the uCMS protocol using FST. Test trials were conducted 24 h after a 5-min pretest session. For that, rats were placed in glass cylinders filled with water (23°C; 50 cm deep) for 5 min. An increase in immobility time was taken as a measure of depressive-like behavior.

Serum corticosterone levels measurement

Corticosterone levels were measured in the rat's blood serum using a [^{125}I] radioimmunoassay (RIA) kit (MP Biomedicals), according to manufacturer's instructions. Blood sampling (tail venipuncture) was performed during the diurnal nadir (N, 08:00-09:00) at the sixth week of the uCMS protocol.

Bromodeoxyuridine (BrdU) administration

BrdU is a thymidine analogue that can be incorporated into newly synthesized DNA during the S-phase of the cell cycle. To assess proliferation and cell cycle exit, BrdU (intraperitoneal; 100 mg/kg in 0.9% saline solution; Sigma-Aldrich) was administered to the rats 24 h before sacrifice.

Determination of 5-HT and 5-HIAA concentrations in the hDG by HPLC-ED (preliminary data)

Animals (n=2) were rapidly decapitated, and brains immediately snap-frozen in liquid nitrogen. Hippocampal DGs were dissected and stored at -80° C until use. Perchloric acid (0.2 N) was added to each sample, and after disruption and sonication (5 min on ice), samples were centrifuged at 5000 g. The resulting supernatant was filtered through a Spin-X high-performance liquid chromatography (HPLC) column (Costar) to remove debris, as previously described (Borges et al., 2013). Levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) were measured by HPLC with electrochemical detection using a Gilson instrument, fitted with an analytical column (Supelco Supelcosil LC-18 3 mM, flow rate: 1.0 mL/min), as previously described (Borges et al., 2013). Briefly, 150 mL supernatant aliquots were injected into the system, using a mobile phase of 0.7 M aqueous potassium phosphate (pH 3.0) in 10% methanol, 1-heptanesulfonic acid (222 mg/L), and Na-EDTA (40 mg/L). A standard curve using known concentrations was run simultaneously.

Neurospheres culture

Postnatal day 5 Wistar Han rats were rapidly decapitated using a scissors and their brains collected. The meninges were removed, the hemispheres separated and the hippocampus macrodissected in ice-cold DMEM+10% FBS. After mechanical trituration and washes in DMEM+10% FBS, hippocampal cells were seeded in 12-well plates (NUNC) in 3 mL of neurospheres medium (DMEM-F12-GlutaMAX™, B27 supplement 2%, Pen-Strep 1%, HEPES buffer 8 mM, bFGF and EGF (10 ng/μL)). Cells were maintained at 37° C, in 5% CO_2 and humid atmosphere. Every 2 days, Dexamethasone (DEX, Fortecortin, Merck; 1 μM) was added to the medium. In the last 4 days of culturing, serotonin (Serotonin hydrochloride, 5-HT 1 μM, Sigma) was added to the culture. Neurospheres were harvested at day 7 for further analysis.

Immunostaining procedures

Animal model

Rats (n=5/group) were deeply anesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and transcardially perfused with 0.9% saline solution followed by ice-cold 4% paraformaldehyde (PFA). Brains were removed and post-fixed in 4% PFA. Twenty-four hours later, brains were washed with phosphate-buffered saline (PBS) solution, and transferred into a 30% sucrose solution until they sank. Brains were then embedded in OCT compound (Thermo Scientific) and frozen using liquid nitrogen and 2-methyl-butane. Brains were maintained at -20° C until being cut in a cryostat. Coronal cryosections (20 µm) were double-stained for BrdU (mouse monoclonal; 1:50; Dako), and Ki67 (rabbit polyclonal; 1:300; Merck Millipore). Briefly, tissue sections were permeabilized in 1x PBS/0.5% Triton-X 100 for 10 min followed by washes with 1x PBS. Antigen-retrieval was performed with sodium citrate buffer for 15 min in the microwave. Sections were then washed in 1x PBS and acidified with HCl for 30 min prior to overnight incubation with primary antibodies (1x PBS, 10% Fetal Bovine Serum, FBS) at 4°C. After the primary antibody step, sections were washed in 1x PBS followed by secondary antibodies incubation (1:1000; anti-mouse Alexa-fluor® 488; anti-rabbit Alexa-fluor® 568; Life Technologies, Thermo Fisher Scientific). Finally, all sections were stained with 4',6- diamidino-2-phenylindole (DAPI; 1 mg/mL). For each animal, BrdU positive, Ki67 positive and double positive cells within the SGZ of the dentate gyrus (DG) were counted (6-8 brain sections per animal) using a confocal microscope (Olympus FluoViewTM FV1000). The corresponding DG areas were determined using a motorized microscope (Axioplan 2; Carl Zeiss) and the Neurolucida software (MBF Bioscience) to estimate cell densities. Cell cycle exit was determined as the number of BrdU+Ki67- cells per total BrdU+ cells. Cell counts from each animal were averaged. Results are presented as the average of total cell density counts in each experimental group.

In vitro (Neurospheres culture)

For neurospheres characterization, cells were platted after 7 DIV, in Poly-D-Lysine (PDL)-coated slides for 2 h (neurospheres immunostaining). For differentiation experiments, neurospheres were split at day 7 using Accutase Cell Detachment Solution (Merck Millipore). Viable cells were counted by trypan blue exclusion assay in a hemocytometer and platted in PDL-coated 24-well plates at a density of 30.000 cells/well. Cells were maintained at 37° C in 5% CO_2 and humid atmosphere for 7

days. For proliferation analysis, cells were split and platted in PDL-coated slides for 24 h with 1 μM BrdU (Sigma-Aldrich). All these cultures were fixed in 4% PFA for 10 min at RT and then washed with PBS. PBS-T 0.5% was used to permeabilize cellular membranes for 10 min. Incubation with primary antibodies for Nestin (Millipore, 1:100), GFAP (Dako; 1:200), β-3 tubulin (Sigma-Aldrich; 1:100), Ki67 (Millipore, 1:200) or BrdU (Dako, 1:50) was performed overnight at 4°C. Primary antibodies were detected using subclass specific secondary antibodies (1:1000; anti-mouse Alexa-fluor® 488; anti-rabbit Alexa-fluor® 568; Life Technologies, Thermo Fisher Scientific), incubated for 2h at RT. Finally, a 10 min incubation with DAPI (Invitrogen) was performed. Slides were then washed in PBS 1x and mounted with PermaFluor mountant medium (Thermo Scientific). Sections were analyzed using an Olympus BX-61 Fluorescence Microscope (Olympus).

For proliferation analysis BrdU positive, Ki67 positive and BrdU/Ki67 double positive cells were counted. For this purpose, two or three coverslips and ten randomly selected microscope fields per condition were analyzed. Results are shown as the average number of BrdU+ or Ki67+ cells per DAPI.

Cell cycle distribution

To estimate the distribution of neural progenitor cells (NPCs) within G1, S, and G2/M phases of the cell cycle, propidium iodide (PI; Invitrogen) staining was performed. PI is a fluorescent molecule (excitation max.=535 nm; emission max.=617 nm) and an intercalating agent that can be used to stain cells. PI is used to quantitatively assess DNA content as it binds stoichiometrically to DNA by intercalating between the bases, with little or no sequence preference. In this way, cells with increasing DNA content will take up proportionally more dye and fluoresce more brightly.

Briefly, animals (n=3/experimental group) were deeply anesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and transcardially perfused with 0.9% saline solution. Hippocampal DG was macrodissected and dissociated in cold 1x PBS using a 70 μm cell strainer and a syringe plunger. The resulting cellular suspension was centrifuged and cells were resuspended in 70% ethanol and kept at -20° C for at least 24 h, for fixation.

Regarding *in vitro* cultures, neurospheres (n=5) were split using Accutase cell detachment solution for 10 min (Merck Millipore) followed by mechanical dissociation with a micropipette (P200) and

then centrifuged for 5 min at 1000 rpm. After dissociation, single cells were resuspended in 70% ethanol and kept at -20° C for at least 24 h, for fixation.

After fixation, both hDG cells and *in vitro* neurospheres-derived NPCs were washed in PBS 1x and then incubated with PI staining solution (PI in PBS/0.1% Triton-X 100 and RNAse A (20 mg/mL; Invitrogen) for 1 h at RT. The cell cycle distribution analysis was performed using a FACS Diva flow cytometry system (BD Biosciences). Cells were first gated on the forward/side scatter plot to eliminate debris. Then, singlets were gated according to DNA content represented by PI intensity (area vs width). A histogram was used to determine events representing cells with distinct DNA content; double DNA content (double fluorescence intensity) corresponded to cells in G2/M phase compared to the G0/G1 phase and cells with intermediate DNA content (intermediate fluorescence) corresponded to the S phase of the cell cycle. At least 10.000 PI stained events were collected for each analysis.

Metabolic viability

Metabolic viability of the hippocampal-derived neurospheres was assessed by the MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2Htetrazolium) test (Promega). After 7 DIV, neurospheres were incubated in culture medium containing MTS (5:1) in a humidified atmosphere at 37° C and 5% CO_2. After three hours of incubation, 100 μl of solution from each sample (n=1) were transferred to a 96-well plate and the optical density was determined at 490 nm.

Annexin V/PI

Cell death of the hippocampal-derived neurospheres was assessed using the Annexin V Apoptosis Detection Kit APC (eBioscience) and flow cytometry analysis, according to the manufacturer's recommendations. Briefly, cells were dissociated as previously described, washed in 1x PBS and resuspended in 1x Binding buffer. Then, the fluorochrome-conjugated Annexin V was added to the cell suspension and incubated for 15 min at RT. After that, cells were washed in 1x Binding buffer and the PI staining solution was added. Cells were immediately analyzed in the flow cytometer.

Gene expression analyses

RNA purification

Total RNA was isolated from the macrodissected DG of animals (n = 4-5/group) or from hippocampal neurospheres (n = 4/group) using the Direct-zol™ RNA MiniPrep (Zymo Research), according to the manufacturer's instructions. RNA quantification and quality assessment was performed using a NanoDrop spectrophotometer. A260/230 and A260/280 ratios between 2.0 and 2.2 were accepted as good RNA quality.

cDNA synthesis and real-time RT-PCR analysis

Total RNA (500 ng) was reverse-transcribed using qScript™ cDNA SuperMix (Quanta Biosciences™). For real time RT-PCR, oligonucleotide primers for selected genes of interest were used. Primers sequences can be found in **Supplementary Table 1**. 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). Target gene expression levels were normalized against the housekeeping gene Beta-2-Microglobulin (B2M). The relative expression was calculated using the $\Delta\Delta$Ct method. Results are presented as mean relative gene expression levels (fold change) after normalization to B2M mRNA levels and to CT samples.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6.0 software (GraphPad Software, Inc.). The underlying assumptions of all statistical procedures were assessed. The normal distribution was tested using the Kolmogorov–Smirnov test. Student's t-test was used to assess differences between Control and uCMS groups, and between uCMS and uCMS+FLX groups. Pearson correlation was used to assess correlation between the number of BrdU+ and Ki67+ cells in the DG and the percentage of cells exiting the cell cycle. Test statistics and p-values are shown for each test. Significance was set at $p<0.05$.

RESULTS

Behavioral characterization of the animal model of depression

The animal model of depression used in this study, the unpredictable chronic mild stress (uCMS), uses stress as an etiological factor for the development of depressive and anxiety-like behavior in rodents. Here, we exposed animals to chronic mild stressors for 6 weeks. During the last 2 weeks of this protocol, fluoxetine, a commonly prescribed SSRI, was administered to the animals (**Fig. 1A**). UCMS-exposed animals presented an anhedonic profile as demonstrated by the decreased levels of sucrose preference in the SCT (**Fig. 1B**; SCT: CT=94.10 ± 1.245 % vs uCMS=87.79 ± 1.673 %, t_{18}= 3.029, p=0.0072). Moreover, uCMS animals presented anxiety-like behavior, as revealed by increased latency to feed in the NSF test (**Fig. 1C**; CT=95.38 ± 25.91 s vs uCMS=273.8 ± 25.23 s, t_{18}= 4.934, p=0.0001), and depressive-like behavior in the FST (**Fig. 1D**; CT=143.3 ± 31.81 s vs uCMS=246.0 ± 15.31 s, t_{18}= 2.909, p=0.0094). To validate significant changes in latency to feed assessed by the NSF, alterations in appetite drive must be characterized. Although uCMS-exposed animals presented significantly higher appetite drive when compared to CT, they also showed an increased latency to feed, thus validating the NSF results (**Supplementary Fig. 1**).

Chronic fluoxetine treatment was able to reverse these behavioral deficits to the level of CT animals (**Fig. 1B**, SCT: uCMS+FLX=94.69 ± 1.615 % vs uCMS=87.79 ± 1.673 %, t_{18}= 2.971, p=0.0082; **Fig. 1C**, NSF: uCMS+FLX =141.4 ± 33.81 s vs uCMS=273.8 ± 25.23 s, t_{18}= 3.140, p=0.0057; **Fig. 1D**, FST: uCMS+FLX =144.6 ± 28.5 s vs uCMS=246.0 ± 15.31 s, t_{18}= 3.132, p=0.0058). Additionally, uCMS animals presented significantly increased levels of corticosterone when compared to CT animals (**Fig. 1E**, CT=76.05 ± 15.10 ng/mL vs uCMS=153 ± 28.04 ng/mL, t_{18}= 2.417, p=0.0260). This increase was restored by fluoxetine administration (**Fig. 1E**, uCMS+FLX=85.69 ± 7.223 ng/mL vs uCMS=153 ± 28.04 ng/mL, t_{18}= 2.336, p=0.0313).

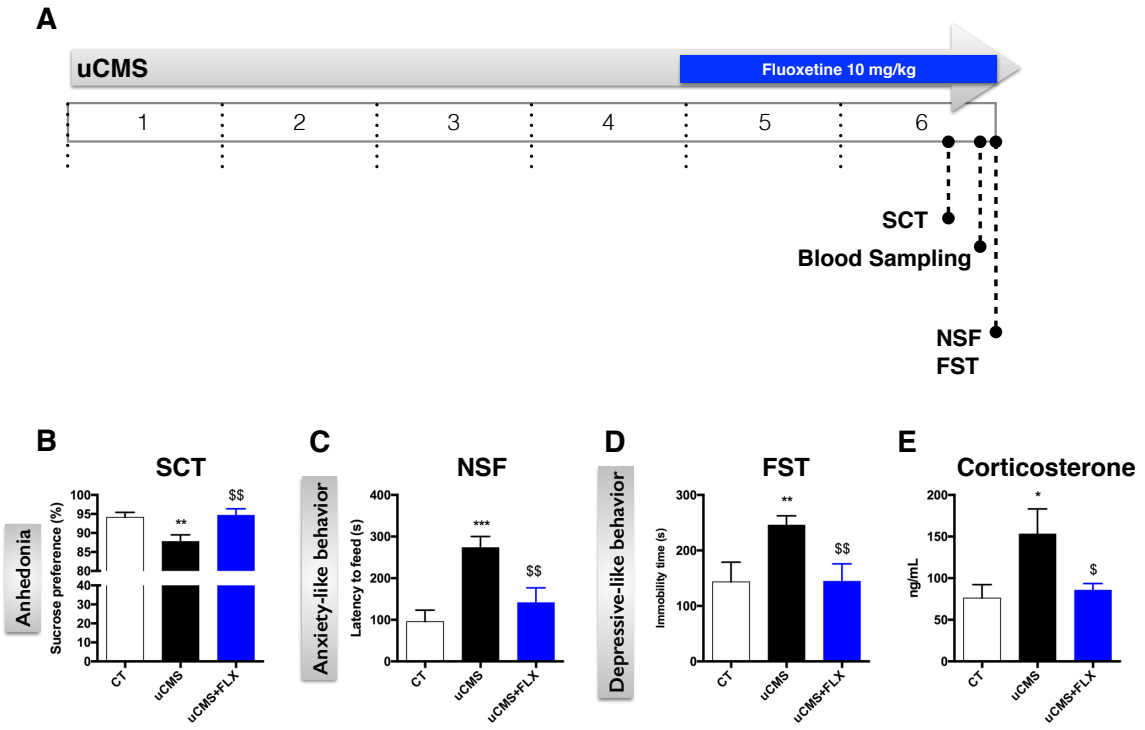


Fig. 1: *Behavioral characterization of the uCMS animal model of depression.* **A.** Schematic representation of the experimental timeline used for the *in vivo* experiments. uCMS was applied for 6 weeks. During the last 2 weeks of uCMS, fluoxetine was administered daily to the animals. SCT, NSF and FST behavioral tests were used to validate the animal model of depression. **B, C, D.** Behavioral characterization of the animal model of depression. UCMS exposure induced an anhedonic profile (**B**), as well as anxiety (**C**) and depressive-like behavior (**D**). These changes were reversed by the treatment with fluoxetine. **E.** Diurnal nadir corticosterone levels measured in the serum of animals. uCMS-exposed animals presented significantly increase levels of corticosterone that were reversed by fluoxetine treatment. Error bars denote SEM. *Denotes the effect of uCMS-exposure; #Denotes the effect of fluoxetine compared with untreated uCMS-exposed animals. */$p<0.05; **/$$p<0.01; ***/$$$p<0.001. Abbreviations: uCMS: unpredictable chronic mild stress; FLX: fluoxetine; SCT: Sucrose consumption test; NSF: Novelty suppressed feeding; FST: Forced swim test.

Proliferation and generation of newborn neurons in the hDG

A reduced number of proliferating cells in the hDG was observed in the uCMS-exposed animals, as assessed using both the exogenous marker BrdU (**Fig. 2A**; CT=14.16 ± 0.9141 vs uCMS=7.643 ± 0.7548, t_8= 5.494, p=0.0006) and the endogenous marker Ki67 (**Fig. 2A**; CT=24.59 ± 3.325 vs uCMS=15.11 ± 1.683, t_8= 2.686, p=0.0250). Fluoxetine treatment was able to reverse these numbers to the level of CT animals (**Fig. 2A**; BrdU: uCMS+FLX=20.14 ± 2.102 vs uCMS=15.11 ± 1.683, t_8= 5.595, p=0.0005; Ki67: uCMS+FLX=27.41 ± 5.306 vs uCMS=15.11 ± 1.683, t_8= 2.621, p=0.0306). Moreover, the number of newly born neurons, assessed by BrdU/DCX double-positive cells (**Fig. 2B)**, was decreased in the uCMS-exposed animals (**Fig. 2C**; CT=14.94 ± 3.124 vs uCMS=6.422 ± 0.9598, t_8= 2.606, p=0.0313) and restored by fluoxetine treatment (uCMS+FLX=11.15 ± 1.068 vs uCMS=6.422 ± 0.9598, t_8= 3.292, p=0.0110).

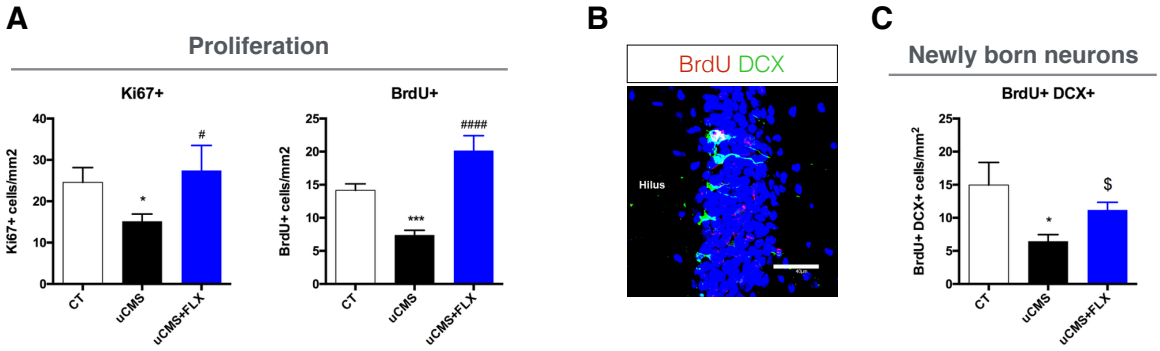


Fig. 2: *Proliferation and generation of new neurons in the hDG.* **A.** BrdU (injected 24 h before sacrifice) and Ki67 immunostaining reveal that uCMS exposure reduces the proliferation in the hDG whereas fluoxetine treatment is able to recover these proliferation levels. **B.** Representative picture of the BrdU/DCX immunofluorescence in the DG (scale bar=40 µM). **C**. The number of BrdU/DCX double-positive cells is decreased in uCMS-exposed animals and restored by the treatment with fluoxetine. Error bars denote SEM. *Denotes the effect of uCMS-exposure; #Denotes the effect of fluoxetine compared with untreated uCMS-exposed animals. */$p<0.05; **/$$p<0.01; ***/$$$p<0.001. Abbreviations: CT: Control; uCMS: unpredictable chronic mild stress; FLX: fluoxetine; BrdU: Bromodeoxyuridine; DCX: doublecortin.

Cell cycle analysis in hDG

Cell cycle distribution analysis using PI staining, revealed that uCMS-exposed animals present a slightly increased proportion of hDG cells in the G0/G1 phase of the cell cycle and a significant decreased proportion in G2/M phases (**Fig. 3A**; G0/G1: CT=77.55 ± 1.050 vs uCMS=80.15 ± 0.5500, t_8= 2.193, p=0.0933; G2/M: CT=16.95 ± 0.850 vs uCMS=14.40 ± 0.300, t_8= 2.829, p=0.0474). Animals treated with fluoxetine present significantly decreased levels of cells in the G0/G1 phase of the cell cycle when compared to uCMS untreated animals (**Fig. 3A**; uCMS+FLX=76.40 ± 0.800 vs uCMS=80.15 ± 0.5500, t_8= 3.863, p=0.0181) but no significant differences in the G2/M phase (**Fig. 3A**; uCMS+FLX=16.45 ± 2.750 vs uCMS=14.40 ± 0.300, t_8= 0.7411, p=0.4998). Additionally, the proportion of cells exiting the cell cycle was evaluated. This ratio gives information about the cells that have entered the cell cycle and incorporated BrdU during S phase (BrdU+ cells) 24h before sacrifice, at the time of BrdU injection, but that were no longer cycling at the moment of sacrifice (Ki67- cells), thus meaning they had exited the cell cycle (**Fig. 3B**). Although not statistically significant, uCMS-exposed animals presented a slightly decreased proportion of cells exiting the cell cycle when compared to CT (**Fig. 3C;** CT=33.25 ± 3.076 vs uCMS=18.43 ± 6.706, t_8= 1.872, p=0.0940). Interestingly, fluoxetine could increase these levels when compared to uCMS untreated animals, although again not statistically significant (**Fig. 3C;** uCMS+FLX=37.5 ± 3.182 vs uCMS=18.43 ± 6.706, t_8=2.179, p=0.0610). To test if the proportion of cells exiting the cell cycle could depend on the number of proliferating cells, we performed a correlation analysis between the proportion of cells exiting the cell cycle and the number of BrdU+ and Ki67+ cells. As expected, there was a significant correlation between the levels of BrdU+ and Ki67+ cells in the hDG (Pearson r: 0.6573, R^2=0.4320, p=0.0078). Importantly, proliferation levels (assessed using both BrdU or Ki67) were not correlated with the proportion of cells exiting the cell cycle (BrdU: Pearson r: 0.3561, R^2=0.1268, p=0.1927; Ki67: Pearson r: 0.3719, R^2=0.1383, p=0.1723; **Supplementary Fig. 2**), thus emerging as an independent measure of the cell cycle dynamics.

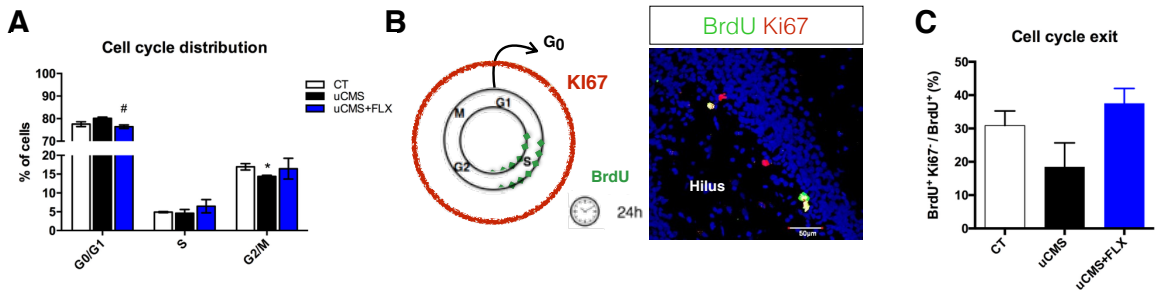


Fig. 3: *Cell cycle analysis in the hDG of the uCMS animal model of depression and after fluoxetine treatment.* **A.** Cell cycle distribution of hippocampal cells shows a slight increase in the proportion of cells from uCMS-exposed animals in G0/G1 phase of the cell cycle and a significant decrease in the G2/M phase, whereas the treatment with fluoxetine significantly decreases the proportion of cells in G0/G1. **B.** Schematic representation of the BrdU administration paradigm and representative picture of the BrdU/Ki67 immunofluorescence in the DG. Scale bar=50 μM. **C**. Although statistical significance was not reached, uCMS-exposed animals presented slightly decreased levels of cell cycle exit compared to CT animals, whereas fluoxetine treatment increased these levels to those of CT animals. Error bars denote SEM. *Denotes the effect of uCMS-exposure; #Denotes the effect of fluoxetine compared with untreated uCMS-exposed animals. */$p<0.05. Abbreviations: CT: control; uCMS: unpredictable chronic mild stress; FLX: fluoxetine.

Analysis of the cell cycle regulators expression in the hDG

To unveil the cell cycle molecules that could be responsible for the changes observed in the cell cycle of hippocampal progenitor cells from uCMS-exposed animals and animals treated with fluoxetine, we analyzed the expression of cyclins and Cdk inhibitors involved in the G1 and S-phases of the cell cycle. Gene expression analysis in the hDG of uCMS animals revealed decreased levels of two G1 cyclins, cyclin D1 and cyclin E (**Fig 4A**; cyclin D1: t_7= 3.517, p=0.0098; cyclin E: t_7= 11.35 p<0.0001), and cyclin A, an S-phase and S/G2 transition cyclin (**Fig. 4A**; cyclin A: t_8= 2.049 p=0.0373). Fluoxetine treatment was only able to significantly restore the decreased levels of cyclin E to those of CT animals (**Fig. 4A**; cyclin E: t_8= 4.691 p=0.0016). No significant differences were observed in the expression of Cdks or Cdk inhibitors (**Fig. 4B** and **4C**).

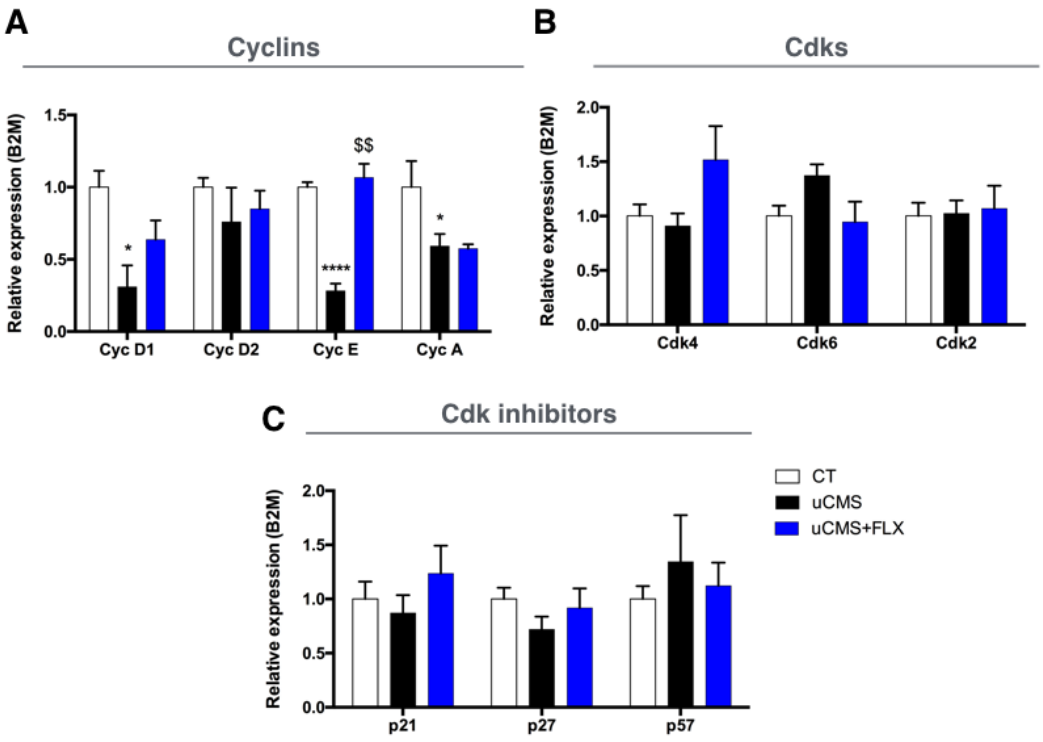


Fig. 4: *Gene expression analysis of cell cycle regulators in the hDG*. **A.** Relative expression levels of G1 phase and S/G2 transition cyclins in the hDG reveal the involvement of cyclin D1, E and A in the regulation of stress and fluoxetine effects. **B, C.** No significant differences were detected in the levels of Cdks (**B**) or Cdk inhibitors (**C**) in the hDG. Error bars denote SEM. *Denotes the effect of uCMS-exposure; #Denotes the effect of fluoxetine compared with untreated uCMS-exposed animals. */$p<0.05; **/$$p<0.01; ***/$$$p<0.001. Abbreviations: CT: control; uCMS: unpredictable chronic mild stress; FLX: fluoxetine.

In vitro experiments

To further understand the molecular regulation of hippocampal progenitor cells proliferation after stress and AD treatment, we used an *in vitro* approach – the neurospheres culture. This approach allowed to study specifically proliferative cells and to further dissect possible mechanisms responsible for the changes observed in our *in vivo* analysis.

Neural progenitor cells proliferation and differentiation

After 7 days *in vitro* (DIV) isolated NPCs grow in sphere-like structures when stimulated with growth factors, EGF and bFGF (**Fig. 5A** and **B**). To assess their ability to differentiate in different neural cell types, neurospheres were split and platted in PDL-coated slides for an additional 7 day-period. Immunostaining analyses show they are able to differentiate in neurons, astrocytes and oligodendrocytes (**Fig. 5B)** thus making them a suitable tool to study proliferation and differentiation phenomena occurring in the hippocampal neurogenic niche.

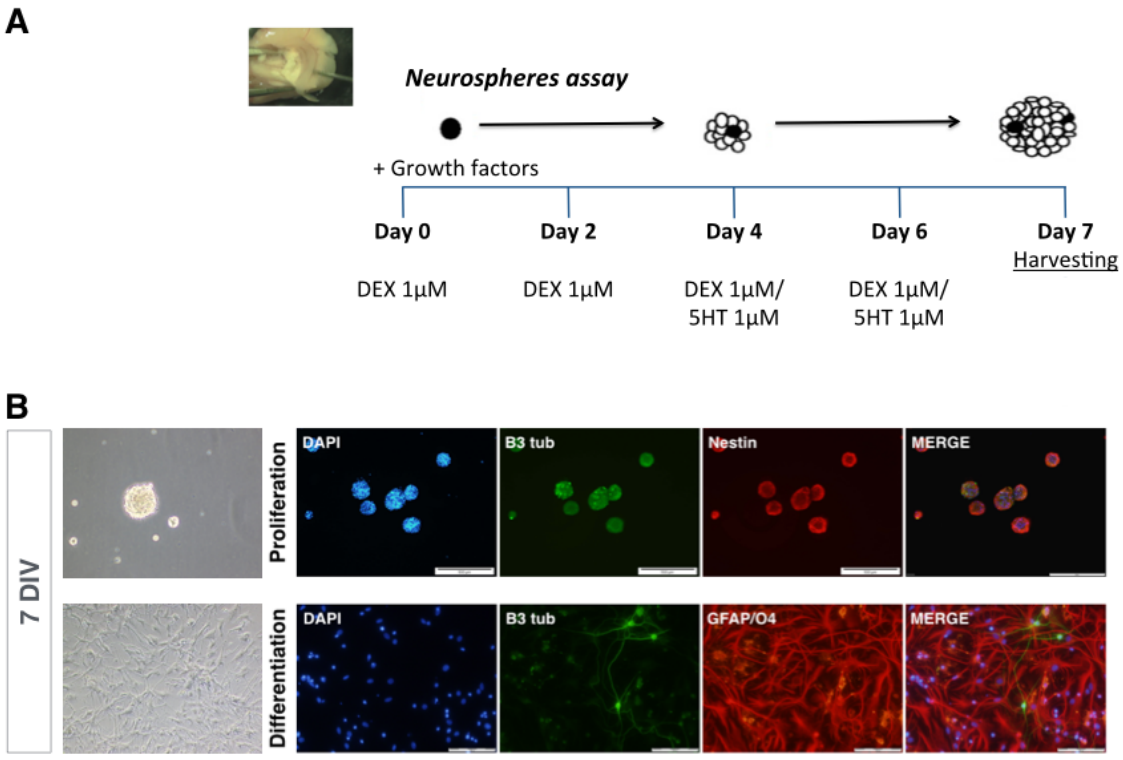


Fig 5: *Neurospheres culture as a model to study the hippocampal progenitor cells proliferation.* **A**. Schematic representation of the neurospheres assay. P5 rat hippocampi were macrodissected and cultured in the presence of growth factors. Cells were stimulated with DEX 1μM every two days. At days 4 and 6, cells were additionally treated with 5-HT. Neurospheres were harvested at day 7. **B**. Representative pictures of bright field and immunofluorescence of neurospheres expansion and differentiation into neural phenotypes (scale bar=500 μm). Abbreviations: DEX – Dexamethasone; 5-HT- serotonin; DIV – days *in vitro*.

To mimic the effects of elevated GC levels observed in uCMS-exposed rats (**Fig. 1E**) we stimulated the NPCs with DEX, a GR agonist. NPCs were grown in the presence of 1 μM DEX for 7 days. DEX was added to the culture every second day (**Fig. 5A**). During the last 4 days of culturing, part of the cells were additionally stimulated with 1 μM of 5-HT to mimic the pro-serotonergic effects of SSRIs treatment (**Fig. 5A**), also observed in our preliminary analysis of depressive-like animals treated fluoxetine (**Supplementary Fig. 3**).

Proliferation and cell cycle regulation in DEX-stimulated neurospheres and after 5-HT treatment

DEX treatment for 7 days promoted decreased proliferation of NPCs as assessed by BrdU immunostaining (**Fig. 6A**; CT=0.17 ± 0.02 vs DEX=0.05 ± 0.02, t_2= 4.243, p=0.025). Although the number of Ki67+ cells was similar between DEX-treated and untreated cells (**Fig. 6B**), the number of BrdU+Ki67+ double-positive cells was decreased in the DEX-treated NPCs (**Fig. 6C;** CT=0.1200 ± 0.0100 vs DEX=0.0250 ± 0.0150, t_2= 5.270, p=0.0342). 5-HT treatment was able to significantly reverse the number of BrdU+Ki67+ double-positive cells (**Fig. 6C;** DEX+5-HT=0.08 ± 0.01528 vs DEX=0.0250 ± 0.0150, t_3= 2.426, p=0.045).

Cell cycle distribution, as assessed by PI staining, revealed a G1 phase arrest in DEX-treated cells, shown by a significant increased percentage of cells in G0/G1 phase and a decreased percentage of cells in the S phase (**Fig. 6D**; G0/G1: CT=78.55 ± 2.100 vs DEX=84.72 ± 1.230, t_8= 2.535, p=0.0350; S: CT=18.220 ± 1.100 vs DEX=10.10 ± 0.200, t_8= 7.263, p<0.0001), that was only partly, and not significantly, rescued by 5-HT treatment (**Fig. 6D**; G0/G1: DEX=84.72 ± 1.230 vs DEX+5-HT=81.93 ± 1.200, t_8= 1.624, p=0.1431; S: DEX=10.10 ± 0.200 vs DEX+5-HT=11.17 ± 1.400, t_8= 0.7566, p=0.4710). Moreover, preliminary data suggest that DEX treatment promotes decreased metabolic viability (**Fig. 6E**) and increased cell death (**Fig. 6F**).

Since GR and 5-HT receptors expression has been previously associated with the proliferation of NPCs in the hippocampal neurogenic niche and with the action of ADs (Banasr et al., 2004, Samuels et al., 2015), we sought to analyze their expression, both in the hDG of rats and *in vitro*. No statistically significant differences were detected in the hDG; however, there was a trend for increased levels of 5-HT1a and 5-HT2a receptors expression after chronic fluoxetine treatment (**Supplementary Fig. 4A)**. *In vitro*, 5-HT treatment significantly increased the expression of 5-HT2a when compared to DEX-treated cells (t_4=11.37; p=0.0003; **Supplementary Fig. 4B**).

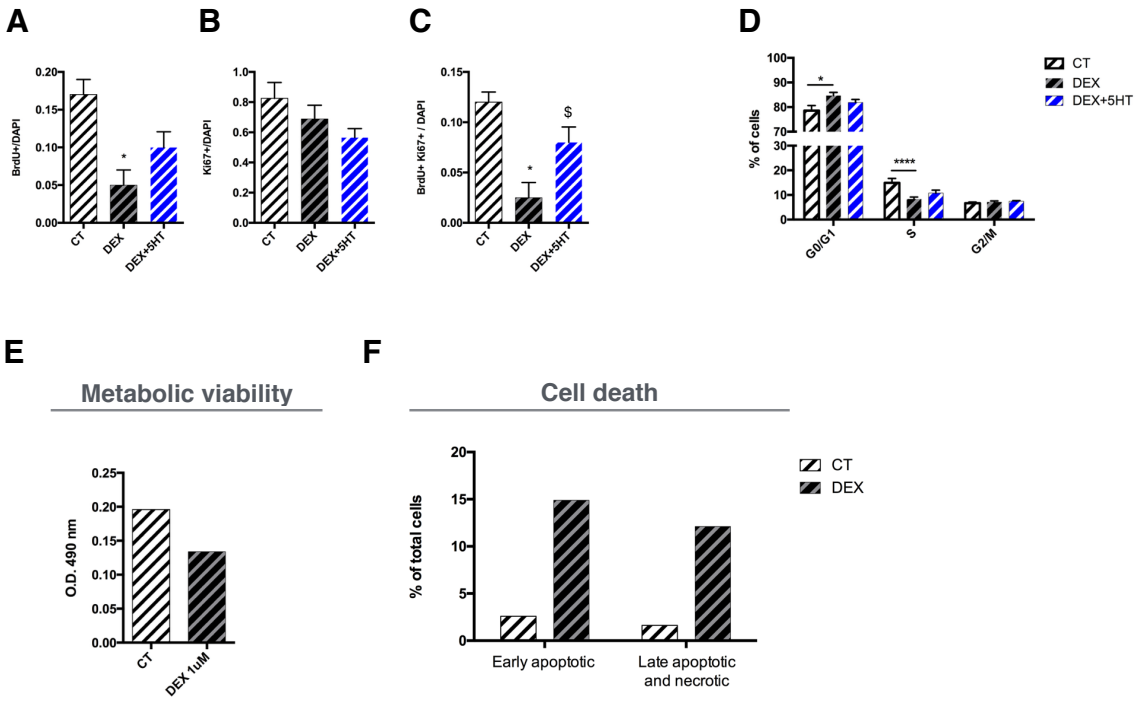


Fig. 6: *Proliferation, cell cycle regulation and cell death in DEX-stimulated neurospheres and after 5-HT treatment*. **A, B, C**. Proliferation assessed by BrdU (24 h), Ki67 and BrdU/Ki67 immunostaining in neurospheres-derived NPCs, reveals decreased levels of BrdU+ and BrdU+Ki67+ cells in DEX-treated cells and a reversion of the number of BrdU+Ki67+ cells by 5-HT treatment. **D**. Cell cycle distribution analysis using PI staining suggests a G1 phase arrest of DEX-treated cells that is only partly reversed by 5-HT. **E**. Metabolic viability assessed by MTS test (n=1); DEX-treatment decreases metabolic viability. **F**. Cell death analysis by Annexin V/PI staining shows increased cell death in DEX-treated cells (n=1). Error bars denote SEM. *Denotes the effect of DEX-stimulation; #Denotes the effect of 5-HT compared to DEX-treated cells. */$p<0.05; **/$$p<0.01; ***/$$$p<0.001. Abbreviations: CT: control; DEX – Dexamethasone; 5-HT-serotonin.

Analysis of the cell cycle regulators expression in DEX-stimulated neurospheres and after 5-HT treatment

As DEX was able to promote an arrest in the cell cycle progression we sought to explore the expression of cyclins, Cdks and Cdk inhibitors in these cells. DEX treatment significantly decreased

the expression of cyclin D1 (t_6=2.801, p=0.0311) and cyclin D2 (t_6=2.565, p=0.0426). Cdk6 expression was also significantly decreased in DEX-treated cells (t_6=2.518, p=0.0454). Interestingly, and despite the modest effect on reversing the DEX-induced changes in proliferation and cell cycle distribution, 5-HT treatment restored the levels of cyclin D1 and Cdk6 to those of CT cells (**Fig. 7A B;** cyclin D1: t_6=3.490, p=0.0130; Cdk6: t_6=3.158, p=0.0196). Moreover, the levels of the Cdk inhibitor p27Kip1 were significantly increased in the DEX-exposed cells (**Fig. 7C**; t_6=2.727, p=0.0339), but were not reversed by 5-HT treatment (**Fig. 7C**).

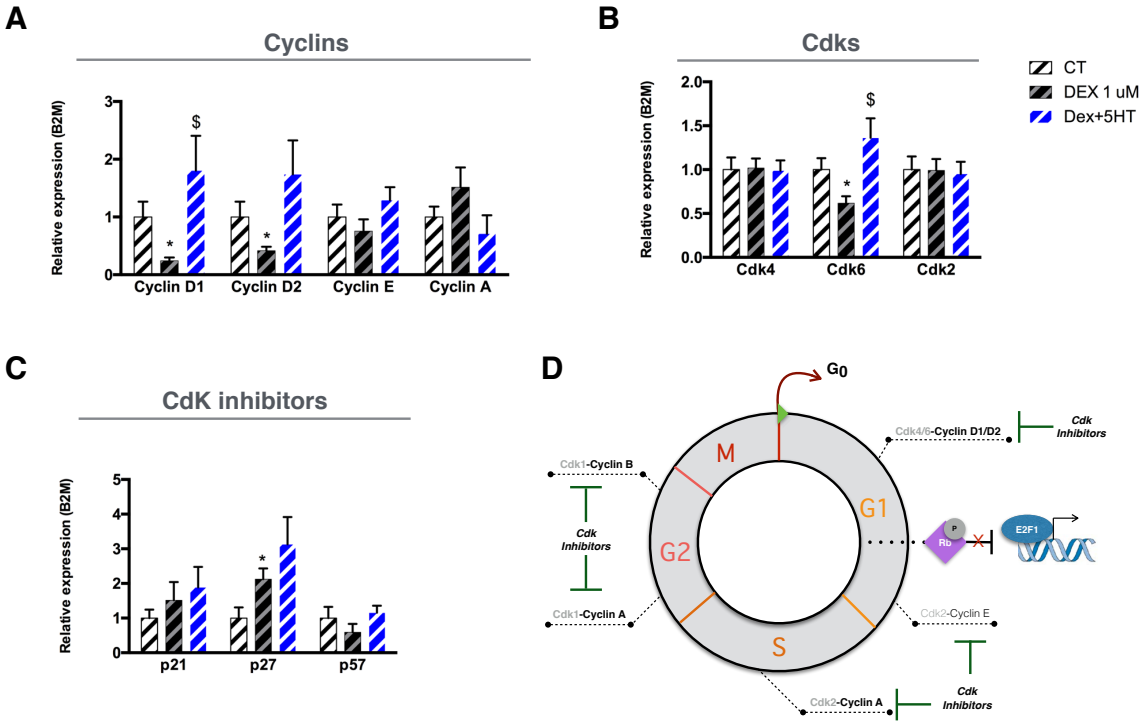


Fig. 7: *Expression of cell cycle markers in vitro after DEX and 5-HT treatment.* **A**. Gene expression analysis of cyclins disclosed decreased levels of cyclin D1 and D2 in DEX-treated cells, compared to CT. 5-HT treatment was able to significantly reverse the levels of cyclin D1 expression to those of CT. **B**. Expression analysis of Cdks revealed decreased levels of Cdk6 expression in DEX-exposed cells and reversion by 5-HT. **C.** Gene expression analysis of Cdk inhibitors reveals increased expression of p27Kip1 in the DEX-treated cells. **D.** Schematic representation of the classical cell cycle. Error bars denote SEM. *Denotes the effect of DEX-stimulation; #Denotes the effect of 5-HT compared DEX-treated cells. */$p<0.05; **/$$p<0.01; ***/$$$p<0.001. Abbreviations: CT: control; DEX – dexamethasone; 5-HT-serotonin; Rb: retinoblastoma protein.

DISCUSSION

The generation of new neurons in the adult hippocampus in the context of depression and antidepressant treatment has been widely investigated. In fact, many studies by our group and others support a role for adult cell genesis in the onset and remission from depressive-like behaviors (Santarelli et al., 2003, Sahay and Hen, 2007, Snyder et al., 2011, Mateus-Pinheiro et al., 2013a, Mateus-Pinheiro et al., 2013b, Khemissi et al., 2014). Still, the molecular changes responsible for the regulation of these proliferative phenomena at the level of the cell cycle are largely unknown.

In this way, the present work aimed to characterize the cell cycle changes occurring in the hippocampal progenitor cells in the context of depression and antidepressant treatment. For that we used two approaches: 1) analysis of the hDG tissue from uCMS untreated animals and uCMS-animals treated with 10 mg/kg fluoxetine for 2 weeks, and 2) an *in vitro* approach, using an hDG primary culture - the neurospheres culture – stimulated with DEX, a GR agonist, and 5-HT. This multidimensional approach allowed us to explore this question from two distinct, but complementary, perspectives taking advantage of each of the models. Recognizing the importance of the niche, in our *in vivo* model we evaluated the cell cycle of these cells in their physiological context; however this approach has some experimental limitations related to the fact that the hDG is mainly composed of post-mitotic cells. Complementarily, the *in vitro* approach allowed to further dissect the mechanisms by which the cell cycle can be regulated, by specifically studying hippocampal-derived proliferative cells. Herein, we used DEX, to mimic the increased GC levels observed after stress and in depressive patients (Sousa and Almeida, 2012), and confirmed in our model. Additionally, we used 5-HT, a neurotransmitter involved in the action of SSRIs (Stahl, 2013). Preliminary data from our model suggest that 5-HT is increased after chronic administration of fluoxetine as previously described for SSRIs (Mikail et al., 2012) and that the expression of 5-HT receptors is increased after 5-HT administration.

Our *in vivo* model - the uCMS animal model of depression - recapitulates many of the core behavioral and neuroplastic changes that occur in human depression, and is responsive to antidepressant therapy (Willner, 2005, Pittenger and Duman, 2008). It is thus a suitable tool to study the relationship between chronic stress, a potent precipitating factor for depression, and the molecular, cellular and behavioral changes occurring in a depressive state. We characterized the behavioral profile of these animals, and as previously described (Bessa et al., 2009a, Bessa et al.,

2009b, Patricio et al., 2015), disclosed changes in anhedonia, depressive-like behavior and anxiety-like behavior. Chronic administration of fluoxetine reversed these behavioral deficits in uCMS animals. Moreover, we observed a decrease in proliferation and in the number of newly-born neurons in the hDG of uCMS-exposed animals that was reversed by fluoxetine treatment. Our cell cycle analysis data suggested that the decreased number of proliferating cells in the DG of uCMS-exposed animals may result from a delayed progression or cell cycle arrest in the G1 phase that can be overpassed by fluoxetine treatment. Firstly, the reduced numbers of Ki67+ cells in uCMS animals may suggest that stem cells are less recruited to enter the cell cycle, probably due to decreased proliferative cues (Patricio et al., 2013) and neurotrophic factors (Castren and Rantamaki, 2010). Moreover, when cells enter the G1 phase of the cell cycle they are less likely to go through S, G2 and M phases and give rise to a newborn cell, as demonstrated by the decreased numbers of BrdU+ positive cells and decreased proportion of cells in G2/M phases. Moreover, there is indication of a slight decrease in the proportion of cells exiting the cell cycle in uCMS animals further supporting this view. Future experiments assessing the cell cycle length of these cells could help to further elucidate this phenomenon. These changes in cell cycle kinetics were accompanied by decreased expression of G1 and S/G2 phase transition cyclins, namely cyclin D1, E and A. Nonetheless, these cyclins have been shown to be expressed in post-mitotic cells and to perform alternative functions in that context (Glickstein et al., 2007, Ikeda and Ikeda, 2015). As in this study we analyzed whole DG samples, and could not discriminate between cell types, data must be carefully interpreted. Further studies evaluating the protein expression of these cyclins using co-labelling methods should help disclosing their implications in the regulation of the cell cycle in progenitor cells *in vivo*.

Our *in vitro* results corroborate the cell cycle arrest hypothesis, with DEX treatment having similar anti-proliferative and G1 arrest effects on hNPCs. Interestingly, no changes were detected in the number of Ki67+ cells. This result may be in line with a G1 phase arrest hypothesis, as previous studies in cancer cells have shown that G1 arrested cells may express Ki67 (Loddo et al., 2009). As previously described in several models (Sapolsky, 2000, Li et al., 2012, He et al., 2016), our preliminary data further suggest a role for DEX in promoting cell death of hNPCs which may occur as a consequence of the G1 phase arrest. Supporting this hypothesis are the previous findings in T-lymphoma cells; in these cells DEX-mediated cell cycle arrest was shown to precede apoptosis (Kullmann et al., 2013). Future work in our model may disclose if cell death is also occurring *in vivo* and the putative role of antidepressants in preventing this effect. As observed in the hDG of uCMS-

exposed animals, DEX treatment decreased the expression of cyclin D1 in proliferating cells *in vitro*. Additionally, it decreased the levels of cyclin D2 and Cdk6, while increasing p27 expression. GCs have been consistently reported as regulators of proliferation (Quiros et al., 2008, Wagner et al., 2009, Ekthuwapranee et al., 2015). Moreover, GRs are expressed in the majority of neuronal developmental stages, including in stem cells and transit amplifying progenitors (tAPs) but not in committed neuronal progenitors (Egeland et al., 2015). In line with this, our findings suggest that the regulation of proliferation by stress occurs most likely in proliferating stem cells or tAPs at the level of the G1 phase and that these changes may be in part mediated by GCs. GCs classically exert their action on gene expression by activation of cytoplasmatic glucocorticoid receptors (GRs) that bind to glucocorticoid response elements (GREs). Using an *in silico* tool (Grant et al., 2011), we found a previously identified GRE sequence 5' - TGTCCT - 3' (Scheidereit et al., 1983, Scheidereit et al., 1986, Kullmann et al., 2013) in the rat cyclin D1 promoter, at position -389 relative to the transcription start site (**Supplementary Table 2**). Future studies are needed to investigate whether this is a functional GRE in this context.

Although 5-HT seems to be partly involved in the proliferative actions of SSRI antidepressants, as observed by its modest effect in the reversion of the DEX-induced phenotype, our data suggest the need of other factors for the beneficial effects of fluoxetine observed *in vivo*. One could speculate that this might have occurred as a consequence of the 5-HT concentration and duration of treatment, or due to a limitation of the *in vitro* model that is overly simplistic when compared to the *in vivo* setting. In fact, additional factors may be required for 5-HT to trigger paracrine and/or downstream effects, such as increase in the expression of neurotrophic factors or activation of signalling pathways. Preliminary data (not shown) suggest that increasing 5-HT concentration does not change this effect, thus indicating that indeed other factors are needed to fully mimic the proliferative effects of SSRIs *in vitro*.

Together, our results confirm previous suggestions of a G1 phase arrest of the cell cycle after chronic stress (Heine et al., 2004); in that study Heine et al. disclosed an increased number of p27Kip1+ cells in the hippocampal SGZ after exposure to an unpredictable stress paradigm for 3 weeks. Although in our *in vivo* model we could not disclose changes in this Cdk inhibitor, probably due to experimental limitations, we found increased expression of p27Kip1 after DEX treatment in the *in vitro* experiments. Even though distinct methodologies were used, these results further support a GC-mediated cell cycle arrest and the involvement of both cyclins and Cdk inhibitors.

Interestingly, we could not detect significant differences in the expression of p21Cip1, a Cdk inhibitor described to be involved in the pro-neurogenic effects of antidepressants in unstressed rodents (Pechnick et al., 2011).

Future studies should focus on particular cell types and use other complementary approaches to definitely disclose the underpinnings of cell cycle regulation in the context of depression and antidepressants action. Moreover, the recently described differential impact of stress and antidepressants in the neurogenic process along the septo-temporal axis of the DG (Tanti and Belzung, 2013, Tanti et al., 2013), urges the need to explore putative differential cell cycle control mechanisms in the dorsal versus ventral DG. Of potential importance would be also to assess the effects of different classes of antidepressants in the cell cycle regulation.

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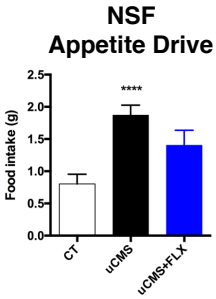
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4.1. Supplementary Information

Cell cycle regulation of the hippocampal progenitor cells in a rat model of depression and after fluoxetine treatment

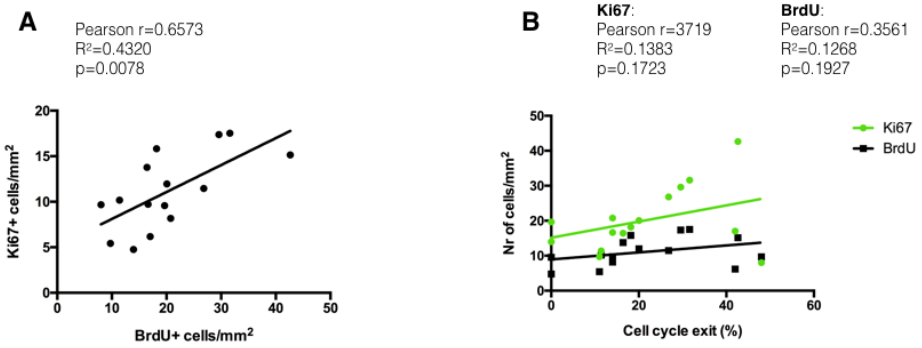
SUPPLEMENTARY DATA

Supplementary Figure 1



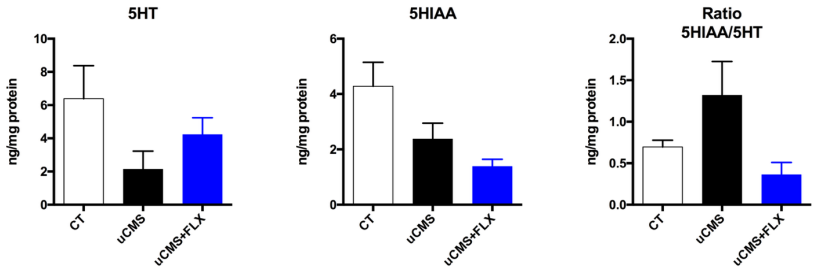
Supplementary Fig. 1: *Food intake after the Novelty Suppressed Feeding Test.* In order to validate significant changes in latency to feed assessed by this behavioral paradigm, alterations in appetite drive must be characterized. In this way, a significant increase in latency to feed (anxious-like behavior) can only be validated when not accompanied by decreased appetite drive. Conversely, a significant decrease in latency to feed may only be considered when not associated with a significant increase in appetite drive. Although uCMS-exposed animals presented significantly higher appetite drive when compared to CT (CT=0.8025 ± 0.1409 g vs uCMS=1.872 ± 0.1463 g, t_{18}= 5.264, p<0.0001), they also showed an increased latency to feed, thus not configuring any of the above-mentioned situations. Error bars denote SEM. *Denotes the effect of uCMS-exposure; #Denotes the effect of fluoxetine compared with untreated uCMS-exposed animals. */$p<0.05; **/$$p<0.01; ***/$$$p<0.001. Abbreviations: uCMS - unpredictable chronic mild stress; FLX - fluoxetine.

Supplementary Figure 2



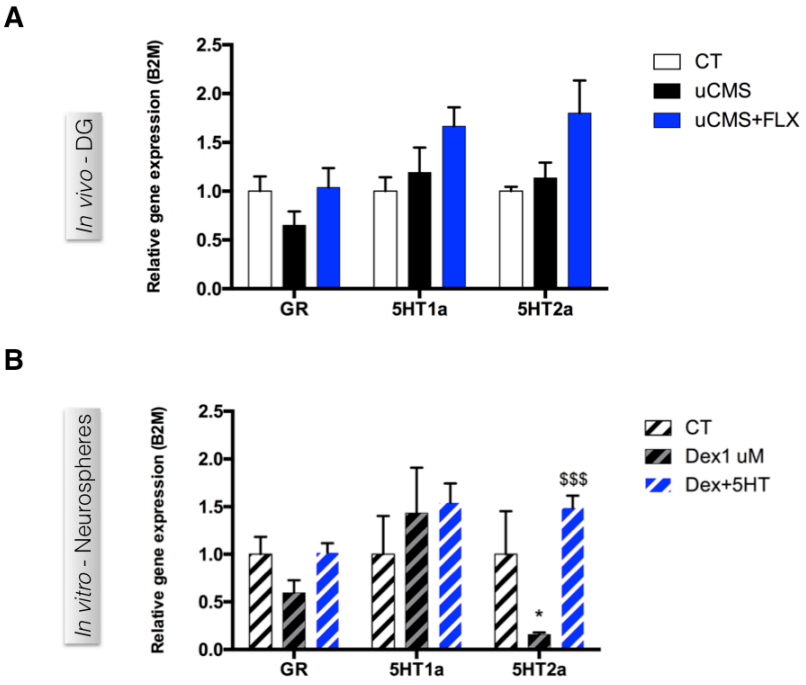
Supplementary Fig. 2: A. Pearson correlation between the numbers of Ki67 and BrdU positive cells per mm^2 in the DG. **B.** Pearson correlation between the numbers of BrdU positive or Ki67 positive cells per mm^2 and the percentage of cells that have exited the cell cycle.

Supplementary Figure 3



Supplementary Fig. 3: Preliminary data on the levels of 5-HT, 5HIAA and turnover ratio in the DG of CT, uCMS and uCMS+FLX animals, measured by HPLC (n=2). Abbreviations: uCMS - unpredictable chronic mild stress; FLX - fluoxetine; 5HT – serotonin; 5HIAA - 5-Hydroxyindoleacetic acid.

Supplementary Figure 4



Supplementary Fig. 4: Expression of the glucocorticoid receptor and 5-HT receptors in the DG (**A**) and in neurospheres (**B**), measured by qRT-PCR. Error bars denote SEM. *Denotes the effect of DEX; #Denotes the effect of DEX+5HT compared with DEX only. */$p<0.05; **/$$p<0.01; ***/$$$p<0.001. Abbreviations: DEX - dexamethasone; 5HT – serotonin; GR – glucocorticoid receptor; 5HT1a – serotonin receptor 1a; 5HT2a – serotonin receptor 2a.

Supplementary Table 1: Sense and antisense sequences of oligonucleotide primers used in the quantitative real time polymerase chain reaction (qRT-PCR) and their corresponding product sizes.

Gene symbol	Sense	Antisense	Product size (bp)
Ccnd1 (Cyclin D1)	CGCGTACCCTGACACCAATCT	CTTCTGCTCCTCGCAGACCTCTA	172
Ccnd2 (Cyclin D2)	ACACCGATGTGGATTGTCTCAAAG	TCAACATCCCGCACGTCTGTA	154
Ccne1 (Cyclin E)	AGCAGTCAGCCTTGGGATGAT	AGGCTCTGGGCGGTCTGATTT	139
Ccna1 (Cyclin A)	GGCCTTGTTCTCGAGACTTCTATTC	CCCAAATCTTCCAGCATGGTCTTA	182
Cdkn1a (P21Cip1)	GCAGACCAGCCTAACAGATTTCTA	TCCTGACCCACAGCAGAAGAAG	109
Cdkn1b (P27Kip1)	CAGACGTAAACAGCTCCGAATTAAG	GCGCAATGCTACATCCAATGCT	221
CDKN1C (P57Kip2)	GGGGCCTCTCATCTCTGACTTCT	GCAAGTTCTCTCTGGCCGTTAGG	186
Cdk2	TCAACGCAGAGGGGTCCATCAA	CGGGTCACCATTTCGGCAAAG	193
Cdk4	GGTGCCTATGGGACGGTGTA	ACAGACATCCATCAGCCGTACAA	192
Cdk6	GCCTTGCCCGCATCTACAGTTTT	GGAGTCCGATGACGTCCAAGATT	219
Htr1a (5-HT1a)	TCATCGGCTCCTTGGCGGTT	CCGGGGCGTCCTTTTGTTCA	233
Htr2a (5-HT2a)	ACCGACATGCCTCTCCATTCTTC	CAAAGGCCACCGGTACCCATACA	244
Nr3c1 (GR)	AGGCCGGTCAGTGTTTTCT	CAATCGTTTCTTCCAGCACA	234
B2M	GTGCTTGCCATTCAGAAAACTCC	AGGTGGGTGGAACTGAGACA	136

Supplementary Table 2: Adapted table from FIMO motif search tool output for cyclin D1 promoter region (2000 kb upstream transcription start site (TSS)).

DATABASE AND MOTIFS

DATABASE sequences.fa
Database contains 1 sequences, 2000 residues
MOTIFS motifs.meme (DNA)

MOTIF	WIDTH	BEST POSSIBLE MATCH
1	15	AGAACAnnnTGTTCT

Random model letter frequencies (from non-redundant database):
A 0.275 C 0.225 G 0.225 T 0.275

SECTION I: HIGH-SCORING MOTIF OCCURENCES

- There were 1 motif occurrences with a p-value less than 0.0001.
- The p-value of a motif occurrence is defined as the probability of a random sequence of the same length as the motif matching that position of the sequence with as good or better a score.
- The score for the match of a position in a sequence to a motif is computed by summing the appropriate entries from each column of the position-dependent scoring matrix that represents the motif.
- The q-value of a motif occurrence is defined as the false discovery rate if the occurrence is accepted as significant.
- The table is sorted by increasing p-value.

Motif	Sequence Name	Strand	Start	End	p-value	q-value	Matched Sequence
1	ENSRNOG00000020918 ENSRNOT00000088588	+	1611	1625	6.21e-05	0.247	AGTATAGGGTGTCCT

5. General Discussion

5. GENERAL DISCUSSION

Although much improvement has been done on the comprehension of depression's pathophysiology, this hasn't been reflected yet in the clinical setting, as depression prevails as one of the world's major public health concerns (Collins et al., 2011, Lepine and Briley, 2011). Since the discovery and establishment of the first class of antidepressants, in the 1950's, many other drugs with similar antidepressant properties have emerged (Millan et al., 2015). Strikingly, the advent of novel antidepressants development has dropped massively in recent years (Hyman, 2013), and the field is still lacking effective drugs for a great proportion of patients. This certainly poses a major problem for an ever growing "depressed society". Indeed, much remains to be understood regarding the pathophysiology of depression compared to other medical conditions. That, and our limited understanding of the action of antidepressants have been motivating the concerted actions of researchers towards a better definition of this still underestimated disease. Many strategies have been used to pursue this aim, based on both preclinical and clinical approaches. One of these strategies is to use unbiased genome-wide approaches in brain regions involved in depression in order to obtain a comprehensive picture of the possible underlying regulatory mechanisms operating in depression and after antidepressant treatment.

In this thesis we have focused on the molecular regulation of the hippocampal DG in the context of depression and antidepressant treatment. The hippocampus is a prominent target for dysregulation in depression (Campbell and Macqueen, 2004, MacQueen and Frodl, 2011). Moreover, neural plasticity events in the hippocampal DG, including dendritic remodelling and cell genesis, are thought to mediate some of the behavioral correlates observed in depression onset and remission (Pittenger and Duman, 2008). To explore these regulatory mechanisms we used an animal model of depression – the unpredictable chronic mild stress (uCMS) model – as a tool to attain insights on the pathways and molecules that are changed after chronic stress exposure, a major etiological factor for depression, and after antidepressant treatment. Additionally, *in vitro* methodologies were used to complement these analyses. Two major studies were developed: in the first one, we conducted microarray analyses of the hippocampal DG of control rats, untreated depressive-like rats, and depressive-like animals that were chronically treated with antidepressants from different classes. These included one representative of TCAs (imipramine), one of SSRIs (fluoxetine), the atypical

tianeptine, and a more recently introduced antidepressant, agomelatine. Our aim was to understand if different classes of currently used antidepressants exert their beneficial effects in hippocampal neural plasticity via the same mechanisms or if they use differential molecular strategies to achieve the same final outcome. The second study aimed to evaluate the cell cycle mechanisms implicated in the anti-proliferative effects of chronic stress exposure, and in the pro-proliferative effects of fluoxetine, in the adult hippocampal neurogenic niche. With this work we expected to expand the knowledge on the molecular mechanisms regulating the two major forms of neuroplastic phenomena, synapto-dendritic plasticity and cell genesis, in the hippocampal DG of the depressed-like rat and after the action of different classes of antidepressants.

In this general discussion I will first focus on each of these two works separately, their strengths, major implications to the field, but also discuss some of their limitations. To finish, I will examine some points and major hurdles that may have been limiting the understanding of the neurobiology of depression, and propose some avenues for new developments in this field.

5.1 Antidepressants: are they all the same?

Newer antidepressants were developed to be more selective for their neurochemical targets, being thus undeniably safer than those developed back in the 1950's. Nevertheless, they lack mechanistic novelty in the way that, despite the existence of a few exceptions, most first and second line antidepressants primarily target monoaminergic neurotransmission. Interestingly, we have been watching the dawn of new hypotheses on the pathophysiology of depression and appreciating that antidepressants mechanisms of action go considerably beyond those initially assumed. This propels further investigation on the current pharmacotherapies but also the continuation of the efforts for finding novel therapeutic targets.

The first study presented on this thesis (Chapter 3) showed that distinct antidepressants produce comparable behavioral and neuroplastic effects, with the exception of agomelatine, a melatonergic agonist and 5-HT2C antagonist, that could not fully reverse either the behavioral or the neuroplasticity impairments in the hippocampal DG produced by chronic stress exposure. Its effects were only observed in the SCT, a test that assesses anhedonia. Few studies have shown that this antidepressant could indeed reverse the chronic stress effects at the cellular (Banasr et al., 2006, Dagyte et al., 2011) and behavioral levels (Papp et al., 2003, Boulle et al., 2014). The inability of

agomelatine to produce beneficial effects in our particular experimental framework may be due to the treatment dosage. In fact, in these studies agomelatine was used in higher doses (Boulle et al., 2014) or for longer periods (Papp et al., 2003, Banasr et al., 2006, Dagyte et al., 2011). These differences may have accounted for the disparate results. In this way, and despite some contradictory reports (Lam, 2010), our work suggests that agomelatine may have later onset of action when compared with the remaining antidepressants used in this study. Interestingly, and although agomelatine has been evaluated as an effective drug for the treatment of depression, some recent meta-analyses, based on published and unpublished data, show that the total effect size of agomelatine is rather small, compared to other antidepressant drugs (Singh et al., 2012, Koesters et al., 2013, Gahr, 2014, Taylor et al., 2014).

We next analyzed the transcriptional changes induced by chronic stress exposure and by the treatment with the four antidepressants. To the best of our knowledge, this study was the first to assess the transcriptional profile of four representative antidepressants from different classes, in the hippocampal DG using an animal model of depression. Others have performed this type of transcriptome analysis in the hippocampus and other brain regions of naive animals treated with antidepressants (Landgrebe et al., 2002, Conti et al., 2007, Sillaber et al., 2008, Lee et al., 2010, Gaska et al., 2012, Korostynski et al., 2013). However, the translational potential of studying the molecular effects of antidepressants in animals that do not display a depressive-like phenotype is rather limited and may lead to erroneous interpretations. Although animal models have indisputable limitations and do not fully recapitulate the human disease, analysing the impact of different antidepressant drugs in a pathological context akin to depression, will probably return more relevant data to the field.

Our gene expression analysis in the DG of chronically stressed rats revealed significant changes in only 93 transcripts and small magnitudes of response, similarly to previously published results (Surget et al., 2009, Datson et al., 2012). These were not unexpected results since we are looking at a snapshot after 6 weeks of chronic stress exposure; it is thus possible that some compensatory mechanisms may have developed during that time. Interestingly, antidepressants treatment produced changes in many more transcripts compared to uCMS alone. These results may suggest that antidepressant treatment recruits additional systems and pathways. Moreover, for those antidepressants that were able to fully reverse the behavioral and cellular deficits induced by uCMS exposure (fluoxetine, imipramine and tianeptine) a relatively small percentage of the uCMS-induced transcriptomic changes were reversed (25, 22 to 32%, respectively). Although modest, this reversion

of the uCMS-induced changes may be crucial for the beneficial effects of antidepressants; agomelatine, which did not fully rehabilitated the behavior and dendritic plasticity, reversed less percentage of these changes (12%). On the other hand, the relatively small proportion of reversed changes in all antidepressant treatments reveals that antidepressants are not restoring completely the system back to the physiological control condition, but rather inducing a new "antidepressant-induced" state, by activating different pathways needed to restore behavior and neuroplasticity in the hippocampal DG. Another hypothesis is that this analysis corresponds to an early stage of antidepressants action and that more time is needed for the system to normalize to the control situation. In this respect, longitudinal studies could provide important readouts on the time course of the molecular modifications. Additionally, there may not exist a causal relationship between neural plasticity changes and all the molecular alterations observed, since antidepressants have several pharmacological targets; thus, we cannot exclude that some of the observed changes may be epiphenomena related to side effects of the drugs.

In a previous study, a transcriptome analysis in the DG, cingulate cortex and amygdala (Surget et al., 2009) after chronic fluoxetine treatment, revealed very dissimilar molecular signatures among these brain regions. This finding encourages additional studies using different classes of antidepressants in order to attain a broader perspective on their effects in other brain regions involved in the pathophysiology of depression, such as the NAc, amygdala and PFC.

Although valuable information can be extracted from these transcriptomic analyses, one limitation of these studies relates to the fact that many of the molecules are regulated at the post-transcriptional level, emphasizing the need for future confirmation of the changes at the protein level. In line with this, proteomics and metabolomics studies are now emerging to improve the knowledge on the complex networks involved in the pathophysiology of depression and in the actions of antidepressants (Kaddurah-Daouk et al., 2013, Webhofer et al., 2013, Zhu et al., 2013, Carboni, 2015).

One limitation of our analysis is that we could not directly assess changes occurring in each cell type present in the DG. Our results reflect the complex interactions between stem cells, progenitors, neurons and glial cells in the adult DG. In order to gain more information on the impact of these antidepressants on different cell populations we performed an *in silico* cell type enrichment analysis. This analysis suggested that different antidepressants preferentially target specific cell types, which may result on dissimilar outcomes in terms of plasticity and behavior. Whereas fluoxetine impacted preferentially on neurons, tianeptine and imipramine didn't show any cellular preference, impacting

similarly on neurons, astrocytes and oligodendrocytes. Conversely, agomelatine impacted more on neurons and oligodendrocytes. These results are in line with other previously published data, showing that stress, depression and distinct antidepressants impact on different cell types (Czeh et al., 2013, Etievant et al., 2013, Mateus-Pinheiro et al., 2013, Sanacora and Banasr, 2013, Miyata et al., 2015), challenging the instated "neurocentric" view of depression. Future studies addressing the relevance of different cell types, as well as their complex interactions in the hippocampus and other brain regions, may further contribute to change this view.

Ours and other transcriptomic studies have served to further unveil the underpinnings of depression's pathophysiology and antidepressants effects. But will this preclinical data ever be translated into more clinically relevant applications? The data gathered so far suggests that indeed different classes of antidepressants may share core mechanisms of action but also present distinct pathways through which they exert their positive effects. In our study, we found that for example fluoxetine exhibited a prominent anti-inflammatory profile. In line with these results, it has been shown that SSRIs and SNRIs are able to prevent depressive-like behavior and decrease the serum levels of pro-inflammatory cytokines induced by LPS administration (Ohgi et al., 2013). Moreover, the levels of C-reactive protein (CRP), a common marker of systemic inflammation, are decreased after antidepressant treatment (O'Brien et al., 2006) and may predict antidepressant response (Hashimoto, 2015). That said, one might hypothesize that by exploring the particular features of each class of antidepressants we may find the most adequate therapeutic strategy for each patient in an attempt to promote a faster remission, towards a more personalized psychiatry.

5.2 Epigenetic mechanisms as mediators of stress and antidepressant response - Possible biomarkers of disease and treatment

Many of the genes identified by microarray analysis in the context of stress, depression and antidepressant treatments are epigenetic regulators (McEwen et al., 2015a, McEwen et al., 2016). Indeed, environmental stimuli, such as chronic stress, are suggested to interact with susceptibility genes via epigenetic mechanisms, producing long-lasting changes in the brain that may at least partly explain the heterogeneity of depression aetiology (Tsankova et al., 2007, Mateus-Pinheiro et al., 2011, Menke and Binder, 2014). In the past few years, microRNAs (miRNA) have emerged as an important form of epigenetic regulation of gene expression (Dalton et al., 2014), namely in the

context of depression. Among other epigenetic regulators found in our microarray analysis, two miRNAs called our attention, miR-409 and miR-411 (Chapter 3). These miRNAs were significantly up-regulated by uCMS exposure and their expression was reversed after antidepressant treatment. MiR-411 expression, in particular, was reversed by fluoxetine, imipramine and tianeptine, but not agomelatine. Interestingly, agomelatine was the only antidepressant unable to reverse the dendritic plasticity atrophy in the dorsal DG, observed in depressive-like animals. This result and the fact that stress and antidepressants may impact differently along the septo-temporal axis of the hippocampus (Tanti and Belzung, 2013b, Pinto et al., 2015) encouraged us to further explore the expression of mir-411 separately in the dorsal and ventral DG (Appendix III). Combining our expression results with *in silico* analysis of the predicted targets for this miRNA, we hypothesize that miR-411-5p may be an important mediator of the effects of stress and antidepressants, namely fluoxetine, in neural plasticity changes. At this point, we do not have enough experimental support to confirm if these changes in expression are causally related to the dendritic plasticity mechanisms. Future functional assays, using pre-miRs and anti-miRs, will help to disclose if there is any causal role in this context. Adding to this, we explored the potential of this miRNA as a biomarker of diagnosis and treatment response. A few miRNAs have been recently suggested as possible biomarkers for psychiatric disorders (Bocchio-Chiavetto et al., 2013, Hansen and Obrietan, 2013, Scott et al., 2015). The expectations on the use of peripheral biomarkers as adjuvant tools for the diagnosis of psychiatric disorders are rising (Schmidt et al., 2011), in part due to their stability in bodily fluids that can be collected by minimally invasive procedures, such as blood, serum and CSF (Scott et al., 2015). Our preliminary data shows that fluoxetine treatment decreased the levels of miR-411-5p in the blood when compared to untreated depressive-like rats. Although these results partly corroborate our findings in the dorsal DG, caution must be taken when extrapolating the results obtained in peripheral samples to brain tissue. In fact, the origin of such miRNAs in the blood is undetermined and may solely be associated with changes in blood cells (Scott et al., 2015). Nevertheless, some studies have found suggestive gene expression similarities between blood and brain samples (Liew et al., 2006, Jasinska et al., 2009). In summary, this and other studies encourage further investigation of miRNAs as putative biomarkers of disease and treatment in depression. In the future, it is conceivable that a biomarker panel would help satisfying the unmet needs in depression diagnosis and treatment. **Figure 1** represents the experimental approach that led to the identification of this miRNA and the intended future experimental strategy.

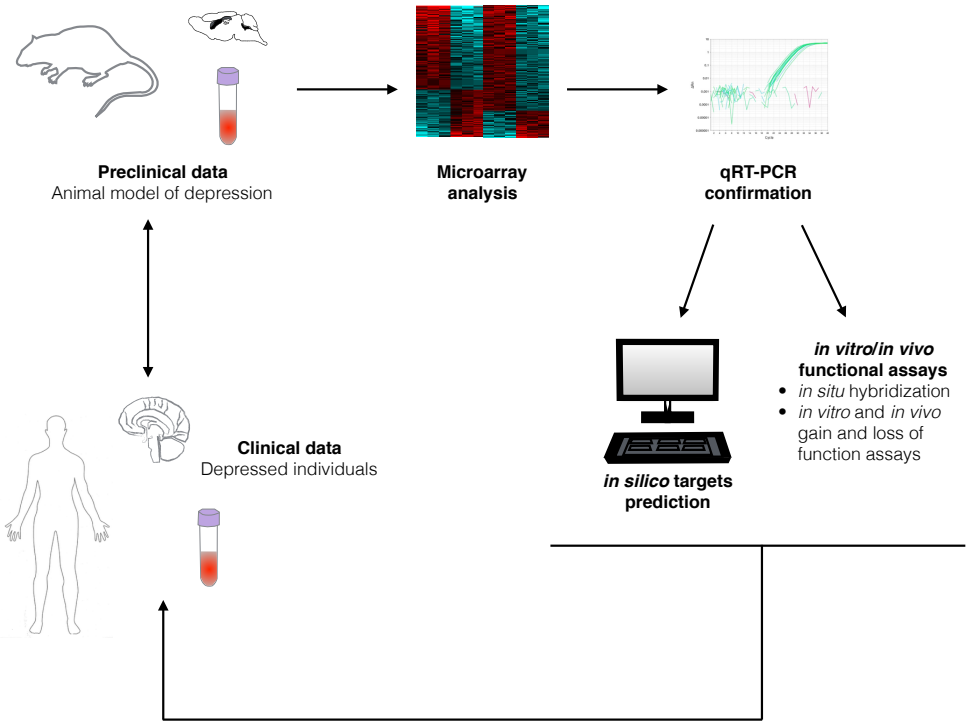


Figure 1: Schematic representation of the experimental approach used to identify miR-411 and future strategies to further unveil the relevance of this miRNA for the neuroplasticity changes occurring in depression and after antidepressants, and its potential application as a biomarker of disease.

5.3 Cytogenesis in the adult hippocampus: How is proliferation regulated in depression and by antidepressants?

In the fourth chapter of this thesis, we assessed the cell cycle mechanisms regulating hippocampal proliferation in depressive-like conditions and after antidepressant treatment.

As previously addressed in this thesis, there has been a large body of research focusing on the relevance of hippocampal DG adult-born neurons for the pathophysiology of depression and the action of antidepressants. Though the involvement of new neurons in the vulnerability to develop depression is still a matter of intense debate, the requirement of these new cells for the remission from depression is fairly consensual in the field (Sahay and Hen, 2007, Eisch et al., 2008, Balu and Lucki, 2009, Tanti and Belzung, 2013a).

Understanding the molecular factors regulating the generation of new neurons in the adult hippocampal DG, namely at the cell cycle level, may further guide the comprehension of the proliferation stimuli, opening new avenues for the development of more targeted therapeutic strategies to restore cell genesis and improve remission. In recent years, there has been a great deal of works addressing the regulation of neural progenitor cells (NPCs) proliferation in the adult hippocampal DG in basal conditions. Strikingly, in respect to the cell cycle regulation, most of the studies have been performed in constitutive KO animal models (Kowalczyk et al., 2004, Vandenbosch et al., 2007, Pechnick et al., 2008, Qiu et al., 2009, Pechnick et al., 2011, Andreu et al., 2015). Some of these studies have shown that the single targeted deletion of cell cycle regulators does not yield any particular defects in hippocampal neurogenesis, most likely due to molecular redundancies and compensatory mechanisms occurring during development (Beukelaers et al., 2012). By using such approaches, we may be underappreciating the relevance of each of these molecules in the fine-tuning of NPCs proliferation. This highlights the need for more targeted methodologies, such as conditional KO models, to exclude these possible compensatory effects that may be affecting the interpretation of results in the adult brain.

A major hurdle when studying the processes that control the cell cycle processes in the adult hippocampus is the small proportion of proliferating cells at a given time. The adult hippocampal DG is mainly composed of post-mitotic cells, thus limiting the ability to dissect the cell cycle phenomena. In an attempt to overcome this limitation, we used two complementary approaches to explore the regulatory mechanisms governing the cell cycle in depressive-like conditions and after antidepressant treatment. Considering the relevance of the niche, we used an *in vivo* approach - the uCMS rat model of depression. Additionally, and given the above-mentioned limitations of studying such discrete population of dividing cells *in vivo*, we took advantage of an *in vitro* system – the neurospheres culture – to study specifically the hippocampal proliferating cells. Both *in vivo* and *in vitro* results suggested the G1 phase of the cell cycle as a key target for the modulatory effects of stress, namely via the regulation of cyclin D1 expression. Furthermore, our results indicate that this restriction of cell cycle progression in the G1 phase after chronic stress may be at least partly mediated by GCs, as stimulating the DG progenitor cells *in vitro* with dexamethasone (DEX), produced similar effects to those observed in the hDG of rats after chronic stress exposure. Interestingly, we found a putative GRE in the promoter region of the rat cyclin D1. Future studies will be crucial to elucidate the functionality of this GRE and its contribution to the decreased expression

of cyclin D1. Previous studies have shown that GCs decrease embryonic stem cells proliferation by promoting ubiquitin-mediated degradation of cyclin D1 (Sundberg et al., 2006), suggesting an additional GC-mediated post transcriptional regulatory mechanism for decreasing cyclin D1 expression. The mechanism by which fluoxetine exerted its pro-cytogenic effects was also addressed. Again, fluoxetine seemed to impact preferentially at the level of the G1 phase of the cell cycle. Nevertheless, the molecules involved in this regulation were not completely unveiled. In order to mimic the fluoxetine treatment effects we stimulated the progenitor cells *in vitro* with 5-HT. Interestingly, 5-HT did not completely reverse the DEX-induced impairments in proliferation, suggesting the need of additional effector molecules or more complex cellular interactions for the pro-proliferative effects induced by fluoxetine treatment. In the future, the treatment of these cells with norfluoxetine, the active metabolite of fluoxetine, could be of interest to further disclose these mechanisms. A previous study, using a reporter mouse line, has shown that chronic fluoxetine treatment specifically targets the transit amplifying neural progenitors (tANPs), increasing the number of symmetric divisions of these cells (Encinas et al., 2006). Though the cell cycle mechanisms responsible for that observation were not explored, their data support our *in vitro* approach, which is representative of proliferating progenitor cells in the DG. In that study, however, fluoxetine-treated animals hadn't been previously challenged with any stressor. Therefore, the results may not be representative of the mechanisms regulating the depressive-like condition we were trying to mimic in this study. Interestingly, not only fluoxetine but actually most of the currently available antidepressant drugs are able to positively modulate hippocampal cytogenesis (Miller and Hen, 2015). Also in our uCMS animal model, we could detect increased proliferation levels after treatment with either imipramine, tianeptine or agomelatine (data not shown). In line with this, we are now also evaluating the cell cycle regulatory mechanisms responsible for this increased proliferation.

Future studies to confirm our findings comprise laser capture microdissection techniques to allow for the specific isolation of the subgranular zone (SGZ), where progenitor cells reside, and transgenic reporter mice lines with fluorescently labelled subpopulations of cells, namely proliferating progenitor cells, for cell sorting and time-lapse *in vitro* analysis.

5.4 Paving the way to a new era in depression diagnosis and treatment

Depression is a complex and heterogeneous clinical entity (Smith et al., 2013) that may overlap with other neuropsychiatric disorders, such as anxiety disorders (Hettema, 2008). Moreover, the diagnosis of depression is based on relatively subjective evaluations of symptoms, relying exclusively on clinical presentation information. In this sense, the stratification of patients based on specific pathophysiological characteristics may open new avenues for the treatment of depression. But do we have enough preclinical and clinical data to support the use of pathophysiological biomarkers for this categorization of depressive patients? And if so, can we take advantage of the differential characteristics and potential effects of currently available antidepressants? At this point certainly not, but it is reasonable to envision that this is the way the field will evolve. This would indubitably have major implications for the diagnosis and treatment, towards a more personalized approach of such heterogeneous disorder. The reason why some patients respond well to some of the currently available therapeutic options while others don't remains pretty much unclear. Analysis of the molecular and cellular profile of different antidepressants, like the one presented in this thesis, may boost this knowledge. Recent data suggest that, for instance, peripheral markers, such as cortisol levels (Ventura-Junca et al., 2014), and inflammatory response molecules levels (Hashimoto, 2015), as well as neuroimaging findings (MacQueen and Frodl, 2011) may allow the identification of patients at risk for treatment non-response to first line treatments. Importantly, merging these complementary diagnosis tools with the understanding of the unique properties of each antidepressant may guide the therapeutic choice and provide these patients with the most suitable therapeutic intervention.

A substantial problem concerning currently available antidepressant therapies is their delayed onset of action. In the past, the identification of therapies that provide a rapid alleviation of depressive symptoms, such as electroconvulsive therapy (ECT) has challenged the way we view the treatment of depression. Still, this treatment may cause undesirable side effects mostly concerning cognition (Kennedy and Giacobbe, 2007), and better, safer strategies are needed. More recently, other therapeutic strategies, also providing an immediate alleviation of depression symptoms, are under investigation. These include the fast acting antidepressant drug ketamine (Naughton et al., 2014), and deep brain stimulation (DBS) (Schlaepfer et al., 2013, Taghva et al., 2013, Schlaepfer et al., 2014) that have been used to treat refractory patients (Berman et al., 2000, Giacobbe and Kennedy, 2006, Anderson et al., 2012, Naughton et al., 2014). In any case, more data on response duration

and safety profile is needed to allow its routine implementation in clinical practice. Interestingly, though their mechanism of action is generally distinct, they both ultimately target neural plasticity mechanisms.

Treatment resistance in depression represents another major challenge for the future of research in this field (Rush et al., 2006) and demands great efforts towards the development of novel therapeutic options. The oversimplification of depression pathophysiology as solely a neurochemical imbalance disorder has biased the research of new antidepressants for decades. Fortunately, it is now recognized that depression is a systems disorder affecting specific neural circuits, and that the currently available pharmacological approaches have a rather limited action. Neurochemical and neuroimmune imbalances, changes in the levels of neurotrophins and neuroendocrine changes have been identified in depressive patients and animal models of depression (Krishnan and Nestler, 2008, Willner et al., 2013). Interestingly, impairments in these systems may converge in neural plasticity changes, including dendritic plasticity and cell genesis (Pittenger and Duman, 2008, Lucassen et al., 2014), which are probably the main gateway for the network disruption found in depression. Specifically targeting these networks may foster the development of more effective treatment options for refractory patients. In practical terms, three primary strategies are being followed in order to fulfil the unmet needs in the treatment of depression: 1) improvement of pharmacological modulation of monoaminergic neurotransmission; 2) identification of novel targets for antidepressant drug development; and 3) direct modulation of neuronal activity using focal brain stimulation (Holtzheimer and Nemeroff, 2008). Regarding novel putative targets and therapeutic strategies under investigation, one may highlight the modulation of glutamatergic signalling (Banasr et al., 2010, Naughton et al., 2014), the resynchronization of circadian rhythms (Bunney et al., 2015, Edgar and McClung, 2013), and the prominent, previously underestimated, role of the gut microbiome (Dinan et al., 2013, Moloney et al., 2014).

Taking into account the higher prevalence of depression in women compared to men (American Psychiatric Association, 2013), and the evident bias on research towards male preclinical studies, it is urgent to re-orientate the focus in the field and invert this trend. Likewise, emerging data have disclosed sex differences, for instance, in the response to stress at several levels including, differential molecular and cellular responses, activation of distinct brain circuits, different neural plasticity mechanisms, and therefore different behavioral outcomes (Dalla et al., 2008, Bangasser and Valentino, 2012, Hodes et al., 2015, McEwen et al., 2015b). Moreover, preclinical studies have

shown that males and females respond differently to chronic antidepressant treatment (Dalla et al., 2010). Adding to this, unpublished work from our group suggests that cell genesis ablation in the hippocampus of female rats impacts differently on behavior compared to males. It is thus determinant not only to investigate the basis of depression susceptibility and pathophysiology in females but also to include them in preclinical drug screening studies.

The field of depression research has undeniably blossomed in the past few decades. Recognizing that depression has a biological basis represented a major step for investigation in this field, as it signified the shift to a neuroscience-based approach of this medical condition. The great amount of gathered information brought greater complexity to the field but also novel perspectives on how to improve diagnosis and treatment. Indeed, in order to provide patients with better treatment alternatives we must first aim for improved diagnoses. This improvement may rely on the utilization of more objective tools that inherently depend on the how well we understand the biological basis of depression. Hopefully, by integrating preclinical and clinical data, we will reach a stage where we can specifically pinpoint the underlying imbalances and try to develop integrated therapeutic strategies to restructure faulty circuits in the depressed brain.

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APPENDICES

APPENDIX I - Re-cycling Paradigms: Cell Cycle Regulation in Adult Hippocampal Neurogenesis and Implications for Depression

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Re-cycling Paradigms: Cell Cycle Regulation in Adult Hippocampal Neurogenesis and Implications for Depression

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Abstract Since adult neurogenesis became a widely accepted phenomenon, much effort has been put in trying to understand the mechanisms involved in its regulation. In addition, the pathophysiology of several neuropsychiatric disorders, such as depression, has been associated with imbalances in adult hippocampal neurogenesis. These imbalances may ultimately reflect alterations at the cell cycle level, as a common mechanism through which intrinsic and extrinsic stimuli interact with the neurogenic niche properties. Thus, the comprehension of these regulatory mechanisms has become of major importance to disclose novel therapeutic targets. In this review, we first present a comprehensive view on the cell cycle components and mechanisms that were identified in the context of the homeostatic adult hippocampal neurogenic niche. Then, we focus on recent work regarding the cell cycle changes and signaling pathways that are responsible for the neurogenesis imbalances observed in neuropathological conditions, with a particular emphasis on depression.

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Introduction

The view that no new neurons can be added to the adult brain was deconstructed over the past years. Neurogenesis in the adult smammalian brain is now a widely accepted neuroplastic event [1–3] that enables the brain to adapt to intrinsic and extrinsic stimuli. In fact, during the past few years, a large amount of data has provided evidence that the production, differentiation and survival of neurons in the adult brain have significant implications for several physiological processes, such as memory and learning [4–6]. Moreover, many studies have linked neurogenesis deregulation with the emergence of several pathological features in neuropsychiatric disorders. However, the role of neurogenesis in these disorders is yet to be completely established. Presently, much effort is devoted to the generation of behavioral and molecular data that establish a mechanistic link between neurogenesis and disease-state, so that ultimately directed therapeutic interventions can be designed.

Adult stem cells are responsible for tissue integrity, by adding new cells to the networks or by promoting the capacity to repopulate mature differentiated tissues as their constituting cells die due to damage or degeneration. The complex process of producing new cells throughout an organism's lifespan may rely on a common denominator—the cell cycle regulatory machinery (Fig. 1). However, the specificity of this transversal phenomenon in each tissue or cell type offers a wide spectrum of responses to a particular stem cell niche. In this review we aim to provide a comprehensive and integrated view of the cell cycle regulation in

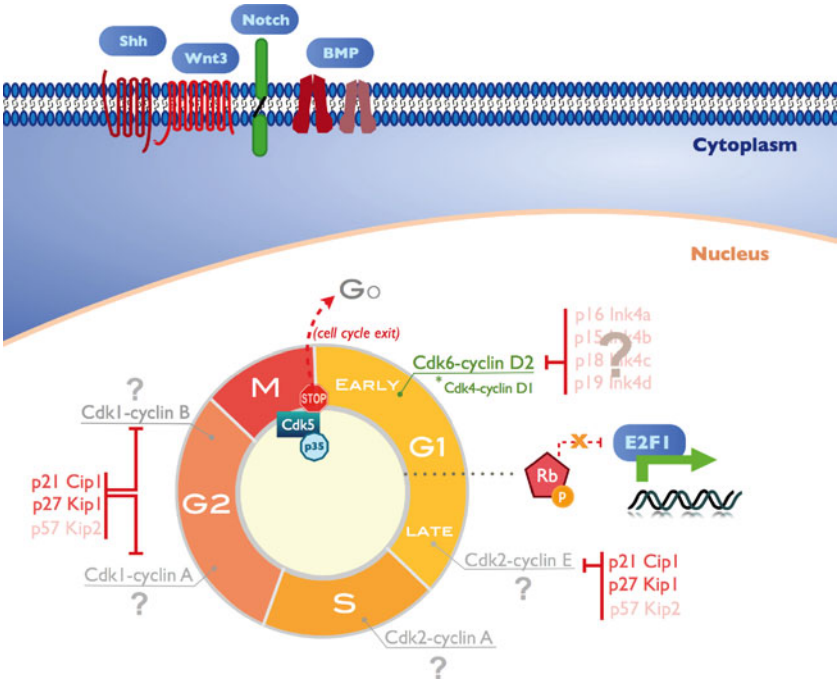


Fig. 1 Cell cycle regulation in the adult hippocampal neurogenic niche. Some niche-specific cell cycle regulators in the adult hippocampus have been identified. Cdk6-cyclin D2 and Cdk4-cyclin D1 complexes promote the expansion of the neural progenitor pool. P21 and p27 Cdk inhibitors have a role in proliferation arrest, both at the G1 and G2 phase. E2F1 has an important role in the neurogenic process by inducing the expression of genes involved in cell proliferation and differentiation. Cdk5 activity is associated with cell cycle reentry inhibition in postmitotic neurons. Signaling pathways, such as Notch, BMP, Shh and Wnt, are also involved in proliferation regulation, and in the balance between proliferation induction and stem cell quiescence maintenance. Nevertheless, several key molecules remain to be identified in this process in the context of adult hippocampal neurogenic niche (represented by a question mark). Rb retinoblastoma protein

the adult hippocampal neurogenic niche both in basal conditions and in disease (namely, in depression). With this, we offer a perspective on how the cell cycle machinery may constitute an interesting and still largely unrecognized link between alterations in postnatal hippocampal neurogenesis and disease, highlighting its relevance for the discovery of new molecular targets for the treatment of neurobiological disorders.

Neurogenesis in the Adult Mammalian Brain

Adult neurogenesis is the process by which neural progenitors divide mitotically to produce new neurons in the adult brain. This complex process involves several steps beyond cell division; namely, the commitment of the new cell to a neuronal phenotype, the migration and maturation of the cells, and the establishment of appropriate synaptic contacts that culminate with a full integration on the pre-existent network. Well described, adult neurogenesis is known to occur at least in two different regions of the mammalian brain: the subependymal zone (SEZ) of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) [7]. In both regions, astroglial cells act as the source of adult progenitor cells [8, 9]. The neuroblasts born in the SEZ migrate along the rostral migratory stream (RMS) becoming mostly mature GABAergic granule and periglomerular interneurons in the olfactory bulb (OB). The cells born in the adult SGZ migrate into the granular cell layer (GCL) of the DG and differentiate into glutamatergic granule cells [7]. Additionally, although disputable [10, 11], several reports describe the generation of new neurons in other regions of the adult brain, including the cortex [12, 13], the amygdala [14–16], the hypothalamus [17, 18], the striatum [19, 20] and the substantia nigra [21, 22]. However, in all these areas, neurogenesis appears to occur at very low levels or under non-physiological conditions [23].

Adult Hippocampal Neurogenesis

Neurogenesis in the adult DG occurs from a progenitor population residing in a narrow layer of about three nuclei wide, the SGZ. The first type of progenitor cells, defined as multipotent, are the neural stem cells (NSCs or type_1 cells). These cells express nestin and glial fibrillary acidic protein (GFAP), among other markers, and can be divided in two subtypes based on their orientation in the SGZ: radial astrocytes/NSCs (rA) and horizontal astrocytes/NSCs (hA). Radial NSCs, morphologically characterized by having a single radial process, are slow dividing cells, whereas horizontal NSCs have a short horizontal process and

divide faster than rA [24, 25]. Either one or both of these cell subtypes will then divide asymmetrically into one daughter cell and one progenitor cell, both of which already committed to a neuronal lineage. These latter cells, designated by transit amplifying neural progenitors (tANPs, or type_2 cells), are mitotically active and divide to give rise to neuroblasts (also known as type_3 cells). This last stage corresponds to a transition from a slow proliferating neuroblast, which is exiting the cell cycle, to a postmitotic immature neuron, that will migrate a short distance into the GCL. Neuroblasts transiently express markers of the neuronal lineage, such as the calcium-binding protein calretinin, doublecortin (DCX) and polysialylated-neural cell adhesion molecule (PSA-NCAM) [10, 26]. The newborn cells will then fully maturate into granule neurons, elongating their axons towards the hippocampal Cornus Ammonis 3 (CA3) area [23] and making the appropriate axonal connections [26]. These adult-born neurons become integrated in the pre-existing neuronal network 4 to 8 weeks after their birth [3, 27–29] (Fig. 2a).

Importantly, not all cells expressing immature neuronal markers develop into fully mature neurons [30]. In fact, if not recruited to perform any function, the great majority of these newly-born cells are eliminated by apoptosis once they exit cell cycle [31, 32]; a mechanism that until recently was largely undescribed. However, a recent report by Lu *et al.* [33], has very elegantly shown that DCX-positive neuronal progenitors present a phagocytic activity in the DG as well as in the SEZ, with important implications for the neurogenic process [33].

The generation of new neurons in the hippocampal niche of the adult brain, depends on the harmonization of several processes and cellular activities, which include proliferation, cell cycle exit, activation of survival/death pathways, migration through the GCL and differentiation/maturation of the newborn neurons [34]. These processes are regulated by both intrinsic and extrinsic factors that are ultimately responsible for the modulation of the neurogenic phenomenon. While there are numerous focused studies on several of these steps, little is known about the cell cycle regulation in the context of adult hippocampal neurogenesis and its repercussions for disease states. Here, to provide an integrated view, we will consider the "expanded cell cycle" [35], taking into consideration some of the most well-described mitogenic signals and the interacting signaling pathways.

Cell Cycle Regulation in the Adult Hippocampal Neurogenic Niche

Providing important clues on the regulation of the adult neurogenic process, the expression of cell cycle proteins and their regulation have been extensively explored in the context of embryonic development [36–40]. On one hand,

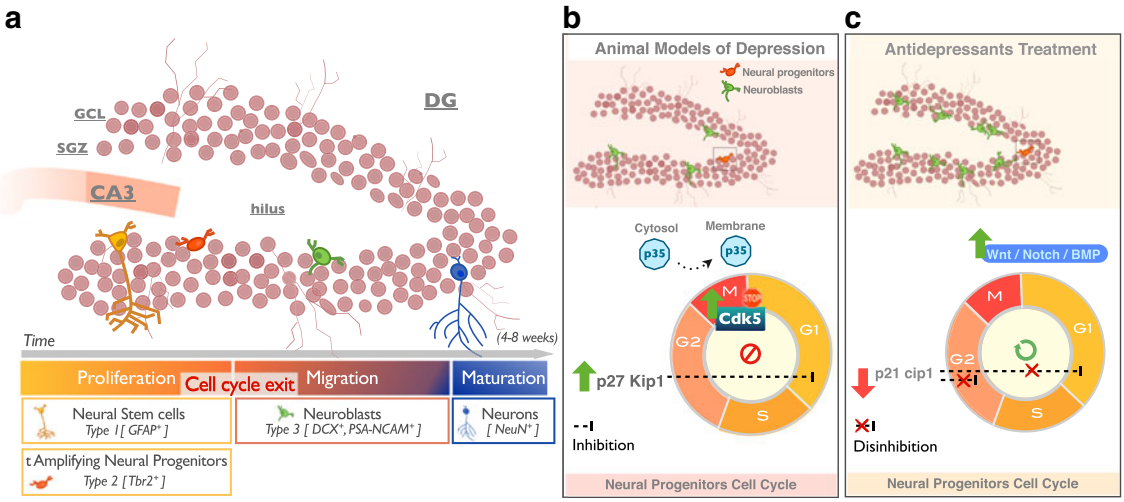


Fig. 2 **a** Neurogenesis in the hippocampus comprises several steps, including proliferation of neural stem cells and transit amplifying neural progenitors in the subgranular zone (SGZ), cell cycle exit, neuroblasts migration throughout the granule cell layer (GCL), and maturation of the newborn neurons. **b,_c** Cell cycle regulators implicated in neurogenesis imbalances observed in animal models of depression and in the pro-neurogenic effects of antidepressant drugs and other stimuli. **b** Neurogenesis imbalances have been observed in animal models of depression. These imbalances are attributed to an increased expression of p27 Cdk inhibitor (green arrow) in the DGs of animal models of depression. P27 inhibits neural progenitor cells proliferation in this neurogenic niche. Cdk5 is involved in the development of depressive-like signs in an animal model of depression. The increased activity of Cdk5 (green arrow), together with the translocation of p35 activator to the membrane, was observed in chronic mild stress (CMS) exposed animals. **c** The pro-neurogenic actions of antidepressant drugs and stimuli, such as physical exercise, have also been correlated with alterations in the "expanded cell cycle."Antidepressants are able to specifically inhibit p21 expression (red arrow) in the DG, while increasing neurogenesis. Additionally, signaling pathways with recognized effects over the cell cycle regulation, such as Wnt, Notch and BMP, were implicated in the modulation of adult hippocampal neurogenesis in the context of depression and antidepressant stimuli

some of the mechanisms are common to both embryonic and adult brain; however, there are essential differences between them especially regarding niche properties. Whereas during development, the cellular environment is highly specialized to support proliferation, in the adult hippocampus the environmental context includes a population of fully mature and functionally active neurons [7, 37], thus providing a different set of both intrinsic and extrinsic signals. In fact, in the adult mammalian brain, the vast majority of neuronal cells are in a quiescent differentiated state (G0 phase of the cell cycle), which is probably promoted by an increase in the expression of region-specific Cdk inhibitors [34, 41, 42]. Nonetheless, the expression of cell cycle proteins in the postnatal brain and their definite role in this neurogenic niche are still being unveiled [36].

Contrary to the traditional concept that postmitotic mature neurons are stably maintained in a quiescent differentiated state, recent, albeit still controversial, evidence has demonstrated that in some disease conditions, such as Alzheimer's disease [43, 44], traumatic brain injury [45] and cerebral hypoxia-ischemia [46], these cells are capable of responding to mitogenic signals and reenter the cell cycle [35, 47]. However, apparently these neurons neither finish dividing nor revert to their G0 quiescent state, ultimately undergoing apoptotic cell death and suggesting that they lack the factors needed for cell cycle progression [35].

It is important to mention, at this point, that the expression of cell cycle proteins in neuronal populations is not always associated with cell proliferation or cell cycle reentry. Indeed, a small number of studies have demonstrated that the expression of key cell cycle components may be associated with other neuronal processes, such as neuronal migration, dendrite morphogenesis, synaptic maturation and plasticity [48, 49]. Nonetheless, a few cell cycle molecules and signaling pathways have been implicated in the regulation of adult hippocampal neurogenesis. Moreover, their deregulation is often the cause for neurogenesis imbalances observed in several disorders, such as depression. As such, we will first provide a brief overview on these molecules and its functions in the context of adult hippocampal neurogenesis.

Cell Cycle Regulators

The cell cycle consists of a succession of events that lead to cell division. It comprises four distinct consecutive phases: the first gap (G1) phase, during which cells prepare for DNA replication in the synthetic (S) phase, followed by a second gap (G2) phase and mitosis (M). A highly coordinated network of molecules mediates progression through these four phases. There are two major classes of cell cycle regulators that cooperate in order to promote cell cycle progression: cyclins and cyclin-dependent kinases (Cdks). Cdks are serine/threonine kinases stably expressed during cell cycle progression that must bind to cyclins, their regulatory subunits, whose expression levels vary throughout the cell cycle phases, to form active catalytic heterodimers [50, 51]. Each Cdk is able to associate with different cyclins, which will in turn determine the proteins to be phosphorylated by a specific Cdk–cyclin complex (Fig. 1).

Several studies support the view that most cell cycle regulators are functionally redundant [52] and the need for a particular molecule is dependent on the cell type and on the niche [53]. This holds also true for the hippocampal neurogenic niche. As an example, studies on the role of Cdk4 and Cdk6 in the adult hippocampus unraveled a crucial role for Cdk6, but not Cdk4, in controlling the expansion of neuronal committed progenitors and thus the rate of neuronal production [54]. In fact, the absence of Cdk6 was shown to induce the lengthening of the G1 phase causing premature cell cycle exit and differentiation [54]. These findings lead to the "cell cycle length hypothesis" [55], which states that proliferative divisions exhibit a short G1 phase whereas neurogenic divisions are characterized by longer G1 phases. In a molecular perspective, it is proposed that the ability of a cell fate determinant to induce any cellular response is related with the time it has to act during G1 [55, 56]. Additionally, overexpression of the Cdk4–cyclin D1 complex in the adult mouse hippocampal niche was shown to increase the expansion of neural progenitor cells (NPCs), in a specific and cell–autonomous manner, while inhibiting neurogenesis [57]. Indeed, stopping the overexpression of the Cdk4–cyclin D1 complex was accompanied by an overproduction of new neurons, corroborating its effect on the expansion of the neural progenitors at the expense of neuronal differentiation. Moreover, no effects were observed in the survival and maturation patterns of DCX+ immature neurons [57]. Altogether, results suggest that the Cdk4–cyclin D1 complex is able to decrease the cell cycle length of cells that characteristically present longer cell cycles, whereas it is not able to change the short-length cycles [57]. In line, it is proposed [55, 58] that beyond the cell cycle length itself, it is the length relative variation that may be underlying the changes in the fate of a given cell [57]; however, more studies are needed to clarify this subject.

Cyclins, another important class of cell cycle regulators, are also implicated in the adult neurogenic phenomenon. Indeed, three types of cyclins D (D1, D2 and D3), which control Cdk4/6 activity, were already identified in mammals [59]. Although most cells express more than one cyclin D, several studies have demonstrated cell type-dependent roles for each of them [60–62]. One paradigmatic example of such specificity in the adult brain is the demonstration that cyclin D2, but not cyclin D1, knock-out (KO) mice, have a marked reduction of cell proliferation in the DG, measured by BrdU incorporation [59]. Other studies have corroborated this inability of cyclin D1 to promote neurogenesis in the hippocampus in the absence of cyclin D2 [63, 64].

Cell cycle progression is also negatively regulated, at a post-translational level, by two families of cyclin-dependent kinase inhibitors: the Inhibitor of kinase 4/Alternative reading frame (Ink4/ARF) family and the CDK inhibitory protein/Kinase inhibitory protein (Cip/Kip) family. These intracellular proteins are responsible for slowing or arresting the progression through the cell cycle, by blocking imperative events. The expression pattern and function of some of these Cdk inhibitors in the context of adult neurogenesis have also been characterized. P27kip1 (p27) is an important Cdk inhibitor, that induces cell cycle exit in proliferative cells [65]. In accordance, p27 expression decreases when cells are exposed to mitogenic signals, allowing their entrance in the S phase [66]. In the context of adult hippocampal neurogenesis, p27 protein is expressed in the SGZ of mice [67] and rats [64]; interestingly, many of the p27 cells co-express DCX in this region [67]. *In vitro* assays, using cultured NPCs, showed rapid increases in p27 expression following differentiation by growth factor withdrawal, thus confirming its role in cell cycle arrest and NPCs differentiation [67]. Additionally, deletion of p27 promoted an increase in the proliferative pool of NPCs [67], in accordance with previously published results in the SEZ [68]. These data were corroborated by *in vivo* assays showing an increased number of BrdU positive cells in the SGZ and in the SEZ of p27 KO mice. These results, together with the increased levels of proliferating cell nuclear antigen (PCNA) expression in the KO animals, further suggest that the absence of p27 promotes NPCs proliferation in both adult neurogenic niches [67]. Adding to the p27 studies, Pechnick *et al*. [69, 70] carried out two separate studies exploring the function of p21cip1 (p21) in the mouse hippocampus. Using a BrdU incorporation paradigm (one injection every 2h in a total of three injections, and sacrifice 24 h after the first injection), the authors showed, both *in vivo* and *in vitro*, that the absence of p21 lead to an increased proliferation of hippocampal neurons. Ultimately, the work suggests that p21 is responsible for blocking cell cycle progression in the adult hippocampal SGZ [69, 70]. Interestingly, p21 expression is restricted to neuronal committed progenitors [70], unlike p27 that reveals no distinction on lineage preference [67]. Strikingly, the results are not in agreement with a previous report in which p21 deletion showed no impact on the proliferation of neural progenitors in basal conditions [71]. However, it is worth mentioning that a different BrdU incorporation paradigm was used in this latter study. Indeed, the use of BrdU incorporation approaches has limitations as a direct measure of proliferation because does not always discriminates among effects that may underlie an increased S phase labeling; for example, G1/G2 phase shortening, increase in the growth fraction and lengthening of S phase [56]. The appropriate controls, the use of additional thymidine analogs and endogenous markers of proliferation should improve the analyses and may give a broader picture on the cell cycle regulatory mechanisms.

A final word to mention E2F1, an element that is part of a broad family of transcription factors involved in the regulation of cell cycle progression [42], but also with important roles in the adult neurogenic process. Contrary to what is described for most members of this family, E2F1 has been reported to induce cell death, by forcing postmitotic cells to re-entry cell cycle [42, 72]. In the context of adult neurogenesis, E2F1 was shown to be important for cell proliferation and differentiation. Using a single BrdU injection paradigm, 2h before sacrifice, Cooper-Kuhn *et al*. [72] showed that E2F1-deficient mice have decreased cell proliferation and diminished neurogenesis, both in the hippocampal DG and the SVZ. These authors also described a decrease of about 60-70 % in apoptotic cells, in the hippocampal neurogenic niche of E2F1-deficient mice compared to wild-type (WT), further corroborating the role of this gene in regulating cell death in the context of adult neurogenesis [72]. Figure 1 depicts the cell cycle regulators described in the context of adult hippocampal neurogenesis.

Signaling Pathways

Cell cycle entry promotion and initial progression through G1 phase is induced by mitogens or growth factors present in the extracellular environment [64, 73, 74]. The interplay between cell cycle regulation and cell fate determination is also a topic of great relevance [56]. In particular, the G1 phase length is crucial for the switch from proliferation to differentiation and is modulated by cell cycle regulators and cell fate determinants [56]. Thus, signaling from the niche is suggested to be responsible for key processes in the regulation of adult neurogenesis homeostasis, including: the balance between quiescence versus proliferation, the mode of cell division, and the prevention of stem cell depletion [75, 76]. In this section we will briefly describe some of the signaling pathways activated in the adult hippocampal neurogenic niche.

The role of Notch signaling in NPCs in the adult hippocampus was investigated *in vivo* through inducible gain- and loss-of-function experiments. Activated Notch1 overexpression induced proliferation of endogenous progenitors, whereas inhibition or ablation of Notch1 signaling promoted cell cycle exit, inducing the transition from neural stem or progenitor cells to transit-amplifying cells or neurons [77]. On the other hand, in maturing neurons, Notch1 proved to be relevant for survival and structural plasticity modulation [77].

Bone morphogenetic proteins (BMPs) are other key regulatory components of the adult hippocampal neurogenic niche, restricting the proliferation of the stem cell pool,

through BMP receptor-IA (BMPR-IA) activation, and thus maintaining the equilibrium between stem cell proliferation and quiescence [75]. Downregulation of endogenous BMP signaling promoted an increased proliferation of SOX2+ cells by recruiting quiescent radial cells into the cell cycle. Moreover, the canonical BMP signaling pathway is reactivated shortly after neuronal fate commitment, possibly to promote cell cycle exit of newly born neurons [75].

Sonic hedgehog (Shh) is an evolutionarily conserved secreted protein that plays an important role in many aspects of developmental control [78], as well as in adult hippocampal neurogenesis [79]. Shh signaling pathway was shown to induce a dose-dependent proliferative response in progenitors *in vitro*, whereas inhibition of Shh signaling reduced proliferation *in vivo*. These studies confirmed Shh signaling pathway as an important regulator of adult hippocampal neural progenitors [79], suggesting also its involvement in cell cycle regulation.

Like Shh, Wnt proteins are also well-known key regulators of NSC behavior during embryonic development [80]. Wnt signaling has been reported as a regulator of adult hippocampal neurogenesis [81], through the activation of the proneural gene NeuroD1 [82]. Activation of NeuroD1 is important for the generation of granule cells in the hippocampus and cerebellum [83], possibly by promoting cell cycle exit.

Altogether, these data highlight the complex orchestration of the cell cycle process in the context of adult hippocampal neurogenesis, as well as the interplay between cell cycle regulators and upstream molecular signaling pathways. Interestingly, there is a certain level of functional redundancy among cell cycle regulatory components, possibly as an evolutionary mechanism to prevent severe damage upon deficiency of one of these molecules. On the other hand, most of these studies point to tissue and cell specificity as a hallmark of these systems, proving that these regulators may operate at different levels of the cell cycle and implying the need for their fine tuning in the homeostatic control of adult hippocampal neurogenesis.

Cell Cycle Regulation in Neuropathological Scenarios

The molecular mechanisms and pathways regulating adult hippocampal neurogenesis in response to deleterious stimuli, and the contrasting actions of pro-neurogenic drugs, are still largely undisclosed. It is legitimate to consider that these alterations in adult neurogenesis may be attributable to direct or indirect changes in cell cycle regulatory mechanisms. As such, the cell cycle machinery is possibly a convergent pathway through which intrinsic and extrinsic factors, such as stress and toxins, manifest their effects. Indeed, cell cycle deregulation in the context of adult neurogenesis has been associated with the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease [84] and Parkinson's disease [85], neuropsychiatric diseases, as is the case of schizophrenia [86] and major depression [87], and injury, namely stroke [88, 89]. These changes in cell cycle dynamics, as observed in several disease states [90–92], further reinforce the need for additional studies examining the role of core cell cycle players as targets for disruption. Next, we will briefly explore the case of depression as a paradigmatic example of how cell cycle deregulation can lead to the development of pathological traits.

Major Depression

Major depression is a chronic and debilitating disease, and one of the most common psychiatric disorders in modern society. It is estimated that about 16 % of the population will be affected by this disease once or several times during lifetime [93]. Like other psychiatric disorders, depression is a complex and heterogeneous clinical entity [94], dependent on the interaction between genetic susceptibility [95, 96] and environmental factors [97]. Depressive patients present symptoms of depressed_mood, learned helplessness, anhedonia and impaired cognition, and present a high comorbidity with anxiety disorders [94].

Strikingly, depression is characterized by several pathophysiological alterations in the brain such as differences in size of specific brain regions, changes in neuronal morphology, neurochemical and signaling alterations, and changes in genetic and epigenetic regulation [98, 99]. Knowledge of the etiopathogenesis of depression has progressed substantially in the last years [100], in part due to studies employing animal models. Animal models of depression use known etiological factors (etiological validity) to induce behavioral and neurobiological symptoms in animals similar to those of the human disease (face validity). Moreover, a valid animal model for the formulation of hypotheses and for the development of novel therapeutic strategies should respond to clinically effective treatments (predictive validity) [94, 101]. There are several animal models of depression: chronic mild stress (CMS), social stress, early life stress, fear conditioning and olfactory bulbectomy [97]. Although none of them can fully recapitulate the complexity and heterogeneity of the human disease, they are considered robust approaches to study the depression in humans. For example, the CMS animal model presents alterations in the three behavioral domains known to be affected in depressive patients, i.e., mood, anxiety and cognition [94]. Despite this large contribution of data from studies in animal models of depression, and from post-mortem studies of human brains, the neurobiological basis of this disorder is still poorly defined. Importantly, the fact that approximately half of the patients presenting clinical depression show incomplete remission or relapse after treatment with the currently

available antidepressants [97] further reinforces the need for finding new molecular targets and more efficient treatments.

There are currently several leading hypotheses that attempt to elucidate the neural and molecular mechanisms of depression. The monoamine hypothesis of depression [102] has been the most prevalent. The main support of this hypothesis is the fact that most classic antidepressants induce an increase of the serotonin and noradrenaline levels [103]. More recently, additional studies have shown that other mechanisms are implicated in the neurobiology of this disorder; this is mostly based on the observation that other factors are altered in depressed individuals [87, 104, 105] and on the efficacy of new antidepressants, in which the mechanisms of action do not rely on the monoamine transmission systems [106–109]. Thus, several other hypotheses on the etiology of depression have been put forward, including: the neurotrophin hypothesis, the cytokine hypothesis, the hypothalamic pituitary adrenal (HPA) axis modulation hypothesis and the neurogenic hypothesis. Although none of these are mutually exclusive, in this discussion we focus mostly on the role of adult hippocampal neurogenesis and on the molecular processes that can regulate it at the cell cycle level.

The Neurogenic Hypothesis of Depression and Cell Cycle (De)regulation

Studies showing reduced hippocampal neurogenesis in several animal models of depression [110–112] constitute the basis for the neurogenic hypothesis of depression. Importantly, all major classes of antidepressants [87, 113], and most of the environmental factors that confer antidepressant-like behavioral effects, such as environmental enrichment [114, 115], physical activity [115] and learning [7], are also known to promote hippocampal neurogenesis. These facts have lead to the proposal that neurogenesis may have a role in the etiopathogenesis of depression; however, the currently available data strongly reinforces the need for restructuring this possibly oversimplified view. Indeed, the functional implications of decreased neurogenesis for the precipitation and maintenance of the depressive state are yet to be completely established, as the experimental approaches and time frames of analysis diverge. Some studies have implicated neurogenesis in the emergence of behavioral deficits observed in animal models of depression and in the actions of antidepressants [116–118]. While, other studies showed that at least the short-term mood-improving actions of antidepressants depend on neuronal remodeling in the hippocampus and prefrontal cortex (PFC), rather than on neurogenesis [110]. Moreover, recently published data from our lab showed that the appropriate incorporation of new cells in the adult rat hippocampus is a key factor for the long-term spontaneous recovery from depressive-like behavior as well as for the action of antidepressants [119]. Using a longer experimental time frame, to allow the full differentiation and integration of newborn cells in the pre-existing neuro-glial circuitry, it was possible to fate-map the new cells generated during antidepressants treatment and understand their impact in distinct behavioral dimensions [119]. These findings further reinforce the need for an integrated time-dependent overview of the neurogenic phenomenon with great emphasis on the functional role of newly generated cells in the adult hippocampus. Importantly, most of the stimuli affecting adult neurogenesis, are also responsible for inducing changes at the cell cycle level in the progenitor cells of the hippocampal niche. Some of the most relevant reports on cell cycle regulation in the context of adult hippocampal neurogenesis and stress-related disorders have disclosed a major role for Cdk inhibitors [64, 69, 70]. Heine *et al*. [64] evaluated the role of p27 in the regulation of the cell cycle in the DG of rats following exposure to stress. After 3 weeks of chronic exposure to unpredictable stress, rats presented significantly decreased numbers of proliferating cells, measured by ki67 immunostaining, and increased numbers of p27 positive cells in the SGZ. Notably, this effect was not observed upon acute stress exposure. Moreover, the proliferation levels returned to normal after a 3-week recovery period from chronic stress, suggesting a transient p27-dependent G1 arrest in the SGZ cells of chronically stressed animals [64]. Somehow unexpectedly, neither cyclin-E nor cyclin-D1 protein levels were significantly altered in these animals when compared to controls [64].

Other animal studies have focused their attention on the role of Cdk inhibitors regarding the pro-neurogenic action of antidepressants [69, 70]. Pechnick *et al*. [69]showed that naïve mice chronically treated with imipramine, a tricyclic antidepressant, not only show increased neurogenesis in the DG but also decreased the expression of p21 Cdk inhibitor in the SGZ, when comparing to saline-treated controls. In a more recent study, the same group analyzed the effects of chronic administration of other classes of antidepressants on SGZ p21 expression and neurogenesis; all antidepressants tested (fluoxetine, imipramine and desipramine) were able to specifically inhibit p21 expression in the mice DG and this effect was linked to increased neurogenesis [70]. Unexpectedly, no change was noted in p27 expression following antidepressants administration [70], possibly suggesting specific roles for each of these Cdk inhibitors following different stimuli. It is worth mentioning that Pechnick *et al*. did not include an animal model of depression in their studies, possibly accounting for these results. Together, these findings support the involvement of cell cycle molecules in the mechanistic association between stress and the action of antidepressants, in the context of neurogenesis regulation.

A recent study has implicated the cyclin dependent kinase 5 (Cdk5)/p35 complex in the development of depressive-like behavior and in the action of antidepressants

[120]. Cdk5 still has no recognizable function in the progression of the cell cycle [37, 47], although structurally similar to other Cdks. In fact, Cdk5 expression and activity occur almost exclusively in postmitotic neurons, both in the developing and in the adult brain [37]. This kinase works as a cell cycle inhibitor in postmitotic neurons, repressing aberrant cell cycle reentry, a phenomenon linked to the development of several neurodegenerative disorders [37, 121]. Cdk5 regulation requires activators that are specifically expressed in postmitotic neurons. One of these activators is p35, a regulatory subunit that translocates from the cytosol to the membrane to induce Cdk5 activity [120, 121]. In a recent study, it was reported an increased Cdk5 kinase activity together with the translocation of p35 to the cell membrane, in the DG of rats exposed to CMS. They also observed that inhibition of Cdk5 specifically in the DG, but not in the CA1 or CA3 of the hippocampus, prevented the CMS-induced behavioral impairments, further suggesting the involvement of the Cdk5/p35 complex in the etiology of depressive-like behavior. Remarkably, p35 overexpression blocked the antidepressant behavioral effects of venlafaxine, a selective serotonin reuptake inhibitor (SSRI) antidepressant [120]. These data suggest an association between Cdk5 activity and the development of stress-related disorders [120], similar to what has been previously described for some neurodegenerative disorders [121]. Moreover, the studies may also suggest that the effect of Cdk5 activation is attributable to the impairments typically observed in hippocampal neurogenesis induced by CMS exposure [120]. Complementary studies with analyses of cell proliferation and neurogenesis would help to better define Cdk5 function in the adult hippocampus. Figure. 2b and c show the schematic representations of the adult hippocampal neurogenesis changes observed in animal models of depression and after antidepressant treatment, and the corresponding cell cycle alterations.

Changes in the signaling pathways known to be involved in the modulation of adult hippocampal neurogenesis have also been indirectly associated with the development of depressive-like behavior in animal models. Indeed, Wnt knockdown-mediated neurogenesis ablation was shown to impair several hippocampal-dependent cognitive functions, such as long-term retention of spatial memory and object recognition memory [122]. Importantly, these cognitive behavioral deficits were linked with depression onset and maintenance [94, 123]. Wnt signaling was further implicated in the actions of fluoxetine, an SSRI antidepressant; chronic treatment with this antidepressant was able to stimulate the expression of Wnt3a protein in the hippocampal DG**.** However, Wnt activity appears to be preferentially implicated in fluoxetine's reported induction of neural plasticity and not in its pro-neurogenic actions [124].

Notch and BMP signaling have also been shown to be mediators of the pro-neurogenic actions of physical exercise. Physical exercise is a stimulus with recognized antidepressant effects [125, 126]. Moreover, it has been consistently reported to robustly induce adult hippocampal neurogenesis, by promoting the proliferation of progenitors and the survival and maturation of newborn neurons [127-130]. More recently, some studies investigated the molecular signaling correlates of these cellular events [131-133]. Using thymidine analogs incorporation paradigms, Brandt *et al*. showed that voluntary exercise (i.e., mice that had access to a running wheel) preferentially promotes the proliferation of DCX+type_3 precursor cells and Notch1-dependent cell cycle exit. Since Notch1 is known to induce proliferation and inhibit differentiation in earlier NPCs (type_1 and 2a cells) [77], it is interesting to

Table 1 Summary of the cell cycle and signaling alterations implicated in neurogenesis imbalances observed in animal models of depression and mediating the pro-neurogenic effects of antidepressant drugs and stimuli

Experimental model	Proliferation/neurogenesis in the hippocampal DG	Molecular changes	Reference
Cell cycle regulators			
CUS exposed mice	↓	↑ p27kip1+cells in the SGZ of the DG	[64]
Naïve mice chronically treated with fluoxetine, imipramine and desipramine	↑	↓ p21cip expression in the SGZ of the DG	[69, 70]
CMS exposed rats treated with venlafaxine, mirtazapine, and aripiprazole	(Not assessed)	↑ Cdk5 activity and translocation of p35 activator to the membrane	[120]
Signaling pathways			
Naïve animals chronically treated with fluoxetine	↑	↑ Wnt3a expression	[124]
Voluntary exercise in mice (antidepressant stimulus)	↑	↑ Notch1 activity in DCX+cells (cell cycle exit promotion)	[132]

SGZ subgranular zone, DG dentate gyrus, CUS chronic unpredictable stress, CMS chronic mild stress, DCX doublecortin [64, 69, 70, 120, 124, 132]

notice these contrasting pro-neurogenic functions in more committed progenitors [132]. Altogether the findings support the use of experimental designs that specifically address the role of molecular determinants in each hippocampal cell type. Table 1 summarizes the most relevant studies regarding the cell cycle and signaling alterations implicated in adult hippocampal neurogenesis imbalances in the context of depressive-like behavior.

Conclusions and Perspectives

Sixty years after the first report of ongoing neurogenesis in the adult brain, we are now at the point of evaluating the physiological relevance of the incorporation of new neurons in pre-existing neuronal networks. The integrated studies on adult neurogenesis in its various stages—progenitor cells proliferation, cell cycle exit, migration and differentiation—have brought new players into the complex network of factors and molecular mediators that directly or indirectly participate in the process. Nonetheless, we were not yet able to establish the precise molecular cascades that regulate the homeostasis in adult neurogenic niches. Therefore, the future of this field of research needs to build up an integrated view of the molecular processes, by specifically targeting candidate molecules using conditional approaches to overcome the limitations of full_KO models. This approach will allow the exclusion of possible compensatory mechanisms promoted during embryonic development, a strategy that seems to be of particular importance in the case of cell cycle regulators. Additionally, most of the literature on the regulation of adult neurogenesis relies on the use of thymidine analogs incorporation, such as BrdU. The use of these strategies to study cell cycle regulation in the context of adult hippocampal neurogenesis requires careful interpretation of the data. In this way, the appropriate controls and additional strategies should be considered to ensure that the results definitely reflect the generation of new neural cells. Moreover, caution is needed when comparing different studies, as distinct experimental paradigms may draw contrasting conclusions.

More than a physiological phenomenon, adult hippocampal neurogenesis is a process by which the etiology of many neurodegenerative and neuropsychiatric disorders may be unraveled. More importantly, the neurogenic process is a substrate from which new molecular targets for treating these disorders may arise. The diverse ways of approaching the topic provide unique perspectives on how neurogenesis may be implicated in homeostatic responses and in the development of pathological states. The data reviewed here strongly supports that both direct and indirect cell cycle regulatory events may constitute relevant pieces to elucidate the complex mechanisms underlying the response to anti- and pro-neurogenic stimuli, in both basal conditions and in disease. These reports further emphasize the pertinence of modulating cell cycle regulators as targets for the development of new therapeutic approaches for disorders associated with neuroplastic imbalances.

Particularly in the case of major depression, new theories beyond monoamines have created a broader picture on how etiological factors are translated into disease and the action of antidepressants into the alleviation of the most common symptoms. In this context, much of the mechanisms are now being explored, including those interfering with adult hippocampal neurogenesis. However, the study of depression still represents a challenge for research since it involves the interplay between an individual's genetic predisposition and molecular responses to environment. Certainly, the discovery of new molecular mediators will give us important clues on susceptibility or predisposition targets, promoting the establishment of novel disease models, in a feedback loop that would nourish the field with new perspectives.

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

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Author contribution:

Patrício P. was involved in the concept, design and optimization of the Sweet Drive Test paradigm. She participated in all the behavioral tests and performed the gene expression analysis. Moreover, she was involved in the manuscript writing.

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

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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.

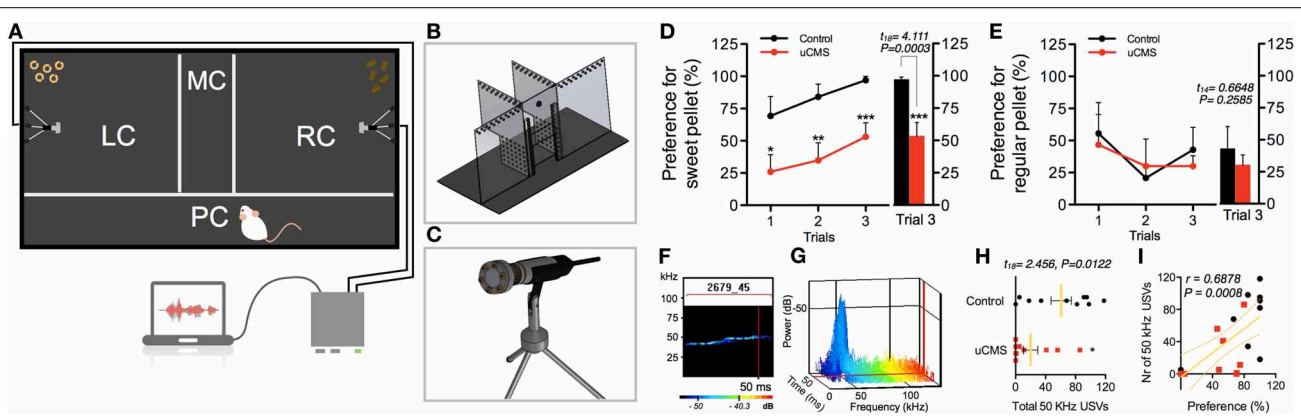


FIGURE 1 | Characterization of anhedonic behavior in rats exposed to uCMS, in the Sweet-Drive Test (SDT) paradigm. (A) Preference for sweet pellets was measured in a compartmentalized SDT apparatus **(B)**, in which ultrasonic vocalizations were recorded using ultrasound microphones **(C)**. **(D,E)** 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

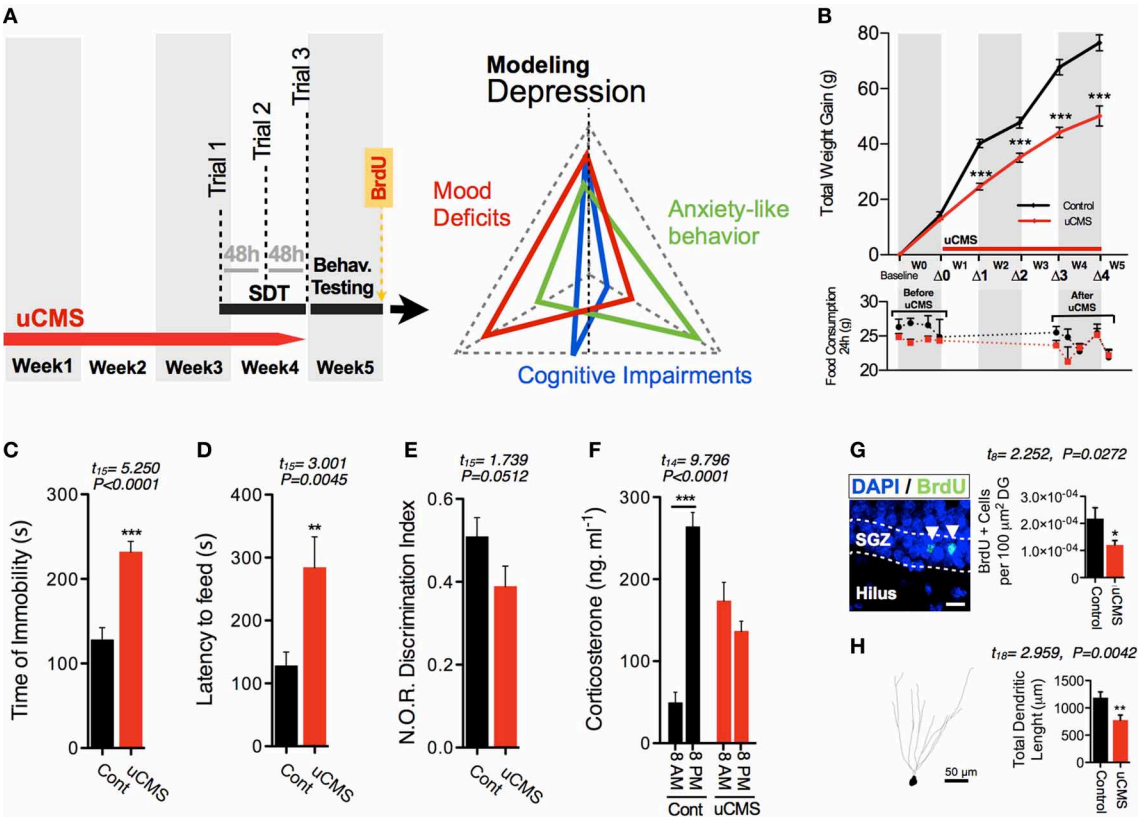


FIGURE 2 | Effects of uCMS exposure in different physiological and behavioral dimensions. (A) After 4 weeks of uCMS exposure, multi-dimensional depressive-like behavior was assessed. **(B)** Weight gain and food intake, before and after 4 weeks of uCMS. Animals exposed to uCMS presented a decreased total 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

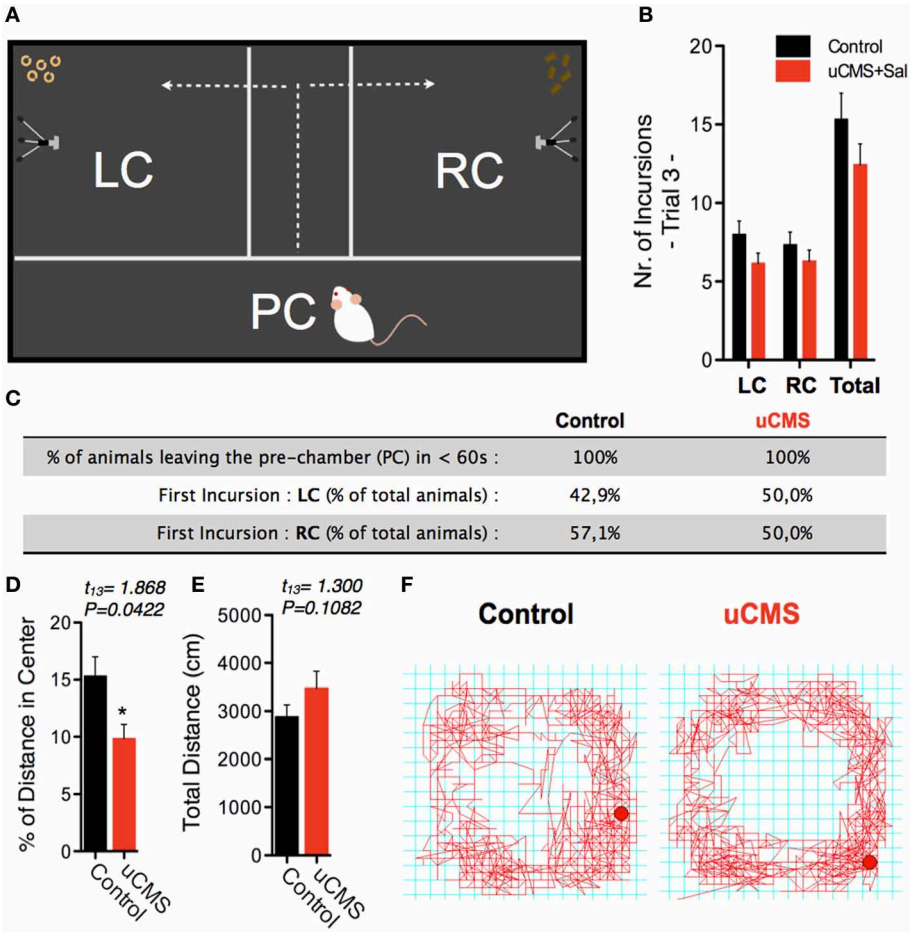


FIGURE 3 | Exploratory parameters in the SDT. (A,B) The average number of incursions into the left-chamber (LC; containing sweet pellets) and into the right-chamber (RC; containing regular pellets), during SDT was assessed and no significant differences were found. **(C)** Additional exploratory parameters 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.

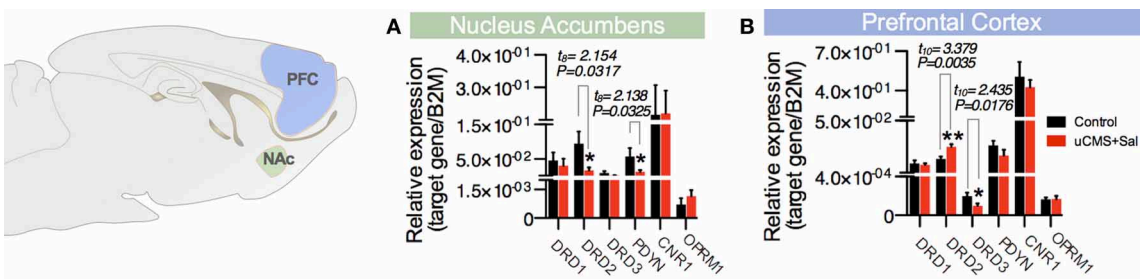


FIGURE 4 | Expression of molecular mediators of hedonia in the (A) nucleus accumbens (NAc) and (B) prefontal cortex (PFC) by RT-PCR: Dopamine Receptors D1, D2, and D3 (Drd1, Drd2, and Drd3), prodynorphin (Pdyn), Cannabinoid Receptor 1 (Cnr1) and Opioid Receptor μ1 (Oprm1). $^{*}P < 0.05$, $^{**}P < 0.01$. 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

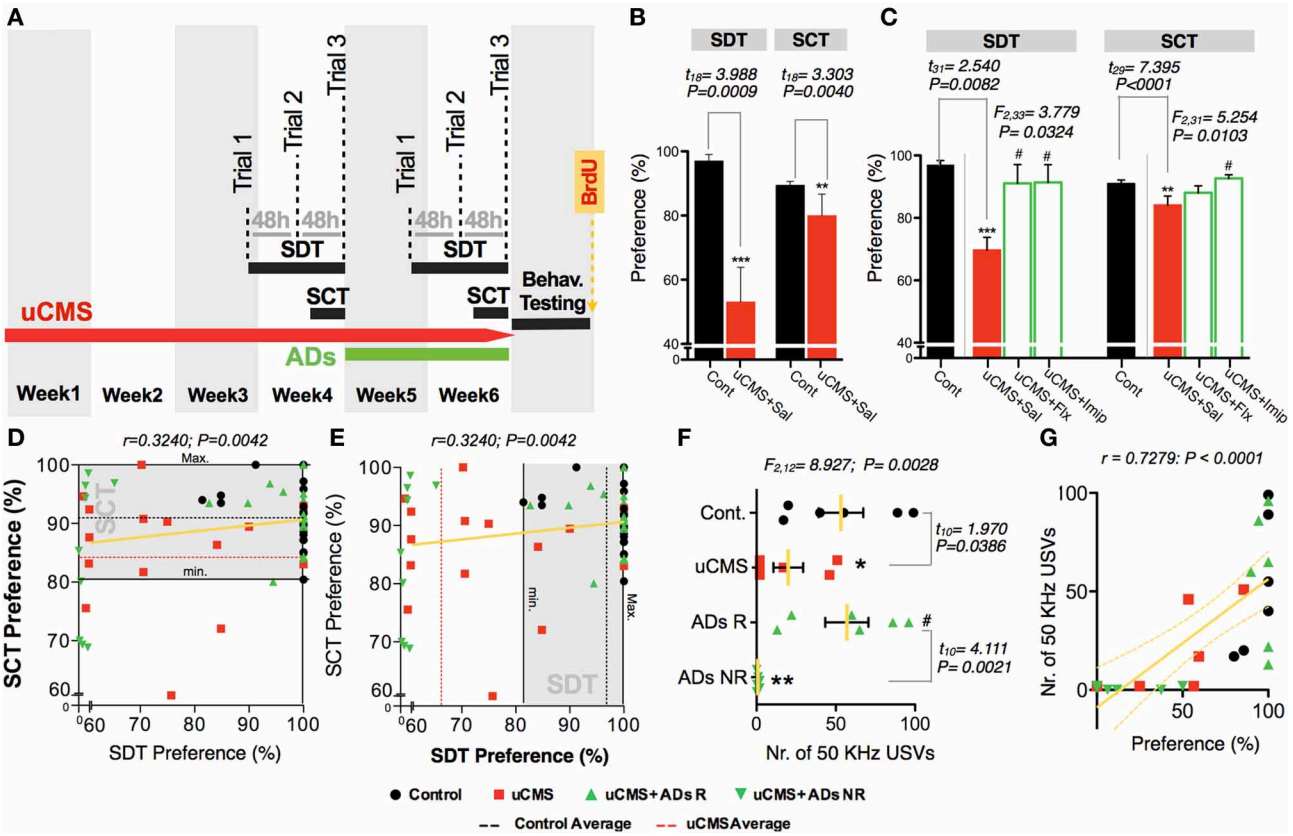


FIGURE 5 | Measuring the effects of uCMS and antidepressant treatment on anhedonic behavior with the Sweet Drive Test (SDT) and the Sucrose Consumption Test (SCT). (A–C) Anhedonic behavior was measured before **(B)** and after **(C)** 2 weeks of antidepressant treatment, in the SDT (data from the 3rd 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

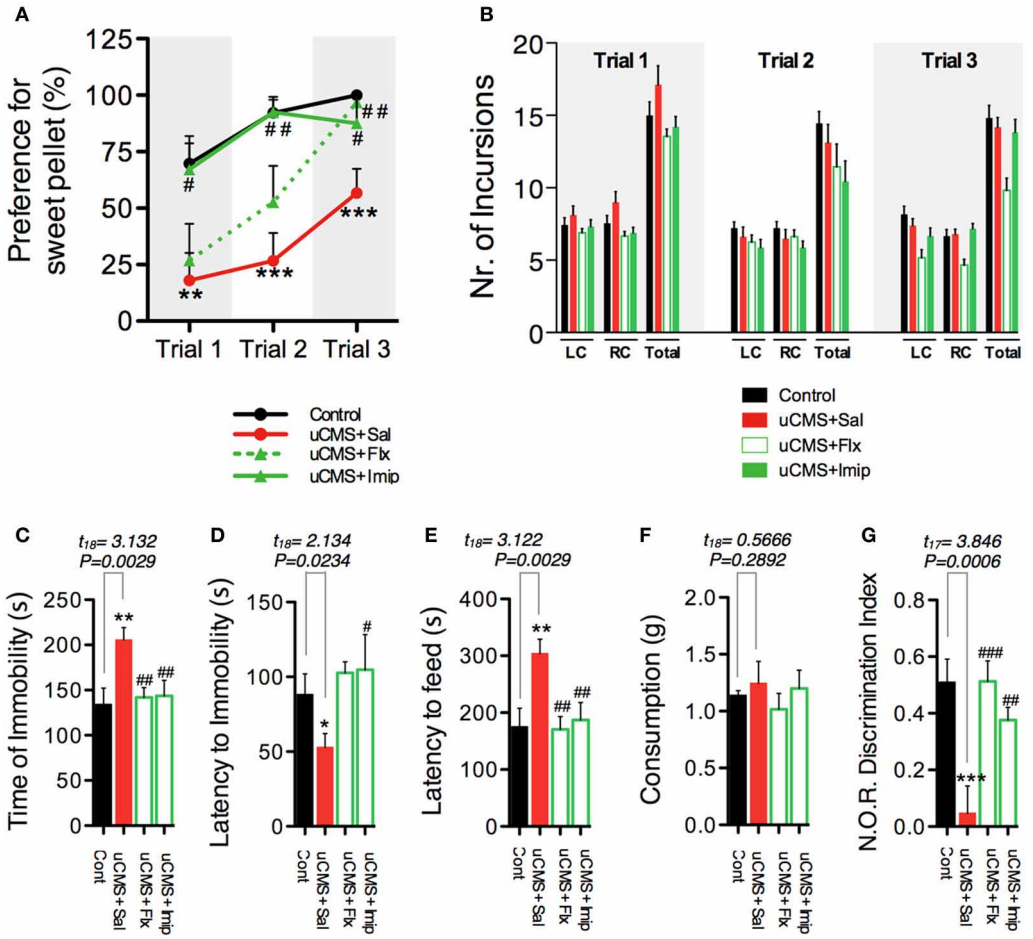


FIGURE 6 | Additional behavioral parameters determined after 6 weeks of uCMS exposure and ADs treatment. (A) uCMS exposure induced a significant decrease in preference for sweet pellets along the trials [Trial 1: $t_{(18)} = 3.011$, $P = 0.0038$; Trial 2: $t_{(18)} = 4.795$, $P < 0.0001$; Trial 3: $t_{(18)} = 4.007$, $P = 0.0004$]. 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

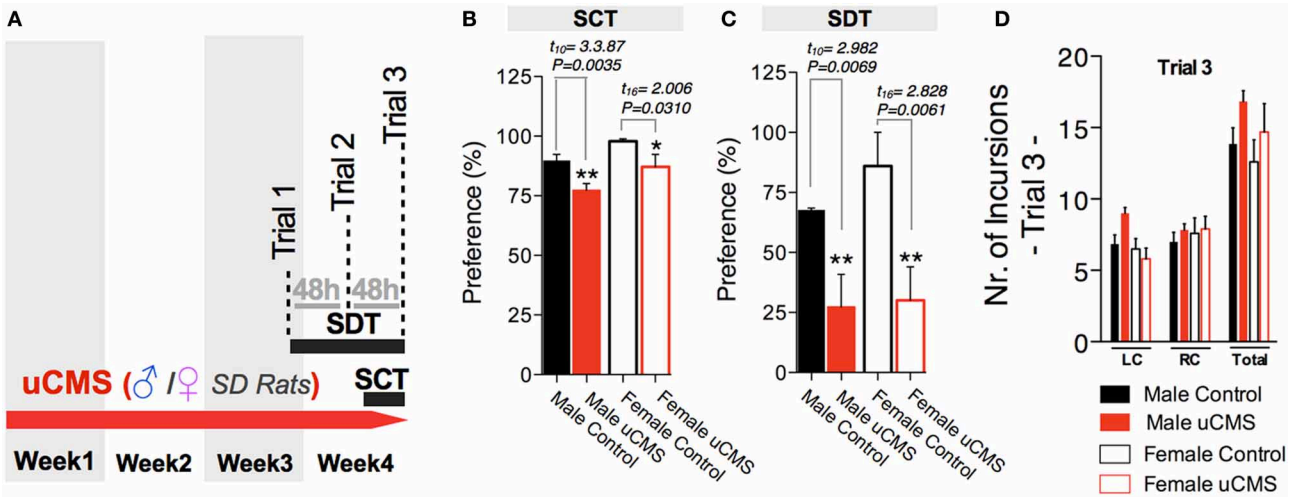


FIGURE 7 | Measuring anhedonic behavior with the Sweet Drive Test (SDT) and the Sucrose Consumption Test (SCT) in Sprague-Dawley (SD) Rats. (A) Using the same experimental approach previously used with Wistar-Han rats, male and female SD rats were tested in the SCT and the SDT. **(B)** uCMS exposure 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

The Supplementary Material for this article can be found online at: at: http://www.frontiersin.org/journal/10.3389/fnbeh.2014.00074/abstract

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APPENDIX III - Relevance of miRNA-411-5p for hippocampal neuroplasticity in the uCMS rat model of depression and after fluoxetine treatment

Preliminary data

PRELIMINARY DATA

Relevance of miR-411-5p for hippocampal neuroplasticity in the uCMS rat model of depression and after fluoxetine treatment

Epigenetic mechanisms are a potential mechanism through which environmental signals, such as stress, exert their effects in gene expression. These mechanisms include histone modification, DNA methylation, and noncoding RNAs (Hsieh and Eisch, 2010). MicroRNAs (miRNAs) are a family of small (19-25 nucleotides) non-coding RNAs that regulate expression at the post-transcriptional level. MiRNAs bind primarily to the 3' UTR of mRNAs leading to mRNA destabilization or repressing translation.

In the past years growing evidence has supported the role of miRNAs as regulators of critical aspects of neuroplasticity and brain function (Dreyer, 2010, Hansen and Obrietan, 2013, O'Carroll and Schaefer, 2013, Issler et al., 2014, Maffioletti et al., 2014). Additionally, alterations in miRNAs levels have been reported in several neuropsychiatric disorders, including depression (Dwivedi, 2014) and as targets for antidepressant treatment (O'Connor et al., 2012; O'Connor et al., 2013). Moreover, the association between changes in miRNAs in body fluids, such as blood and CSF, and brain tissue has generated great interest in the field, as these may represent potential biomarkers for disease (Maffioletti et al., 2014, Scott et al., 2015).

Previous genome-wide analysis of the hippocampal dentate gyrus (DG) detected several differentially expressed miRNAs (Patricio et al., 2015). One of these was mir-411, which was upregulated after chronic stress exposure and restored to the levels of controls after antidepressants treatment (Patricio et al., 2015). Given the important and well established role of the hippocampal DG in the pathophysiology of depression and the recently acknowledged relevance of miRNA regulatory potential, we sought to further validate the expression of this miRNA in an independent set of animals and further explore its function in the context of depression and antidepressant treatment. Given the acknowledged anatomical and functional differentiation between dorsal and ventral hippocampal DG, namely in response to stress and antidepressants, we sought to analyze the expression of miR411-5p in these two subregions, as well as in blood samples from uCMS-exposed

and fluoxetine-treated animals. Additionally, we explored the predicted targets of this miRNA in order to further understand its role in the context of neuroplastic phenomena in the adult DG.

RESULTS

Expression of mir-411-5p in the dorsal and ventral subdivisions of the DG

Given the functional heterogeneity of the hippocampal DG we sought to investigate the differential expression of this miRNA along the septo-temporal axis of this brain region.

Although statistical significance was not reached, miR-411-5p was slightly up-regulated in the dorsal DG (dDG) of uCMS-exposed animals compared to CT animals (t_{15}=1.215; p=0.2430; **Fig. 1A**). Conversely, fluoxetine treatment was able to significantly decrease the levels of this miRNA compared to uCMS-exposed animals (t_{13}=2.410; p=0.0315; **Fig. 1A**). No statistically significant differences were detected in the expression of this miRNA in the ventral DG (vDG; CT vs uCMS: t_{15}=0.8006; p=0.4359 and uCMS+FLX vs uCMS: t_{13}=1.235; p=0.2386; **Fig. 1B**). These results suggest that the previously described changes in the expression of mir-411-5p in total DG (Patricio et al., 2015), most likely reflect changes occurring in the dorsal subdivision.

Expression of mir-411-5p in the blood

A few studies have recently investigated the expression of miRNA in the blood of depressed individuals and after treatment with antidepressants (Belzeaux et al., 2012, Bocchio-Chiavetto et al., 2013, Wang et al., 2015). To further evaluate the potential of this miRNA as a biomarker, we assessed its expression in the blood of the animals. Preliminary results reveal no changes in the expression of this miRNA in the blood of uCMS-exposed animals when compared to controls (t_4=0.3573; p=0.7389; **Fig. 1C**); however, a significant decrease in its expression was disclosed after chronic fluoxetine treatment (t_4=3.792; p=0.0192; **Fig. 1C**).

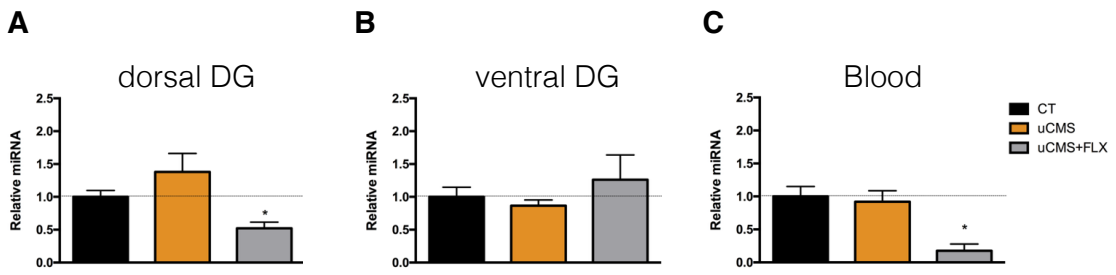


Fig. 1: Expression of miRNA-411-5p in the dorsal (**A**) and ventral (**B**) DG, and in the blood (**C**) of CT, uCMS and uCMS+FLX animals. *Denotes the effect of fluoxetine compared to untreated CMS-exposed animals. T-test, *P<0.05. Abbreviations: dDG – dorsal dentate gyrus; vDG – ventral dentate gyrus; CT – control; uCMS – unpredictable Chronic mild stress; uCMS+FLX – unpredictable chronic mild stress + fluoxetine.

Predicted targets of mir-411-5p and functional annotation analysis

Target prediction analysis identified 82 commonly predicted genes (using data from 4 databases: MirWalk, miRanda, mirDB and TargetScan) that may be regulated by mir-411-5p (**Fig. 2**).

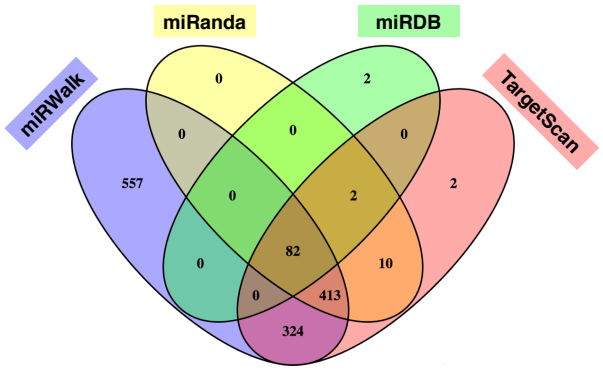


Figure 2: Venn diagram of predicted targets for mir-411-5p using 4 different databases (MirWalk, miRanda, mirDB and TargetScan).

Table 1 presents a list of the 82 commonly predicted targets. Interestingly, *Dusp1*, a negative regulator of the MAP kinase pathway, a major signalling pathway involved in neuronal plasticity,

function and survival, which was previously implicated in the pathophysiology of depression was one of the commonly predicted targets of mir-411-5p (Duric et al., 2010). This gene was also significantly regulated by chronic stress and antidepressants in our previous microarray analysis (Patricio et al., 2015).

Table 1: List of commonly predicted targets of mir-411-5p using 4 different databases (MirWalk, miRanda, mirDB and TargetScan).

Gene symbol	EntrezID	RefseqID
Acad11	315973	NM_001108181
Acox2	252898	NM_145770
Atad5	303348	XM_220750
Atm	300711	NM_001106821
Auh	361215	NM_001108407
Baz1a	314126	NM_001170568
Bnip3	84480	NM_053420
Btaf1	368042	NM_001191917
Camsap1	296580	NM_001168549
Casc4	362204	XM_001077018
Cpt1a	25757	NM_031559
Cript	56725	NM_019907
Cwf19l2	362804	NM_001135003
Dhx36	310461	NM_001107678
Dusp1	114856	NM_053769
Dusp15	362238	NM_001108598
Eif4g2	361628	NM_001017374
Elfn1	288512	NM_001105913
Erap1	80897	NM_030836
Fam110c	500638	NM_001025051
Fam160a1	365834	NM_001134592
Fam45a	308009	NM_001127681
Fasn	50671	NM_017332
Fryl	364144	XM_002724960
Fzd6	282581	NM_001130536
Gclm	29739	NM_017305
Gpr6	83683	NM_031806
Grm2	24415	NM_001105711
Hnrnpul1	361522	NM_001108477
Iqub	296936	NM_001034130
Kdm4b	301128	NM_001044236
Kdr	25589	NM_013062
Klf11	313994	NM_001037354
Lect1	81512	NM_030854

Lmbr1	362295	NM_001191858
Lrif1	310775	NM_001127485
Lrrfip1	367314	NM_001014269
Map2k1	170851	NM_031643
Ms4a2	25316	NM_012845
Mtf2	360905	NM_001100898
Myrf	293736	NM_001170487
Ndrg1	299923	NM_001011991
Nrbp1	619579	NM_001034997
Ogn	291015	NM_001106103
Pcdha1	393085	NM_199503
Pcdha10	116778	NM_053939
Pcdha11	394223	NM_199486
Pcdha12	116779	NM_053940
Pcdha13	116742	NM_053934
Pcdha2	393086	NM_199504
Pcdha3	116780	NM_053941
Pcdha4	116741	NM_053933
Pcdha5	393087	NM_199505
Pcdha6	393088	NM_199506
Pcdha7	393089	NM_199507
Pcdhac1	393091	NM_199509
Pcdhac2	393092	NM_201422
Pex11a	85249	NM_053487
Ppapdc2	619549	NM_001034854
Ptx3	689388	NM_001109536
Pxdc1	361238	NM_001025719
Rab21	299799	NM_001004238
Rbm39	362251	NM_001013207
RGD1560455	302377	NM_001106939
Rps6ka3	501560	NM_001192004
Sema5a	310207	NM_001107659
Serinc5	170907	NM_133395
Sf3b3	292019	NM_001106187
Smarca2	361745	NM_001004446
Snrk	170837	NM_138833
Spry4	291610	NM_001106150
Stx12	65033	NM_022939
Syndig1	362235	NM_001025020
Tmem144	361968	NM_001108551
Tph2	317675	NM_173839
Trmt10b	298081	NM_001013090
Uba3	117553	NM_057205
Ube2e1	100366017	XM_002725032
Xcr1	301086	NM_001106871

Zfand5	293960	NM_001106356
Zfp867	100125361	NM_001109677
Zfr	365703	XM_001056210

Assuming that miRNAs regulatory function is associated with the biological relevance of its targets we performed a functional annotation analysis of mir-411-5p targets, using Panther Classification System. To help cluster the predicted target genes we performed protein classes' analysis and pathway enrichment analysis. The top 10 enriched protein classes are reported in **Table 2**. The most represented categories were Receptor (PC00197), Transferase (PC00220), Nucleic acid binding (PC00171) and Cell adhesion molecule (PC00142).

Table 2: Top 10 enriched protein classes given by Panther Classification System (Mi et al., 2013).

	Category name	**# genes**	**% gene hit against total # genes**	**% gene hit against total # protein class hits**
1	Receptor (PC00197)	17	21.30%	19.30%
2	Transferase (PC00220)	10	12.50%	11.40%
3	Nucleic acid binding (PC00171)	10	12.50%	11.40%
4	Cell adhesion molecule (PC00069)	9	11.30%	10.20%
5	Ligase (PC00142)	5	6.30%	5.70%
6	Hydrolase (PC00121)	5	6.30%	5.70%
7	Oxidoreductase (PC00176)	4	5.00%	4.50%
8	Signalling molecule (PC00207)	4	5.00%	4.50%
9	Transcription factor (PC00218)	4	5.00%	4.50%
10	Kinase (PC00137)	4	5.00%	4.50%

Pathways enrichment analysis revealed two pathways with increased percentage of gene hit, the Cadherin signalling pathway and the Wnt signalling pathway (**Fig. 3**). Interestingly, both pathways

have been implicated in neural plasticity phenomena. Wnt signalling pathway is widely involved in adult neurogenesis and synaptic formation (Lie et al., 2005, Budnik and Salinas, 2011). Cadherins have been shown to play a relevant role in synaptic activities, morphogenesis and neurite extension processes (Suzuki and Takeichi, 2008).

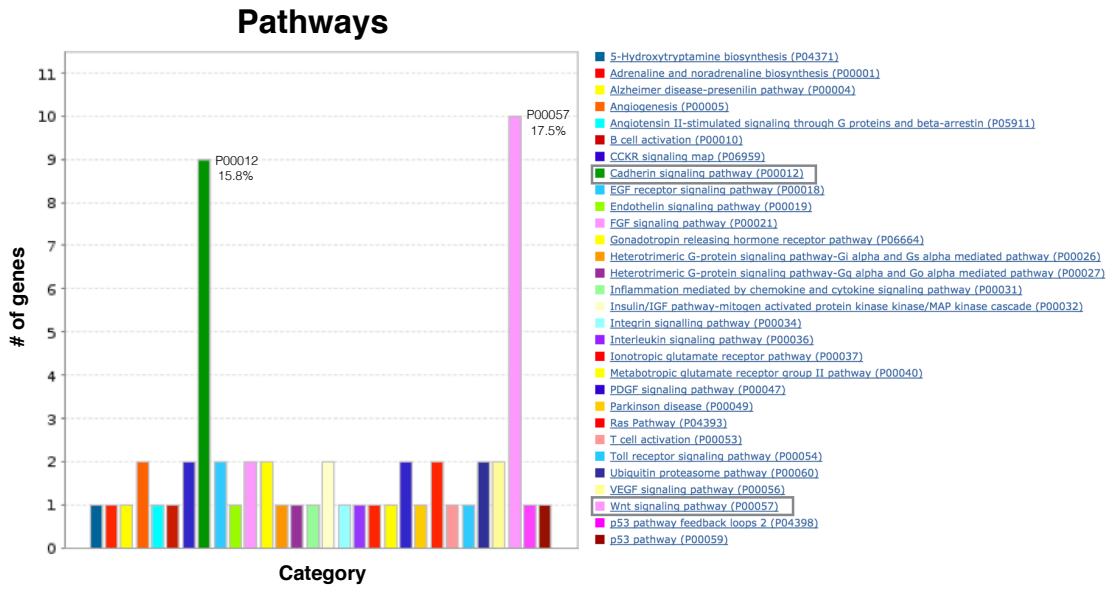


Figure 3: Enriched pathways identified by Panther Classification System in mir-411-5p predicted targets. Percentage on top of the bars represents percentage of gene hit against total number of pathway hits.

In summary, this *in silico* analysis of the protein classes and molecular pathways targeted by miR-411-5p suggest a role for this molecule in the regulation of neural plasticity phenomena. Indeed, our previously published data shows that uCMS exposure produces an atrophy of dendrites in the dorsal part of the hippocampal DG whereas fluoxetine treatment in these animals reverses the dendritic atrophy (Patricio et al., 2015). Here, we show that the atrophy in the dDG of uCMS animals is accompanied by an up-regulation in miR-411-5p, whereas the reversion by fluoxetine is followed by miR-411-5p downregulation. Together, these results suggest an implication of miR-411 in the mediation of neural plasticity-related changes observed in the dorsal DG of these animals. In the future, co-localization experiments using specific *in situ* probes for mir-411 and functional assays

using pre-miRs for upregulation and anti-miRs for downregulation, may further disclose the relevance of this molecule for the neuroplasticity phenomena occurring in this context.

MATERIALS AND METHODS

Animals

Male Wistar rats (200–250g, 2-month old; Charles River Laboratories) were maintained under standard laboratory conditions (lights on: 08:00–20:00 hours; 22 ◦C, relative humidity of 55%, *ad libitum* access to food and water).

Unpredictable Chronic Mild Stress (uCMS)

Rats were randomly assigned to one of the following groups: non-stressed control (CT) vehicle; stress- exposed (uCMS) vehicle; and uCMS fluoxetine (uCMS+FLX) (n=6-9 per group). A validated uCMS protocol was applied for 6 weeks, as previously described (Bessa et al, 2009b). During the last 2 weeks of the uCMS, animals were injected daily with fluoxetine (intraperitoneal injection; 10 mg/kg in ultra-pure water; Kemprotec). Fluoxetine dose was chosen based on previous studies (Bessa et al., 2009, Patricio et al., 2015). All procedures were carried out in accordance with the EU Directive 2010/63/EU on animal care and experimentation.

Behavioral assessments (data not shown)

Behavioral profile to assess the efficacy of the uCMS protocol was performed as previously described (Patricio et al., 2015). Data from sucrose consumption test, sweet drive test and novelty suppressed feeding test confirmed the development of depressive- and anxiety-like behavior. Chronic fluoxetine treatment restored the behavior profile of these animals.

Brain macrodissection

Dorsal and ventral DG were collected 24 h after the last stressor/AD injection. Animals were first anesthetized with pentobarbital and transcardially perfused with 0.9% saline. Immediately after

dissection, tissues were frozen and stored at -80 °C until further analysis. To avoid experimenter-dependent bias, brains were macrodissected by a single investigator.

Blood collection

Blood was collected by tail venipuncture between 8 and 9 am, at the end of the sixth week of the uCMS protocol (n=3 per group).

MiRNA isolation and Real-time PCR

Total RNA, including miRNAs, was isolated from the macrodissected DGs and blood using the Direct-zol™ RNA MiniPrep (ZymoResearch), according to the manufacturer's instructions. RNA samples were treated with qScript™ microRNA cDNA Synthesis Kit (Quanta Biosciences) to generate cDNA. QRT-PCR was performed using PerfeCTa microRNA assay for miRNA-411-5p (Quanta Biosciences) and PerfeCTa Universal PCR primer (Quanta Biosciences). Samples were analyzed using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX, Solis BioDyne), according to the manufacturer's instructions, in an AB7500 fast Real Time PCR system (Applied Biosystems). U6 Small nuclear RNA (RNU6) was used as internal reference (Control Assay, Quanta Biosciences). Results are presented as fold change ($2^{\Delta\Delta_{CT}}$) of control samples.

MiRNA target prediction and functional annotation analysis

For computational prediction of miRNAs target genes, we used MirWalk web platform database tools (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) (Dweep et al., 2011, Dweep and Gretz, 2015). This tool incorporates the databases of other established programs for miRNA targets prediction. In the present study, the following databases were included for targets prediction: mirWalk, miRanda, mirDB and TargetScan. Venn diagram was created with Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The commonly predicted targets by the four databases for the 3'UTR region of the genome were considered for further functional analysis. For functional annotation of miRNA targets PANTHER Classification System was used.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The underlying assumptions of all statistical procedures were assessed. The normal distribution was tested using the Kolmogorov–Smirnov test. Student's t-test was used to assess differences between Control and uCMS groups, and between uCMS and uCMS+FLX groups Test statistics and p-values are shown for each test. Significance was set at $p<0.05$.

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APPENDIX IV – P.1.018 Cell cycle regulation of the progenitor cells from the adult hippocampal dentate gyrus in depression and by antidepressants

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P.1.018 Cell cycle regulation of the progenitor cells from the adult hippocampal dentate gyrus in depression and by antidepressants

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Depression is a complex and multidimensional disorder affecting around 20% of the world population. Alterations in hippocampal dendritic morphology and in adult hippocampal cell proliferation and cell genesis are known to be involved in the pathophysiology of the disorder and in the actions of antidepressants (ADs). Indeed, decreased cell genesis and dendritic atrophy have been detected in the hippocampal neurogenic niche of depressed individuals, whereas ADs treatment was shown to prevent these damages. Previous genome-wide studies by our group, using microarray analysis of macrodissected hippocampal dentate gyrus (hDG) in a rat model of depression (uCMS – unpredictable chronic mild stress), have disclosed differential molecular regulation by different classes of ADs (fluoxetine, imipramine, tianeptine and agomelatine). Moreover, these results were correlated with relevant physiological parameters to unveil the integrated network that maintains homeostasis in the hippocampal niche [1]. Following this study, the cell cycle mechanisms regulating hippocampal cell proliferation and cell genesis were investigated using both *in vivo* (macrodissected hDG) and *in vitro* (P5 rat hippocampal-derived neurospheres culture) approaches. The hDG of uCMS animals presented a slight increase in the percentage of cells in G1 phase of the cell cycle. Accordingly, these cells had decreased expression levels of cyclin D1 compared to control animals, whereas uCMS+fluoxetine-treated animals reversed these levels to those of controls. In order to better characterize these responses, we moved to an *in vitro* system – the neurospheres culture. This system allowed us to enrich our population in hippocampal-derived progenitor cells, as most cells in the hDG are post-mitotic and could be misleading our interpretation of the results. Dexamethasone (glucocorticoid receptor agonist; 1microM) and corticosterone (promotes activation of both glucocorticoid and mineralocorticoid receptors; 1microM) were applied to the neurospheres cultures for 6 days, to mimic the effects of glucocorticoids elevation occurring in the brains of depressed individuals and animal models of depression. Moreover, neurotransmitters involved in the action of monoaminergic ADs (norepinephrine and serotonin) or ADs active metabolites (norfluoxetine and desipramine) were applied to the dexamethasone/corticosterone-treated cells, in the last 4 days of culturing. PI staining was used for cell cycle distribution assessment by flow cytometry. A G1 phase arrest was detected in the Dexamethasone-treated progenitor cells. A partial reversion of this arrest was observed after the addition of neurotransmitters to the cells. The expression of G1 phase cell cycle regulators, such as the cyclin-dependent kinase inhibitors p21 and p27, was significantly increased in the dexamethasone-treated cells. Interestingly, two other atypical cell cycle regulators, Cdk5 and its activator p35, were found to be increased by Dexamethasone exposure. These results

suggest a mechanism for the regulation of hDG progenitor cells proliferation upon glucocorticoids increase. Studies are now being conducted to further elucidate the role of these and other cell cycle molecules in the regulation of proliferative phenomena implicated in the pathophysiology of depression and AD treatment.

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APPENDIX V - S.15.06 Cell cycle regulation of the progenitor cells from the adult hippocampal dentate gyrus in depression and by antidepressants

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S.15.06 Cell cycle regulation of the progenitor cells from the adult hippocampal dentate gyrus in depression and by antidepressants

P. Patricio[1]*, A. Mateus-Pinheiro[1], A. Machado-Santos[1], N. Alves[1], M. Morais[1], J. Bessa[1], N. Sousa[1], L. Pinto[1] [1]*Life and Health Sciences Research Institute ICVS – School of Health Sciences – University of Minho, Neuroscience Research Domain, Braga, Portugal*

Depression is a complex multidimensional disorder affecting around 20% of the world population. Changes in adult hippocampal cell proliferation and genesis are implicated in the pathophysiology of depression and in the antidepressants (ADs) action [1]. Using both in vivo (macrodissected rat hippocampal Dentate Gyrus; hDG) and in vitro (rat hippocampal-derived neurospheres culture) approaches, we aim to unravel the cell cycle mechanisms regulating hippocampal cell proliferation and genesis in the context of depression. Our data shows that the hDG of a rat model of depression (uCMS) presents increased percentage of cells in G1 phase of the cell cycle. Accordingly, these cells presented decreased levels of cyclin D1 expression, compared to control animals; antidepressant treatment reversed these levels to those of control animals. To better characterize these mechanisms the neurospheres culture system was used. Dexamethasone was applied to these cultures to mimic the glucocorticoids elevation occurring in the brains of depressed individuals and animal models of depression. Moreover, neurotransmitters involved in the action of monoaminergic ADs or ADs active metabolites were applied to these cells. A G1 phase arrest was detected in the dexamethasone-treated cells, whereas neurotransmitters partly reversed this arrest. The expression of G1 phase regulators, cyclin-dependent kinase inhibitors p21 and p27, was significantly increased in the dexamethasone-treated cells. Interestingly, two other atypical cell cycle regulators, Cdk5 and its activator p35, were also increased after dexamethasone treatment. Further analysis are being conducted to disclose the regulatory mechanisms involved in hDG progenitor cells proliferation upon glucocorticoids increase and after ADs treatment.

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