Universidade do Minho
Escola de Medicina

Mónica Susana Dias Morais

New insights on the interplay between psychopharmacology and neuroplasticity in psychiatric disorders

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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação do
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“The measure of intelligence is the ability to change.”

Albert Einstein

ABSTRACT

It is now clear that various forms of structural plasticity, including the generation of new neurons and glial cells, may modify pathophysiological processes in neuropsychiatric disorders, namely in depression. In fact, several studies have shown decreased hippocampal neurogenesis in depressed patients, while treatment with different antidepressant drugs in animal models increases neurogenesis in this region, allowing the recovery from emotional and cognitive changes. However, these effects have not been described for all the available classes of antidepressant drugs. Furthermore, the neuroplastic effects of antidepressants in other neurogenic regions such as the hypothalamus have yet to be determined. Despite the importance of these drugs in the recovery from depression, a significant proportion of depressed patients reveal incomplete remission and develop treatment-resistant forms of the disorder. The use of atypical antipsychotics in these cases has been widely used in the clinical setting. However, the neuroplastic effects of these drugs in depression and schizophrenia are still largely unknown. Taking this into consideration we aimed to explore new perspectives on the interplay between psychopharmacology and neuroplasticity in these psychiatric disorders.

To explore the neuroplastic effects of the antidepressant Pirlindole, a MAO-A (monoamine oxidase, type A) inhibitor, we used the unpredictable chronic mild stress (uCMS) animal model of depression. Our results indicate that Pirlindole is able to reverse the behavioural effects of stress exposure, potentiating hippocampal adult neurogenesis and rescuing the stress-induced dendritic atrophy of granule neurons in the dentate gyrus of the hippocampus. These results further reinforce the notion that the modulation of monoaminergic neurotransmission is involved in the neuroplastic effects of currently available antidepressant drugs.

To dissect the potential actions of antidepressants in adult neurogenesis in the hypothalamus we treated animals exposed to uCMS with two different classes of antidepressants: fluoxetine (a selective serotonin reuptake inhibitor) and imipramine (a tricyclic antidepressant). Our results demonstrate that chronic stress and antidepressant treatment can modulate hypothalamic neurogenesis. Moreover, we proved that different classes of antidepressants, with an opposite action on appetite and body weight gain, differentially modulate hypothalamic neurogenesis. This data indicates that in addition to the neuroplastic effects on the hippocampus, stress and antidepressant drugs also modulate hypothalamic adult neurogenesis.

Furthermore, we explored the role of neuroplasticity in the therapeutic actions of atypical antipsychotics in depression. To achieve this, we treated animals exposed to uCMS with a classical (haloperidol) and an atypical (clozapine) antipsychotic. Our data demonstrates that the atypical antipsychotic clozapine improved measures of depressive-like behavior while haloperidol had no beneficial effect, aggravating learned helplessness in the forced swimming test and behavior flexibility in a cognitive task. Importantly, an upregulation of adult neurogenesis and neuronal survival was observed in animals treated with clozapine while haloperidol promoted a downregulation of these processes. These results demonstrate that the atypical antipsychotic is able to reverse the behavioral effects of chronic stress by improving adult neurogenesis, cell survival and neuronal reorganization.

Finally, to understand the impact of different classes of antipsychotics in the negative and cognitive symptoms of schizophrenia, we used a neurodevelopmental model of schizophrenia. Animals expose prenatally to the cytostatic agent methylazoxymethanol (MAM) presented specific cognitive deficits and social impairments. The classical antipsychotic haloperidol presented no beneficial effects in these behavioral dimensions. The atypical antipsychotic clozapine and risperidone revealed a positive effect on both dimensions while aripiprazole presented a significant effect in the social measure. Adult gliogenesis is affected in animals exposed to MAM, being modulated by the atypical antipsychotics used. Neurogenesis is not altered in MAM animals, with haloperidol negatively affecting this phenomenon. In this work, we proved that classical and atypical antipsychotics differentially modulate hippocampal cell genesis possibly contributing to different behavioural actions in hippocampal dependent functions.

Together, these findings contribute to expand our knowledge on the role of psychopharmacological agents (including antidepressants and antipsychotics) on the modulation of different neuroplastic events, including cell genesis and neuronal remodelling. In the future, this knowledge may help to pave the way for new therapeutic interventions both in depression and schizophrenia.

Keywords: depression; schizophrenia; neuroplasticity, neurogenesis; gliogenesis.

RESUMO

É actualmente sabido que várias formas de plasticidade estrutural, incluindo a formação de novos neurónios e células da glia, podem modificar o processo patofisiológico em algumas doenças neuropsiquiátricas, nomeadamente a depressão. De facto, vários estudos têm demonstrado uma redução da neurogénese hipocampal em pacientes com depressão. Para além disso, o tratamento com diferentes fármacos, nomeadamente os antidepressivos, revelou em modelos animais, aumentar a neurogénese nesta região cerebral, permitindo uma recuperação na componente emocional e cognitiva. Contudo, estes efeitos não estão descritos para todas as classes de antidepressivos disponíveis. Além disso, os efeitos dos antidepressivos na neuroplasticidade em outras regiões neurogénicas como o hipotálamo ainda não foram determinados. Apesar da importância destes fármacos na recuperação da depressão, uma proporção significativa de pacientes revela remissão incompleta bem como formas resistentes ao tratamento da doença. Nestes casos, o uso de antipsicóticos atípicos tem sido amplamente utilizado no contexto clínico. No entanto, os efeitos destes fármacos na neuroplasticidade na depressão e esquizofrenia são ainda amplamente desconhecidos. Tendo isto em consideração, propusemos explorar novas perspectivas na inter-relação entre a psicofarmacologia e a neuroplasticidade nestas doenças psiquiátricas.

Para determinar os efeitos do antidepressivo inibidor da MAO-A (monoamina oxidase, tipo A) Pirlindol na neuroplasticidade, utilizamos o modelo animal de exposição a stress crónico moderado e imprevisível (uCMS). Os nossos resultados indicam que o Pirlindol é capaz de reverter os efeitos comportamentais de exposição ao stress, potenciando o aumento na neurogénese no hipocampo adulto e recuperando igualmente a atrofia dendrítica induzida pelo stress em neurónios granulares no giro dentado do hipocampo. Estes resultados reforçam a ideia de que a modulação da neurotransmissão monoaminérgica está envolvida nos efeitos neuroplasticos promovida pelos antidepressivos.

Para dissecar, as acções dos antidepressivos na neurogénese hipotalâmica, utilizamos o modelo animal de depressão uCMS e tratamento com diferentes classes de antidepressivos: fluoxetina (um inibidor selectivo da reabsorção de serotonina) e imipramina (um antidepressivo tricíclico). Os nossos resultados demonstram que o stress crónico e o tratamento com antidepressivos modulam a neurogénese no hipotálamo. Além disso, provou-se que diferentes classes de antidepressivos, com uma acção contrária no apetite e no ganho de peso corporal, modulam a neurogénese no hipotálamo

de uma forma diferencial. Estes resultados, indicam que, para além do hipocampo, o stress e os antidepressivos também modulam a neurogénese no hipotálamo adulto.

Em seguida, exploramos o papel da neuroplasticidade nas acções terapêuticas dos antipsicóticos atípicos na depressão. Para isso, utilizou-se o modelo animal de depressão uCMS e tratamento com o antipsicótico clássico (haloperidol) e o atípico (clozapina). Os nossos dados demonstram que, o antipsicótico atípico clozapina melhorou o comportamento depressivo. Por outro lado, o haloperidol não teve qualquer efeito benéfico, agravando o desalento aprendido no teste de natação forçada e a flexibilidade comportamental numa tarefa cognitiva. Simultaneamente observou-se um aumento da neurogénese adulta e da sobrevivência neuronal em animais tratados com clozapina, enquanto que o haloperidol promoveu uma redução nestes processos. Estes resultados, demonstram que o antipsicótico atípico é capaz de reverter os efeitos comportamentais do stress crónico através da melhoria na neurogénese adulta, da sobrevivência celular e da reorganização neuronal.

Por último, para compreender o impacto das diferentes classes de antipsicóticos nos sintomas negativos e cognitivos da esquizofrenia, utilizamos um modelo de neurodesenvolvimento desta mesma patologia. Os animais expostos no período pré-natal ao agente acetato de metilazoximetanol (MAM) apresentaram défices cognitivos e sociais. O antipsicótico clássico haloperidol não apresentou efeitos benéficos nestas dimensões de comportamento. Por outro lado, os antipsicóticos atípicos, clozapina e risperidona, apresentaram um efeito positivo em ambas as dimensões com o aripiprazol apresentando apenas um efeito estatístico na dimensão social. A gliogénese adulta esta afectada nos animais MAM, sendo esta modulada pelos antipsicóticos atípicos usados. A neurogénese não se encontra alterada neste modelo, sendo, no entanto, negativamente afectada pelo haloperidol. Neste trabalho, provamos que os antipsicóticos clássicos e atípicos modulam diferencialmente a formação de novas células no hipocampo, contribuindo possivelmente para diferentes acções comportamentais, em funções dependentes do hipocampo.

Em suma, estes resultados contribuem para expandir o nosso conhecimento sobre o papel de agentes psicofarmacológicos (incluindo antidepressivos e antipsicóticos) na modulação de diferentes eventos neuroplásticos, incluindo a formação de novas células e a remodelação neuronal. No futuro, este conhecimento poderá ajudar na implementação de novas intervenções terapêuticas tanto na depressão como na esquizofrenia.

Palavras-chave: depressão, esquizofrenia; neuroplasticidade; neurogénese; gliogénese

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ABBREVIATIONS LIST:

5-HT serotonin

5-HT_{1A}

5-HT_{2}

5-HT_{2A}

5-HT_{2C}

A

AVP vasopressin

ACTH adrenocorticotrophic hormone

AD antidepressant

ARC arcuate nucleus

AR: adrenergic receptor

B

BDNF brain-derived neurotrophic factor

BrdU bromodeoxyuridine

C

CA cornu ammonis

CMS chronic mild stress

CNS central nervous system

CNTF ciliary neurotrophic factor

D

DCX doublecortin

DG dentate gyrus

DISC1 disrupted in schizophrenia 1

DMS-5 Diagnostic and Statistical Manual of Mental Disorders (DSM) 5th edition

Drd dopamine receptors

E

E17 embryonic day 17

EPS extrapyramidal symptoms

F

FDA Food and Drug Administration

FST forced swim test

G

GFAP glial fibrillary acidic protein

GR glucocorticoid receptor

H

HPA hypothalamic pituitary adrenal axis

I

i.p intraperitoneally

L

LTP long-term potentiation

LTD long-term depression

M

MAM methylooxymethanol acetate

MAO monoamine oxidase

MAOi monoamine-oxidase inhibitor

MDD major depressive disorder

ME median eminence

MR mineralocorticoid

MT Melatonin

MWM Morris Water Maze

N

NCAM neural cell adhesion molecule

NE norepinephrine

NK natural killer cells

NGF nerve growth factor

NPAS3 Neuronal PAS Domain Protein 3

NMDA *N*-methyl-D-aspartic acid

NPY neuropeptide Y

NOR novel object recognition

NSC neural precursor cell

NSF novelty suppressed feeding

NT-3 neurotrophin 3

NT-4 neurotrophin 4

NT-5 neurotrophin 5

O

OCT optimal cutting temperature

OF open field

P

PCP Phencyclidine

PCR polymerase chain reaction

PFC prefrontal cortex

poly (I:C) polyriboinosinic-polyribocytidilic acid

POMC pro-opiomelanocortin

PPI Prepulse inhibition test

PSA-NCAM Polysialic acid-NCAM

Q

qPCR quantitative real-time polymerase chain-reaction

R

Rima Reversible MAO-A inhibitors

RNA ribonucleic acid

S

SEM standard error of the mean

SEZ subependymal zone

SGZ subgranular zone

SNRI serotonin-norepinephrine reuptake inhibitors

SPT sucrose preference test

SPT sucrose consumption test

SSRI selective serotonin reuptake inhibitor

Syn1 synapsin 1

T

TCA tricyclic agent

TrkB

TRD treatment-resistant depression

U

uCMS unpredictable chronic mild stress

THESIS ORGANIZATION

The present thesis is divided into 6 chapters. The 1st Chapter is an overall introduction to the theme. The chapters concerning the experimental work are presented in chapter 2, 3, 4 and 5 (in the form of research articles). Chapter 6 is dedicated to an overall discussion and conclusions.

The 2nd Chapter is a published article in the Journal of Psychopharmacology.
In this chapter we explore the neuroplastic effects of the antidepressant Pirlindole, a MAO-A (monoamine oxidase, type A) inhibitor using the unpredictable chronic mild stress (uCMS) animal model of depression.

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The 3rd Chapter is a manuscript under preparation.
We analysed the potential actions of antidepressants (fluoxetine and imipramine) in adult hypothalamic neurogenesis.

The 4th Chapter is a submitted article.
Here we explored the role of neuroplasticity in the therapeutic actions of atypical antipsychotics in depression.

The 5th Chapter is a manuscript under preparation.
We try to understand the impact of different classes of antipsychotics in the negative and cognitive symptoms of schizophrenia.

1st CHAPTER

Introduction

1 Introduction

1.1 Adult neuroplasticity

Neuroplasticity is one of the most fascinating abilities of the mammalian brain, representing the capability of the central nervous system to modify and adapt in response to changes in its inputs. It includes different time-dependent events occurring at two different levels: functional and structural. Functional changes at the synaptic level are thought to be more frequent and rapid than the formation of new cellular components in structural plasticity (Bruel-Jungerman *et al*, 2007a; Bruel-Jungerman *et al*, 2007b; Sagi *et al*, 2012). The temporal dynamics of structural plasticity is largely unknown, with cell genesis (including neurogenesis and gliogenesis) occurring in days to weeks whereas the formation of new synapses and dendrites on the existing neurons develops in short periods of time (Bruel-Jungerman *et al*, 2007b).

Synapses are specialised structures that mediate the electro-chemical communication between neurons. Synaptic plasticity is described as a critical process that confers environmental adaptability through modification of the connectivity between neurons and neuronal circuits. The best studied forms of synaptic plasticity are long-term potentiation (LTP) and long-term depression (LTD), which occur both at excitatory and inhibitory synapses throughout the brain (Markram *et al*, 2011; Mendez and Bacci, 2011) and constitute the cellular basis of hippocampal-dependent learning and memory (Ge *et al*, 2010).

Neuronal remodelling comprises the growth and retraction of dendrites and axons; and also the formation and deletion of synapses, including dendritic spines and axonal boutons. Dendritic spines are specialized subcellular compartments where excitatory synapses are located. They are highly dynamic not only during development but also in the mature nervous system and largely heterogeneous in both size and shape. The morphology of dendritic spines can be generally classified into four different classes: mushroom shaped, thin, wide and ramified spines (Harris *et al*, 1992). Spine formation, turnover and morphology are continuously influenced by synaptic activity. Structural plasticity is traditionally studied using post-mortem histological samples (Lamprecht and LeDoux, 2004; Theodosis *et al*, 2008) including Golgi (a widely used technique based on metallic impregnation, introduced by Ramon Cajal in 1873). Essentially, it randomly labels a small number of cells in their entirety so that detailed information regarding dendritic branching, length, and spine density can be measured. Since more than 90% of excitatory synapses are formed on dendritic spines (Nimchinsky *et al*, 2002), dendritic spine numbers obtained from Golgi-stained tissue provide an indirect measure of excitatory

synaptic inputs. However, the quantification of the actual number of synapses can only be achieved using electron microscopic procedures.

There is a clear link between this type of structural plasticity and synaptic plasticity. In fact, the volume of a dendritic spine correlates with the synaptic efficacy of the corresponding synapse (Knott *et al*, 2006; Matsuzaki *et al*, 2001; Zito *et al*, 2009) which, in turn, is influenced by synaptic plasticity. Accordingly, stimuli causing LTP also cause spine enlargements (Okamoto *et al*, 2004; Yang *et al*, 2008) while stimuli causing LTD induce spine shrinkage (Oh *et al*, 2013; Okamoto *et al*, 2004; Zhou *et al*, 2004).

Distinct brain regions undergo different forms of plasticity, namely the hippocampus, amygdala and prefrontal cortex (PFC). Importantly, the plastic changes in these brain regions have been reported to be involved in multiple functional dimensions such as perception, emotional processing and cognition.

1.1.1 Adult Neurogenesis

During the majority of the twentieth century, the adult brain was considered as a static structure, with no capacity self-renewal: no neuronal cells arising *de novo* (Ramon y Cajal 1913). In 1965, Altan and Das provided the first anatomical evidence for the presence of newly generated cells in the postnatal rat hippocampus (Altman and Das, 1965). However, little attention was given to this finding at the time, in part because they were considered to lack functional relevance. In the late 1970s, the issue of adult neurogenesis was revisited with the study of Kaplan & Hinds (1977) showing the survival of newly born hippocampal cells. Additionally, these cells also appeared to receive synaptic inputs (Kaplan and Bell, 1983) and extend axon projections to their target area (Stanfield and Trice, 1988).

The field of adult neurogenesis was revolutionized after the introduction of bromodeoxyuridine (BrdU), a synthetic thymidine analogue that incorporates DNA of dividing cells during the S-phase of the cell cycle (Gratzner, 1982). Since then, immunocytochemistry for BrdU cell detection is commonly used to monitor cell proliferation, new cell survival and differentiation. The introduction of this marker led to important findings in the field, namely the confirmation of adult neurogenesis in mammals, including the human brain (Eriksson *et al*, 1998). Additionally, the combination of retroviral-based lineage tracing (Price *et al*, 1987; Sanes *et al*, 1986) and electrophysiological studies provided the most convincing evidence so far that newborn neurons in the adult mammalian brain are indeed functional and synaptically integrated. Nevertheless, the functional significance of adult neurogenesis is still far from being completely explained.

Adult neurogenesis is defined as a complex and multi-step process by which neural progenitor cells divide mitotically to produce new functional neurons in the adult brain. It has been widely accepted that adult neurogenesis occurs at least in two regions of the mammalian brain, namely the subependymal zone (SEZ) and the subgranular zone of the dentate gyrus (DG) of the hippocampus (Kempermann and Gage, 2000; Kempermann *et al*, 2015). In the SEZ, the neuroblasts generated migrate along the rostral migratory stream to differentiate into interneurons in the olfactory bulb (Luskin, 1993; Whitman and Greer, 2009). In the hippocampus, the newly formed cells migrate into the DG granule cell layer where they differentiate into mature neurons and integrate into the existing hippocampal circuitry (Doetsch and Hen, 2005; Jessberger and Kempermann, 2003; Laplagne *et al*, 2006; Schinder and Gage, 2004; van Praag *et al*, 2002). In rodents, the levels of neurogenesis are higher in the SEZ than in the hippocampus while In humans, adult neurogenesis has been conclusively demonstrated in the hippocampus (Eriksson *et al*, 1998), with no detectable adult olfactory bulb neurogenesis (Bergmann *et al*, 2012; Sanai *et al*, 2011). It may appear that during human evolution, hippocampal neurogenesis has been retained to provide adaptability to hippocampal dependent tasks; in contrast olfactory bulb neurogenesis has decreased with the reduced dependence on olfactionref. The hippocampus is a brain region with a critical role in cognitive function (learning and memory) and emotional processing (Fanselow and Dong, 2010; Kheirbek and Hen, 2010). Interestingly, several studies have described a positive correlation between the levels of neurogenesis and learning, memory and mood regulation. In fact, the exposure of rats to an enriched environment (Kempermann *et al*, 1997a; van Praag *et al*, 2002) or running activity (Brown *et al*, 2003 ; van Praag *et al*, 1999; van Praag *et al*, 2005) was correlated with increased levels of hippocampal neurogenesis, leading to improved performance on a water-maze test of spatial memory (a test highly sensitive to hippocampal impairment).

Besides these two classical neurogenic regions, there is evidence for adult neurogenesis in additional areas, including the striatum (Bedard *et al*, 2006) (Dayer *et al*, 2005 ; Ernst *et al*, 2014) amygdala (Fowler *et al*, 2005; Fowler *et al*, 2002; Gould *et al*, 1999), substantia nigra (Zhao *et al*, 2003) and hypothalamus (Huang *et al*, 1998; Kokoeva *et al*, 2005; Lee *et al*, 2012; Lee *et al*, 2014; Xu *et al*, 2005). However, this has been difficult to replicate consistently other than in the damaged brain. Interestingly, from these brain areas the hypothalamus has been receiving much more attention and was recently described as a region with cell renewal capacity. Previous studies have shown that a subpopulation of tanycytes can behave as neuronal progenitors and are characterized by a distinct

expression of neural progenitors and stem cell markers (Goodman and Hajihosseini, 2015; Lee *et al*, 2012; Robins *et al*, 2013). The hypothalamus is a small brain structure controlling numerous vital physiological functions such as sleep-wake cycles, body temperature, sexual behavior and food intake. In fact, distinct nuclei in the hypothalamus express different neuropeptides implicated in the regulation of food intake, namely the orexigenic factors agouti-related peptide (AgRP), neuropeptide Y (NPY) and anorexigenic factors like pro-opiomelanocortin (POMC). Despite being a brain region involved in several functions, the newborn hypothalamic neurons have been specifically implicated in the regulation of energy balance (Kokoeva *et al*, 2005; Lee *et al*, 2012; Lee *et al*, 2014). In the first study attributing a functional role for hypothalamic neurogenesis, obese mice were injected with a ciliary neurotrophic factor (CNTF) (a drug that induces a decrease in body weight) resulting in a strong increase in hypothalamic neurogenesis. Moreover, if CNTF was co-administrated with an antimitotic agent to suppress neurogenesis, the body weight loss effect was absent, highlighting the importance of newborn hypothalamic neurons in mediating the CNTF effect on body weight regulation (Kokoeva *et al*, 2005). Besides this neurotrophic factor, other growth factors such as BDNF (Pencea *et al*, 2001), EGF and bFGF (Xu *et al*, 2005) stimulate adult hypothalamic neurogenesis (with unknown functional significance). More recently, Lee and colleagues demonstrated that high-fat diet, that leads to an increase in body weight gain, enhances adult neurogenesis in the hypothalamic median eminence (ME). More interestingly, they found a significant attenuation in body weight gain after the inhibition of neurogenesis in these specific hypothalamic nuclei. This study highlights the role of ME neurogenesis on the promotion of body weight gain in a high-fat diet context (Lee *et al*, 2012). Based on these studies we can hypothesize that neurogenesis in the hypothalamus is triggered by different stimulus mainly involved in the modulation of appetite and energy balance control. In addition, the hypothalamic nuclei that respond by creating new neurons are dependent on the type of stimuli applied. Figure 1 shows a schematic representation of the adult neurogenesis process.

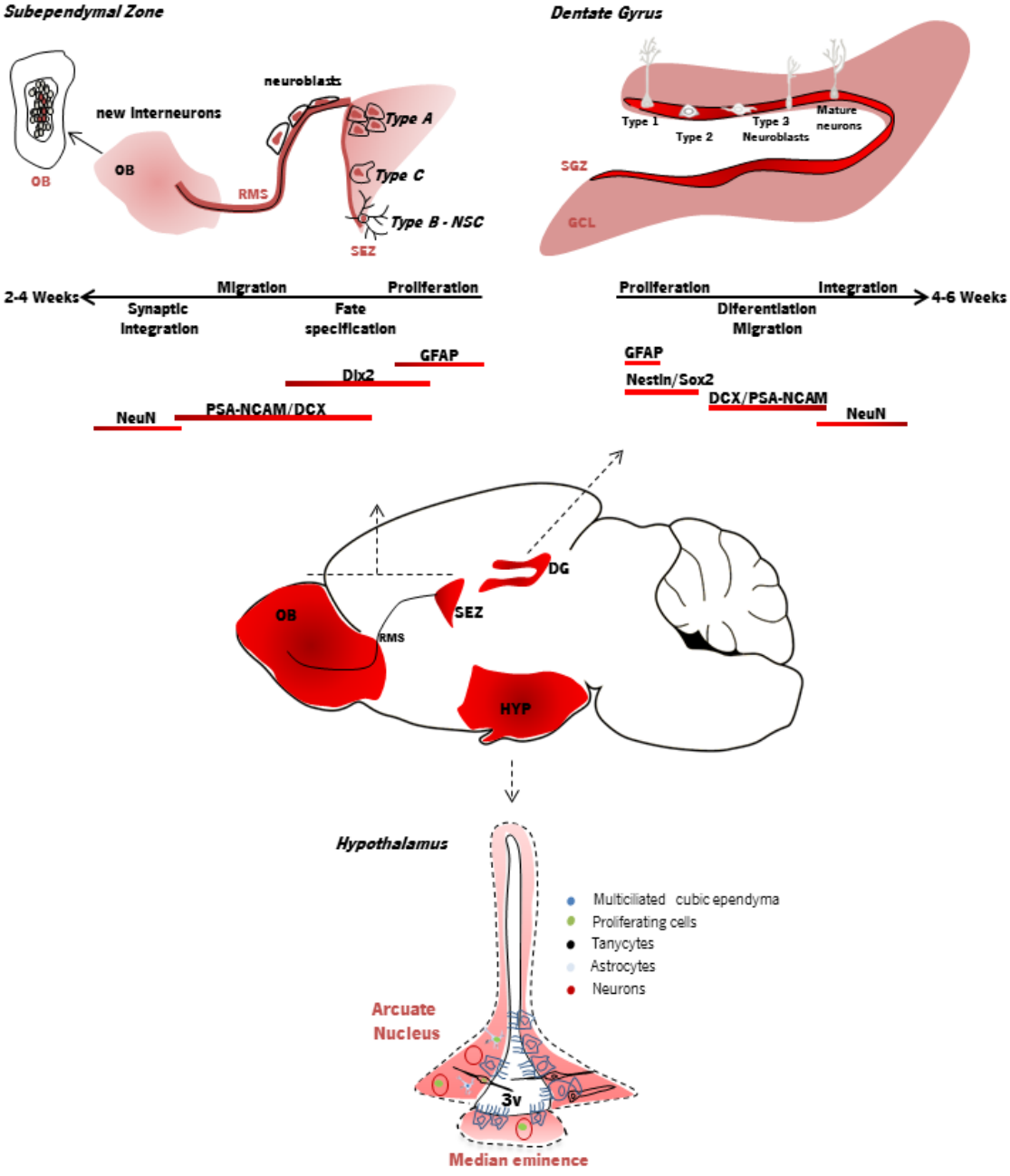

Figure 1 - Schematic representation of the adult neurogenic process in the subependymal zone, dentate gyrus of the hippocampus and arcuate and median eminence nuclei of the hypothalamus.

1.1.1.1 Modulating Factors: The Neurotrophins and HPA axis

Adult neurogenesis is currently viewed as a highly dynamic process regulated by many intrinsic and extrinsic factors. Among these factors, stress (Bessa *et al*, 2009a; Mateus-Pinheiro *et al*, 2013; Morais *et al*, 2014) , age (Kuhn *et al*, 1996) and irradiation (Santarelli *et al*, 2003) are known to be negative regulators of this process. On the other hand, the exposure to odours (Bonzano *et al*, 2014), environmental enrichment (Kempermann *et al*, 1997b), learning (Anderson *et al*, 2011), physical activity (van Praag *et al*, 1999), seizures (Kokaia, 2011; Parent *et al*, 1997) and hypoxia (Zhu *et al*, 2010) are known to be positive regulators of adult neurogenesis.

In recent years, a large number of studies have investigated the role of neurotrophins or neurotrophic factors in adult neurogenesis. Neurotrophins are an important class of extracellular signalling molecules in the brain responsible for axon targeting, neuron growth, maturation of synapses during development and synaptic plasticity. This family includes molecules like the nerve growth factor (NGF) (Levi-Montalcini, 1987), brain-derived neurotrophic factor (BDNF) (Barde *et al*, 1982), as well as neurotrophins 3 (NT-3), 4 (NT-4) and 5 (NT-5) (Hohn *et al*, 1990). BDNF is by far the most well-studied factor of this family, being considered a critical regulator of adult brain plasticity. In addition, in the last decade, an increasing number of studies have associated neurotrophic factors with the pathophysiology of some neuropsychiatric disorders as well as with the mechanisms of action of drugs used for their treatment. Interestingly, BDNF and its receptor tropomyosin receptor kinase (TrkB) have been implicated in the pathophysiology of mood disorders. Indeed, BDNF has gained further interest based on the hypothesis that the action of antidepressant drugs may be related with their effects on hippocampal neurogenesis, being BDNF a central regulator of this process (D'Sa and Duman, 2002; Lepack *et al*, 2014). Support for this 'BDNF hypothesis' has come from a large preclinical literature showing that several forms of stress reduce BDNF-mediated signalling in the hippocampus, whereas chronic treatment with antidepressants increases BDNF (Molendijk *et al*, 2014). Interestingly, similar changes have been observed in the post-mortem hippocampus of humans with depression, as well as in the concentrations of serum BDNF. 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).

Exposure to stress is one of the best-known negative regulators of adult hippocampal neurogenesis (Warner-Schmidt and Duman, 2006). Stress is generally defined as any stimuli that disrupt the body´s

internal control. However, stress is not a single entity and several different types of stressors can be distinguished. In other words, stress can be divided into acute or chronic, it may occur in a single episode or be repetitive in time, can be predictable or unpredictable and also mild or severe (Lucassen *et al*, 2014). The exposure to mild stress for a few hours can enhance cognition by facilitating synaptic plasticity in the hippocampus. In contrast, chronic exposure to stress can have negative effects in neurons due to excessive glucocorticoid exposure (McEwen and Sapolsky, 1995; Sapolsky, 1996).

The hypothalamo-pituitary-adrenal (HPA) axis is one of the main stress response pathways, playing a vital role in mediating and controlling the stress response. The HPA axis activity is governed by the secretion of adrenocorticotrophic hormone-releasing factor (CRF) and vasopressin (AVP) from the hypothalamus, which in turn activate the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary, leading to the stimulation of the secretion of the glucocorticoids (cortisol in humans and corticosterone in rodents) from the adrenal cortex. Corticosteroid actions in the brain are mediated by mineralocorticoid (MR) and glucocorticoid (GR) receptors (Reul and de Kloet, 1985). GR receptors are abundantly expressed throughout the brain but especially enriched in the hippocampus while MR receptors are found primarily in the hippocampus. Regulation occurs through negative feedback after glucocorticoid binding to high-affinity MR and lower affinity GR receptors (de Kloet *et al*, 2005). GR´s help to maintain glucocorticoids levels within physiological limits (Erdmann *et al*, 2008; Kretz *et al*, 1999), and aberrant GR expression has been implicated in hypercortisolism, stress resistance, anxiety and depression (de Kloet *et al*, 2005; Ridder *et al*, 2005; Wei *et al*, 2007). The key biological parameter for measuring stress in an organism is the serum levels of the glucocorticoid hormone. Indeed, high levels of stress lead to high levels of circulating glucocorticoids and may, in the long time, lead to a failure of feedback mechanisms that control glucocorticoid secretion and also GR receptor expression.

Stress has profound effects on synaptic plasticity (Christoffel *et al*, 2011; Popoli *et al*, 2002; Sandi, 2011), with its effects being particularly well-studied in the hippocampus. Stress and glucocorticoid modulation of synaptic plasticity is mediated via activation of MR and GR receptors. Through these receptors, stress and glucocorticoids exert direct effects on neurons and glia cells (Yu *et al*, 2011). The negative effects on synaptic plasticity in the CA1 region of the hippocampus can be prevented or reversed by GR antagonists and monoaminergic antidepressants (Holderbach *et al*, 2007; Krugers *et al*, 2006; Matsumoto *et al*, 2005). Chronic stress is also a risk factor to other diseases such as heart disease, high blood pressure, high cholesterol, type II diabetes and psychiatric disorders. Psychiatric disorders, in particular major depressive disorder (MDD), have been frequently associated with

hyperactivity of the HPA axis and increase levels of glucocorticoid hormones leading ultimately to an impaired HPA axis feedback regulation (Pariante and Lightman, 2008). For instance, a significant percentage of depressed patients have increased levels of cortisol in the saliva, plasma and urine, and increased size and activity of the pituitary and adrenal glands. Moreover, several studies have shown that increased levels of cortisol constitute a risk factor for MDD in risk populations (Goodyer *et al*, 2000; Harris *et al*, 2000). In turn, antidepressants ameliorate many of the neurobiological disturbances in depression, including HPA axis hyperactivity (Surget *et al*, 2011). Figure 2 shows a schematic representation of the HPA axis under physiological stress response.

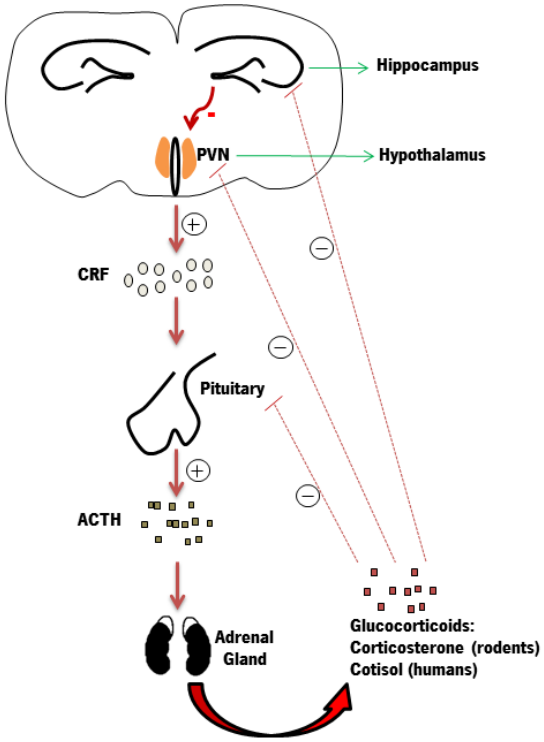

Figure 2 - Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis regulation. Abbreviations: PVN – paraventricular nucleus; CRF - corticotropin releasing factor; ACTH - adrenocorticotropic hormone.

1.2 Psychiatric Disorders

The burden of psychiatric disorders continues to grow with significant impact on health and major social, human rights and economic consequences worldwide. There are different psychiatric disorders,

with different presentations that include depression, bipolar affective disorder, schizophrenia and other psychoses, dementia, intellectual disabilities and developmental disorders. They have multi-factorial aetiologies involving complex interactions between genetic and environmental factors. Increasing evidence demonstrates that neuroplasticity is disrupted in mood disorders including schizophrenia and depression. This connection has been best explored in depression, in which structural plasticity alterations in the hippocampus and PFC have been critically implicated in its pathophysiology. In fact, it is now clear that various forms of structural plasticity, including the generation of new neurons and glial cells in the hippocampus as well as neuronal remodelling in key brain regions (e.g. hippocampus and PFC), may modify pathophysiological processes in depression (Bessa *et al*, 2009a; Mateus-Pinheiro *et al*, 2013; Morais *et al*, 2014). In line with this, treatment with different antidepressant drugs is able to reverse this effect, allowing the recovery from emotional and cognitive changes.

1.2.1 Depression

MDD is a highly prevalent and complex psychiatric disorder that affects multiple behavioral domains, presenting a wide range of symptoms, namely depressed mood, anhedonia, anxiety and cognitive impairments that confer a severe disability and impaired quality of life in patients (Mergl *et al*, 2007; Sheehan, 2002; Villanueva, 2013). The clinical definition and classification of depression has been structured in diagnostic tools such as the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DMS-5). According to DMS-5, patients with MDD must meet at least 5 of these symptoms persisting for at least 2 weeks. Between the symptoms, depressed mood and/or anhedonia (diminished interest or pleasure in all or almost all activities) must be present for a diagnosis. This pathology affects 1.6 to 3.1 times more woman than men with a greater disparity found in the USA and Western Europe. It may appear at any age, presenting a peak of onset in early adulthood that gradually declines with age (American Psychiatry Association, 2013).

Despite causing a substantial impairment in daily functioning, the mechanisms involved in the pathophysiology of MDD and also in the therapeutic actions of antidepressant drugs are still poorly understood. This can be attributed to its complex and heterogeneous nature, in which multiple genetic factors (heritability estimated to be approximately 35%) conferring susceptibility to the disease interact with environmental factors (Flint and Kendler, 2014; Saveanu and Nemeroff, 2012). Several hypotheses have been formulated regarding the aetiology of depression, namely: the monoamine hypothesis, the corticosteroid hypothesis (previously described), the cytokine hypothesis and the neuroplastic hypothesis (Otte *et al*, 2016). One of the first hypotheses described was the monoamine

hypothesis of depression, and almost all the antidepressants available have been developed based on this theory. This hypothesis implicates monoamine deficiency (noradrenalin, dopamine and/or serotonin) as the cause of the disorder; efficient antidepressants are thought to correct these deficits (Delgado, 2000). This simplistic hypothesis fails to explain all aspects of the disorder since antidepressants are able to produce an immediate increase in the levels of monoamines while the therapeutic response requires weeks of continuous administration of antidepressants (Penn and Tracy, 2012). Central monoamine function is still a focus of research, even though more complex pathways have been implicated in depression and antidepressant treatment. Several studies demonstrated the importance of the newly generated neurons and the dendritic reorganization of the pre-existing neurons in the adult hippocampus in the onset and also in the remission from depression (Mateus-Pinheiro *et al*, 2013; Snyder *et al*, 2011; Surget *et al*, 2011).

1.2.1.1 Treatment of depression

In the 1950s, a veritable revolution took place in the fields of psychopharmacology and psychiatry, with the clinical introduction of the main groups of psychoactive drugs still used today. It started with the clinical introduction of the first two antidepressant drugs: iproniazid, a monoamine oxidase inhibitor (MOAI) that had been used in the treatment of tuberculosis, and imipramine, the first drug in the tricyclic antidepressant family. In 1987, the introduction of fluoxetine, a selective serotonin reuptake inhibitor (SSRI), once again revolutionized the therapy for depression, opening a way for new families of antidepressants. The success of the SSRIs and also serotonin-norepinephrine reuptake inhibitors (SNRIs) as first-choice drug was not based on the established differences in efficacy, but rather on a generally more favourable adverse-effect profile, such as the lack of anticholinergic and cardiac effects and a high therapeutic index (the ratio of lethal dose to therapeutic dose). However, the SSRIs and SNRIs are also not devoid of considerable tolerability issues and some patients experience common acute treatment adverse effects such as nausea, insomnia, headaches, dizziness, gastrointestinal symptoms and sexual dysfunction and long-term adverse effects including weight gain, sexual dysfunction and sleep disturbances (Cassano and Fava, 2004). Nevertheless, all the available antidepressants continue to employ the same mechanism of action, that is the modulation of monoaminergic neurotransmission at a synaptic level. Figure 3 shows a chronological representation on how the antidepressant research field has evolved along the years.

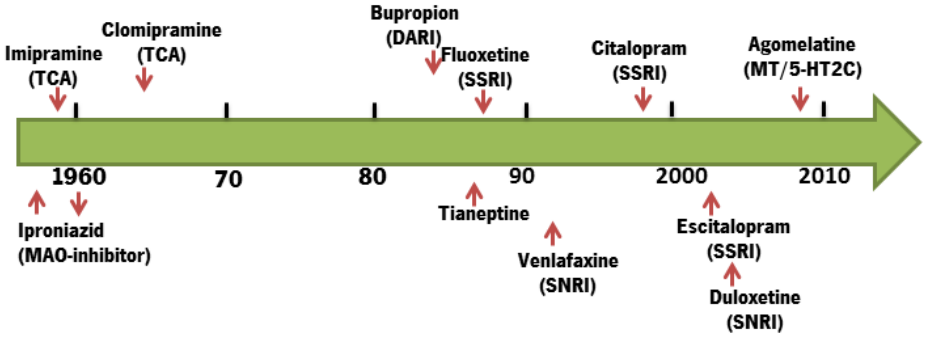

Figure 3 – Schematic representation of the chronology for introduction of medication for the treatment for the major depressive disorder. Abbreviations DARI: dopamine reuptake inhibitor, MAO: monoamine oxidase, MT: melatonin, SNRI serotonin-norepinephrine reuptake inhibitors, TCA: tricyclic agent, SSRI: selective serotonin reuptake inhibitor. Not all drugs are shown.

The first clinical effective antidepressants to be used were inhibitors of the enzyme monoamine oxidase (MAO). They were discovered by accident when an anti-tuberculosis drug (iproniazid) was administrated in patients with tuberculosis and comorbid depression. MAO exists in two subtypes A and B; MAO-A preferentially metabolizes the monoamines serotonin and norepinephrine whereas the MAO-B form preferentially metabolizes trace amines such as phenethylamine. Both MAO-A and MAO-B metabolize dopamine and tyramine. In MAO treatment, MAO-A must be inhibited for antidepressant efficacy. In fact, MAO-A preferentially metabolizes the two monoamines linked to depression and consequently the brain levels of serotonin and norepinephrine increase after MAO-A inhibition. Inhibition of MAO-B is not effective as an antidepressant. When MAO-B is inhibited simultaneously with MAO-A, there is an increase of dopamine as well as serotonin and norepinephrine, resulting in higher antidepressant efficacy. MAOs are considered by many clinicians to be used in treatment-resistant depression, due to the required dietary restrictions and potential fatal drug interaction (Stahl, 2013).

Tricyclic antidepressants were discovered by chance in result of an unsuccessful attempt to improve the antipsychotic effectiveness of phenothiazines (used in the treatment of schizophrenia). Molecular modifications of phenothiazines led to synthesis of imipramine, the first clinically useful tricyclic antidepressant. Tricyclic antidepressants act as strong inhibitors in the reuptake of both norepinephrine and serotonin. Some tricyclics have equal or greater potency for serotonin inhibition; others are more selective for norepinephrine inhibition. However, the majority block both serotonin and norepinephrine

reuptake to some extent. Unfortunately, the tricyclics also block histaminic, cholinergic, and alpha1-adrenergic receptor sites, and this lack of selectivity is what accounts for the unwanted side effects such as weight gain, dry mouth, constipation, drowsiness, and dizziness. The major limitation associated to the use of tricyclics is not their efficacy but the side effects and potential death in overdose (Nojimoto *et al*, 2010) .

Nowadays, SSRIs are the most commonly prescribed antidepressant medications worldwide. They were developed in response to the need for better-tolerated and safer antidepressants, retaining good clinical efficacy. The first SSRI, fluoxetine (Prozac) was released in 1987. Currently, there are six principal drugs included in this class (fluoxetine, sertraline, paroxetine, fluvoxamine, citalopram and escitalopram) all of them sharing the same major pharmacological mechanism (selective and potent inhibition of serotonin transporter). However, each of these drugs has secondary pharmacological targets that may account for their individual difference in terms of efficacy. Fluoxetine, for example, is an SSRI with $5HT_{2C}$ antagonism action. Blocking serotonin action at $5HT_{2C}$ receptor disinhibits the release of norepinephrine and dopamine (Stahl, 2013). Finally, the SNRIs combine the robust serotonin inhibition of the SSRI with various degrees of inhibition of the norepinephrine transporter (Stahl, 2013). In the table 1 is represented a summary of the different antidepressant agents and their major pharmacological targets.

Antidepressant	Class name	Major mechanism (s) of action
Phenelzine	MAO inhibitor	Irreversible MAO inhibition
Pirlindole	MAO inhibitor	Selective and reversible MAO-A inhibition
Imipramine	TCA	5-HT/NA reuptake inhibitor. Antagonist at α1- AR, histamine H1 and muscarinic receptors.
Clomipramine	TCA	5-HT/NE reuptake inhibition. α1-AR, histamine H1, 5-HT2A/2C and muscarinic receptors antagonism.
Fluoxetine	SSRI	5-HT reuptake inhibitor
Citalopram	SSRI	5-HT reuptake inhibitor
Venlafaxine	SNRI	5-HT/NA reuptake inhibitor.
Vortioxetine	-	5-HT reuptake inhibitor, 5-HT1A/1B receptor partial agonist, 5-HT3 and 5-HT7 receptor antagonist
Tianeptine	-	Glutamatergic neurotransmission modulation (indirect).
Agomelatine	-	MT1 and MT2 receptor agonism. 5-HT2C antagonism.

Table 1 - Mechanism of action of some antidepressant agents and major pharmacological targets. Abbreviations: MAO: Monoamine oxidase, TCA: Tricyclic agent, SSRI: Selective serotonin reuptake Inhibitor, SNRI: serotonin-norepinephrine reuptake inhibitor , 5-HT: serotonin, NE: norepinephrine, AR: adrenergic receptor; MT: Melatonin. Adapted from (Millan *et al*, 2015).

Over the last two decades, enormous resources (public and private, academia and industrial) have been dedicated to develope drugs with higher efficacy (Millan *et al*, 2015). However, our insufficient understanding of the pathophysiology of MDD combined with the lack of novel targets has constrained the ability to improve antidepressant therapies. As previously described, antidepressants are the first line for treating depression. However, drug efficacy is unsatisfactory with only one third of the patients presenting remission after treatment with a single drug. The term treatment-resistant depression (TRD) is typically used to describe a form of MDD that has not responded adequately to antidepressant treatment (Fava and Davidson, 1996). In the non-remitted patients, several treatment strategies are adopted including changing antidepressant treatment, combining antidepressants (from the same or from different pharmacological classes), or augmentation strategies that consist in the addition of a

non-antidepressant treatment (e.g. lithium, ketamine and atypical antipsychotics). The anesthetic drug Ketamine (*N*-methyl-D-aspartate (NMDA) antagonist) was observed to induce a rapid antidepressant action in MDD patients (Berman *et al*, 2000; Melo *et al*, 2015) and is currently under intense study. Non-pharmacological treatments may also be used alone or in combination with pharmacological treatments. These include vagus nerve stimulation, electroconvulsive therapy, transcranial magnetic stimulation and deep brain stimulation.

In fact, up to 60% of patients treated with the currently available therapies do not achieve full remission and evolve to treatment resistance (Blier and Blondeau, 2011; Lang and Borgwardt, 2013). Multiple clinical studies have previously highlighted the potential beneficial effects of atypical antipsychotics in treatment-resistant depression (Papakostas *et al*, 2007; Sagud *et al*, 2006; Shelton and Papakostas, 2008). In accordance, different atypical antipsychotic drugs have received approval from the Food and Drug Administration (FDA) for the treatment of antidepressant-resistant forms of major depression (either as monotherapy or augmentation) (Papakostas *et al*, 2004), a fact that supports their potential role in the emotional domain. Studies in animals confirm this view and show that the association of an atypical antipsychotic and a SSRI synergistically increases the release of dopamine in prefrontal areas, thus improving motivation, pleasure, and appetite (Thase *et al*, 2007; Tohen *et al*, 2003). However, the mechanisms involved are still unclear. The possible modulation of neuroplasticity (including adult neurogenesis) by different classes of antipsychotics still remains to be established.

1.2.2 Neuroplasticy as a target for depression

Several hypotheses have been implicated in the pathophysiology of MDD. One of the most popular hypotheses is the so called “neurogenic hypothesis of depression”. This hypothesis implies that adult neurogenesis and other related aspects of hippocampal plasticity are involved in the pathophysiology of MDD and its effective treatment. In fact, several studies have described a downregulation of hippocampal neurogenesis under stressful conditions and an upregulation promoted by different antidepressant drugs (and other antidepressant treatments). Several factors have contributed to the popularity of this hypothesis, namely the temporal dynamics of neuroplastic mechanisms. In fact, the time course of maturation of newly generated neurons in the dentate gyrus parallels the delayed onset of therapeutic action of antidepressants (Schoenfeld and Cameron, 2015). Furthermore, this hypothesis is strengthened by the evidence that other pharmacological classes such as mood stabilizers (lithium and valproate) enhance the proliferation of new cells and also the cell survival of the newly born cells

(Chen *et al*, 2000; Hanson *et al*, 2011a; Hao *et al*, 2004; Silva *et al*, 2008). Non-pharmacological treatments for depression, such as electroconvulsive seizure, also cause an increase in the number of new neurons. Other factors, such as environmental enrichment lead to increased neurogenesis, and interestingly displays a positive effect in stress-induced depressive and anxious behaviours (Jha *et al*, 2011). Exercise is also associated with an increase of neural progenitor cells (NPCs) proliferation and decreased depressive and anxious behaviours (Brandt *et al*, 2010; Olson *et al*, 2006; Yi *et al*, 2009). In addition to these associative studies, others have also suggested that hippocampal neurogenesis is crucial for the manifestation of behavioral mood improvement (David *et al*, 2009; Santarelli *et al*, 2003). In humans, clinical evidences support this hypothesis with a decrease in the hippocampal volume of MDD patients. Also non-treated MDD patients present a decrease in the proliferation compared with treated ones. However, some criticism has been associated with this hypothesis based on the possibility that other factors than decreased number of new neurons contributed for the hippocampal volume changes. In fact, animals exposed to chronic stress or elevated corticosterone concentration presented dendritic atrophy and loss of synapses (Sousa *et al*, 2000; Tata and Anderson, 2010; Vyas *et al*, 2002) and consequently hippocampal volume reduction. Also, the reduced level of neurogenesis (*per se*) in rodents in the absence of stress does not induce depressive-like behaviour. However, reduced neurogenesis can precipitate depression-like symptoms in the context of stress. Regarding post-mortem studies, Reif and colleagues reported no differences on the number of neural progenitors in hippocampus (Reif *et al*, 2006). In contrast, a study by Boldrini et al. found a non-significant trend towards a decrease in hippocampal NSCs in depressed patients (Boldrini *et al*, 2013). Lucassen *et al*., reported a significant reduction of precursor cells in non-treated depressed patients, comparing to age and sex-matched controls (Lucassen *et al*, 2010). In the future, more post-mortem studies should be performed to clarify the importance of this phenomenon in depression pathology.

Considering the limitations associated with human studies, animal models have been widely used to test the neurogenic hypothesis of depression. A downregulation of hippocampal neurogenesis under stress conditions and an upregulation by antidepressants has been clearly demonstrated. Data from our laboratory indicates that antidepressants exert their short-term therapeutic effects by inducing neuronal remodelling of dendrites and synapses (faster morphological changes) in mood-regulating brain regions (hippocampus and PFC) rather than by stimulating neurogenesis (Bessa *et al*, 2009a). At long-term, the generated new neurons (with time to fully mature) and glial cells will have an impact on the emotional and cognitive deficits induced by chronic mild stress (CMS) exposure (Mateus-Pinheiro *et al*, 2013). In

fact, mammalian neurogenesis is a process taking between 4–6 weeks to produce new functional hippocampal neurons; correlating with the minimal time required by antidepressants to exert its action in depression patients. Altogether, strong evidences supports neurogenesis and other aspects of hippocampal plasticity as key players involved in the pathophysiology and treatment of depression (Hanson *et al*, 2011b; Santarelli *et al*, 2003; Snyder *et al*, 2011; Surget *et al*, 2011).

1.3 Animal models for psychiatric disorders

In the recent years several animal models to mimic mental disorders have been developed. These models have been crucial to understand the neurobiological mechanism associated with specific a disorder and also in the developmental of new therapeutic targets. In fact, these models mimicking specific pathology's in a smaller or greater extent (one or several symptoms) reflect the key symptoms observed in human patients suffering from this disease. As expected, none of these models are perfect as none of them reproduces the full clinical picture observed in human disease. Taking this into account, the use of animal models in research to understand the pathological mechanisms and novel drug discovery requires a clear identification of which molecular components of the disease can be represented in a specific model. In my perspective this is the critical point: the correct identification of which dimensions are represented in our animal model and the molecular components altered. While we recognise that symptoms such as guilt, suicidality and sad mood are likely to be purely human features, other aspects of the depressive condition (anhedonia, behavioral despair and other neurovegetative changes such as alterations in sleep and appetite patterns) have been replicated in laboratory animals, and ameliorated with antidepressant treatment (Krishnan and Nestler, 2011; Nestler and Hyman, 2010). A possible model to study depression is the CMS paradigm that involves the exposure to varied intermittent stressors applied over a relatively prolonged time period. Sucrose preference test is normally used to assess the impact of this protocol in hedonic behaviour. Animals exposed to CMS reveal deficits in their motivation to consume a sucrose solution (1–2% of sucrose) measured either as total sucrose intake or as a preference against water (Willner, 2005). CMS exposure has also been shown to result in a number of other emotional changes that are difficult to objectively quantify, such as grooming deficits and changes in aggressive and sexual behaviour. Many of these behavioural phenotypes are reversed by chronic antidepressant treatment (Strekalova *et al*, 2006).

1.4 Schizophrenia

Schizophrenia is a complex neurodevelopmental psychiatric disorder of unknown aetiology involving gene-environment interactions that affects about 1% of the population worldwide (Owen *et al*, 2016a). It typically begins in late adolescence or early adulthood. In terms of symptomatology patients with schizophrenia show abnormal mental functions that have been categorized into: positive, negative and cognitive symptoms. Positive symptoms reflect an excess or distortion of thoughts and perceptions, typically characterized by the development of delusions and hallucinations. Negative symptoms include social withdrawal, loss of motivation, affective blunting and anhedonia, which also characterize other mood disorders such as major depression and account for the significant suicide rates in schizophrenic patients (Freedman, 2003). Cognitive symptoms involve multiple deficits in cognitive and executive processes.

Although with an aetiology still unknown, epidemiology studies have shown that gestational or perinatal disturbances (including maternal starvation (Susser *et al*, 1996) and maternal infection (Brown and Derkits, 2010; Buka *et al*, 2001) increase the risk of developing schizophrenia. These studies give support to the neurodevelopmental theory of schizophrenia and some animal models have been developed to better understand the mechanism associated with this pathology: administration of methylazoxymethanol acetate (MAM), prenatal stress, maternal deprivation, isolation rearing, prenatal immune challenge and maternal malnutrition. In the present work, the neurodevelopmental MAM animal model has been used to study the action of antipsychotic drugs. This model involves the administration of MAM to induce a neurodevelopmental disruption, which in turns produce a schizophrenia-like phenotype in pos-pubertal rats (Flagstad *et al*, 2004; Gastambide *et al*, 2015; Howe *et al*, 2015; Hradetzky *et al*, 2012; Moore *et al*, 2006). The mechanism by which MAM produces this phenotype is not clear. MAM is a cytostatic agent that interferes with mitosis and DNA methylation resulting in behavioural and anatomical brain abnormalities. Behavioural deficits in sensorimotor gating (Le Pen *et al*, 2006), inability to ignore irrelevant stimuli (Flagstad *et al*, 2005), hypersensitivity to amphetamine (Penschuck *et al*, 2006), and social withdrawal (Flagstad *et al*, 2004) with onset in adolescence (Le Pen *et al*, 2006) have been described. Anatomically, reduced thickness of the hippocampus, thalamus and several cortical regions have also been described (Moore H, 1997).

Alterations in the neurotransmitters dopamine, glutamate and serotonin have also been implicated in the aetiology of schizophrenia. Elevated dopamine function is one of the most robust findings in

schizophrenia. In fact, there is a first dopamine hypothesis also described as the "original dopamine hypothesis" stating that an excess of dopamine subcortically is associated with the positive symptoms of schizophrenia. The popularity of this hypothesis was based on two observations: first, sustained exposure to D2 receptor agonists induces schizophrenia-like positive symptoms and second, all drugs with proven antipsychotic effects block D2 receptors to some degree. At the same time, the negative and cognitive symptoms of schizophrenia are thought to arise from a deficit of dopamine in the cortex, and the classical hypothesis was reformulated and termed as "revised dopamine hypothesis". This hypothesis proposes a hyperactive dopamine transmission in the mesolimbic areas and hypoactive dopamine transmission in the prefrontal cortex in schizophrenia patients (Carlsson *et al*, 1999; da Silva Alves *et al*, 2008; Davis *et al*, 1991). For many years, the dopamine hypothesis has strongly influenced the pathophysiological theories of schizophrenia, and most antipsychotics appear to act, at least in part, through inhibition of dopamine D2 receptors in the mesolimbic frontal brain regions. However, nowadays it is clear that schizophrenia is more complex, with dopamine and other neurotransmitters playing critical role in this pathology.

In the 1950´s, the serendipitous discovery of drugs with antipsychotic effects revolutionized the treatment and outcome of schizophrenia (Miyamoto *et al*, 2012). The first generation of antipsychotic drugs, also known as typical antipsychotics (e.g. Haloperidol), with a mechanism of action based on dopamine receptor D2 antagonism, proved to be effective in positive symptoms. However, besides the side effects of eliciting extrapyramidal symptoms (EPS), hyperprolactinemia and metabolic changes, these drugs also exacerbate the negative and cognitive symptoms. In contrast, the second generation of antipsychotics also known as atypical antipsychotics, with less potent D2 antagonism (due, in some cases, to the more rapid dissociation rate from the receptor (Miyamoto S, 200)) and with modulation of serotonin and noradrenaline receptors, maintain their effectiveness against positive symptoms, presenting fewer EPS and beneficial effects on cognitive functions and negative symptoms (Gallhofer *et al*, 1996). Clozapine was introduced in 1975 and is considered the first atypical antipsychotic developed. Clozapine produces no (or few) EPS and has shown to be effective in the management of positive and negative symptoms in chronic and treatment-resistant schizophrenic patients. However, due to the risk of agranulocytosis associated with clozapine, its prescription was restricted to refractory patients (Kilian *et al*, 1999; Rajagopal, 2005; Wong and Delva, 2007).

This fact stimulated the development of novel generation of antipsychotics with a clinical profile similar

to clozapine. Several atypical antipsychotics (including risperidone, olanzapine and ziprasidone) and partial dopamine agonists (aripiprazole) have been created. Interestingly, clozapine remains the most effective agent for the treatment of refractory schizophrenia (Meltzer, 1990); but due to its potentially lethal agranulocytosis-inducing side effect (Kane, 1992) it is usually not considered the first-line treatment. However, clozapine has shown to reduce the risk of suicide in those with schizophrenia (Hennen and Baldessarini, 2005). Risperidone (Leysen *et al*, 1988), like clozapine, presents higher affinity for serotonin 5-HT_2 receptors than dopamine D2 receptors, but they differ in other pharmacologic properties and side effects. Secondary side effects, such as weight gain and the metabolic syndrome, are normally associated with atypical antipsychotics use. Aripiprazole is a third-generation antipsychotic with a different mechanism of action, reducing dopaminergic neurotransmission through D2 partial agonism. As an atypical drug it also modulates receptors of the serotonin system: 5-HT_{1A} partial agonist and serotonin 5-HT_{2A} antagonist. Figure 4 shows a chronological representation on how the research field in treatment for schizophrenia has evolved along the years. In the table 2 is represented a summary of the different antipsychotic agents and their major pharmacological targets.

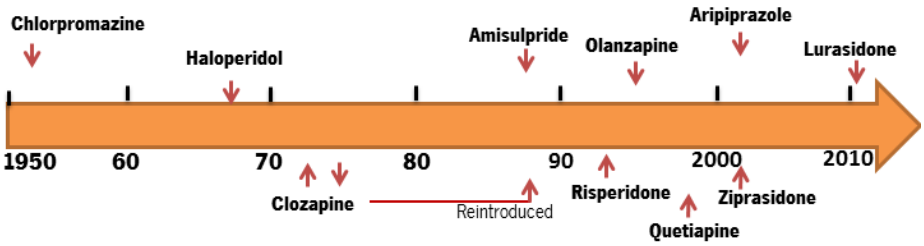

Figure 4 – Schematic representation of the chronology for introduction of medication for the treatment of schizophrenia. Clozapine was withdrawn for safety reasons (agranulocytosis) then re-introduced in light of its unique efficacy and atypical profile. Not all drugs are shown.

Antipsychotic	Class name	Major mechanism (s) of action
Chlorpromizine	Typical agent	Antagonist at D1-D4, 5-T2A/2C, histamine H1, α1-AR and muscarinic receptors.
Haloperidol	Typical agent	Antagonist at D1-D4, 5-HT2A (weak) and α1-AR receptors. Ligand of sigma1 sites. .
Clozapine	Atypical agent	Antagonist atD1-D4, 5-HT2A/2C, 5-HT6/7, α1-AR, α2-AR (modest), histamine H1 and muscarinic M1/M2receptors. Partial agonist at 5-HT1A and M4 receptors.Modulator of glutamatergic and GABA transmission. 5-HT2A>D2 affinity.
Risperidone	Atypical agent	Antagonist at D1-D4,5-HT2A/2C,5-HT7, α1-AR, α2-AR and histamine H1 receptors. 5-HT2A>D2 affinity
Olanzapine	Atypical agent	Antagonist atD1-D4,5-HT2A/2C,5-HT6/7, histamine H1, α1-AR and muscarinic receptors. 5-HT2A>D2 affinity.
Quetiapine	Atypical agent	Antagonist at D1-D4 (weak),5-HT2A/2C, α1- AR, histamine H1 and muscarinic receptors. 5-HT2A>D2 affinity.
Aripiprazole	Atypical agent	Partial agonist at D2, D3 and 5-HT1A receptors. 5-HT2A and (less potent) 5-HT2C and 5-HT7 antagonist.

Table 2 - Mechanism of action of some antipsychotic agents and major pharmacological targets. Adapted from (Millan *et al*, 2015).

1.4.1 Neuroplasticity in Schizophrenia

While the role of adult neurogenesis in depression is still questionable, the link between them is well characterized. However, in schizophrenia, this link is still an open question. In 2006, Reif and colleagues published a study using post-mortem brain tissue of schizophrenic patients, revealing a significant reduction in hippocampal neural stem cell proliferation (Reif *et al*, 2006). Neuronal abnormalities in the olfactory bulb were also reported in post-mortem patient, indicating a possible disturbance of cell proliferation in the SEZ (Arnold *et al*, 2001). In fact, the majority of the schizophrenic patients show defects in olfaction, and first-degree relatives of schizophrenia patients also show such olfactory defects (Moberg *et al*, 1999).

Regarding the genetic component of this psychiatric disorder, some studies have been also developed.

A genetic analysis of a Scottish family with a high prevalence of schizophrenia indicated a mutation in the *disrupted in schizophrenia 1* (*DISC1*) gene (Millar *et al*, 2000). *DISC1* gene encodes for a protein that interacts with different protein partners to promote development and growth (Soares *et al*, 2011). The locations and time course expression of this protein suggest a role in neurogenesis and, in the postnatal brain (localized primarily in the hippocampus) (Austin *et al*, 2003; Schurov *et al*, 2004). Downregulation of *DISC1* gene in mice has been correlated with impairments in cell proliferation in the DG of the hippocampus (Mao *et al*, 2009). DISC1 has been suggested as a modulator of the guidance in the migration of the new neurons in the DG (Namba *et al*, 2011) and, indeed, knockdown of DISC1 protein in mice leads to accelerated maturation and abnormal morphology with less dendritic complexity of the newly generated neurons (Duan *et al*, 2007). These new neurons appear to be misplaced in circuitry and show aberrant physiological characteristics. Another gene associated to schizophrenia is *Neuronal PAS Domain Protein 3* (*NPAS3)*, a neuronal transcription factor known to be involved in a wide array of functions, including neurogenesis (Crews, 1998; Kamnasaran *et al*, 2003). Similarly, *NPAS3* knockout mice show impaired neurogenesis in adulthood (Pieper *et al*, 2005) and diminished social recognition and hyperactivity (Erbel-Sieler *et al*, 2004). In neurodevelopmental animal models of schizophrenia (Phencyclidine (PCP) injection, prenatal injection of polyriboinosinic-polyribocytidilic acid (poly (I:C)), a decrease in hippocampal cell proliferation was also observed, suggesting an involvement of hippocampal neurogenesis in the pathophysiology of schizophrenia. In the hippocampus it was shown that neurons had fewer dendritic spines and reduced dendritic arborisation. Evidence for reduced presynaptic markers was also reported (decrease expression of the presynaptic proteins synapsin and synaptophysin) (Harrison and Eastwood, 2001).

Regarding the effects of antipsychotic medications on adult neurogenesis, the literature is not consensual. Previous studies have shown that haloperidol has no effect on hippocampal neurogenesis (Halim *et al*, 2004; Malberg *et al*, 2000). In the case of atypical antipsychotics, it was reported that risperidone and olanzapine increase neurogenesis in the SEZ, but not in the hippocampus (Wakade *et al*, 2002). However, another study has shown that olanzapine increases hippocampal neurogenesis (Kodama *et al*, 2004). In addition, using animal models of schizophrenia, hippocampal neurogenesis is recovered by atypical antipsychotics (Piontkewitz *et al*, 2012). Taken together, these studies suggest that atypical (but not classical) antipsychotics may increase hippocampal neurogenesis, which may be involved in the mechanisms of action of atypical drugs. These findings suggest a potential role for new neurons in schizophrenia, although a great deal of work is still needed to confirm or refute this

hypothesis.

OBJECTIVES

It is now clear that various forms of structural plasticity, including the generation of new neurons and glial cells, may modify pathophysiological processes in neuropsychiatric disorders, namely in depression. In fact, several studies have shown decreased hippocampal neurogenesis in depressed patients, while treatment with different classes of antidepressant drugs in animal models increases neurogenesis in this region, allowing the recovery from emotional and cognitive changes. However, these effects have not been described for all the available classes of antidepressant drugs. Furthermore, the neuroplastic effects of antidepressants in other neurogenic regions such as the hypothalamus have yet to be determined. Despite the importance of these drugs in the recovery from depression, a significant proportion of depressed patients reveal incomplete remission and develop treatment-resistant forms of the disorder. The use of atypical antipsychotics in these cases has been widely used in the clinical setting. However, the neuroplastic effects of these drugs in depression and schizophrenia are still largely unknown. Taking this into consideration we aimed to explore new perspectives on the interplay between psychopharmacology and neuroplasticity in these psychiatric disorders. More specifically we aim to address:

1- Explore the neuroplastic effects of the MAO-A antidepressant Pirlindole in the unpredictable chronic mild stress (uCMS) animal model of depression;

2- Dissect the actions of chronic stress and antidepressant treatment in hypothalamic neurogenesis in the uCMS animal model;

3- Evaluate the role of neuroplasticity in the therapeutic actions of atypical antipsychotics in depression using the uCMS animal model;

4- Assess the neuroplastic effect of different classes of antipsychotics in the negative and cognitive symptoms of schizophrenia using the MAM animal model.

References

Altman J, Das GD (1965). Post-natal origin of microneurones in the rat brain. *Nature 1965 Aug 28;207(5000):953-6*.

Anderson ML, Sisti HM, Curlik DM, 2nd, Shors TJ (2011). Associative learning increases adult neurogenesis during a critical period. *The European journal of neuroscience* **33**(1): 175-181.

Arnold SE, Han LY, Moberg PJ, Turetsky BI, Gur RE, Trojanowski JQ*, et al* (2001). Dysregulation of olfactory receptor neuron lineage in schizophrenia. *Archives of general psychiatry* **58**(9): 829-835.

Austin CP, Ma L, Ky B, Morris JA, Shughrue PJ (2003). DISC1 (Disrupted in Schizophrenia-1) is expressed in limbic regions of the primate brain. *Neuroreport* **14**(7): 951-954.

Barde YA, Edgar D, Thoenen H (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J* **1**(5): 549-553.

Bedard A, Gravel C, Parent A (2006). Chemical characterization of newly generated neurons in the striatum of adult primates. *Exp Brain Res* **170**(4): 501-512.

Bergmann O, Liebl J, Bernard S, Alkass K, Yeung MS, Steier P*, et al* (2012). The age of olfactory bulb neurons in humans. *Neuron* **74**(4): 634-639.

Berman RM, Cappiello A, Anand A, Oren DA, Heninger GR, Charney DS*, et al* (2000). Antidepressant effects of ketamine in depressed patients. *Biological psychiatry* **47**(4): 351-354.

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA*, et al* (2009). The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Molecular psychiatry* **14**(8): 764-773, 739.

Blier P, Blondeau C (2011). Neurobiological bases and clinical aspects of the use of aripiprazole in treatment-resistant major depressive disorder. *J Affect Disord128 Suppl 1:S3-10 doi: 101016/S0165-0327(11)70003-9*.

Boldrini M, Santiago AN, Hen R, Dwork AJ, Rosoklija GB, Tamir H*, et al* (2013). Hippocampal granule neuron number and dentate gyrus volume in antidepressant-treated and untreated major depression. *Neuropsychopharmacology* **38**(6): 1068-1077.

Bonzano S, Bovetti S, Fasolo A, Peretto P, De Marchis S (2014). Odour enrichment increases adult-born dopaminergic neurons in the mouse olfactory bulb. *The European journal of neuroscience* **40**(10): 3450-3457.

Brandt MD, Maass A, Kempermann G, Storch A (2010). Physical exercise increases Notch activity, proliferation and cell cycle exit of type-3 progenitor cells in adult hippocampal neurogenesis. *The European journal of neuroscience* **32**(8): 1256-1264.

Brown AS, Derkits EJ (2010). Prenatal infection and schizophrenia: a review of epidemiologic and

translational studies. *The American journal of psychiatry* **167**(3): 261-280.

Brown J, Cooper-Kuhn CM, Kempermann G, Van Praag H, Winkler J, Gage FH*, et al* (2003). Enriched environment and physical activity stimulate hippocampal but not olfactory bulb neurogenesis. *Eur J Neurosci17(10):2042-6*.

Bruel-Jungerman E, Davis S, Laroche S (2007a). Brain Plasticity Mechanisms and Memory: A Party of Four. *The Neuroscientist* **13**(5): 492-505.

Bruel-Jungerman E, Rampon C, Laroche S (2007b). Adult hippocampal neurogenesis, synaptic plasticity and memory: facts and hypotheses. *Rev Neurosci 18(2):93-114*.

Buka SL, Tsuang MT, Torrey EF, Klebanoff MA, Bernstein D, Yolken RH (2001). Maternal infections and subsequent psychosis among offspring. *Archives of general psychiatry* **58**(11): 1032-1037.

Carlsson A, Waters N, Carlsson ML (1999). Neurotransmitter interactions in schizophrenia–therapeutic implications. *Biological psychiatry* **46**(10): 1388-1395.

Cassano P, Fava M (2004). Tolerability issues during long-term treatment with antidepressants. *Annals of clinical psychiatry : official journal of the American Academy of Clinical Psychiatrists* **16**(1): 15-25.

Chen G, Rajkowska G, Du F, Seraji-Bozorgzad N, Manji HK (2000). Enhancement of hippocampal neurogenesis by lithium. *Journal of neurochemistry* **75**(4): 1729-1734.

Christoffel DJ, Golden SA, Russo SJ (2011). Structural and synaptic plasticity in stress-related disorders. *Reviews in the neurosciences* **22**(5): 535-549.

Crews ST (1998). Control of cell lineage-specific development and transcription by bHLH-PAS proteins. *Genes & development* **12**(5): 607-620.

D'Sa C, Duman RS (2002). Antidepressants and neuroplasticity. *Bipolar disorders* **4**(3): 183-194.

da Silva Alves F, Figee M, van Amelsvoort T, Veltman D, de Haan L (2008). The revised dopamine hypothesis of schizophrenia: evidence from pharmacological MRI studies with atypical antipsychotic medication. *Psychopharmacology bulletin* **41**(1): 121-132.

David DJ, Samuels BA, Rainer Q, Wang JW, Marsteller D, Mendez I*, et al* (2009). Neurogenesis-dependent and -independent effects of fluoxetine in an animal model of anxiety/depression. *Neuron* **62**(4): 479-493.

Davis KL, Kahn RS, Ko G, Davidson M (1991). Dopamine in schizophrenia: a review and reconceptualization. *The American journal of psychiatry* **148**(11): 1474-1486.

Dayer AG, Cleaver KM, Abouantoun T, Cameron HA (2005). New GABAergic interneurons in the adult neocortex and striatum are generated from different precursors. *J Cell Biol 168(3):415-27*.

de Kloet ER, Joels M, Holsboer F (2005). Stress and the brain: from adaptation to disease. *Nat Rev*

Neurosci **6**(6): 463-475.

Delgado PL (2000). Depression: the case for a monoamine deficiency. *The Journal of clinical psychiatry* **61 Suppl 6**: 7-11.

Doetsch F, Hen R (2005). Young and excitable: the function of new neurons in the adult mammalian brain. *Current Opinion in Neurobiology* **15**(1): 121-128.

Duan X, Chang JH, Ge S, Faulkner RL, Kim JY, Kitabatake Y*, et al* (2007). Disrupted-In-Schizophrenia 1 regulates integration of newly generated neurons in the adult brain. *Cell* **130**(6): 1146-1158.

Erbel-Sieler C, Dudley C, Zhou Y, Wu X, Estill SJ, Han T*, et al* (2004). Behavioral and regulatory abnormalities in mice deficient in the NPAS1 and NPAS3 transcription factors. *Proceedings of the National Academy of Sciences of the United States of America* **101**(37): 13648-13653.

Erdmann G, Berger S, Schutz G (2008). Genetic dissection of glucocorticoid receptor function in the mouse brain. *Journal of neuroendocrinology* **20**(6): 655-659.

Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn A-M, Nordborg C, Peterson DA*, et al* (1998). Neurogenesis in the adult human hippocampus. *Nat Med* **4**(11): 1313-1317.

Ernst A, Alkass K, Bernard S, Salehpour M, Perl S, Tisdale J*, et al* (2014). Neurogenesis in the striatum of the adult human brain. *Cell* **156**(5): 1072-1083.

Fanselow MS, Dong H-W (2010). Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures? *Neuron* **65**(1): 7-19.

Fava M, Davidson KG (1996). Definition and epidemiology of treatment-resistant depression. *The Psychiatric clinics of North America* **19**(2): 179-200.

Flagstad P, Glenthoj BY, Didriksen M (2005). Cognitive deficits caused by late gestational disruption of neurogenesis in rats: a preclinical model of schizophrenia. *Neuropsychopharmacology* **30**(2): 250-260.

Flagstad P, Mork A, Glenthoj BY, van Beek J, Michael-Titus AT, Didriksen M (2004). Disruption of neurogenesis on gestational day 17 in the rat causes behavioral changes relevant to positive and negative schizophrenia symptoms and alters amphetamine-induced dopamine release in nucleus accumbens. *Neuropsychopharmacology* **29**(11): 2052-2064.

Flint J, Kendler KS (2014). The genetics of major depression. *Neuron* **81**(3): 484-503.

Fowler CD, Johnson F, Wang Z (2005). Estrogen regulation of cell proliferation and distribution of estrogen receptor-alpha in the brains of adult female prairie and meadow voles. *The Journal of comparative neurology* **489**(2): 166-179.

Fowler CD, Liu Y, Ouimet C, Wang Z (2002). The effects of social environment on adult neurogenesis in the female prairie vole. *Journal of neurobiology* **51**(2): 115-128.

Freedman R (2003). Schizophrenia. *The New England journal of medicine* **349**(18): 1738-1749.

Gallhofer B, Bauer U, Lis S, Krieger S, Gruppe H (1996). Cognitive dysfunction in schizophrenia: comparison of treatment with atypical antipsychotic agents and conventional neuroleptic drugs. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* **6 Suppl 2**: S13-20.

Gastambide F, Taylor AM, Palmer C, Svard H, Karjalainen M, Janhunen SK*, et al* (2015). Alterations in spatial memory and anxiety in the MAM E17 rat model of hippocampal pathology in schizophrenia. *Psychopharmacology* **232**(21-22): 4099-4112.

Ge Y, Dong Z, Bagot RC, Howland JG, Phillips AG, Wong TP*, et al* (2010). Hippocampal long-term depression is required for the consolidation of spatial memory. *Proceedings of the National Academy of Sciences of the United States of America* **107**(38): 16697-16702.

Goodman T, Hajihosseini MK (2015). Hypothalamic tanycytes-masters and servants of metabolic, neuroendocrine, and neurogenic functions. *Frontiers in neuroscience* **9**: 387.

Goodyer IM, Herbert J, Tamplin A, Altham PM (2000). Recent life events, cortisol, dehydroepiandrosterone and the onset of major depression in high-risk adolescents. *The British journal of psychiatry : the journal of mental science* **177**: 499-504.

Gould E, Reeves AJ, Graziano MS, Gross CG (1999). Neurogenesis in the neocortex of adult primates. *Science* **286**(5439): 548-552.

Gratzner HG (1982). Monoclonal antibody to 5-bromo- and 5-iododeoxyuridine: A new reagent for detection of DNA replication. *Science* **218**(4571): 474-475.

Groves JO (2007). Is it time to reassess the BDNF hypothesis of depression? *Molecular psychiatry* **12**(12): 1079-1088.

Halim ND, Weickert CS, McClintock BW, Weinberger DR, Lipska BK (2004). Effects of chronic haloperidol and clozapine treatment on neurogenesis in the adult rat hippocampus. *Neuropsychopharmacology* **29**(6): 1063-1069.

Hanson ND, Nemeroff CB, Owens MJ (2011a). Lithium, but not fluoxetine or the corticotropin-releasing factor receptor 1 receptor antagonist R121919, increases cell proliferation in the adult dentate gyrus. *The Journal of pharmacology and experimental therapeutics* **337**(1): 180-186.

Hanson ND, Owens MJ, Nemeroff CB (2011b). Depression, antidepressants, and neurogenesis: a critical reappraisal. *Neuropsychopharmacology* **36**(13): 2589-2602.

Hao Y, Creson T, Zhang L, Li P, Du F, Yuan P*, et al* (2004). Mood stabilizer valproate promotes ERK pathway-dependent cortical neuronal growth and neurogenesis. *J Neurosci* **24**(29): 6590-6599.

Harris KM, Jensen FE, Tsao B (1992). Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic

physiology and long-term potentiation. *J Neurosci 1992 Jul;12(7):2685-705*.

Harris TO, Borsanyi S, Messari S, Stanford K, Cleary SE, Shiers HM*, et al* (2000). Morning cortisol as a risk factor for subsequent major depressive disorder in adult women. *The British journal of psychiatry : the journal of mental science* **177**: 505-510.

Harrison PJ, Eastwood SL (2001). Neuropathological studies of synaptic connectivity in the hippocampal formation in schizophrenia. *Hippocampus* **11**(5): 508-519.

Hennen J, Baldessarini RJ (2005). Suicidal risk during treatment with clozapine: a meta-analysis. *Schizophrenia research* **73**(2-3): 139-145.

Hohn A, Leibrock J, Bailey K, Barde YA (1990). Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. *Nature* **344**(6264): 339-341.

Holderbach R, Clark K, Moreau JL, Bischofberger J, Normann C (2007). Enhanced long-term synaptic depression in an animal model of depression. *Biological psychiatry* **62**(1): 92-100.

Howe WM, Tierney PL, Young DA, Oomen C, Kozak R (2015). MAM (E17) rodent developmental model of neuropsychiatric disease: disruptions in learning and dysregulation of nucleus accumbens dopamine release, but spared executive function. *Psychopharmacology* **232**(21-22): 4113-4127.

Hradetzky E, Sanderson TM, Tsang TM, Sherwood JL, Fitzjohn SM, Lakics V*, et al* (2012). The methylazoxymethanol acetate (MAM-E17) rat model: molecular and functional effects in the hippocampus. *Neuropsychopharmacology* **37**(2): 364-377.

Huang L, DeVries GJ, Bittman EL (1998). Photoperiod regulates neuronal bromodeoxyuridine labeling in the brain of a seasonally breeding mammal. *Journal of neurobiology* **36**(3): 410-420.

Jessberger S, Kempermann G (2003). Adult-born hippocampal neurons mature into activity-dependent responsiveness. *European Journal of Neuroscience* **18**(10): 2707-2712.

Jha S, Dong B, Sakata K (2011). Enriched environment treatment reverses depression-like behavior and restores reduced hippocampal neurogenesis and protein levels of brain-derived neurotrophic factor in mice lacking its expression through promoter IV. *Translational psychiatry* **1**: e40.

Kamnasaran D, Muir WJ, Ferguson-Smith MA, Cox DW (2003). Disruption of the neuronal PAS3 gene in a family affected with schizophrenia. *Journal of medical genetics* **40**(5): 325-332.

Kane JM (1992). Clinical efficacy of clozapine in treatment-refractory schizophrenia: an overview. *The British journal of psychiatry Supplement*(17): 41-45.

Kaplan MS, Bell DH (1983). Neuronal proliferation in the 9-month-old rodent â€" Radioautographic study of granule cells in the hippocampus. *Experimental Brain Research* **52**(1): 1-5.

Kempermann G, Gage FH (2000). Neurogenesis in the adult hippocampus. *Novartis Found Symp 231:220-35; discussion 235-41, 302-6*.

Kempermann G, Kuhn HG, Gage FH (1997a). More hippocampal neurons in adult mice living in an enriched environment. *Nature* **386**(6624): 493-495.

Kempermann G, Kuhn HG, Gage FH (1997b). More hippocampal neurons in adult mice living in an enriched environment. *Nature* **386**(6624): 493-495.

Kempermann G, Song H, Gage FH (2015). Neurogenesis in the Adult Hippocampus. *Cold Spring Harb Perspect Biol 1;7(9):a018812 doi: 101101/cshperspecta018812*.

Kheirbek MA, Hen R (2010). Dorsal vs Ventral Hippocampal Neurogenesis: Implications for Cognition and Mood. *Neuropsychopharmacology* **36**(1): 373-374.

Kilian JG, Kerr K, Lawrence C, Celermajer DS (1999). Myocarditis and cardiomyopathy associated with clozapine. *Lancet (London, England)* **354**(9193): 1841-1845.

Knott GW, Holtmaat A, Wilbrecht L, Welker E, Svoboda K (2006). Spine growth precedes synapse formation in the adult neocortex in vivo. *Nat Neurosci* **9**(9): 1117-1124.

Kodama M, Fujioka T, Duman RS (2004). Chronic olanzapine or fluoxetine administration increases cell proliferation in hippocampus and prefrontal cortex of adult rat. *Biological psychiatry* **56**(8): 570-580.

Kokaia M (2011). Seizure-induced neurogenesis in the adult brain. *The European journal of neuroscience* **33**(6): 1133-1138.

Kokoeva MV, Yin H, Flier JS (2005). Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science* **310**(5748): 679-683.

Kretz O, Reichardt HM, Schutz G, Bock R (1999). Corticotropin-releasing hormone expression is the major target for glucocorticoid feedback-control at the hypothalamic level. *Brain research* **818**(2): 488-491.

Krishnan V, Nestler EJ (2011). Animal models of depression: molecular perspectives. *Current topics in behavioral neurosciences* **7**: 121-147.

Krugers HJ, Goltstein PM, van der Linden S, Joels M (2006). Blockade of glucocorticoid receptors rapidly restores hippocampal CA1 synaptic plasticity after exposure to chronic stress. *The European journal of neuroscience* **23**(11): 3051-3055.

Kuhn HG, Dickinson-Anson H, Gage FH (1996). Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J Neurosci* **16**(6): 2027-2033.

Lamprecht R, LeDoux J (2004). Structural plasticity and memory. *Nat Rev Neurosci* **5**(1): 45-54.

Lang UE, Borgwardt S (2013). Molecular mechanisms of depression: perspectives on new treatment strategies. *Cell Physiol Biochem 31(6):761-77 doi: 101159/000350094 Epub 2013 May 31*.

Laplagne DA, Esposito MS, Piatti VC, Morgenstern NA, Zhao C, van Praag H*, et al* (2006). Functional convergence of neurons generated in the developing and adult hippocampus. *PLoS Biol* **4**(12): e409.

Le Pen G, Gourevitch R, Hazane F, Hoareau C, Jay TM, Krebs MO (2006). Peri-pubertal maturation after developmental disturbance: a model for psychosis onset in the rat. *Neuroscience* **143**(2): 395-405.

Lee DA, Bedont JL, Pak T, Wang H, Song J, Miranda-Angulo A*, et al* (2012). Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. *Nat Neurosci* **15**(5): 700-702.

Lee DA, Yoo S, Pak T, Salvatierra J, Velarde E, Aja S*, et al* (2014). Dietary and sex-specific factors regulate hypothalamic neurogenesis in young adult mice. *Frontiers in neuroscience* **8**: 157.

Lepack AE, Fuchikami M, Dwyer JM, Banasr M, Duman RS (2014). BDNF release is required for the behavioral actions of ketamine. *The international journal of neuropsychopharmacology* **18**(1).

Levi-Montalcini R (1987). The Nerve Growth Factor: 35 Years Later (Nobel Lecture). *Angewandte Chemie International Edition in English* **26**(8): 707-716.

Leysen JE, Gommeren W, Eens A, de Chaffoy de Courcelles D, Stoof JC, Janssen PA (1988). Biochemical profile of risperidone, a new antipsychotic. *The Journal of pharmacology and experimental therapeutics* **247**(2): 661-670.

Lucassen PJ, Pruessner J, Sousa N, Almeida OF, Van Dam AM, Rajkowska G*, et al* (2014). Neuropathology of stress. *Acta neuropathologica* **127**(1): 109-135.

Lucassen PJ, Stumpel MW, Wang Q, Aronica E (2010). Decreased numbers of progenitor cells but no response to antidepressant drugs in the hippocampus of elderly depressed patients. *Neuropharmacology* **58**(6): 940-949.

Luskin MB (1993). Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. *Neuron* **11**(1): 173-189.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* **20**(24): 9104-9110.

Mao Y, Ge X, Frank CL, Madison JM, Koehler AN, Doud MK*, et al* (2009). Disrupted in Schizophrenia 1 Regulates Neuronal Progenitor Proliferation via Modulation of GSK3β/β-Catenin Signaling. *Cell* **136**(6): 1017-1031.

Markram H, Gerstner W, Sjostrom PJ (2011). A history of spike-timing-dependent plasticity. *Frontiers in synaptic neuroscience* **3**: 4.

Mateus-Pinheiro A, Pinto L, Bessa JM, Morais M, Alves ND, Monteiro S*, et al* (2013). Sustained remission from depressive-like behavior depends on hippocampal neurogenesis. *Translational*

psychiatry **3**: e210.

Matsumoto M, Tachibana K, Togashi H, Tahara K, Kojima T, Yamaguchi T*, et al* (2005). Chronic treatment with milnacipran reverses the impairment of synaptic plasticity induced by conditioned fear stress. *Psychopharmacology* **179**(3): 606-612.

Matsuzaki M, Ellis-Davies GCR, Nemoto T, Miyashita Y, Iino M, Kasai H (2001). Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. *Nat Neurosci* **4**(11): 1086-1092.

McEwen BS, Sapolsky RM (1995). Stress and cognitive function. *Curr Opin Neurobiol* **5**(2): 205-216.

Melo A, Kokras N, Dalla C, Ferreira C, Ventura-Silva AP, Sousa N*, et al* (2015). The positive effect on ketamine as a priming adjuvant in antidepressant treatment. *Translational psychiatry* **5**: e573.

Meltzer HY (1990). Monitoring clozapine. *Hospital & community psychiatry* **41**(12): 1356-1357.

Mendez P, Bacci A (2011). Assortment of GABAergic plasticity in the cortical interneuron melting pot. *Neural Plast 2011;2011:976856 doi: 101155/2011/976856 Epub 2011 Jul 11*.

Mergl R, Seidscheck I, Allgaier AK, Moller HJ, Hegerl U, Henkel V (2007). Depressive, anxiety, and somatoform disorders in primary care: prevalence and recognition. *Depress Anxiety* **24**(3): 185-195.

Millan MJ, Goodwin GM, Meyer-Lindenberg A, Ove Ogren S (2015). Learning from the past and looking to the future: Emerging perspectives for improving the treatment of psychiatric disorders. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* **25**(5): 599-656.

Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, Semple CA*, et al* (2000). Disruption of two novel genes by a translocation co-segregating with schizophrenia. *Human molecular genetics* **9**(9): 1415-1423.

Miyamoto S, Miyake N, Jarskog LF, Fleischhacker WW, Lieberman JA (2012). Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. *Molecular psychiatry* **17**(12): 1206-1227.

Moberg PJ, Agrin R, Gur RE, Gur RC, Turetsky BI, Doty RL (1999). Olfactory dysfunction in schizophrenia: a qualitative and quantitative review. *Neuropsychopharmacology* **21**(3): 325-340.

Molendijk ML, Spinhoven P, Polak M, Bus BA, Penninx BW, Elzinga BM (2014). Serum BDNF concentrations as peripheral manifestations of depression: evidence from a systematic review and meta-analyses on 179 associations (N=9484). *Molecular psychiatry* **19**(7): 791-800.

Moore H GA (1997). Anatomical changes in limbic structures produced by methylazoxymethanol acetate (MAM) during brain development are associated with changes in physiological interactions among afferents to the nucleus accumbens. *Soc Neurosci Abstr 23: 2378*.

Moore H, Jentsch JD, Ghajarnia M, Geyer MA, Grace AA (2006). A neurobehavioral systems analysis of adult rats exposed to methylazoxymethanol acetate on E17: implications for the neuropathology of schizophrenia. *Biological psychiatry* **60**(3): 253-264.

Morais M, Santos PA, Mateus-Pinheiro A, Patricio P, Pinto L, Sousa N*, et al* (2014). The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition. *Journal of psychopharmacology (Oxford, England)* **28**(12): 1178-1183.

Namba T, Ming GL, Song H, Waga C, Enomoto A, Kaibuchi K*, et al* (2011). NMDA receptor regulates migration of newly generated neurons in the adult hippocampus via Disrupted-In-Schizophrenia 1 (DISC1). *Journal of neurochemistry* **118**(1): 34-44.

Nestler EJ, Hyman SE (2010). Animal models of neuropsychiatric disorders. *Nat Neurosci* **13**(10): 1161-1169.

Nimchinsky EA, Sabatini BL, Svoboda K (2002). Structure and function of dendritic spines. *Annu Rev Physiol64:313-53*.

Nojimoto FD, Mueller A, Hebeler-Barbosa F, Akinaga J, Lima V, Kiguti LR*, et al* (2010). The tricyclic antidepressants amitriptyline, nortriptyline and imipramine are weak antagonists of human and rat alpha1B-adrenoceptors. *Neuropharmacology* **59**(1-2): 49-57.

Oh WC, Hill TC, Zito K (2013). Synapse-specific and size-dependent mechanisms of spine structural plasticity accompanying synaptic weakening. *Proceedings of the National Academy of Sciences of the United States of America* **110**(4): E305-312.

Okamoto K, Nagai T, Miyawaki A, Hayashi Y (2004). Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity. *Nat Neurosci 7(10):1104-12 Epub 2004 Sep 7*.

Olson AK, Eadie BD, Ernst C, Christie BR (2006). Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. *Hippocampus* **16**(3): 250-260.

Otte C, Gold SM, Penninx BW, Pariante CM, Etkin A, Fava M*, et al* (2016). Major depressive disorder. *Nature reviews Disease primers* **2**: 16065.

Owen MJ, Sawa A, Mortensen PB (2016). Schizophrenia. *Lancet (London, England)* **388**(10039): 86-97.

Papakostas GI, Petersen TJ, Nierenberg AA, Murakami JL, Alpert JE, Rosenbaum JF*, et al* (2004). Ziprasidone augmentation of selective serotonin reuptake inhibitors (SSRIs) for SSRI-resistant major depressive disorder. *The Journal of clinical psychiatry* **65**(2): 217-221.

Papakostas GI, Shelton RC, Smith J, Fava M (2007). Augmentation of antidepressants with atypical antipsychotic medications for treatment-resistant major depressive disorder: a meta-analysis. *The Journal of clinical psychiatry* **68**(6): 826-831.

Parent JM, Yu TW, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH (1997). Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci* **17**(10): 3727-3738.

Pariante CM, Lightman SL (2008). The HPA axis in major depression: classical theories and new developments. *Trends in neurosciences* **31**(9): 464-468.

Pencea V, Bingaman KD, Wiegand SJ, Luskin MB (2001). Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *J Neurosci* **21**(17): 6706-6717.

Penn E, Tracy DK (2012). The drugs don't work? antidepressants and the current and future pharmacological management of depression. *Therapeutic advances in psychopharmacology* **2**(5): 179-188.

Penschuck S, Flagstad P, Didriksen M, Leist M, Michael-Titus AT (2006). Decrease in parvalbumin-expressing neurons in the hippocampus and increased phencyclidine-induced locomotor activity in the rat methylazoxymethanol (MAM) model of schizophrenia. *The European journal of neuroscience* **23**(1): 279-284.

Pieper AA, Wu X, Han TW, Estill SJ, Dang Q, Wu LC*, et al* (2005). The neuronal PAS domain protein 3 transcription factor controls FGF-mediated adult hippocampal neurogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America* **102**(39): 14052-14057.

Piontkewitz Y, Bernstein HG, Dobrowolny H, Bogerts B, Weiner I, Keilhoff G (2012). Effects of risperidone treatment in adolescence on hippocampal neurogenesis, parvalbumin expression, and vascularization following prenatal immune activation in rats. *Brain, behavior, and immunity* **26**(2): 353-363.

Popoli M, Gennarelli M, Racagni G (2002). Modulation of synaptic plasticity by stress and antidepressants. *Bipolar disorders* **4**(3): 166-182.

Price J, Turner D, Cepko C (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proceedings of the National Academy of Sciences* **84**(1): 156-160.

Rajagopal S (2005). Clozapine, agranulocytosis, and benign ethnic neutropenia. *Postgraduate medical journal* **81**(959): 545-546.

Reif A, Fritzen S, Finger M, Strobel A, Lauer M, Schmitt A*, et al* (2006). Neural stem cell proliferation is decreased in schizophrenia, but not in depression. *Molecular psychiatry* **11**(5): 514-522.

Reul JM, de Kloet ER (1985). Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* **117**(6): 2505-2511.

Ridder S, Chourbaji S, Hellweg R, Urani A, Zacher C, Schmid W*, et al* (2005). Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive

reactions. *J Neurosci* **25**(26): 6243-6250.

Robins SC, Stewart I, McNay DE, Taylor V, Giachino C, Goetz M*, et al* (2013). alpha-Tanycytes of the adult hypothalamic third ventricle include distinct populations of FGF-responsive neural progenitors. *Nature communications* **4**: 2049.

Sagi Y, Tavor I, Hofstetter S, Tzur-Moryosef S, Blumenfeld-Katzir T, Assaf Y (2012). Learning in the fast lane: new insights into neuroplasticity. *Neuron 73(6):1195-203 doi: 101016/jneuron201201025 Epub 2012 Mar 21*.

Sagud M, Mihaljevic-Peles A, Muck-Seler D, Jakovljevic M, Pivac N (2006). Quetiapine augmentation in treatment-resistant depression: a naturalistic study. *Psychopharmacology* **187**(4): 511-514.

Sanai N, Nguyen T, Ihrie RA, Mirzadeh Z, Tsai H-H, Wong M*, et al* (2011). Corridors of migrating neurons in the human brain and their decline during infancy. *Nature* **478**(7369): 382-386.

Sandi C (2011). Glucocorticoids act on glutamatergic pathways to affect memory processes. *Trends in neurosciences* **34**(4): 165-176.

Sanes JR, Rubenstein JL, Nicolas JF (1986). Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *The EMBO Journal* **5**(12): 3133-3142.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S*, et al* (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301**(5634): 805-809.

Sapolsky RM (1996). Why stress is bad for your brain. *Science* **273**(5276): 749-750.

Saveanu RV, Nemeroff CB (2012). Etiology of depression: genetic and environmental factors. *The Psychiatric clinics of North America* **35**(1): 51-71.

Schinder AF, Gage FH (2004). A Hypothesis About the Role of Adult Neurogenesis in Hippocampal Function. *Physiology* **19**(5): 253-261.

Schoenfeld TJ, Cameron HA (2015). Adult neurogenesis and mental illness. *Neuropsychopharmacology* **40**(1): 113-128.

Schurov IL, Handford EJ, Brandon NJ, Whiting PJ (2004). Expression of disrupted in schizophrenia 1 (DISC1) protein in the adult and developing mouse brain indicates its role in neurodevelopment. *Molecular psychiatry* **9**(12): 1100-1110.

Sheehan DV (2002). Establishing the real cost of depression. *Manag Care* **11**(8 Suppl): 7-10; discussion 21-15.

Shelton RC, Papakostas GI (2008). Augmentation of antidepressants with atypical antipsychotics for treatment-resistant major depressive disorder. *Acta Psychiatr Scand* **117**(4): 253-259.

Silva R, Mesquita AR, Bessa J, Sousa JC, Sotiropoulos I, Leao P*, et al* (2008). Lithium blocks stress-

induced changes in depressive-like behavior and hippocampal cell fate: the role of glycogen-synthase-kinase-3beta. *Neuroscience* **152**(3): 656-669.

Snyder JS, Soumier A, Brewer M, Pickel J, Cameron HA (2011). Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature* **476**(7361): 458-461.

Soares DC, Carlyle BC, Bradshaw NJ, Porteous DJ (2011). DISC1: Structure, Function, and Therapeutic Potential for Major Mental Illness. *ACS chemical neuroscience* **2**(11): 609-632.

Sousa N, Lukoyanov NV, Madeira MD, Almeida OF, Paula-Barbosa MM (2000). Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neuroscience* **97**(2): 253-266.

Stahl SM (2013). Stahl's essential psychopharmacology: Neuroscientific basis and practical applications. *Cambridge: Cambridge University Press*.

Stanfield BB, Trice JE (1988). Evidence that granule cells generated in the dentate gyrus of adult rats extend axonal projections. *Experimental Brain Research* **72**(2): 399-406.

Strekalova T, Gorenkova N, Schunk E, Dolgov O, Bartsch D (2006). Selective effects of citalopram in a mouse model of stress-induced anhedonia with a control for chronic stress. *Behavioural pharmacology* **17**(3): 271-287.

Surget A, Tanti A, Leonardo ED, Laugeray A, Rainer Q, Touma C*, et al* (2011). Antidepressants recruit new neurons to improve stress response regulation. *Molecular psychiatry* **16**(12): 1177-1188.

Susser E, Neugebauer R, Hoek HW, Brown AS, Lin S, Labovitz D*, et al* (1996). Schizophrenia after prenatal famine. Further evidence. *Archives of general psychiatry* **53**(1): 25-31.

Tata DA, Anderson BJ (2010). The effects of chronic glucocorticoid exposure on dendritic length, synapse numbers and glial volume in animal models: implications for hippocampal volume reductions in depression. *Physiology & behavior* **99**(2): 186-193.

Thase ME, Friedman ES, Biggs MM, Wisniewski SR, Trivedi MH, Luther JF*, et al* (2007). Cognitive therapy versus medication in augmentation and switch strategies as second-step treatments: a STAR*D report. *The American journal of psychiatry* **164**(5): 739-752.

Theodosis DT, Poulain DA, Oliet SpHR (2008). Activity-Dependent Structural and Functional Plasticity of Astrocyte-Neuron Interactions. *Physiological Reviews* **88**(3): 983-1008.

Tohen M, Vieta E, Calabrese J, Ketter TA, Sachs G, Bowden C*, et al* (2003). Efficacy of olanzapine and olanzapine-fluoxetine combination in the treatment of bipolar I depression. *Archives of general psychiatry* **60**(11): 1079-1088.

van Praag H, Kempermann G, Gage FH (1999). Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci2(3):266-70*.

van Praag H, Schinder AF, Christie BR, Toni N, Palmer TD, Gage FH (2002). Functional neurogenesis in the adult hippocampus. *Nature* **415**(6875): 1030-1034.

van Praag H, Shubert T, Zhao C, Gage FH (2005). Exercise Enhances Learning and Hippocampal Neurogenesis in Aged Mice. *The Journal of Neuroscience* **25**(38): 8680-8685.

Villanueva R (2013). Neurobiology of major depressive disorder. *Neural Plast* **2013**: 873278.

Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. *J Neurosci* **22**(15): 6810-6818.

Wakade CG, Mahadik SP, Waller JL, Chiu FC (2002). Atypical neuroleptics stimulate neurogenesis in adult rat brain. *Journal of neuroscience research* **69**(1): 72-79.

Warner-Schmidt JL, Duman RS (2006). Hippocampal neurogenesis: opposing effects of stress and antidepressant treatment. *Hippocampus* **16**(3): 239-249.

Wei Q, Hebda-Bauer EK, Pletsch A, Luo J, Hoversten MT, Osetek AJ*, et al* (2007). Overexpressing the glucocorticoid receptor in forebrain causes an aging-like neuroendocrine phenotype and mild cognitive dysfunction. *J Neurosci* **27**(33): 8836-8844.

Whitman MC, Greer CA (2009). Adult neurogenesis and the olfactory system. *Progress in Neurobiology* **89**(2): 162-175.

Willner P (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology* **52**(2): 90-110.

Wong J, Delva N (2007). Clozapine-induced seizures: recognition and treatment. *Canadian journal of psychiatry Revue canadienne de psychiatrie* **52**(7): 457-463.

Xu Y, Tamamaki N, Noda T, Kimura K, Itokazu Y, Matsumoto N*, et al* (2005). Neurogenesis in the ependymal layer of the adult rat 3rd ventricle. *Experimental neurology* **192**(2): 251-264.

Yang Y, Wang X-b, Frerking M, Zhou Q (2008). Spine Expansion and Stabilization Associated with Long-term Potentiation. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**(22): 5740-5751.

Yi SS, Hwang IK, Yoo KY, Park OK, Yu J, Yan B*, et al* (2009). Effects of treadmill exercise on cell proliferation and differentiation in the subgranular zone of the dentate gyrus in a rat model of type II diabetes. *Neurochemical research* **34**(6): 1039-1046.

Yu S, Yang S, Holsboer F, Sousa N, Almeida OF (2011). Glucocorticoid regulation of astrocytic fate and function. *PloS one* **6**(7): e22419.

Zhao M, Momma S, Delfani K, Carlen M, Cassidy RM, Johansson CB*, et al* (2003). Evidence for neurogenesis in the adult mammalian substantia nigra. *Proceedings of the National Academy of*

Sciences of the United States of America **100**(13): 7925-7930.

Zhou Q, Homma KJ, Poo M-m (2004). Shrinkage of Dendritic Spines Associated with Long-Term Depression of Hippocampal Synapses. *Neuron* **44**(5): 749-757.

Zhu XH, Yan HC, Zhang J, Qu HD, Qiu XS, Chen L*, et al* (2010). Intermittent hypoxia promotes hippocampal neurogenesis and produces antidepressant-like effects in adult rats. *J Neurosci* **30**(38): 12653-12663.

Zito K, Scheuss V, Knott G, Hill T, Svoboda K (2009). Rapid Functional Maturation of Nascent Dendritic Spines. *Neuron* **61**(2): 247-258.

SECTION II – EXPERIMENTAL WORK

2nd CHAPTER

The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition

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The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition

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Abstract

There is accumulating evidence that adult neurogenesis and dendritic plasticity in the hippocampus are neuroplastic phenomena, highly sensitive to the effects of chronic stress and treatment with most classes of antidepressant drugs, being involved in the onset and recovery from depression. However, the effects of antidepressants that act through the selective inhibition of monoamine oxidase subtype A (MAO-A) in these phenomena are still largely unknown. In the present study, adult neurogenesis and neuronal morphology were examined in the hippocampus of rats exposed to chronic mild stress (CMS) and treated with the selective reversible MAO-A inhibitor (RIMA) drug, pirlindole and the selective serotonin reuptake inhibitor (SSRI), fluoxetine. The results provide the first demonstration that selective MAO-A inhibition with pirlindole is able to revert the behavioural effects of stress exposure while promoting hippocampal adult neurogenesis and rescuing the stress-induced dendritic atrophy of granule neurons.

Keywords

Depression, pirlindole, fluoxetine, stress, neuroplasticity, hippocampus, neurogenesis

Introduction

Major depression is a highly prevalent mood disorder (Kessler and Walters, 1998) associated with a significant social and economic impact (Sheehan, 2002). However, the precise physiopathological mechanisms involved in the aetiology of this disorder and in the therapeutic actions of antidepressant (AD) drugs are still largely unknown (Berton and Nestler, 2006).

There is increasing evidence that the generation of new neurons and the dendritic reorganization of pre-existing neurons in the adult hippocampus are complementary neuroplastic phenomena involved not only in the onset of but also in the remission from depression (Mateus-Pinheiro et al., 2013; Snyder et al., 2011; Surget et al., 2011). The potentiation of adult neurogenesis in the dentate gyrus of the hippocampus has been extensively described with the administration of different classes of AD drugs, namely with non-selective monoamine oxidase (MAO) inhibitors (Malberg et al., 2000), tricyclic antidepressants (TCAs) (Sairanen et al., 2005) and selective serotonin reuptake inhibitors (SSRIs) (Santarelli et al., 2003). These observations suggest that the different pharmacological interventions targeting monoaminergic neurotransmission have a common effect in adult neurogenesis. However, the effects of ADs in the reversal of stress-induced morphological changes of granule neurons in the hippocampus have only been described with TCAs and SSRIs (Bessa et al., 2009a; Jayatissa et al., 2006; Surget et al., 2011). Thus, it remains to be established whether adult neurogenesis and dendritic reorganization of pre-existing hippocampal granule neurons after stress exposure are involved in the behavioural actions of selective reversible MAO-A inhibitors (RIMA).

In the present study, we evaluated the behavioural effects of the RIMA pirlindole (Macedo et al., 2011) and the SSRI fluoxetine in the chronic mild stress (CMS) animal model. In addition, we examined whether stress-induced changes in neurogenesis and neuronal plasticity within the hippocampus are influenced by these commonly used ADs. Our results demonstrate that, like fluoxetine, pirlindole is able to reverse the behavioural effects of stress exposure while potentiating hippocampal adult neurogenesis and rescuing the stress-induced dendritic atrophy of granule neurons.

Experimental procedures

Animals

Ninety-six male Wistar rats (Charles-River Laboratories, Barcelona, Spain), weight 300-400 g, age 2 months, were used in this study. Animals were housed (three per cage) under standard laboratory conditions (12 h light: 12 h dark cycle, lights on at 8 a.m., 22ºC, relative humidity 55%; free access to food and water). Animals

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were assigned to one of the six treatment groups. A control group (*n*=16) and five treatment groups exposed to CMS: vehicle (*n*=16), fluoxetine 10 mg/kg (*n*=16), pirlindole 5 mg/kg (*n*=16), pirlindole 15 mg/kg (*n*=16) and pirlindole 30 mg/kg (*n*=16). All procedures were carried out in accordance with the Portuguese national authority for animal experimentation, Direção Geral de Veterinária (ID: DGV9457) and in accordance with the guidelines for the care and handling of laboratory animals in Directive 2010/63/EU of the European Parliament and Council.

Drugs

The drugs used were fluoxetine (10 mg/kg; Kemprotec, Middlesbrough, UK) and pirlindole (5, 15 and 30 mg/kg; Grupo Tecnimede, Sintra, Portugal). Compounds were dissolved in sterile distilled water with sonication and administered intraperitoneally (1 mL/kg) to animals, daily at 20:00. During the last 3 weeks of CMS, animals were given daily injections with vehicle (*n*=16), fluoxetine 10 mg/kg (*n*=16), pirlindole 5 mg/kg (*n*=16), pirlindole 15 mg/kg (*n*=16) and pirlindole 30 mg/kg (n=16). The daily doses of AD drugs administered chosen were based on their therapeutic effects as described in the literature (Bruhwyler et al., 1997; Song et al., 2006).

Chronic mild stress

A slightly modified version of an unpredictable CMS protocol was used (Bessa et al., 2009b; Willner, 2005). It consisted of chronic exposure to unpredictable mild stressors (confinement in a restricted space for 1 h, placement in a tilted cage [30º] for 4 h, housing on damp bedding for 8 h, overnight illumination, food deprivation for 18 h followed by exposure to inaccessible food for 1 h, water deprivation for 18 h followed by exposure to an empty bottle for 1 h, overcrowding for 4 h, exposure to noise for 4 h, exposure to strobe lights for 4 h and reversed light/dark cycle for 48 h every 7 days) over 7 weeks (Supplementary Table 1).

Sucrose preference test

Anhedonia was assessed weekly during exposure to CMS using the sucrose preference test (SPT). Briefly, animals were allowed to habituate to the sucrose solution in three baseline trials of 1 h exposure to 1% sucrose solution or tap water, following 18 h of food and water deprivation 1 week before the CMS protocol, to establish baseline preference levels. To test sucrose preference, animals that were food- and water-deprived for 18 h (Supplementary Table 1) were presented with two pre-weighed bottles containing 1% sucrose solution or tap water for a period of 1 h. Sucrose preference was calculated according to the formula: sucrose preference = [sucrose intake / (sucrose intake + water intake)] X 100, as previously described (Bekris et al., 2005).

Forced swimming test

Depressive-like behaviour was evaluated in the forced swimming test (FST) on the last day of exposure to CMS. Twenty-four hours after a pre-test session (10 min), rats were placed in transparent glass cylinders (64 cm height and 22 cm diameter) filled with water (25ºC; depth 30 cm) for a period of 5 min. Test sessions were assessed using a camera connected to a video tracking system (Viewpoint, Lyon, France); the system automatically calculated immobility time and latency to immobility. Depressive-like behaviour was defined as an increase in time of immobility and a decrease in latency to immobility (Castagné et al., 2011).

Immunostaining procedures

For cell proliferation and cell phenotype analysis, eight animals from each experimental group were injected with a single dose of BrdU (100 mg/kg, Sigma-Aldrich, St Louis, USA) 24 h before sacrifice (Landgren and Curtis, 2011). At the end of the experimental procedures, animals were sacrificed under anaesthesia. Serial coronal sections (20 µm) were cut and stained for BrdU (1:50; Dako, Glostrup, Denmark). Sections were then double-stained with PSA-NCAM for neuroblasts (1:500; Millipore, Billerica, MA, USA) or GFAP for glial cells (1:200; Dako). Proliferation densities were estimated in the subgranular zone (SGZ) of the dentate gyrus as a ratio between the total number of immunostained cells and the area of the SGZ, using an Olympus BX51 optical microscope and *Newcast* software (Visiopharm, Hoersholm, Denmark). For each animal, eight sections were analysed. The double staining with neuronal (PSA-NCAM) or glial (GFAP) markers (von Bohlen, 2011) were performed using a confocal microscope (Olympus FV1000) and an optical microscope (Olympus BX51), respectively.

Structural analysis

For the 3D morphometric analysis, six animals from each treatment group were transcardially perfused with 0.9% saline and processed (Gibb and Kolb, 1998). Briefly, brains were immersed in Golgi-Cox solution for 21 days (Glaser and Van der Loos, 1981), transferred to a 30% sucrose solution and cut on a vibratome. Coronal sections (200 µm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinised in 18.7% ammonia, developed in Dektol (Kodak), fixed in Kodak Rapid Fix, dehydrated and xylene-cleared before coverslipping. Dendritic arborisation and spine numbers and shape of granule neurons were analysed using a motorized microscope (Axioplan 2, Carl Zeiss) and Neurolucida software (Microbrightfield, Willinston, USA). A 3D analysis of the reconstructed neurons was performed using NeuroExplorer software (MicroBrightfield, Inc.). Forty neurons were studied for each animal and measurements from individual neurons were averaged for each animal. Several aspects of dendritic morphology were examined, namely, the total dendritic length, dendritic spine density, spine morphology and Sholl analysis to evaluate the spatial arrangement of dendritic material by quantifying the number of dendritic intersections at concentric 20-µm intervals from the soma (Harris et al., 1992).

Statistical analysis

After confirmation of homogeneity, appropriate statistical tests were applied to the data. Repeated measures ANOVA was used to analyse the results of the sucrose consumption test. One-way ANOVA was used to evaluate the impact of stress and the effect of ADs in further behavioural and structural data. Differences between groups were then determined by Tukey's honestly

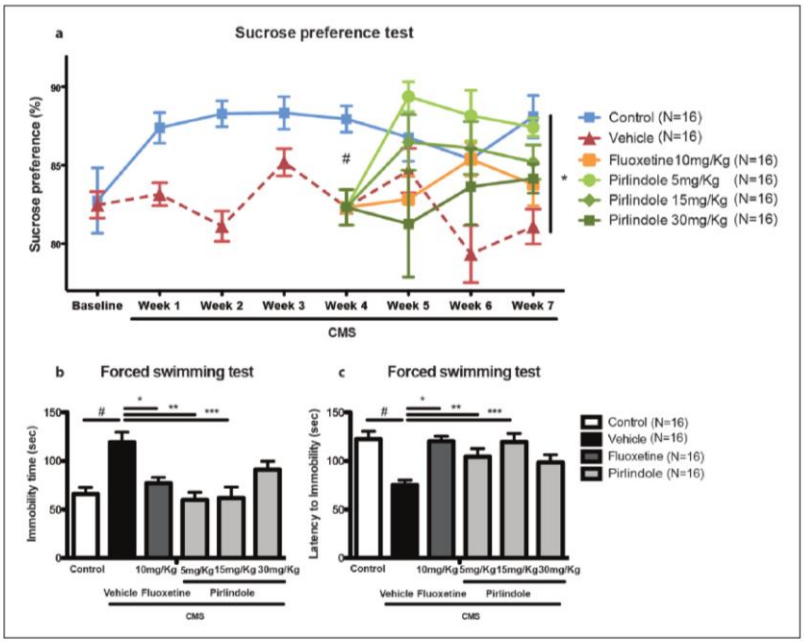

Figure 1. Behavioural effects of fluoxetine and pirlindole on hedonic and depressive-like behaviour. (a) Sucrose preference in the SPTs performed during CMS. #P=0.003, *P<0.001. (b) Immobility time and (c) latency to immobility time in the forced swimming test performed after CMS and drug administration. #P<0.001, *P<0.05; **P<0.05, ***P< 0.001. Data presented as mean + s.e.m.

significant difference test (Tukey HSD) post-hoc analysis. Statistical significance was accepted for P<0.05. Results are expressed as mean + s.e.m.

Results

Behavioural results

The analysis of the SPT during the first 4 weeks of the CMS protocol revealed a significant decrease of sucrose preference in animals exposed to chronic stress treated with vehicle ($F_{1,94}$=9.147; P=0.003) confirming the induction of an anhedonic behavioural phenotype. During the last 3 weeks of the CMS protocol, a significant global effect of treatment was observed ($F_{5,90}$=5.964; P<0.001). Post-hoc analysis revealed significant differences between the animals treated with vehicle and animals treated with pirlindole 5 mg/kg (P=0.001). However, no significant differences were observed with fluoxetine or with higher doses of pirlindole (Figure 1a). Concerning behaviour in the FST, a significant increase in immobility time ($F_{1,32}$=23.839; P<0.001) and decrease in latency to immobility time ($F_{1,32}$=23.834; P<0.001) was observed in stress-exposed animals (Figure 1b,c). AD treatment proved to be a significant factor in recovery from depressive-like behaviour in the FST ($F_{4,80}$=7.433; P<0.001). Fluoxetine reversed the stress-induced behaviour in immobility time (P=0.012) and in latency to immobility time (P<0.001). Similarly, treatment with pirlindole induced a significant decrease in immobility time at dosages of 5 mg/kg (P<0.001) and 15 mg/kg (P<0.001) and an increase in latency to immobility time at dosages of 5 mg/kg (P=0.043) and 15 mg/kg (P<0.001). No significant effects were observed at a dosage of pirlindole 30 mg/kg.

Cell proliferation, neurogenesis and glial phenotype

Cell proliferation analysis (Figure 2a,b) revealed a non-significant decrease in the density of BrdU-positive cells in animals exposed to CMS ($F_{1,16}$=2.090; P=1.170). However, an overall effect of AD treatment was observed ($F_{4,40}$=2.697; P=0.046) with a significant increase associated with fluoxetine treatment (P=0.029) in comparison with vehicle-treated animals. Adult neurogenesis assessed by co-labelling of BrdU with neuroblast marker PSA-NCAM (Figure 2c,d) was significantly decreased with stress exposure ($F_{1,16}$=6.728; P=0.021). A significant effect of AD treatment was observed ($F_{4,40}$=5.235; P=0.002). Both fluoxetine (P=0.033) and pirlindole at all dosages tested (5 mg/kg, P=0.035; 15 mg/kg, P=0.005; 30 mg/kg, P=0.005), increased adult neurogenesis in the SGZ. No significant effects of stress exposure or AD treatment were observed in glial lineage in the SGZ as assessed by co-labelling of BrdU with glial cell marker GFAP (Figure 2e,f).

Structural analysis

The three-dimensional morphometric analysis of Golgi-impregnated neurons in the dentate gyrus (Figure 3) revealed that

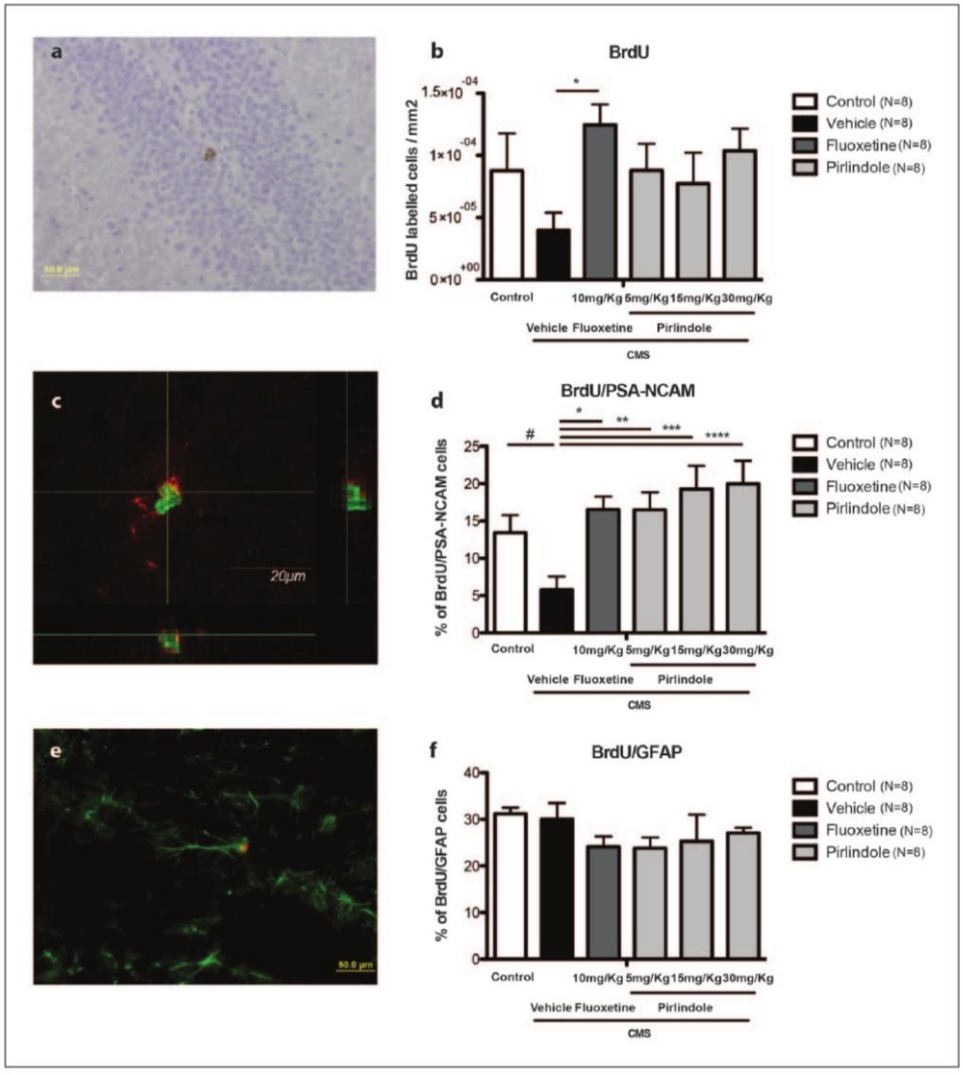

Figure 2. Cell proliferation, neurogenesis and glial phenotype. (a) Proliferative niche of BrdU-labelled cells in the subgranular zone (SGZ) obtained with optical microscopy. (b) The density of BrdU-labelled cells in the SGZ of the dentate gyrus. **P*<0.05. (c) Niche of newly formed neurons in the SGZ, obtained by confocal microscopy. Green: BrdU-positive cells; red: PSA-NCAM-positive cells. (d) The percentage of BrdU-immunopositive cells that were co-labelled with antibodies against PSA-NCAM in the SGZ. #*P*<0.05, **P*<0.05; ***P*<0.05, ****P*=0.005, *****P*=0.005. (e) Newly formed glial cells in the SGZ, obtained by optical microscopy. Green: GFAP-positive cell; red: BrdU-positive cell. (f) Percentage of BrdU-positive cells that were co-labelled with glial marker GFAP in the SGZ. Data represented as mean + s.e.m.

exposure to CMS induced atrophy in granule neurons, with a significant decrease in their total dendritic length ($F_{1,12}$=11.358; *P*=0.007). Importantly, this atrophic effect of chronic stress was reversed after administration of ADs ($F_{4,30}$=5.422; *P*=0.003). Both fluoxetine (*P*=0.002) and pirlindole at dosages of 30 mg/kg (*P*=0.040) significantly increased total dendritic length. No significant effects of stress exposure or antidepressant treatment were observed in spine densities, spine morphology or Sholl analysis (Supplementary Figure 1).

Discussion

The results of this study reveal that the RIMA pirlindole is able to reverse stress-induced anhedonia and confirm the previously described AD effects of pirlindole in the FST (Bruhwyler et al., 1998). This concordance between the reversal of anhedonic behaviour in the SPT and the decrease in immobility in the FST, similar to the one observed with the SSRI fluoxetine, confirms the AD effects of pirlindole in this validated animal model of

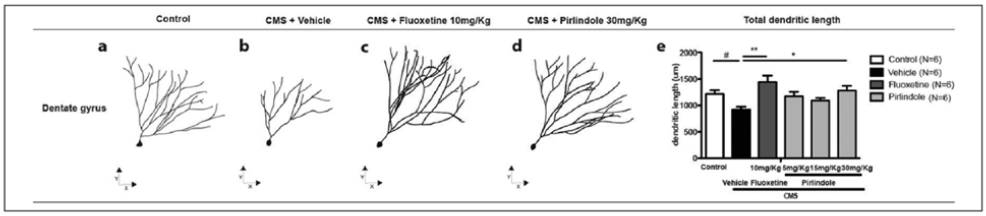

Figure 3. 3D morphometric analysis of Golgi-impregnated neurons using computer-assisted reconstructions of hippocampal granule neurons. Representative neurons of different experimental groups: (a) Control, (b) CMS+vehicle (c) Fluoxetine 10 mg/kg and (d) pirlindole 30 mg/kg. (e) Total dendritic length of neurons in the dentate gyrus of the hippocampus. #P<0.01, *P<0.05; **P<0.005. Data represented as mean + s.e.m.

depression (Bessa et al., 2009b). However, the fact that sucrose preference was evaluated after food deprivation does not exclude the possibility that metabolic demands may be involved in these behavioural results in the SPT. Importantly, the treatment with fluoxetine and pirlindole effectively restored the generation of new neurons in the SGZ without affecting glial cells and reversed the atrophic changes induced by chronic stress.

Interestingly, the results suggest that the effects of pirlindole on depressive-like behaviour are dose-dependent, with the lower dosages showing the ability to reverse the stress-induced changes in the sucrose preference and forced swimming test. However, the effects of this drug in the modulation of hippocampal adult neurogenesis are present at all the doses used. This observation is in accordance with the notion that the mood-improving effects of antidepressants are not exclusively dependent on the ability to modulate the generation of new hippocampal neurons (Bessa et al., 2009a).

The observation that the impact of CMS and ADs was significant on hippocampal neurogenesis but not on cell proliferation suggests that these processes may be differently regulated. This is in accordance with previous studies regarding the effects of CMS and ADs on the proliferation, differentiation and survival of newly born hippocampal cells (Lee et al., 2006; Mateus-Pinheiro et al., 2013). Furthermore, the fact that the highest dose of pirlindole failed to reveal significant AD effects while reversing the effects of stress on dendritic length, suggests a possible dissociation between the behavioural and neuroplastic effects of this drug.

In conclusion, this study provides the first demonstration that MAO-A selective inhibition reverses the deleterious neuroplastic effects of chronic stress in the hippocampus by restoring adult neurogenesis and by rescuing dendritic atrophy of granule neurons. Taking into account the fact that the subtype A of MAO preferentially metabolizes serotonin and noradrenaline (Syha and Schraven, 1990), these results further reinforce the notion that the modulation of monoaminergic neurotransmission is a critical factor for the neuroplastic effects of currently available antidepressant drugs.

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Conflict of interest

Filipe A, Pedroso P and Almeida S are employees of Grupo Tecnimede.

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References

Bekris S, Antoniou K, Daskas S and Papadopoulou-Daifoti Z (2005) Behavioral and neurochemical effects induced by chronic mild stress applied to two different rat strains. *Behav Brain Res* 161: 45–59.

Berton O and Nestler EJ (2006) New approaches to antidepressant drug discovery: beyond monoamines. *Nat Rev Neurosci* 7: 137–151.

Bessa JM, Ferreira D, Melo I, et al. (2009a) The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Mol Psychiatry* 14: 764–773.

Bessa JM, Mesquita A, Oliveira M, et al. (2009b) A trans-dimensional approach to the behavioural aspects of depression. *Front Behav Neurosci* 3:1.

Bruhwyler J, Liégeois JF and Géczy J (1997) Pirlindole: a selective reversible inhibitor of monoamine oxidase A. A review of its preclinical properties. *Pharmacol Res* 36(1): 23–33.

Bruhwyler J, Liégeois JF, Gérardy J, et al. (1998) Comparative study of pirlindole, a selective RIMA, and its two enantiomers using biochemical and behavioural techniques. *Behav Pharmacol* 9(8): 731–7.

Castagné V, Moser P, Roux S, et al. (2011) Rodent models of depression: forced swim and tail suspension behavioral despair tests in rats and mice. *Curr Protoc Neurosci* 2011 Apr; Ch 8:Unit 8.10A.

Gibb R and Kolb B (1998) A method for vibratome sectioning of Golgi-Cox stained whole rat brain. *J Neurosci Methods* 79: 1–4.

Glaser EM and Van der Loos H (1981) Analysis of thick brain sections by obverse–reverse computer microscopy: application of a new, high clarity Golgi-Nissl stain. *J Neurosci Methods* 4: 117–125.

Harris KM, Jensen FE and Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation. *J Neurosci* 12: 2685–26705.

Jayatissa MN, Bisgaard C, Tingstro A, et al. (2006) Hippocampal cytogenesis correlates to escitalopram-mediated recovery in a chronic mild stress rat model of depression. *Neuropsychopharmacology* 31: 2395–2404

Kessler RC and Walters EE (1998) Epidemiology of DSM-III-R major depression and minor depression among adolescents and young adults in the National Comorbidity Survey. *Depress Anxiety* 7: 3–14.

Landgren H and Curtis MA (2011) Locating and labeling neural stem cells in the brain. *J Cell Physiol* 226(1): 1–7.

Lee KJ, Kim SJ, Kim SW, et al. (2006) Chronic mild stress decreases survival, but not proliferation, of new-born cells in adult rat hippocampus. *Exp Mol Med* 38(1):44–54.

Macedo A, Leiria E and Filipe A (2011) Pirlindole in the treatment of depression: a meta-analysis. *Clin Drug Invest* 31(1): 61–71.

Malberg JE, Eisch AJ, Nestler EJ, et al. (2000) Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* 20(24): 9104–10.

Mateus-Pinheiro A, Pinto L, Bessa JM, et al. (2013) Sustained remission from depressive-like behavior depends on hippocampal neurogenesis. *Transl Psychiatry* 15: 3.

Sairanen M, Lucas G, Ernfors P, et al. (2005) Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. *J Neurosci* 25: 1089–1094.

Santarelli L, Saxe M, Gross C, et al. (2003) Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* 301: 805–809.

Sheean DV (2002) Establishing the real cost of depression. *Manag Care* 11: 7–10.

Snyder JS, Soumier A, Brewer M, et al. (2011) Adult hippocampal neurogenesis buffers stress responses and depressive behaviour. *Nature* 476: 458–461.

Song L, Che W, Min-Wei W, et al. (2006) Impairment of the spatial learning and memory induced by learned helplessness and chronic mild stress. *Pharmacol Biochem Behav* 83: 186–193.

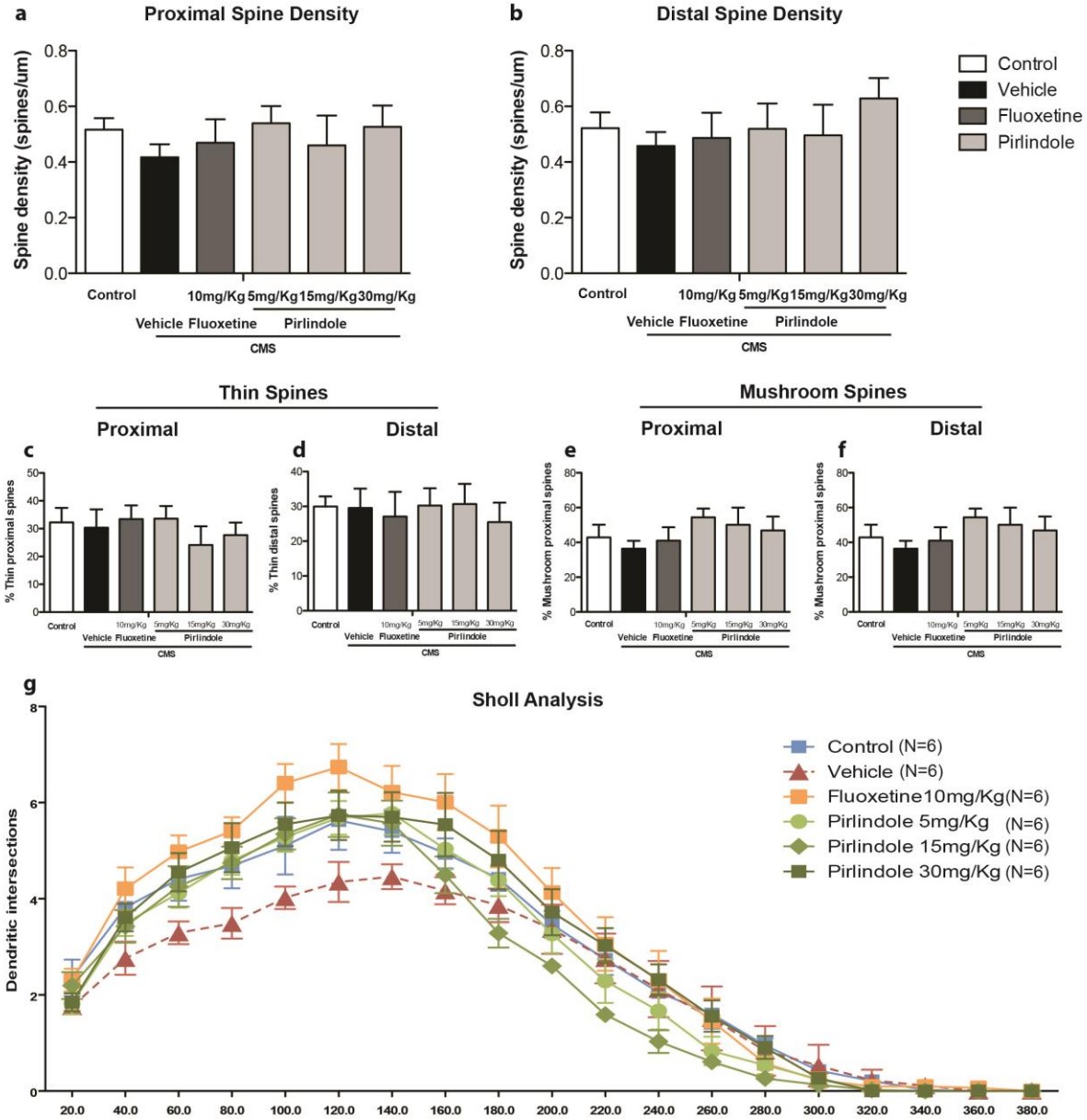
Surget A, Tanti A, Leonardo ED, et al. (2011) Antidepressants recruit new neurons to improve stress response regulation. *Mol Psychiatry* 16: 1177–1188.

Syha K and Schraven E (1990) Effects of a chronic administration of the new antidepressant pirlindole on biogenic amine receptors in rat prefrontal cortex and hippocampus. Comparison with desipramine, nomifensine and zimelidine. *Arzneimittelforschung* 40(8): 843–847.

von Bohlen and Halbach O (2011) Immunohistological markers for proliferative events, gliogenesis, and neurogenesis within the adult hippocampus. *Cell Tissue Res* 345(1): 1–19.

Willner P (2005) Chronic mild stress (CMS) revisited: consistency and behavioral-neurobiological concordance in the effects of CMS. *Neuropsychobiology* 52: 90–110.

Supplementary figures and table



Supplementary Figure 1. (a) Proximal and (b) distal spine densities of granule neurons in the SGZ of the hippocampus. Morphological classification of dendritic spines (c,d,e,f) and sholl analysis (g). Data represented as mean ± s.e.m

<table>
<tr><th></th><th>Monday</th><th>Tuesday</th><th>Wednesday</th><th>Thursday</th><th>Friday</th><th>Saturday</th><th>Sunday</th></tr>
<tr><td>8 a.m.</td><td rowspan="5">Tilted Cage</td><td>Inaccessible Food</td><td>Empty Bottle</td><td rowspan="3">Noise exposure</td><td rowspan="3">Strobe Lights</td><td rowspan="6">Reversed light/dark</td><td rowspan="6">Reversed light/dark + Food and water deprivation</td></tr>
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<tr><td>Overnight</td><td>Food Deprivation</td><td>Water Deprivation</td><td>Overnight illumination</td><td>Wet Bed</td><td>Reversed light/dark</td><td>+ Food and water deprivation</td><td>Sucrose Preference test</td></tr>
</table>

3rd CHAPTER

Modulation of hypothalamic neurogenesis by stress and antidepressants: the relevance in energy balance regulation in depression

M. Morais, A. Pinheiro, P. Patricio, L. Pinto, N. Sousa , J. Bessa

Modulation of hypothalamic neurogenesis by stress and antidepressants: the relevance in energy balance regulation in depression

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Abstract

Major depression is associated with critical changes in appetite and body weight, which are differentially regulated by distinct classes of antidepressants. The hypothalamus is the key brain region involved in energy balance regulation and has been described as a novel neurogenic region. However, the possible modulation of hypothalamic neurogenesis by stress and antidepressant treatment is yet to be explored. In the present study, we aimed to address this by exploring hypothalamic neurogenesis in rats submitted to chronic mild stress (CMS) and treated with the antidepressants fluoxetine and imipramine. These analyses were performed in the arcuate (ARC) and median eminence (ME) nuclei of the hypothalamus, neurogenic regions that have recently been implicated in energy balance regulation. Additionally, the relevance of the functional phenotype of these newborn neurons was assessed by the co-expression with NPY, POMC and leptin receptors. At the end of treatment, the behavioural dimensions commonly affected in depression were assessed. During the entire experimental protocol body weight from each rat was collected once a week. Total food intake (during day and night period) was also quantified for each individual animal.

The results revealed that stress and antidepressant treatment induced significant changes in food intake and body weight gain. Animals exposed to stress presented no differences in the total food intake but revealed significant body weight loss. Treatment with antidepressants differentially regulated these phenomena. While fluoxetine reduced total food intake and body weight gain, imipramine restored total food intake and increased body weight gain. In addition, the circadian disruption of feeding patterns in stressed animals was reversed by both antidepressants. Regarding the impact in the brain, the results revealed that stress and antidepressants differentially modulate hypothalamic neurogenesis in the ARC and ME nuclei. Stressed animals displayed an increase in newborn neurons in the ARC and a decrease in the ME. Interestingly, only imipramine was able to revert these neuroplastic effects.

In summary, this work demonstrates that CMS and antidepressant treatment can modulate hypothalamic neurogenesis in two different hypothalamic nuclei involved in energy homeostasis. Furthermore, a differential effect in hypothalamic neurogenesis was observed with different classes of antidepressants.

Introduction

Major depression and antidepressant treatment are associated with critical changes in appetite and body weight, which are typically included in the neurovegetative symptoms of depression. More frequently, there is a weight loss during the installation of depression and a weight gain during the treatment with antidepressant drugs. However, this effect is dependent on the type of antidepressant used (Davison, 2013; Deshmukh and Franco, 2003; Vanina *et al*, 2002). Fluoxetine, a serotonin selective reuptake inhibitor (SSRI) (Michelson *et al*, 1999), is associated with weight loss while imipramine, a tricyclic agent, is associated with weight gain. In the central nervous systems (CNS), the hypothalamus plays a central role in the regulation of these alterations (Kishi and Elmquist, 2005; Shimogori *et al*, 2010) and the specific hypothalamic neuronal populations express different types of neuropeptides, defined as anabolic and catabolic neurons, acting in a cooperative way to modulate food intake. The anabolic neurons co-express the orexigenic neuropeptides, agouti-related protein (AgRP) and neuropeptide Y (NPY) (Mercer *et al*, 2011), and their up-regulation promotes an increase in food intake. The catabolic neurons express the anorexigenic neuropeptides proopiomelanocortin (POMC) (Boston *et al*, 1997) acting to decrease food intake. The levels of expression of neuropeptides by these two different neuronal populations are regulated by metabolic peripheral signals including hormones (such as leptin and insulin) and gastrointestinal peptides (ghrelin) (Morton *et al*, 2006; Schwartz *et al*, 2000). Consequently, to understand the complexity of body weight regulation the interplay between the CNS and peripheral tissues should be considered.

One of the theories proposes to be involved in the pathophysiology and treatment of depression is the "neurogenic hypothesis of depression". This hypothesis proposes a decrease in hippocampal neurogenesis as a precipitant factor to depression while efficient antidepressant treatment is able to reverse this effect. Previous work from our team has already addressed the importance of adult hippocampal neurogenesis in the onset and remission from depression (Bessa *et al*, 2009a; Mateus-Pinheiro *et al*, 2013; Morais *et al*, 2014). However, until now the importance of this phenomenon in the hypothalamic structure was not been studied. The hypothalamus was recently described as a brain region with cell renewal capacity, with newborn hypothalamic neurons being described as critical players in the regulation of energy balance (Kokoeva *et al*, 2005; Lee *et al*, 2012; Lee *et al*, 2014). The first study describing a functional role for this newly formed hypothalamic neurons was published by Kokoeva *et al*. in 2005 (Kokoeva *et al*, 2005). In this study, obese mice that were infused with a ciliary neurotrophic factor (CNTF) (a drug that induces a decrease in body weight) displayed a strong increase

in hypothalamic neurogenesis. By using a co-administration of CNTF with an antimitotic drug to inhibit neurogenesis in obese mice they determined that the action of the drug on body weight reduction is compromised by neurogenesis inhibition (Kokoeva *et al*, 2005). More recently, Lee and colleagues demonstrated that high-fat diet (that leads to an increase in body weight gain) enhances adult neurogenesis in the hypothalamic median eminence (ME). More interestingly, they found a significant attenuation in body weight gain after the inhibition of neurogenesis in these specific hypothalamic nuclei. This study highlights the role of ME neurogenesis on the promotion of body weight gain in a high-fat diet context (Lee *et al*, 2012). Based on these studies we can appreciate that neurogenesis in the hypothalamus is triggered by different stimulus mainly involved on the modulation of appetite and energy balance control. In addition, the hypothalamic nuclei that respond by creating new neurons are dependent on the type of stimuli applied.

In the present study, we aimed to explore the link between the changes in energy balance (body weight and food intake) in the context of depression (and antidepressant treatment) and the possible modulation of hypothalamic cell genesis (the formation of new neurons and astrocytes). Additionally, we also aimed to explore if these newborn cells exhibit a functional phenotype relevant to energy-balance regulation, namely if they express POMC/NPY neuropeptides and leptin receptors. These analyses were performed in the arcuate (ARC) and median eminence (ME) nuclei of the hypothalamus, already describe as neurogenic regions implicated in energy balance regulation. To address these questions an unpredictable chronic mild stress (uCMS) paradigm was implemented during 9 weeks to induce core symptoms of depressive-like behaviour in rats (Bessa *et al*, 2009a; Bessa *et al*, 2009b; Mateus-Pinheiro *et al*, 2013). During the last 3 weeks of uCMS, two different antidepressants, fluoxetine and imipramine, were daily administered. During the entire experimental protocol, food intake (during day and night period) and body weight gain were assessed once a week. At the end of treatment, animals were sacrificed and the brain was collected for cell genesis analysis and gene expression studies.

Materials and methods

Animals

Male Wistar rats (Charles-River Laboratories, Barcelona, Spain), weighing 300–400 g and aged 3 months were used in this study. Animals were housed (two per cage) under standard laboratory conditions (12h light/ 12 h dark cycle, at 22°C, relative humidity of 55%; free access to food and water). Animals were assigned to one of two main treatment groups (control and uCMS). All procedures were carried out in accordance with European Union Directive 86/609/EEC and NIH guidelines on animal care and experimentation.

Unpredictable chronic mild stress protocol

Unpredictable chronic mild stress (uCMS) was implemented based on a slightly modified protocol (Willner, 2005), already validated in our laboratory (Bessa *et al*, 2009b). Briefly, animals were random- and uninterruptedly exposed to a variety of mild stressors (confinement to a restricted space for 1h; overnight food deprivation followed by 1h of exposure to inaccessible food; overnight water deprivation followed by 1h of exposure to an empty bottle; overnight damp bedding; inverted light/dark cycles; exposure to stroboscopic lights and noise exposure) during 9 weeks. During the last 3 weeks of uCMS, animals were given daily injections of saline, fluoxetine and imipramine.

Body weight and food intake measures

During the entire experimental protocol body weight from each rat was collected once a week. Longitudinal weight gain was normalized to weight at the beginning of the experimental protocol. Total food intake (during day and night period) was also quantified for each animal. Total food intake was quantified during 24h once a week.

Drugs

The drugs used were fluoxetine (10mg/kg; Kemprotec, Middlesborough, UK) and imipramine (10mg/kg; Sigma-Aldrich, St Louis, MO, USA). Fluoxetine and imipramine were administered intraperitoneally (i.p.;1ml/kg). Compounds were dissolved in 5% DMSO in 0.9% saline.

Behavioural Tests

Sucrose preference test

To assess anhedonia, the sucrose preference test was conducted weekly during all the experimental procedure. Briefly, animals were allowed to habituate to the sucrose solution for 1 week before the uCMS protocol to establish baseline values for sucrose preference. To test sucrose preference, animals that were food- and water-deprived for 24h and then presented with two pre-weighed bottles containing 2% of sucrose solution or tap water for a period of 1 h. Sucrose preference was calculated according to the formula: sucrose preference = [sucrose intake/(sucrose intake + water intake)] × 100, as previously described (Bekris *et al*, 2005). Anhedonia was defined as a reduction in sucrose preference relative to baseline levels.

Forced swimming test

Behavior despair was assessed through the forced swimming test. Twenty-four hours after a pre-test session (10min), rats were placed in cylinders filled with water (25°C; depth 30cm) for a period of 5min. Test sessions were assessed using a camera connected to a video tracking system (Viewpoint); the system automatically calculated immobility time and latency to immobility. Behavioral despair was defined as an increase in time of immobility and a decrease in latency to immobility.

Novelty suppressed feeding

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.

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) at the end of the uCMS protocol.

Tissue processing and immunohistochemical analysis

For immunofluorescence analysis, animals were anaesthetized with sodium pentobarbital (Eutasil, 60 mg/Kg i.p.; Ceva Saúde Animal, Portugal) and perfused transcardially with 0,9% of NaCl followed by 4% of paraformaldehyde. Brains were following immersed in sucrose solution (30%), preserved with OCT compound and snap-frozen. Serial coronal sections (20 μm), extending over the entire length of the telencephalon, were cut on a cryostat and stored at -20°C. The impact of uCMS and antidepressant treatment on hypothalamic neurogenesis was assessed by double staining using a marker to assess cell proliferation ki-67 (rabbit Ki-67 antibody; 1:300; Millipore) and a neuronal marker for immature and mature neurons Hu (mouse monoclonal anti-Hu; 1:200; Molecular Probes). Gliogenesis was assessed using ki-67 (rabbit Ki-67 antibody; 1:300; Millipore) and a glia cell marker (mouse GFAP Ab-6 (ASTRO6); 1:200; NeoMarkers). Immunofluorescence to POMC (chicken polyclonal to POMC; 1:200; Abcam), NPY (Guinea pig polyclonal to Neuropeptide Y; 1:500; Abcam) and leptin receptor (Chicken polyclonal to Leptin Receptor, 1:200; Abcam) were also performed. All these analyses were performed using a confocal microscope (Olympus FV1000).

qPCR measurements

Total RNA was isolated from hypothalamus using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 500 ng of total RNA was reverse-transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Quantitative real-time PCR analysis was used to measure the expression levels of the leptin receptor, proopiomelanocortin (POMC) and neuropeptide Y (NPY) mRNA transcript. The reference gene, hypoxanthine guanine phosphoribosyl transferase (Hprt), was used as internal standard for normalization. Oligonucleotide primers for leptin receptor (sense CCGCTGGGTTTGCGTATGGA and antisense AGACGATTTCAGCAGCCTCTCT) NPY (sense TGGACTGACCCTCGCTCTAT and antisense TGTCTCAGGGCTGGATCTCT), POMC (sense TCCATAGACGTGTGGAGCTG and antisense GACGTACTTCCGGGGATTTT) and Hprt (sense GCAGACTTTGCTTTCCTTGG and antisense TCCACTTTCGCTGATGACAC) were designed using the Primer3 software. Reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, LLC, CA, USA) using PerfeCTa SYBRGreen SuperMix, Low ROX (Quanta Biosciences). The relative expression was calculated using the DDCt method. Results are presented as fold-change of mRNA levels between the respective experimental groups after normalization to Hprt levels.

Statistical analysis

After confirming the homogeneity of the data distribution, the appropriate statistical tests were performed using SPSS. Repeated measures ANOVA were used to analyze the results of sucrose preference test**,** body weight gain and food intake during day and night period. One-way ANOVA was used to evaluate the impact of CMS and of the treatment with the drugs in FST, NSF, corticosterone levels in the day and night period and the results of neurogenesis and gene expression. Differences between groups were then determined by Tukey's honestly significant difference test (Tukey HSD) post hoc analysis. Statistical significance was accepted for $P < 0.05$.

Results

Behavioral results

In the present study we explored the possible role of antidepressant drugs in the modulation of hypothalamic neurogenesis. To tackle this question we used a well-established animal model of depression (the uCMS protocol) in which animals were treated with two different antidepressant drugs, fluoxetine and imipramine (Figure 1a). Anhedonia was assessed weekly in the SPT, performed during all the experimental protocol. Animals exposed to uCMS revealed a significant decrease in sucrose preference when compared with control animals ($F_{1,46}$=52,853; P<0.0001), indicating an anhedonic phenotype. Chronic administration of antidepressants in the last 3 weeks of the uCMS protocol lead to a significant global effect reverting the depressive like phenotype ($F_{3,44}$=39,693; P<0.0001) (figure 1b). Post-hoc analysis revealed significant differences between the animals treated with vehicle and animals treated with fluoxetine and imipramine (P<0.0001).

Exposure to the uCMS protocol induced a significant increase in the immobility time in the FST ($F_{1,22}$=27.951; P<0.0001). Antidepressant treatment reversed this phenotype ($F_{2,33}$=25,916, P<0.0001) (Figure 1c). Exposure to chronic stress significantly increased the latency to feed in the NSF paradigm ($F_{1,22}$=6,780; p=0,016). A significant effect of antidepressant treatment was observed ($F_{2,33}$=10,131; P<0.0001). Both imipramine and fluoxetine normalized the anxious phenotype induced by uCMS protocol (P=0.001) (Figure 1e). Corticosterone levels were measured in the serum. At the 9th week of the protocol, control animals presented a functional circadian regulation of the corticosterone production (nadir vs zenith t_{12}=-5,120; p<0.0001) whereas uCMS-exposed animals presented no circadian regulation (nadir vs zenith t_{12}=0,019; p=0,985). The chronic treatment with fluoxetine and imipramine reverted the uCMS effects, presenting a circadian regulation of the corticosterone secretion (nadir vs zenith: fluoxetine: t_{12}=-3,527; p=0,005; imipramine: t_{12}=-4,289; p=0,001). Considering the difference between groups in the two different time points, basal and peak, we found a significant effect in the basal ($F_{3,44}$=4,240; p=0,01) but not in the peak ($F_{3,44}$=2,14; p=0,109). At the basal level we observed a difference between uCMS vs control (p=0,009) and uCMS vs imipramine (p=0,05) (Figure 1e).

Body weight and food intake

Regarding the body weight gain during the experimental protocol before treatment, all animals presented an increase (figure 2a). However, uCMS animals showed a significant reduction in weight

gain when compared with control group (before ($F_{1,46}$=26,05; p<0.0001) and after treatment ($F_{3,44}$=97,536; p<0.0001)). During the last three weeks of the uCMS protocol, the group of animals treated with antidepressants presented a significant *decrease* in *body weight gain* (compared with uCMS animals) (p<0.0001). Comparing the body weight gain curve profile, between the two antidepressants used, we observed a more pronounce effect (a decrease) in fluoxetine than imipramine (p=0,001) group. Total food intake was also analyzed during all the experimental protocol. uCMS animals presented no difference in the total food intake during all the experimental protocol when compared with controls. Regarding the period of chronic antidepressant treatment, in the first week, fluoxetine and imipramine treated animals presented a significant decrease in the total food intake compared with uCMS (p<0.0001). This effect in the reduction of the total food intake persists in the second (p<0.0001) and third (p=0,001) week in the fluoxetine-treated animals. Imipramine-treated animals only presented differences in the first week of treatment (p=0,004), but not in the second (p=0,272) and third week (p=0,585) compared with uCMS animals. Comparing both treatments (fluoxetine versus imipramine), fluoxetine treatment elicited a more pronounce effect in the decrease of food intake in the first (p=0.004), second (p=0.014) and third week (p= 0.01) of treatment. We also measured the amount of food intake during the day and night period during all the experimental protocol (figure 2d). As expected, control animals presented a normal circadian rhythms of feeding (eating more during the night period). uCMS animals exhibited a different curve profile, showing a disruption in the normal pattern of feeding. This disruption in the circadian rhythms of feeding was visible after the 3rd week of the uCMS protocol and was maintained until the end of the study with no significant differences between the food intake during the night and day period (fig f) (control versus uCMS $F_{1,46}$=413,936; p<0.0001). At the end of the experimental protocol (3rd week of treatment), control animals presented a clear difference between the food intake at day and night period (t=-7,879; p=0,001). uCMS animals maintained the pattern of feeding behavior, presenting no differences between the food intake at the day and night (t=-1,967; p=0,106) period. Antidepressant treatment, namely fluoxetine (t=-2,595; p= 0.049) and imipramine (t=-2,425, p= 0.06), was able to restore the normal pattern of feeding. Regarding the effect in the day and night period in all the groups, no differences were observed in the day ($F_{3,20}$=1,391; p=0,274). In the night an overall effect was observed ($F_{3,20}$=7,272; p=0,002). Differences were observed between control and fluoxetine (p=0,002) and imipramine treated animals (p=0,019) and an non statistical difference were observed between uCMS and fluoxetine treatment (p=0,06).

Impact of antidepressants on cell genesis

The possible modulation of adult hypothalamic neurogenesis by uCMS and antidepressant treatment was following analyzed. Two different nuclei of the hypothalamus were studied, namely the arcuate (ARC) and median eminence (ME) nuclei. For that, brain sections of the different groups of animals containing hypothalamus were analyzed for neurogenesis and also gliogenesis (more specifically astrocytes). Regarding neurogenesis in the ARC nucleus of the hypothalamus (figure 3a), uCMS animals presented a significant increase compared with controls ($F_{1,6}$=12,155; p=0,013). Regarding the effect of chronic treatment with antidepressants, an overall effect was observed ($F_{2,9}$=15,298; p=0,001). Fluoxetine treated animals presented the same levels of neurogenesis compared to uCMS (p=0,597); the chronic treatment with imipramine lead to a significant decrease on neurogenesis in this specific hypothalamic nuclei compared with uCMS (p=0,006) and fluoxetine (p=0.001) treated animals (figure 3b). Concerning neurogenesis in the ME nucleus, uCMS animals presented no differences compared with control group ($F_{1,5}$=2,662; p=0,164) (although a tendency to a decrease was observed). Fluoxetine treated animals presented no differences when compared to uCMS (p=0,973) while imipramine induced a significant increase in the formation of new neurons in this specific nucleus (figure 3c) (p=0,003).

Regarding the formation of new astrocytes, a non-significant increase was observed in the uCMS group (control versus uCMS animals). This effect was observed in the ARC ($F_{1,6}$=4,196; p=0.086) but not in the ME ($F_{1,5}$=3,571; p=0,117). The same effect was observed in animals treated with fluoxetine and imipramine (presenting no differences compared to uCMS) both in the ARC and ME nuclei.

After the observation that stress and antidepressants modulate hypothalamic neurogenesis, we explored if these new cells express appetite-related neuropeptides (NPY and POMC) and leptin receptors. Considering the formation of new NPY cells (figure 4a), an increase in the percentage of cells that express NPY was observed in uCMS animals (compared with control) ($F_{1,10}$=7,429; p=0,023). Antidepressant treatment modulated the gene expression of NPY ($F_{2,14}$= 5,444; p=0,018); with fluoxetine and imipramine treated animals presenting no differences compared with uCMS. However, a difference between both antidepressants was observed with imipramine treated animals presenting a decrease in the number of new cells expressing NPY (p=0,014). These results were only observed in the ARC nucleus of the hypothalamus. No new cells expressing NPY were observed in the ME nucleus of the hypothalamus. We also analyzed if these newly born cells express POMC. No new cells expressing

POMC were observed in all the different groups, in both nuclei under study (figure 4c). Additionally, we analyzed if this new proliferating cells expressed leptin receptors. No expression of leptin receptors was observed in the ARC and ME nuclei of the hypothalamus in all the different groups (figure 4d).

Gene expression studies

The mRNA expression levels of leptin receptors, NPY and POMC was measured by qPCR in the dissected hypothalamus. uCMS animals presented a decrease in the levels of leptin receptors compared with control ($F_{1,10}$=5,146, p=0,049). The chronic administration of fluoxetine and imipramine did not induce any alteration in the levels of leptin receptors . Considering the levels of NPY, no differences were observed between control and uCMS ($F_{1,10}$=1,893; p=0,199). Regarding the POMC levels, we observed a decrease after exposition to uCMS ($F_{1,8}$=1,893; p=0,017). Chronic treatment with antidepressants (fluoxetine and imipramine) induced no alteration in its levels (figure 4f).

Discussion

In the present study we show for the first time that chronic stress and antidepressant treatment modulate hypothalamic cell genesis and that these newly formed cells express neuropeptides implicated in the regulation of appetite and energy balance. We also demonstrated that different classes of antidepressants have a different action in the modulation of hypothalamic neurogenesis.

The recent discoveries that the postnatal hypothalamus is able to produce new neurons and that this phenomenon is altered in response to different types of diet have opened new lines of research. In the present study we aimed to understand if the alterations observed in energy balance induced by the exposition to chronic stress as well as treatment with different antidepressants may be associated with variations in hypothalamic neurogenesis. To address this question we used the uCMS animal model of depression, and we chronically treated these animals with two different classes of antidepressants, fluoxetine and imipramine (Bessa *et al*, 2009b). By using different behavioral paradigms, we confirmed the induction of a depressive-like phenotype (SPT, FST and NSF) that was reversed with the administration of fluoxetine and imipramine. Additionally, exposure to uCMS indiced a disruption in the diurnal pattern of corticosterone production, with antidepressant treatment leading to a re-synchronization of the diurnal pattern of corticosterone secretion as previously described (Patricio *et al*, 2015).

Reduction in the body weight gain is a well-known consequence of exposure to chronic stress. In the present study we confirmed this effect, with uCMS animals presenting a clear decrease in the body weight gain comparing with control animals. Regarding the impact of antidepressants, a different curve profile was observed when comparing fluoxetine with imipramine treated animals. As previously described, fluoxetine treated animals exhibited a more pronounced effect on the reduction of the body weight gain compared with imipramine animals (Gutierrez *et al*, 2002).

In terms of total food intake, our uCMS animals presented no differences compared with controls. However, some groups have also observed a decrease in food intake (Farhan *et al*, 2014). Furthermore, we have observed a disruption in the circadian rhythms of feeding, with uCMS animals eating the same during the day and night period. Both antidepressants were able to restore the normal pattern of feeding, eating the treated animals more during their active period (night period). The positive impact of antidepressant drugs in the normalization of the circadian rhythms of feeding could be due to the reestablishment of the diurnal pattern of corticosterone secretion by treatment. Again, and in

accordance with body weight gain data, imipramine-treated animals ate more during all the treatment weeks.

Considering the impact of chronic stress on hypothalamic neurogenesis, more specifically in the ARC nucleus of the hypothalamus, an increase was observed (compared with the control group). In the ME, a decrease on neurogenesis was observed after exposition to uCMS protocol. These results showed for the first time a modulation of hypothalamic neurogenesis by the chronic stress. Furthermore, we observed the involvement of two hypothalamic nuclei, responding differently to the same stimulus. Regarding the impact of antidepressant drugs on the modulation of hypothalamic neurogenesis, fluoxetine treated animals presented the same levels of neurogenesis as uCMS. However, imipramine treated animals presented an opposite impact, decreasing the levels of neurogenesis in the ARC and increasing the levels on the ME nucleus. Interestingly, these two antidepressants have a different impact on food intake and body weight gain and modulate differently the hypothalamic neurogenesis (ARC and ME). Considering the function of these two hypothalamic nuclei, the ARC is a well-established nucleus that plays a crucial role in the regulation of energy balance by expressing different appetite-related neuropeptides, namely NPY and POMC. The function of the ME nucleus is not so well established, however considering that ME lies outside the blood-brain barrier and consequently can sense more easily the alteration in the circulating peripheral signals make this nucleus particularly attractive. The recent paper of Lee *et al.* attributes also a functional role for this nucleus in the increase of body weight with high-fat diet context (Lee *et al*, 2012; Lee *et al*, 2014). Another important finding was also more recently published describing that high-fat diet enhances ME neurogenesis in females but not in males, suggesting a sex-specific modulation of neurogenesis in this nuclei. Additionally, they observed an opposite effect on neurogenesis (a decrease) in the ARC nuclei in both sexes under the same stimulus (high-fat diet) (Lee *et al*, 2014). This was an interesting finding, since the ME and ARC nuclei are in close contact, but can respond differently and in an opposite way to the same stimulus. In the present study we observed the same opposing effects, with ARC and ME nuclei responding differently to the same stimuli (stress and antidepressant treatment). In the future, more studies should be performed to understand the meaning of this different modulation. Another study was published by Sousa-Ferreira *et al*. assessing the impact of fluoxetine on cell proliferation and differentiation using fetal hypothalamic neuroprogenitor cells as an in vitro model. They observed an increase on cell proliferation (using ki-67 as a marker to assess cell proliferation), showing by this way the ability of

fluoxetine to modulate cell proliferation (Sousa-Ferreira *et al*, 2014). This in vitro data correlates with our in vivo study since fluoxetine is able to modulate neurogenesis.

In the ARC nucleus of the hypothalamus, the expression of different neuropeptides (orexigenic and anorexigenic) involved in the modulation of food intake were also analyzed. We found newly formed cells expressing these neuropeptides, highlighting the possible role of hypothalamic neurogenesis in the adaptation to different energy status (induced by stress and antidepressant treatment). Considering the observed increase in the newly NPY cells by uCMS and fluoxetine treatment, we hypothesized a potential compensatory mechanism to counterbalance the impact of stress and fluoxetine treatment in the decrease of body weight gain.

The expression of different genes involved in energy balance control was also analyzed in the hypothalamus. Our data showed a modulation of leptin receptors by uCMS protocol (a decrease was observed). Other groups have already described a reduction in the leptin levels after uCMS and chronic social defeat models of depression (Lu *et al*, 2006). No alterations in the levels of leptin receptors were observed by antidepressant treatment. Regarding the gene expression levels of NPY, no differences were observed indicating that stress and fluoxetine treatment may act specifically in the arcuate nucleus of the hypothalamus. Previously, in an *in vitro* study using fetal hypothalamic neuroprogenitor cells, an increase in the mRNA levels of the orexigenic neuropeptide NPY was reported with fluoxetine treatment (Sousa-Ferreira *et al*, 2014). No differences were observed in the mRNA levels of POMC after fluoxetine administration. In our study we only observed a decrease the levels POMC after uCMS (that was maintained with antidepressant treatment). However, we should consider that we are using a more complex model and treating animals that present a depressive-like phenotype with antidepressants.

In summary, this work demonstrated that chronic stress, as a precipitant factor for depression, can change hypothalamic neurogenesis in two different hypothalamic nuclei involved in the regulation of energy balance. Antidepressant treatment can also modulate hypothalamic neurogenesis, and the type of modulation induced is dependent on the class of antidepressant used. In the future, more studies should be performed to understand the functional implications of cell genesis in the hypothalamus to the behaviour control of hypothalamic function.

References

Bekris S, Antoniou K, Daskas S, Papadopoulou-Daifoti Z (2005). Behavioural and neurochemical effects induced by chronic mild stress applied to two different rat strains. *Behavioural brain research* **161**(1): 45-59.

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA*, et al* (2009a). The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Molecular psychiatry* **14**(8): 764-773, 739.

Bessa JM, Mesquita AR, Oliveira M, Pego JM, Cerqueira JJ, Palha JA*, et al* (2009b). A trans-dimensional approach to the behavioral aspects of depression. *Front Behav Neurosci* **3**: 1.

Boston BA, Blaydon KM, Varnerin J, Cone RD (1997). Independent and additive effects of central POMC and leptin pathways on murine obesity. *Science (New York, NY)* **278**(5343): 1641-1644.

Davison KM (2013). The relationships among psychiatric medications, eating behaviors, and weight. *Eating behaviors* **14**(2): 187-191.

Deshmukh R, Franco K (2003). Managing weight gain as a side effect of antidepressant therapy. *Cleveland Clinic journal of medicine* **70**(7): 614, 616, 618, passim.

Farhan M, Ikram H, Kanwal S, Haleem DJ (2014). Unpredictable chronic mild stress induced behavioral deficits: a comparative study in male and female rats. *Pakistan journal of pharmaceutical sciences* **27**(4): 879-884.

Gutierrez A, Saracibar G, Casis L, Echevarria E, Rodriguez VM, Macarulla MT*, et al* (2002). Effects of fluoxetine administration on neuropeptide y and orexins in obese zucker rat hypothalamus. *Obesity research* **10**(6): 532-540.

Kishi T, Elmquist JK (2005). Body weight is regulated by the brain: a link between feeding and emotion. *Mol Psychiatry* **10**(2): 132-146.

Kokoeva MV, Yin H, Flier JS (2005). Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science* **310**(5748): 679-683.

Lee DA, Bedont JL, Pak T, Wang H, Song J, Miranda-Angulo A*, et al* (2012). Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. *Nat Neurosci* **15**(5): 700-702.

Lee DA, Yoo S, Pak T, Salvatierra J, Velarde E, Aja S*, et al* (2014). Dietary and sex-specific factors regulate hypothalamic neurogenesis in young adult mice. *Frontiers in neuroscience* **8**: 157.

Lu XY, Kim CS, Frazer A, Zhang W (2006). Leptin: a potential novel antidepressant. *Proceedings of the National Academy of Sciences of the United States of America* **103**(5): 1593-1598.

Mateus-Pinheiro A, Pinto L, Bessa JM, Morais M, Alves ND, Monteiro S*, et al* (2013). Sustained remission from depressive-like behavior depends on hippocampal neurogenesis. *Translational psychiatry* **3**: e210.

Mercer RE, Chee MJ, Colmers WF (2011). The role of NPY in hypothalamic mediated food intake. *Frontiers in neuroendocrinology* **32**(4): 398-415.

Michelson D, Amsterdam JD, Quitkin FM, Reimherr FW, Rosenbaum JF, Zajecka J*, et al* (1999). Changes in weight during a 1-year trial of fluoxetine. *The American journal of psychiatry* **156**(8): 1170-1176.

Morais M, Santos PA, Mateus-Pinheiro A, Patricio P, Pinto L, Sousa N*, et al* (2014). The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition. *Journal of psychopharmacology (Oxford, England)* **28**(12): 1178-1183.

Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW (2006). Central nervous system control of food intake and body weight. *Nature* **443**(7109): 289-295.

Patricio P, Mateus-Pinheiro A, Irmler M, Alves ND, Machado-Santos AR, Morais M*, et al* (2015). Differential and converging molecular mechanisms of antidepressants' action in the hippocampal dentate gyrus. *Neuropsychopharmacology* **40**(2): 338-349.

Schwartz MW, Woods SC, Porte D, Jr., Seeley RJ, Baskin DG (2000). Central nervous system control of food intake. *Nature* **404**(6778): 661-671.

Shimogori T, Lee DA, Miranda-Angulo A, Yang Y, Wang H, Jiang L*, et al* (2010). A genomic atlas of mouse hypothalamic development. *Nature neuroscience* **13**(6): 767-775.

Sousa-Ferreira L, Aveleira C, Botelho M, Alvaro AR, Pereira de Almeida L, Cavadas C (2014). Fluoxetine induces proliferation and inhibits differentiation of hypothalamic neuroprogenitor cells in vitro. *PloS one* **9**(3): e88917.

Vanina Y, Podolskaya A, Sedky K, Shahab H, Siddiqui A, Munshi F*, et al* (2002). Body weight changes associated with psychopharmacology. *Psychiatric services (Washington, DC)* **53**(7): 842-847.

Willner P (2005). Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. *Neuropsychobiology* **52**(2): 90-110.

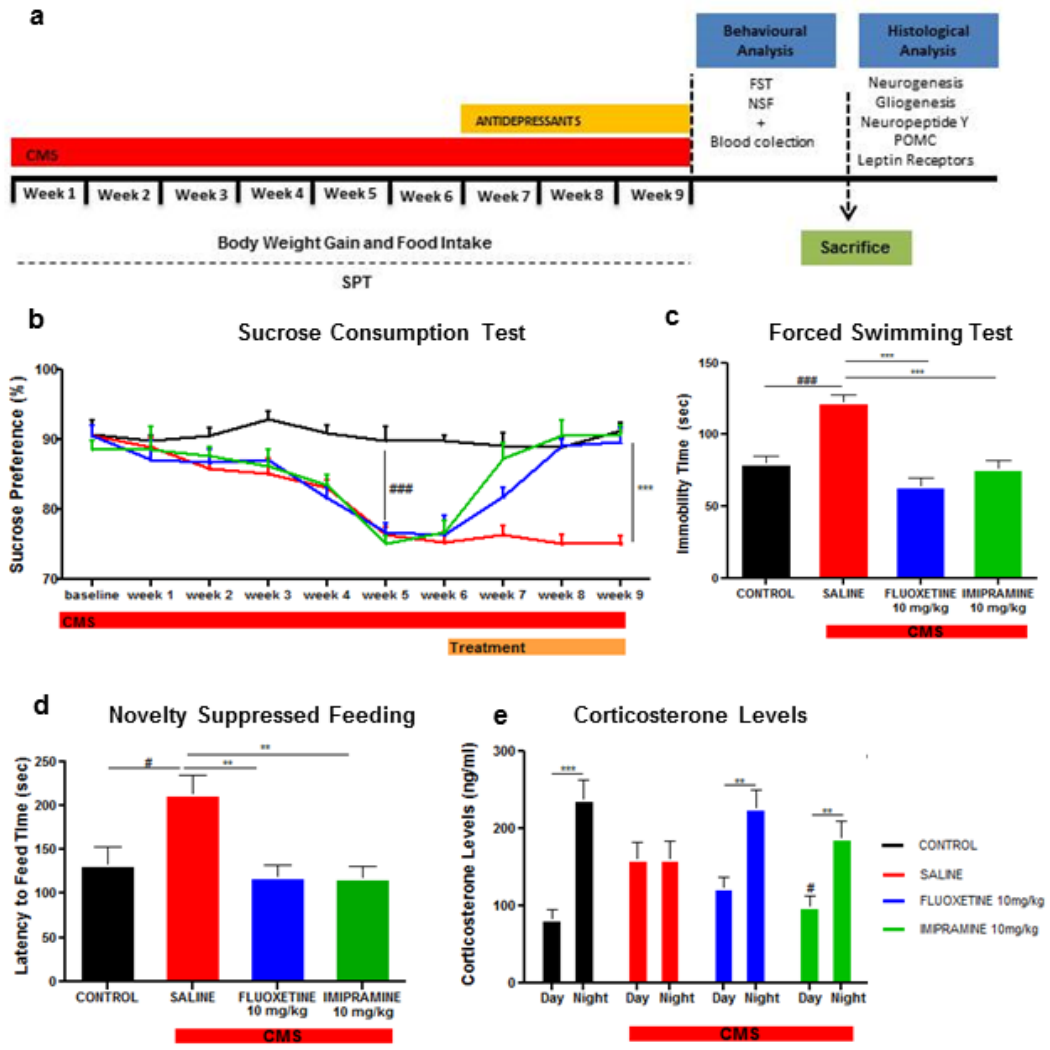

Figure 1- Behavioral effects of the uCMS and antidepressant treatment on mood and anxiety. (a) uCMS protocol was applied to the rats for 9 weeks; two different antidepressants (fluoxetine and imipramine) were administrated in the last three weeks of the uCMS protocol. (b) Sucrose Consumption Test was performed during all experimental protocol to evaluate anhedonia. (c) Learned helplessness was evaluated in the Forced Swim Test. (d) Anxiety was analysed in the Novelty Supressed Feeding (e) Corticosterone levels were measured in the blood. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals. *,# p<0,05; **,## p<0,01;***,### p<0,001.

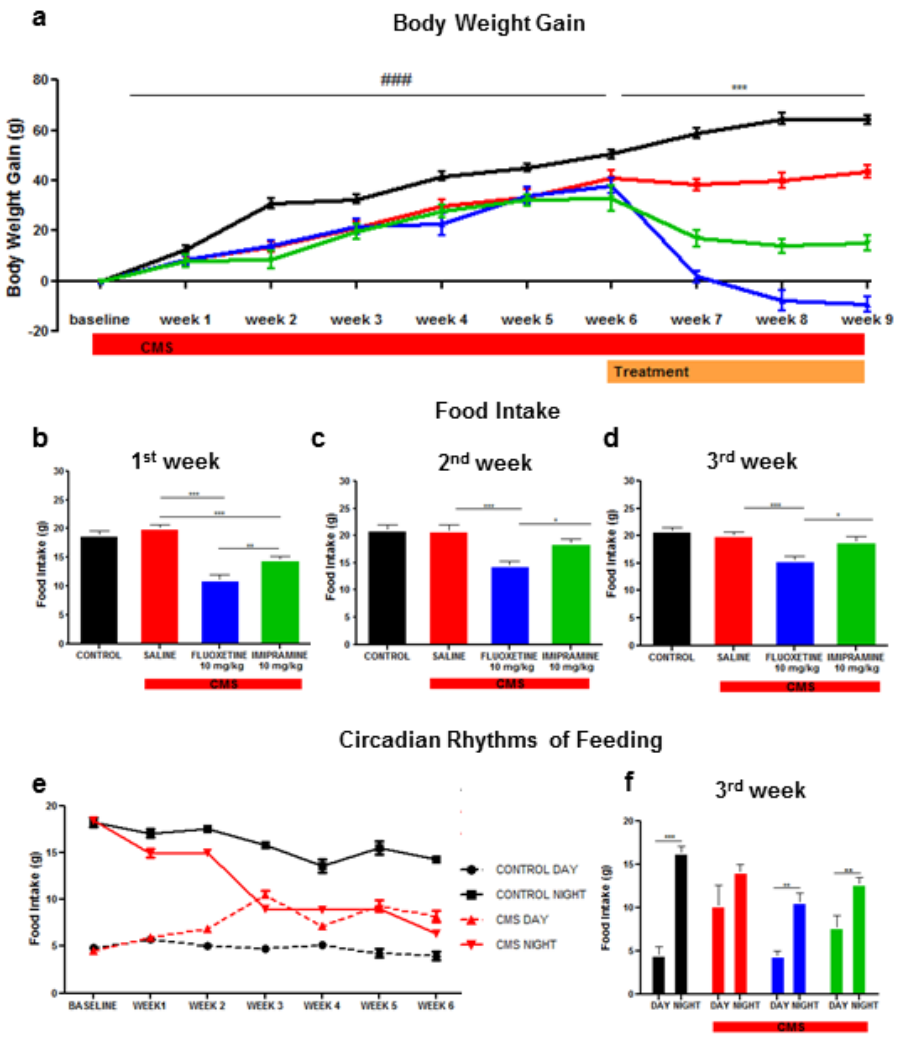

Figure 2- Effects of the uCMS and antidepressant treatment on (a) body weight gain and (b, c, d) appetite during the treatment period. The circadian rhythms of feeding were measured (e) during uCMS protocol and in (f) in the last week of treatment. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals.

*,# p<0,05; **,## p<0,01;***,### p<0,001.

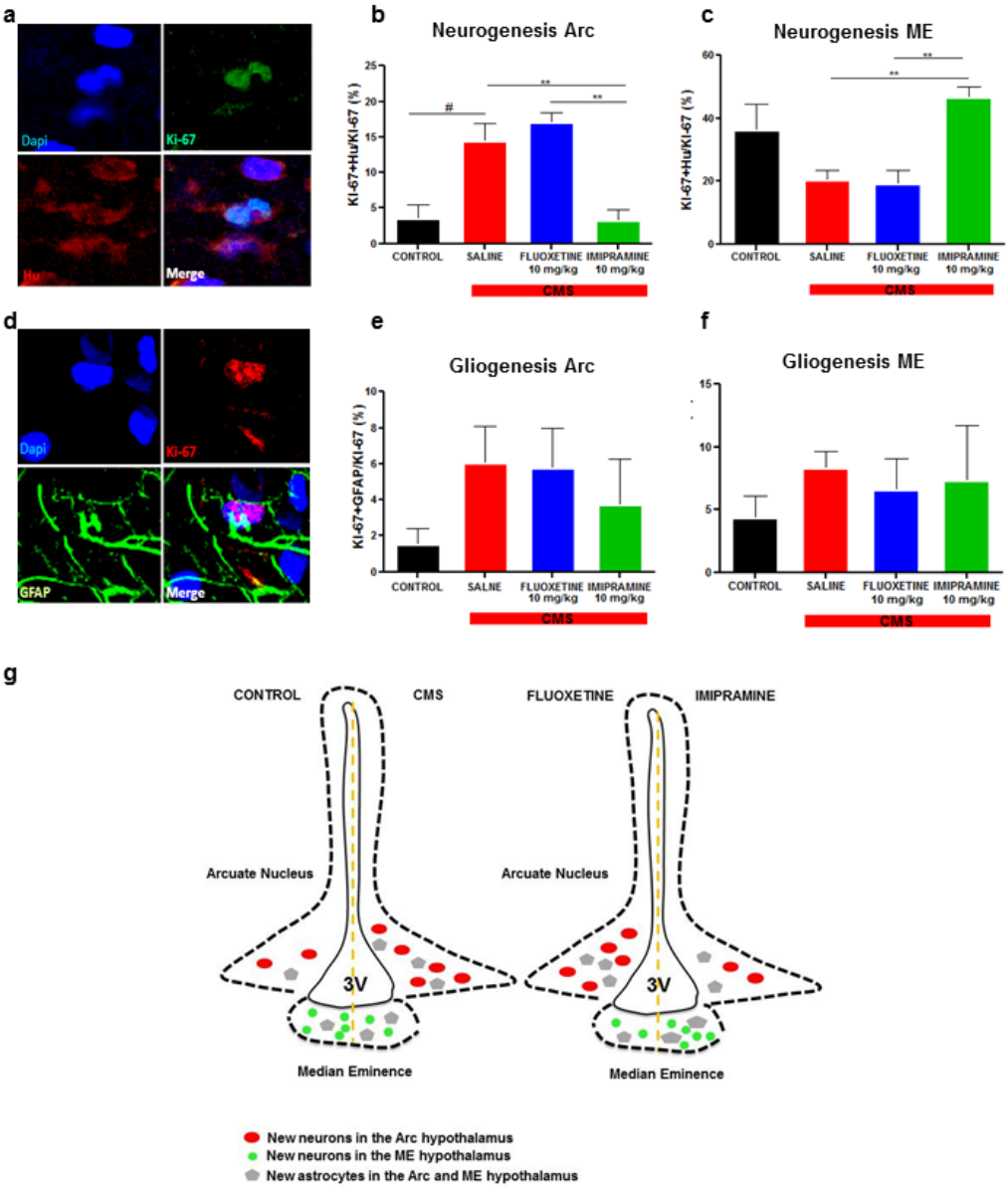

Figure 3- Antidepressant treatment effects on the newly born cells. (a) Proliferative ki-67 cells in the hypothalamus expressing a neuronal marker (HU). (b) The percentage Ki-67 that was co-labelled with Hu in the ARC nucleus of the hypothalamus and (c) ME. (d) Newly formed astrocytes in the hypothalamus. (e) The percentage of ki-67^{+} cells that was co-labelled with the antibody against GFAP in the ARC and (f) ME. (g) Schematic representation of the neuro/gliogenesis process observed in the present study. Data represented as mean + sem. $^{\#}$denotes the effect of CMS-exposure; Data represented as mean + sem. $^{\#}$denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals. $^{*,\#}$p<0,05; $^{**,\#\#}$p<0,01;$^{***,\#\#\#}$ p<0,001.

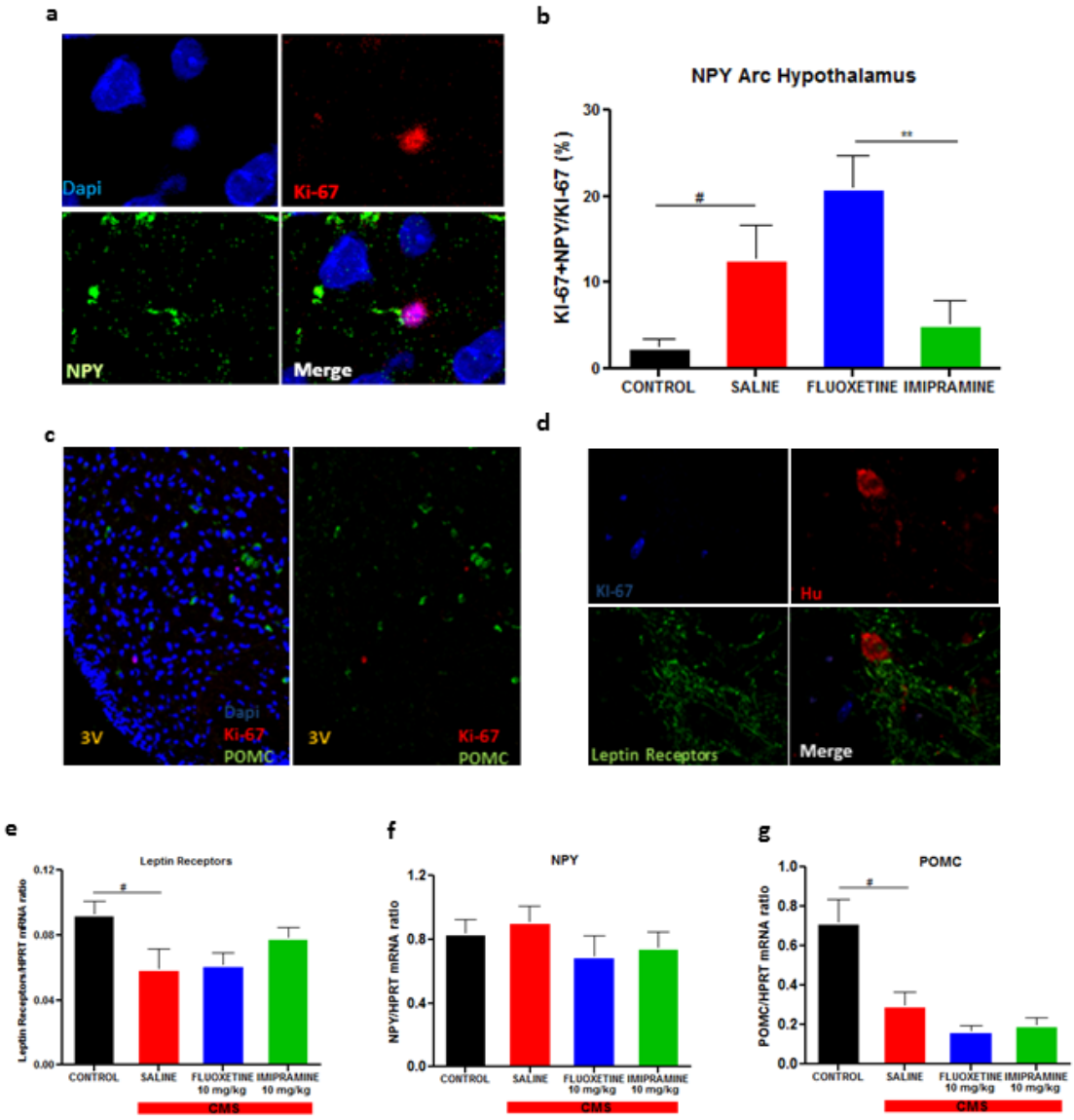

Figure 4- Antidepressant treatment effects on the newly born cells. (a, b) Newly born cell expressing NPY. New cells do not express (c) POMC and (d) NPY. The gene expression levels of (e) leptin receptors (f) NPY and (g) POMC in the hypothalamus. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals.

*,# p<0,05; **,## p<0,01;***,### p<0,001.

4th CHAPTER

The modulation of adult neuroplasticity is involved in the mood improving actions of atypical antipsychotics in an animal model of depression

Mónica Morais, Patrícia Patrício, António Mateus-Pinheiro, Nuno D Alves, Ana R Machado-Santos, Joana S Correia, Joana Pereira, Luísa Pinto, Nuno Sousa, João M Bessa

The modulation of adult neuroplasticity is involved in the mood improving actions of atypical antipsychotics in an animal model of depression

Running title: The neuroplastic effects of antipsychotics in depression

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Abstract

Depression is a prevalent psychiatric disorder with an increasing impact in global public health. However, a large proportion of patients treated with currently available antidepressant drugs fail to achieve remission. Recently, antipsychotic drugs have received approval for the treatment of antidepressant-resistant forms of major depression. The modulation of adult neuroplasticity, namely hippocampal neurogenesis and neuronal remodeling, has been considered to play a key role in the therapeutic effects of antidepressants. However, the impact of antipsychotic drugs on these neuroplastic mechanisms remains largely unexplored. In this study, an unpredictable chronic mild stress protocol was used to induce a depressive-like phenotype in rats. In the last 3 weeks of stress exposure, animals were treated with two different antipsychotics: haloperidol (a classical antipsychotic) and clozapine (an atypical antipsychotic). We demonstrated that clozapine improved both measures of depressive-like behavior (behavior despair and anhedonia) while haloperidol had no significant effect in anhedonia and aggravated learned helplessness in the forced swimming test and behavior flexibility in a cognitive task. Importantly, an upregulation of adult neurogenesis and neuronal survival was observed in animals treated with clozapine while haloperidol promoted a downregulation of these processes. Furthermore, clozapine was able to reestablish the stress-induced impairments in neuronal structure and gene expression in the hippocampus and prefrontal cortex. These results demonstrate the modulation of adult neuroplasticity by antipsychotics in an animal model of depression, revealing that the atypical antipsychotic drug clozapine reverts the behavioral effects of chronic stress by improving adult neurogenesis, cell survival and neuronal reorganization.

Introduction

Major depression is a highly prevalent and complex psychiatric disorder that affects multiple behavioral domains, presenting a wide range of symptoms, namely depressed mood, anhedonia, anxiety and cognitive impairments that confer a severe disability and impaired quality of life in patients.(Mergl *et al*, 2007; Sheehan, 2002; Villanueva, 2013) Strikingly, up to 60% of patients treated with the currently available therapies do not achieve full remission and evolve to treatment resistance.(Blier *et al*, 2011; Lang *et al*, 2013) Taking this into account it is essential to explore new strategies to achieve full remission and to prevent the recurrence of depressive episodes. Multiple clinical studies have previously highlighted the potential beneficial effects of atypical antipsychotics in treatment-resistant depression.(Papakostas *et al*, 2007; Sagud *et al*, 2006; Shelton *et al*, 2008) In accordance, different atypical antipsychotic drugs have received approval from the Food and Drug Administration (FDA) for the treatment of antidepressant-resistant forms of major depression (either as monotherapy or augmentation)(Papakostas *et al*, 2004), a fact that supports their potential role in the emotional domain. Studies in animals confirm this view and show that the association of an atypical antipsychotic and a selective serotonin reuptake inhibitor (SSRI) synergistically increases the release of dopamine in prefrontal areas, thus improving motivation, pleasure, and appetite.(Thase *et al*, 2007; Tohen *et al*, 2003) However, until now the mechanisms by which atypical antipsychotics work in the treatment of this disorder remains unclear.

Antipsychotic drugs are generally classified into classical and atypical. The mechanism of action of classical antipsychotics (e.g. haloperidol), is based on dopamine receptor type 2 (D2) antagonism and have proven to be effective in the positive symptoms of schizophrenia. However, besides eliciting extrapyramidal symptoms (EPS), hyperprolactinemia and metabolic changes, these drugs may exacerbate the negative and cognitive symptoms. In contrast, second generation antipsychotics also known as atypical antipsychotics, (e.g. clozapine), with less potent D2 antagonism and with modulation of serotonin and noradrenaline receptors, maintain their effectiveness against positive symptoms, with fewer EPS and with no impairments on cognitive function and negative symptoms.(Ginovart and Kapur, 2012; Miyamoto *et al*, 2005)

Adult neuroplasticity, namely hippocampal neurogenesis and neuronal morphology have been implicated in the action of antidepressants.(Malberg *et al*, 2000; Morais *et al*, 2014; Patricio *et al*, 2015; Pittenger and Duman, 2008; Santarelli *et al*, 2003) This hypothesis is supported by animal and human studies describing a downregulation of hippocampal neurogenesis and neuronal morphological

complexity under stressful conditions, which is reverted by antidepressant drugs.(Morais *et al*, 2014; Sapolsky, 2004; Snyder *et al*, 2011; Surget *et al*, 2011) Furthermore, these neuroplastic changes have been associated with the expression of neurotrophic factors, cell adhesion molecules and synaptic proteins.(Bessa *et al*, 2009a; Duman, 2004; Opal *et al*, 2014; Pittenger *et al*, 2008) However, the importance of these mechanisms in the therapeutic effects of antipsychotics in depression has never been explored.

In the present study, we evaluated the behavioral effects of different classes of antipsychotics in the chronic mild stress (CMS) animal model of depression. Rats were exposed to the CMS paradigm for 7 weeks to induce core symptoms of depressive-like behavior.(Bessa *et al*, 2009a; Bessa *et al*, 2009b) During the last 3 weeks of CMS, two different antipsychotic drugs, haloperidol and clozapine, were daily administered. Anhedonia was assessed using the sucrose preference test (SPT), during the experimental protocol. Behavior despair was evaluated with the forced swimming test (FST). Cognitive function was assessed by different tasks designed to assess spatial working, reference memory and behavioral flexibility. To explore adult neuroplasticity we examined whether stress-induced changes in neurogenesis at short-term(Bessa *et al*, 2009a; Morais *et al*, 2014) and long-term(Mateus-Pinheiro *et al*, 2013) are influenced by the different classes of antipsychotic drugs. Furthermore, dendritic arborization and complexity was analyzed in Golgi impregnated neurons in the hippocampus and prefrontal cortex (PFC). Finally, the expression of genes involved in neuroplasticity and in antipsychotic action was evaluated in these brain regions.

Materials and Methods

Animals

Male Wistar rats (n=79, Charles-River Laboratories), weighing 200–300g and with 3 months of age were group-housed (three per cage) under 12h light: 12h dark cycles, at 22°C, relative humidity of 55% and with food and water ad libitum. These animals were randomly assigned to four main experimental groups – a control group without stress exposure treated with saline (n=17) and four groups exposed to CMS and treated with either saline (n=17), haloperidol (0,05mg/kg, n=15), clozapine (2,5mg/kg, n=15) and fluoxetine (10mg/kg, n=15). All procedures were carried out in accordance with European Union Directive 86/609/EEC and NIH guidelines on animal care and experimentation.

Chronic mild stress protocol

Chronic mild stress was implemented based on a slightly modified protocol,(Willner, 2005) already validated in our laboratory.(Bessa *et al*, 2009a) Briefly, the animals were random- and uninterruptedly exposed to a variety of mild stressors (confinement to a restricted space for 1h; overnight food deprivation followed by 1h of exposure to inaccessible food; overnight water deprivation followed by 1h of exposure to an empty bottle; overnight damp bedding; inverted light/dark cycles; exposure to stroboscopic lights during 1h and noise exposure during 1h during 7 weeks. During the last 3 weeks of CMS, animals were given daily injections of saline, haloperidol, clozapine and fluoxetine.

Drugs

The antipsychotics used in this study were haloperidol (0,05 mg/kg; Sigma-Aldrich, St Louis, MO, USA), clozapine (2,5 mg/kg; Kemprotec, Middlesborough, UK) and fluoxetine (10mg/kg; Kemprotec, Middlesborough, UK). Compounds were dissolved in distilled water and administered intraperitoneally (i.p.) (1 ml/kg) during the last 3 weeks of the CMS protocol. All injections were performed at 18:00. To assess cell proliferation, neurogenesis and gliogenesis all animals received an injection of Bromodeoxyuridine (BrdU) (100mg/kg, i.p.) 24h before sacrificed. To assess cell and neuronal survival all the animals were injected with BrdU (50mg/kg/day, i.p) during 5 days and sacrificed 1 month later.

Behavioral Tests

Sucrose preference test

To assess anhedonia, the SPT was conducted weekly during all the experimental procedure. Briefly, animals were allowed to habituate to the sucrose solution for 1 week before the CMS protocol to establish baseline values for sucrose preference. To test sucrose preference, animals that were subjected to food and water deprivation for 24h and then presented with two pre-weighed bottles containing 2% of sucrose solution or tap water for a period of 1h. Sucrose preference was calculated according to the formula: sucrose preference = [sucrose intake/(sucrose intake + water intake)] × 100, as previously described.(Bessa *et al*, 2009a) Anhedonia was defined as a reduction in sucrose preference relative to baseline levels.

Forced swimming test

Behavior despair was assessed through the FST on the last day of exposure to CMS. Twenty-four hours after a pre-test session (10 min), the FST was conducted by placing rats in cylinders filled with water (25 °C; depth 30 cm) for a period of 5min. Test sessions were assessed using a camera connected to a video tracking system (Viewpoint); the system automatically calculated immobility time and latency to immobility. Behavior despair was defined as an increase in time of immobility and a decrease in latency to immobility.

Morris Water Maze

Cognitive function was evaluated in different tasks of the Morris Water Maze (MWM): spatial working, reference memory and behavioral flexibility. The MWM was conducted in a circular black tank (diameter: 170 cm; depth: 50 cm), divided in quadrants by imaginary lines, and filled with water (22°C) to a depth of 31 cm. During testing, a black platform (12 × 12 cm; invisible to the rats) was placed at a height of 30 cm. The room was dimly lit and extrinsic visual clues were glued to the walls. Data were collected using a video tracking system (Viewpoint).(Cerqueira *et al*, 2007)

The working memory task was used to evaluate the cognitive domain that relies on the interplay between the hippocampal and PFC function.(Cerqueira *et al*, 2007) In this task the position of the platform is kept constant during the four trials of each day, but varies on each successive day such that all four quadrants are used. Rats are placed, facing the wall of the maze, at a different starting point (north, east, south, or west) at the beginning of each of the four daily trials. A trial is considered

complete when the rat escapes onto the platform; when this escape fails to occur within 120 s, the animal is gently guided to the platform and an escape latency of 120 s is recorded for that trial. Rats are allowed to spend 30s on the escape platform before being positioned at a new starting point. Length of the path described (distance swam) and time spent to reach the platform (escape latency) are recorded in the consecutive trials.

After the working memory procedure, animals were tested in the spatial learning test, an hippocampal-dependent task. In this task, animals were tested for three consecutive days (four trials per day, with a maximum of 2 min per trial). The escape platform was placed in the centre of an arbitrarily-defined quadrant, assigned to a specific test subject. Test sessions begun with rats being placed, facing the wall of the maze, in a defined start position and finished once the escape platform had been reached. This procedure was continued in a clock-wise fashion over the subsequent trials. The distance travelled and the time spent to reach the platform was recorded. When the escape platform was not reached within 2 min, the experimenter guided the animal to the platform. At the end of each test session, animals were dried and allowed to rest for 30 s before being returned to the maze for the remaining test sessions of that day.

After the reference memory evaluation, animals were tested in a reverse learning task (a PFC-dependent function) in which the escape platform was positioned in a new (opposite) quadrant and rats were tested in a four-trial paradigm, as described above. For this task, distance and time spent swimming in each quadrant were recorded. The difference between distances travelled in the quadrant containing the newly-positioned platform ("new") and the quadrant that previously contained the platform ("old") was calculated as a measure of reversal performance. The total distance swum was evaluated as a measure of locomotor activity. All behaviour data analysis was performed with the experimenter blinded to the group under analysis.

Tissue processing and immunohistochemical analysis

Animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Safoni) and perfused with saline and rapidly decapitated. Serial coronal sections (20 μm) were cut on a cryostat and stored at -20°C. For the short-term analysis we evaluate the impact of antipsychotics immediately after chronic treatment on cell proliferation by counting the total number of $BrdU^+$ cells (1:100, Abcam, Cambridge, UK) in the hippocampus using Olympus BX51 optical microscope and Newcast software (Visiopharm). BrdU is incorporated into DNA during the S-phase of the mitotic process, thus allowing the assessment

of cell proliferation. For hippocampal analysis the densities were estimated in the subgranular zone of the dentate gyrus. To assess the impact of these drugs on hippocampal neurogenesis and gliogenesis we performed a double staining for BrdU and polysialylated neuronal cell adhesion molecule (PSA-NCAM) (for neuroblasts; 1:200; Millipore, USA) and glial fibrillary acidic protein (GFAP) (for glia; 1:200; Sigma-Aldrich) using a confocal microscope (Olympus FV1000). For the long-term analysis, we counted the number of $BrdU^+$ cells that survived 4 weeks later after the last BrdU injection and the neuronal phenotype (NeuN, for mature neurons; 1:100; Chemicon, Temecula, CA, USA) of these cells. The schematic representation of the experimental design is described in Figure 1a. To minimize bias, each slide was coded to keep the experimenter blind to the experimental group.

Neuronal Morphology

Three-dimensional morphometric analysis was performed on Golgi-Cox stained material obtained from rats 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 (randomly selected) were analysed in the hippocampal dentate gyrus and PFC. For each selected neuron, dendritic branches were reconstructed at x1000 (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 mm was determined. To minimize bias, each slide was coded to keep the experimenter blind to the experimental group.

Gene expression

Total RNA was isolated from hippocampus and PFC using Trizol reagent (Invitrogen, Carlsbad, CA, USA). 500 ng of total RNA was reverse-transcribed using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Quantitative real-time PCR analysis was used to measure the expression levels of the neural cell adhesion molecule 1(Ncam1), synapsin 1 (Syn1) and brain-derived neurotrophic factor (BDNF) in the hippocampus and PFC. In the PFC we also analysed gene expression levels of different dopamine receptors (Drd1, Drd2 and Drd3). Target gene expression levels were normalized

against the housekeeping gene Beta-2-Microglobulin (B2M). Sense and antisense sequences can be found in Supplementary Table S1. Reactions were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, LLC, CA, USA) using PerfeCTa SYBRGreen SuperMix, Low ROX (Quanta Biosciences). The relative expression was calculated using the DDCt method. Results are presented as fold-change of mRNA levels between the respective experimental groups after normalization to B2M levels.

Statistical analysis

Adequate sample size was determined a priori using G-Power software v3.1.9.2, based on results of a previous pilot experiment suggesting a $\eta 2p$ of 0.424 for the effect of treatment in the FST and assuming a 95% power and 5% probability of type I errors. After confirming the homogeneity of the data distribution, the appropriate statistical tests were performed using SPSS software. Equality of variances was tested with an F test. Repeated measures ANOVA was used to analyse the results of SPT, reference memory, working memory and sholl analysis. Paired sample t-test was used to analyse behavioral flexibility. One-way ANOVA was used to evaluate the impact of CMS and antipsychotic treatment in the FST, neuronal morphology, gene expression and immunostaining results. Differences between groups were then determined by Tukey's honestly significant difference test (Tukey HSD) post hoc analysis. All values were calculated as means + standard error of the mean (SEM). Statistical significance was accepted for $P < 0.05$.

Results

Behavioral results

Anhedonia was assessed weekly in the sucrose preference test. Analysis of the test during the first 4 weeks of the CMS protocol revealed a significant decrease of sucrose preference in animals exposed to chronic stress ($F_{1,62}$=17,470; P<0.001, Figure 1b) confirming the induction of an anhedonic behavioral phenotype. During the last 3 weeks of the CMS protocol, a significant global effect of treatment was observed ($F_{2,44}$=3.378; P=0.043, Figure 1b). Post-hoc analysis revealed significant differences between animals treated with vehicle and animals treated with clozapine (P=0.034) with no significant differences when compared with haloperidol-treated animals (p=0,344) (Figure 1b).

CMS also induced increased immobility in the FST ($F_{1,32}$=11,390; p=0,002, Figure 1c), a measure of behavioral despair, which is another hallmark symptom of depressive-like behavior. The chronic treatment with antipsychotics induces an overall effect ($F_{2,44}$=12,249; P<0,001, Figure 1c). Treatment with clozapine reversed the stress-induced behavior in the immobility time (p=0,037). In haloperidol-treated animals we observed an increase in the immobility time compared to CMS animals (p=0,037, Figure 1c), indicating an aggravation of the depressive-like phenotype.

Cognitive function was assessed in the different tasks of the MWM. In the working memory task, we observed no differences between all the groups analysed (Figure 1d).

The evaluation of spatial learning in the MWM also failed to reveal any significant differences between control and CMS animals ($F_{1,32}$=0,015; p=0,902, Figure 1e). Accordingly, neither clozapine nor haloperidol induced changes in performance in the spatial learning task in the MWM. On the other hand, performance in the reverse learning task, to test behavior flexibility, was significantly impaired in animals exposed to CMS, as indicated by the lower percentage of distance swum in the “new” quadrant compared with the percentage spent in the “old” quadrant (t_{16}=2,637; p=0,018, Figure 1f). This impairment was reversed by clozapine treatment with animals spending approximately the same time in “old” and “new” quadrant as the control group (t_{16}=0,990; p=0,076, Figure 1f). The chronic treatment with haloperidol was not able to reverse the impairment induced by CMS exposure (t_{14}=6,484; p<0,001). We also treated a subset of animals with the antidepressant drug fluoxetine. As expected, fluoxetine treatment was able to reverse the negative effects induced by the exposition to the CMS protocol (Supplementary Figure S1). Additionally, we analysed the impact of antipsychotic drugs in control animals (Supplementary Figure S2); we found no differences between groups, indicating that the

chronic treatment with antipsychotics in control animals does not induce significant changes in all the behaviour domains analysed.

Cell proliferation and differentiation

The possible modulation of adult hippocampal neurogenesis by CMS and antipsychotic treatment was analyzed in two different time points –immediately after the chronic treatment ("short-term) and 4 weeks after the cessation of chronic treatment ("long-term"). We first analyzed the short-term effects on hippocampal cell proliferation by determining the number of BrdU-labeled cells/area in the subgranular zone of the dentate gyrus (DG). The density of $BrdU^+$ cells was significantly reduced in animals exposed to CMS ($F_{1,8}$=11,033, p=0,011, Figure 1a, 1b). Chronic treatment with different classes of antipsychotics had a different impact on cell proliferation ($F_{1,12}$=10,737, p=0,002, Figure 1b). Treatment with clozapine promoted an increase in cell proliferation (p=0,004, Figure 1b), while haloperidol had no effect (p=0,983, Figure 1b). To determine the cell fate of the $BrdU^+$ cells, we co-labelled these cells with cell-specific markers, including PSA-NCAM and GFAP to assess neurogenesis and astrogliogenesis, respectively. In the case of neurogenesis, the percentage of $BrdU^+$ cells that co-labelled with PSA-NCAM was significantly reduced ($F_{1,8}$=9,436; p=0,015, Figure 1c, 1d) in rats exposed to CMS. Regarding the effect of chronic treatment with antipsychotics an overall effect was observed ($F_{2,12}$=17,011; p<0,001, Figure 1d); with clozapine-treated animals presenting an increase (p=0,001, Figure 1d) in the levels of neurogenesis and with no effect in the haloperidol treated group (p=0,790, Figure 1d). Astrogliogenesis (which may include a small percentage of neural progenitor cells) measured by the percentage of $BrdU^+$ cells co-labelled with GFAP was not significantly altered by stress exposure or administration of the different antipsychotic drugs (Figure 1E, 1F).

To assess the role of CMS and antipsychotic drugs in cell survival in the DG (long-term cell analysis) we analysed the number of $BrdU^+$ cells that incorporated BrdU and survived after 4 weeks. CMS animals presented a decrease in cell survival in the DG ($F_{1,8}$=16,104; p=0,004, Figure 2g). Regarding the action of the different antipsychotics used we observed no effect in the haloperidol-treated group (p=0,290, Figure 2g) and a beneficial effect promoted by the clozapine treatment (p=0,018, Figure 2g) with an increase in cell survival. These results are translated in terms of neuronal survival assessed by the quantification of the BrdU cells that express the neuronal marker NeuN (Figure 2h). As previously described we found a decrease in neuronal survival in animals submitted to chronic stress ($F_{1,8}$=7,684; p=0,024, Figure 2i). Regarding the action of the different antipsychotics used we observed an overall

effect of treatment ($F_{1,12}$=5,338; p=0,022, Figure 2i) with an increase in neuronal survival promoted by clozapine (p=0,021, Figure 2i). No effect was observed in animals chronically treated with haloperidol (p=0,666, Figure 2i).

Structural analysis

The three-dimensional morphometric analysis of Golgi-impregnated neurons in the dentate gyrus revealed that exposure to CMS induced atrophy in granule neurons of the DG, with a significant decrease in their total dendritic length ($F_{1,10}$=11,358; p=0,007, Figure 3a, 3b). Only clozapine treatment reversed this structural change (p=0,003, Figure 3a). Sholl analysis revealed no statistical significant differences between the experimental groups (Figure 3c, 3d).

Significant dendritic atrophy in total dendritic length was also observed in pyramidal neurons in the PFC with CMS exposed animals presenting shorter neurons ($F_{1,10}$=17,960; p=0,002, Figure 3e, 3f); this atrophic effect of chronic stress was reversed by treatment with clozapine (p=0,002, Figure 3e) but not by haloperidol (p=0,920, Figure 3e) treatment. In addition, Sholl analysis revealed less complex neurons in CMS-exposed animals when compared with controls ($F_{1,10}$=7,871; p=0,019, Figure 3g, 3h). The effect of CMS was normalized by clozapine (p=0,017, Figure 3h) but not by haloperidol (p=0,233, Figure 3h) treatment.

Gene expression studies

We analysed the expression of different genes described to be involved in neuronal plasticity in the hippocampus and in the PFC. The expression of BDNF was significantly reduced in the hippocampus of CMS rats ($F_{1,8}$=5,510, p=0,047, Figure 4a). Chronic treatment with both antipsychotics was not able to restore the expression of this gene to control levels (Figure 4a). We found no significant statistical differences in the expression of NCAM1 and SYN1 in CMS animals (Figure 4b, 4c) and the chronic treatment with haloperidol and clozapine did not alter their expression (Figure 4b, 4c).

Moreover, animals exposed to CMS revealed significantly reduced levels of BDNF ($F_{1,8}$=29,305; p=0,001, Figure 4d), NCAM1 ($F_{1,8}$=17,184; p=0,003, Figure 4e) and SYN1 ($F_{1,8}$=18,336; p=0,003, Figure 4f) in the PFC. Treatment with clozapine was able to promote an increase in the expression of BDNF (p=0,027, Figure 4d), NCAM1(p=0,048, Figure 4f) and SYN1(p=0,031, Figure 4g) while the treatment with haloperidol revealed a non-significant increase in the gene expression of BDNF (Figure 4d). We also analysed the gene expression of different dopamine receptors in the PFC (Figure 4g, 4h

and 4i). We found Drd2 mRNA levels to be decreased in stressed animals ($F_{1,8}$=6,512; p=0,034, Figure 4h), with no differences in Drd1 and Drd3. Only clozapine treatment was able to restore the gene expression of Drd2 (p=0,019, Figure 4h).

Discussion

Antipsychotic drugs have been generally classified into two distinct pharmacological classes: the classical and the atypical antipsychotics. The classical haloperidol acts as a D2 antagonist with high affinity for the receptors. Clozapine, as an atypical antipsychotic drug, exhibits lower affinity for D2 receptors and displays multiple modulating actions, namely in serotonin and noradrenaline receptors.(Miyamoto *et al*, 2005) Herein we show that different classes of antipsychotics have different effects on adult neuroplasticity, namely on the process of adult hippocampal neurogenesis and neuronal morphology. More so, we found that only clozapine-treated animals, which presented a rescue in neuroplasticity, were able to recover from the depressive-like phenotype and from the cognitive deficits in behavioural flexibility induced by exposure to CMS. Contrarily, haloperidol-treated animals showed impairments in neuroplasticity, presenting important deficits in emotional and cognitive behaviour.

It is now clear that various forms of structural plasticity, including the generation of new neurons and glial cells, may modify the pathophysiology of some neuropsychiatric disorders.(Mateus-Pinheiro *et al*, 2013; Santarelli *et al*, 2003; Surget *et al*, 2011) This idea has been widely explored in depression. In fact, multiple studies have shown a decrease in hippocampal neurogenesis in animal models of depression, while treatment with different antidepressants increases neurogenesis in this region.(Malberg *et al*, 2000; Morais *et al*, 2014; Santarelli *et al*, 2003) Considering that atypical antipsychotics can be effective in the treatment of refractory depression,(Papakostas *et al*, 2007) we hypothesized that these drugs may also regulate neuronal plasticity, in which neurogenesis is included. Our data is in accordance with this hypothesis; we observed that chronic treatment with clozapine is able to promote an increase in neurogenesis and that this effect persists even after the end of the treatment, with clozapine-treated animals presenting an increased neuronal survival. In contrast, the number of newly-born cells and the number of surviving neurons were negatively affected by the chronic treatment with haloperidol. The same effect was described by Maeda and colleagues in an animal model of schizophrenia with impairments in adult neurogenesis.(Maeda *et al*, 2007) However, literature is not consensual regarding this topic with significant discrepancies regarding the effects of haloperidol in neuroplasticity.(Backhouse *et al*, 1982; Dawirs *et al*, 1998; Malberg *et al*, 2000; Wang *et al*, 2004) These conflicting results might be due to differences in experimental designs, drug dosages, and the species studied. Another possible explanation for these discrepancies is the use of control animals to test these drugs, in which there are no prior deficits in behavior or in the levels of

neurogenesis.(Wakade *et al*, 2002; Wang *et al*, 2004) Our present results clearly demonstrate an opposite action of these classes of antipsychotic drugs on adult neurogenesis without affecting the glial cell lineage.

Previous studies from our lab have demonstrated the importance of structural changes in the hippocampus and PFC in the pathophysiology of depression.(Bessa *et al*, 2009a; Mateus-Pinheiro *et al*, 2013; Patricio *et al*, 2015) Antidepressants drugs, independent of their mechanism of action, are able to restore these alterations.(Bessa *et al*, 2009a) Based on these observations, it became critical to understand the impact of antipsychotic drugs in this type of event. In the present study, animals exposed to CMS presented a decrease in the dendritic length in hippocampal granule neurons; importantly, treatment with clozapine, but not haloperidol, was able to reverse this effect. In the PFC, exposure to CMS induced a decrease in total dendritic length and in neuronal complexity; again, only clozapine was able to promote a significant recovery. Our observations are consistent with clinical data suggesting that the structural changes observed in schizophrenia can be attenuated by atypical antipsychotics.(Lieberman *et al*, 2005) Furthermore, previous studies have demonstrated that treatment with an atypical (olanzapine), but not a classical (haloperidol), antipsychotic reversed dopamine denervation-induced changes in dendritic length in the PFC.(Wang and Deutch, 2008) The present results clearly demonstrate the different impact of classic and atypical antipsychotics in neuronal remodelling in the hippocampus and PFC. This is in accordance with our previous study describing neuronal remodelling as an important neuroplastic event for the mood-improving actions of antidepressants.(Bessa *et al*, 2009a)

In addition, we analysed the expression of neuroplasticity-related genes, such as Syn1, NCAM1 and BDNF in the hippocampus and PFC. We observed a decrease in the expression of these genes in the PFC of animals exposed to CMS, but also that chronic treatment with clozapine was able to promote a recovery in gene expression, thereby supporting the neuroplastic effects of this drug. Interestingly, we found that animals with a decrease in the expression of neuroplasticity-related genes presented deficits in a cognitive task depend (reversed learning task) on this brain region. We also analysed the possible modulation of the different dopamine receptors namely Drd1, Drd2 and Drd3 in the PFC. Indeed, CMS exposure induced a significant decrease in Drd2 receptor expression, while the atypical antipsychotic clozapine was able to reverse this effect. These results are in accordance with previous studies that have shown that clozapine exerts its therapeutic effects in part by increasing dopaminergic

neurotransmission in the PFC, while haloperidol had no significant effects on the cortical release of dopamine.(Youngren *et al*, 1999)

Antipsychotic medications are the most commonly prescribed drugs to treat schizophrenia. In 2006, Reif and colleges found a significant reduction in hippocampal neural stem cell proliferation in schizophrenic patients.(Reif *et al*, 2006) Moreover, dendritic changes in frontal cortical pyramidal cells are amongst one of the most replicated findings in post-mortem studies of schizophrenia.(Black *et al*, 2004; Broadbelt *et al*, 2002; Kalus *et al*, 2000; Kolluri *et al*, 2005). These studies highlight the role of neuroplasticity on schizophrenia. Our present results demonstrate a beneficial effect on adult neuroplasticity with chronic treatment with the atypical antipsychotic clozapine. This effect could be one of the possible mechanisms that may contribute to the action of atypical antipsychotics not only in the positive symptoms of schizophrenia but also in the negative and cognitive symptoms. In contrast, the efficacy of haloperidol treatment is mainly against the positive symptoms of schizophrenia, exacerbating the negative and cognitive symptoms. Interestingly, our results clearly indicate the absence of a positive effect on adult neuroplasticity after haloperidol treatment. Despite the diverse pharmacological profiles (monoamine oxidase inhibitors, tricyclic antidepressants, serotonin-selective reuptake inhibitors and serotonin–norepinephrine reuptake inhibitors), all antidepressant drugs result in similar behavioral and neuroplastic outcomes, suggesting similar mechanisms of action. In contrast, classical and atypical antipsychotics are strikingly different as evidenced by their actions, mechanisms, effects and side effects. For instance, the atypical antipsychotic clozapine has a more complex pharmacological lengthprofile than the classical haloperidol, presenting binding affinities for various neurotransmitter receptors, including several serotonin and noradrenaline receptors.(Meltzer *et al*, 1989) Our present results suggest the potential importance of serotonergic and noradrenergic system modulation in the beneficial effects of atypical antipsychotics on neuroplasticity that should be addressed in future studies.

In conclusion, the present study demonstrates the modulation of adult neuroplasticity by antipsychotics, revealing that the atypical antipsychotic drug clozapine reverts the behavioral effects of chronic stress while improving adult neurogenesis, cell survival and neuronal reorganization. These observations may pave the way to new therapeutic interventions not only in treatment-resistant depression but also in schizophrenia.

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Conflict of Interest

The authors declare no conflict of interest.

References

Backhouse, B., O. Barochovsky, C. Malik, A. J. Patel and P. D. Lewis (1982). "Effects of haloperidol on cell proliferation in the early postnatal rat brain." Neuropathol Appl Neurobiol **8**(2): 109-116.

Bessa, J. M., D. Ferreira, I. Melo, F. Marques, J. J. Cerqueira, J. A. Palha, O. F. Almeida and N. Sousa (2009). "The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling." Mol Psychiatry **14**(8): 764-773, 739.

Bessa, J. M., A. R. Mesquita, M. Oliveira, J. M. Pego, J. J. Cerqueira, J. A. Palha, O. F. Almeida and N. Sousa (2009). "A trans-dimensional approach to the behavioral aspects of depression." Front Behav Neurosci **3**: 1.

Black, J. E., I. M. Kodish, A. W. Grossman, A. Y. Klintsova, D. Orlovskaya, V. Vostrikov, N. Uranova and W. T. Greenough (2004). "Pathology of layer V pyramidal neurons in the prefrontal cortex of patients with schizophrenia." Am J Psychiatry **161**(4): 742-744.

Blier, P. and C. Blondeau (2011). "Neurobiological bases and clinical aspects of the use of aripiprazole in treatment-resistant major depressive disorder." J Affect Disord.128 Suppl 1:S3-10. doi: 10.1016/S0165-0327(11)70003-9.

Broadbelt, K., W. Byne and L. B. Jones (2002). "Evidence for a decrease in basilar dendrites of pyramidal cells in schizophrenic medial prefrontal cortex." Schizophr Res **58**(1): 75-81.

Cerqueira, J. J., F. Mailliet, O. F. Almeida, T. M. Jay and N. Sousa (2007). "The prefrontal cortex as a key target of the maladaptive response to stress." J Neurosci **27**(11): 2781-2787.

Dawirs, R. R., K. Hildebrandt and G. Teuchert-Noodt (1998). "Adult treatment with haloperidol increases dentate granule cell proliferation in the gerbil hippocampus." J Neural Transm (Vienna) **105**(2-3): 317-327.

Duman, R. S. (2004). "Role of neurotrophic factors in the etiology and treatment of mood disorders." Neuromolecular Med **5**(1): 11-25.

Ginovart, N. and S. Kapur (2012). "Role of dopamine D(2) receptors for antipsychotic activity." Handb Exp Pharmacol(212): 27-52.

Kalus, P., T. J. Muller, W. Zuschratter and D. Senitz (2000). "The dendritic architecture of prefrontal pyramidal neurons in schizophrenic patients." Neuroreport **11**(16): 3621-3625.

Kolluri, N., Z. Sun, A. R. Sampson and D. A. Lewis (2005). "Lamina-specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia." Am J Psychiatry **162**(6): 1200-1202.

Lang, U. E. and S. Borgwardt (2013). "Molecular mechanisms of depression: perspectives on new treatment strategies." Cell Physiol Biochem. 31(6):761-77. doi: 10.1159/000350094. Epub 2013 May 31.

Lieberman, J. A., G. D. Tollefson, C. Charles, R. Zipursky, T. Sharma, R. S. Kahn, R. S. Keefe, A. I. Green, R. E. Gur, J. McEvoy, D. Perkins, R. M. Hamer, H. Gu and M. Tohen (2005). "Antipsychotic drug effects on brain morphology in first-episode psychosis." Arch Gen Psychiatry **62**(4): 361-370.

Maeda, K., H. Sugino, T. Hirose, H. Kitagawa, T. Nagai, H. Mizoguchi, K. Takuma and K. Yamada (2007). "Clozapine prevents a decrease in neurogenesis in mice repeatedly treated with phencyclidine." J Pharmacol Sci **103**(3): 299-308.

Malberg, J. E., A. J. Eisch, E. J. Nestler and R. S. Duman (2000). "Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus." J Neurosci **20**(24): 9104-9110.

Mateus-Pinheiro, A., L. Pinto, J. M. Bessa, M. Morais, N. D. Alves, S. Monteiro, P. Patricio, O. F. Almeida and N. Sousa (2013). "Sustained remission from depressive-like behavior depends on hippocampal neurogenesis." Transl Psychiatry **3**: e210.

Meltzer, H. Y., B. Bastani, L. Ramirez and S. Matsubara (1989). "Clozapine: New research on efficacy and mechanism of action." European archives of psychiatry and neurological sciences **238**(5): 332-339.

Mergl, R., I. Seidscheck, A. K. Allgaier, H. J. Moller, U. Hegerl and V. Henkel (2007). "Depressive, anxiety, and somatoform disorders in primary care: prevalence and recognition." Depress Anxiety **24**(3): 185-195.

Miyamoto, S., G. E. Duncan, C. E. Marx and J. A. Lieberman (2005). "Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs." Mol Psychiatry **10**(1): 79-104.

Morais, M., P. A. Santos, A. Mateus-Pinheiro, P. Patricio, L. Pinto, N. Sousa, P. Pedroso, S. Almeida, A. Filipe and J. M. Bessa (2014). "The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition." J Psychopharmacol **28**(12): 1178-1183.

Opal, M. D., S. C. Klenotich, M. Morais, J. Bessa, J. Winkle, D. Doukas, L. J. Kay, N. Sousa and S. M. Dulawa (2014). "Serotonin 2C receptor antagonists induce fast-onset antidepressant effects." Mol Psychiatry **19**(10): 1106-1114.

Papakostas, G. I., T. J. Petersen, A. A. Nierenberg, J. L. Murakami, J. E. Alpert, J. F. Rosenbaum and M. Fava (2004). "Ziprasidone augmentation of selective serotonin reuptake inhibitors (SSRIs) for SSRI-resistant major depressive disorder." J Clin Psychiatry **65**(2): 217-221.

Papakostas, G. I., R. C. Shelton, J. Smith and M. Fava (2007). "Augmentation of antidepressants with atypical antipsychotic medications for treatment-resistant major depressive disorder: a meta-analysis." J Clin Psychiatry **68**(6): 826-831.

Patricio, P., A. Mateus-Pinheiro, M. Irmler, N. D. Alves, A. R. Machado-Santos, M. Morais, J. S. Correia, M. Korostynski, M. Piechota, R. Stoffel, J. Beckers, J. M. Bessa, O. F. Almeida, N. Sousa and L. Pinto (2015). "Differential and converging molecular mechanisms of antidepressants' action in the hippocampal dentate gyrus." Neuropsychopharmacology **40**(2): 338-349.

Pittenger, C. and R. S. Duman (2008). "Stress, depression, and neuroplasticity: a convergence of mechanisms." Neuropsychopharmacology **33**(1): 88-109.

Reif, A., S. Fritzen, M. Finger, A. Strobel, M. Lauer, A. Schmitt and K. P. Lesch (2006). "Neural stem cell proliferation is decreased in schizophrenia, but not in depression." Mol Psychiatry **11**(5): 514-522.

Sagud, M., A. Mihaljevic-Peles, D. Muck-Seler, M. Jakovljevic and N. Pivac (2006). "Quetiapine augmentation in treatment-resistant depression: a naturalistic study." Psychopharmacology (Berl) **187**(4): 511-514.

Santarelli, L., M. Saxe, C. Gross, A. Surget, F. Battaglia, S. Dulawa, N. Weisstaub, J. Lee, R. Duman, O. Arancio, C. Belzung and R. Hen (2003). "Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants." Science **301**(5634): 805-809.

Sapolsky, R. M. (2004). "Is impaired neurogenesis relevant to the affective symptoms of depression?" Biological Psychiatry **56**(3): 137-139.

Sheehan, D. V. (2002). "Establishing the real cost of depression." Manag Care **11**(8 Suppl): 7-10; discussion 21-15.

Shelton, R. C. and G. I. Papakostas (2008). "Augmentation of antidepressants with atypical antipsychotics for treatment-resistant major depressive disorder." Acta Psychiatr Scand **117**(4): 253-259.

Snyder, J. S., A. Soumier, M. Brewer, J. Pickel and H. A. Cameron (2011). "Adult hippocampal neurogenesis buffers stress responses and depressive behaviour." Nature **476**(7361): 458-461.

Surget, A., A. Tanti, E. D. Leonardo, A. Laugeray, Q. Rainer, C. Touma, R. Palme, G. Griebel, Y. Ibarguen-Vargas, R. Hen and C. Belzung (2011). "Antidepressants recruit new neurons to improve stress response regulation." Mol Psychiatry **16**(12): 1177-1188.

Thase, M. E., E. S. Friedman, M. M. Biggs, S. R. Wisniewski, M. H. Trivedi, J. F. Luther, M. Fava, A. A. Nierenberg, P. J. McGrath, D. Warden, G. Niederehe, S. D. Hollon and A. J. Rush (2007). "Cognitive therapy versus medication in augmentation and switch strategies as second-step treatments: a STAR*D report." Am J Psychiatry **164**(5): 739-752.

Tohen, M., E. Vieta, J. Calabrese, T. A. Ketter, G. Sachs, C. Bowden, P. B. Mitchell, F. Centorrino, R. Risser, R. W. Baker, A. R. Evans, K. Beymer, S. Dube, G. D. Tollefson and A. Breier (2003). "Efficacy of olanzapine and olanzapine-fluoxetine combination in the treatment of bipolar I depression." Arch Gen Psychiatry **60**(11): 1079-1088.

Villanueva, R. (2013). "Neurobiology of major depressive disorder." Neural Plast **2013**: 873278.

Wakade, C. G., S. P. Mahadik, J. L. Waller and F. C. Chiu (2002). "Atypical neuroleptics stimulate neurogenesis in adult rat brain." J Neurosci Res **69**(1): 72-79.

Wang, H. D. and A. Y. Deutch (2008). "Dopamine depletion of the prefrontal cortex induces dendritic spine loss: reversal by atypical antipsychotic drug treatment." Neuropsychopharmacology **33**(6): 1276-1286.

Wang, H. D., F. D. Dunnavant, T. Jarman and A. Y. Deutch (2004). "Effects of antipsychotic drugs on neurogenesis in the forebrain of the adult rat." Neuropsychopharmacology **29**(7): 1230-1238.

Willner, P. (2005). "Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS." Neuropsychobiology **52**(2): 90-110.

Youngren, K. D., F. M. Inglis, P. J. Pivirotto, H. P. Jedema, C. W. Bradberry, P. S. Goldman-Rakic, R. H. Roth and B. Moghaddam (1999). "Clozapine Preferentially Increases Dopamine Release in the Rhesus Monkey Prefrontal Cortex Compared with the Caudate Nucleus." Neuropsychopharmacology **20**(5): 403-412.

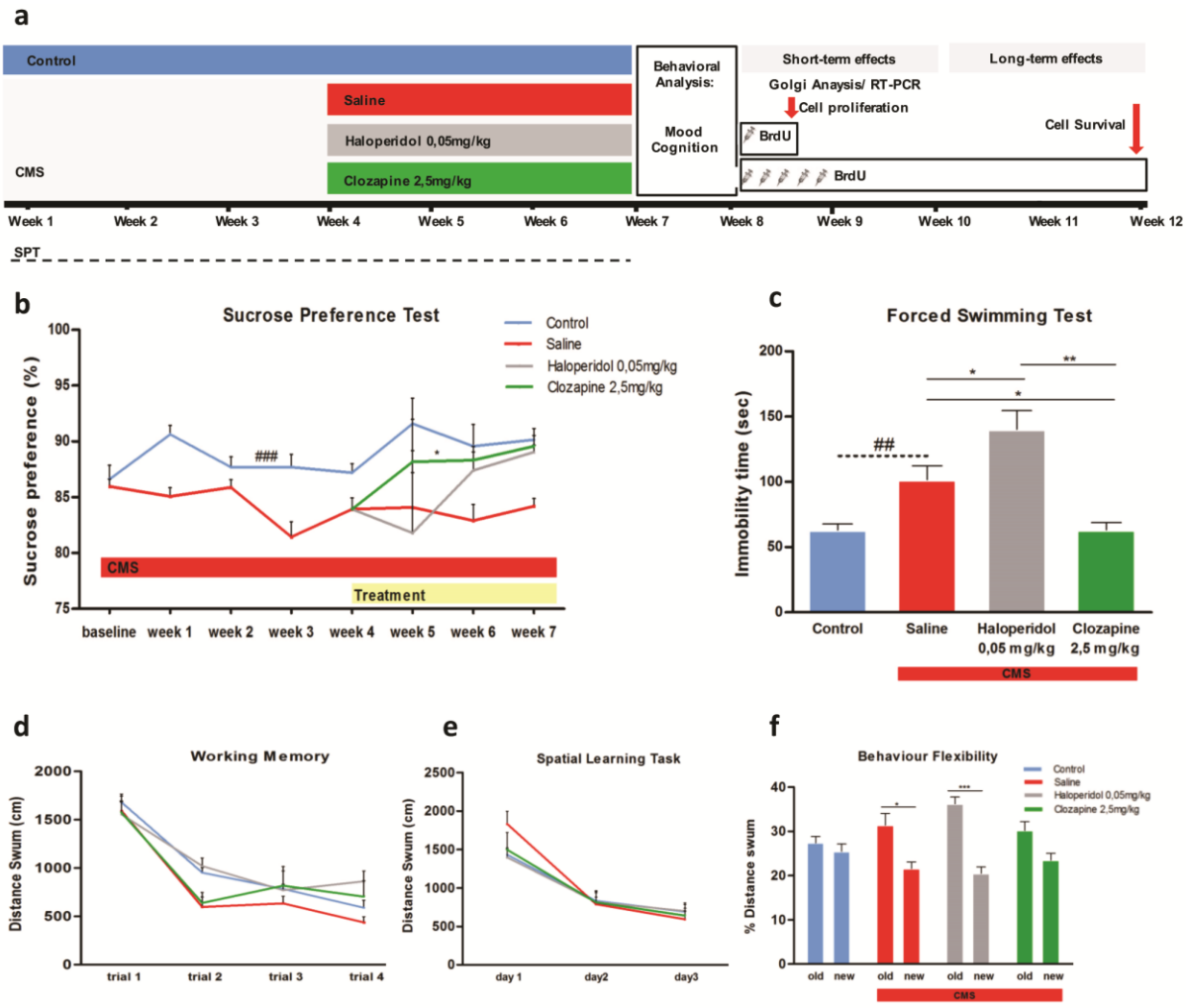


Figure 1- Behavioral effects of the chronic mild stress (CMS) and antipsychotic treatment on mood and cognition. (a) CMS protocol was applied to the rats for 7 weeks; two different antipsychotics (haloperidol and clozapine) were administrated in the last three weeks of the CMS protocol. To analyse the impact of antipsychotics on cell proliferation we injected BrdU 24h before sacrificed (short-term effects). To analyse the impact on neuronal survival we injected BrdU during 5 days, the sacrifice was performed 4 weeks later (long-term effects). Animals used for morphological and gene expression analysis were sacrificed immediately after performing the behaviour analysis. (b) Sucrose Preference Test was performed during all experimental protocol to evaluate anhedonia. (c) Learned helplessness was evaluated in the Forced Swim Test. Cognition was analysed in the different tasks of the Morris Water Maze (d) Working Memory (e) Spatial Learning Task and (f) Behaviour Flexibility. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals.*p<0,05; **,##p<0,01;***p<0,001. n=15-17 animals per group.

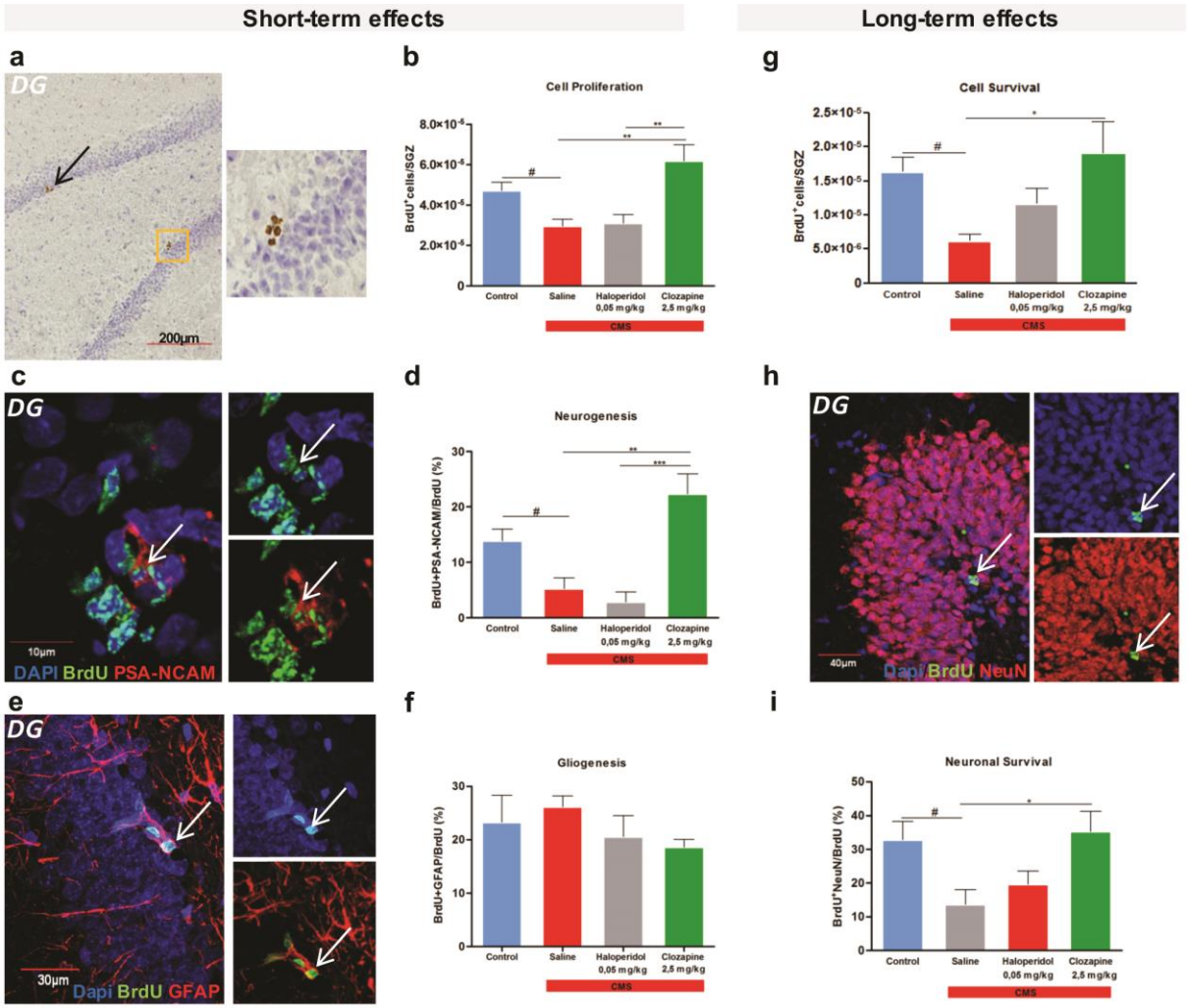


Figure 2- Antipsychotic treatment effects on the newly born cells and neuronal survival. (a) Proliferative niche of BrdU-labelled cells in the subgranular zone (SGZ) obtained with optical microscope. (b) The density of BrdU-labelled cells in the SGZ of the dentate gyrus. (c) Niche of newly formed neurons in the SGZ, obtained by confocal microscopy. (d) The percentage of $BrdU^+$ cells that were co-labelled with the antibody against PSA-NCAM. (e) Newly formed glial cells in the SGZ, obtained by confocal microscope. (f) Percentage of $BrdU^+$ cells that were co-labelled with glial marker GFAP in the SGZ. (g) The density of BrdU-labelled cells in the dentate gyrus that survives after 4 weeks. (h) Newly formed neurons in the dentate gyrus that survives after 4 weeks. (i) The percentage of $BrdU^+$ cells that were co-labelled with the antibody against NeuN. (a)-(f) short-term and (g)-(i) long-term effects of antipsychotics on neuro- and glio-genesis. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals.$^{\#,*}$ $p<0,05$; $^{**}p<0,01$;$^{***}p<0,001$. n=5 animals per group.

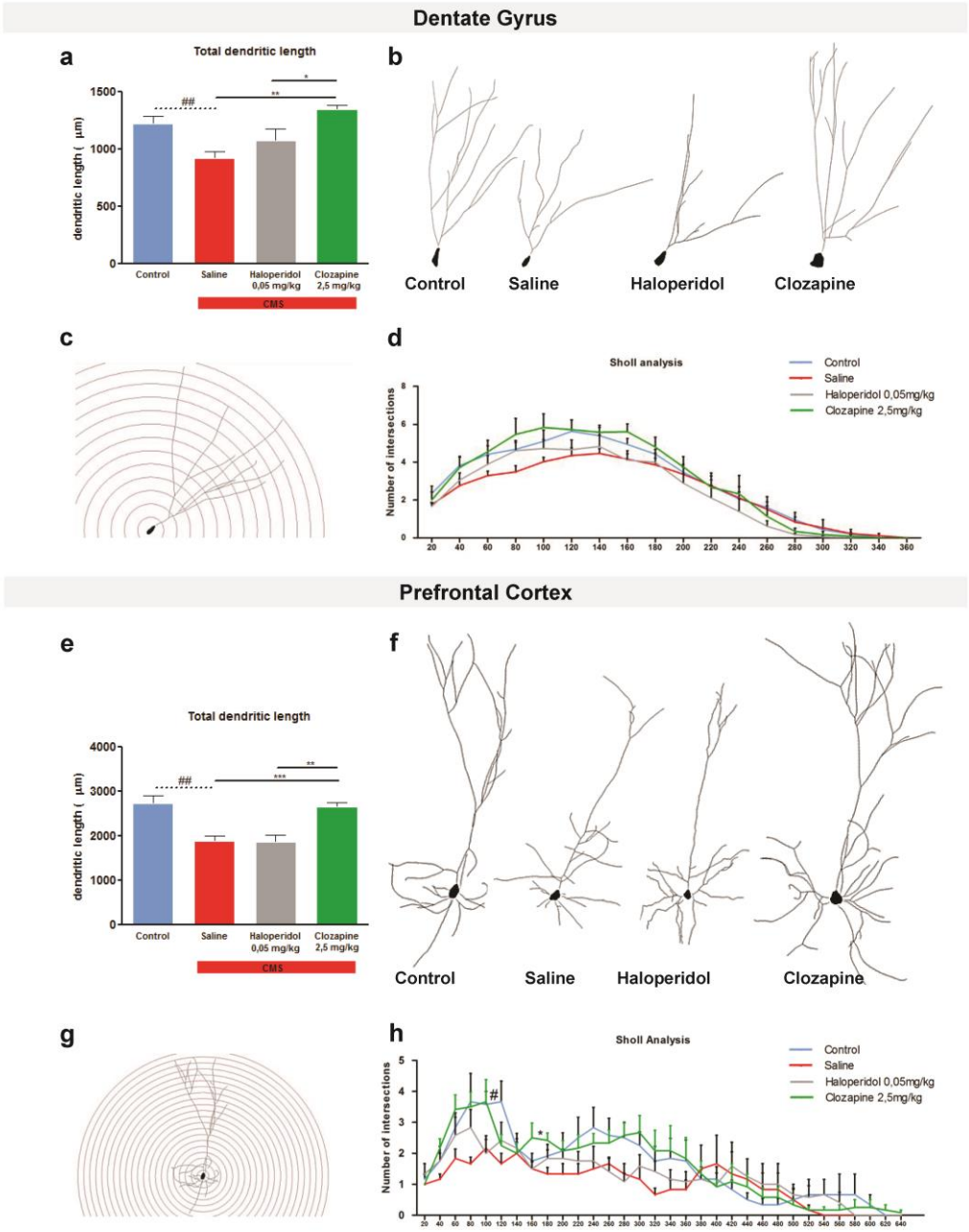


Figure 3- Three-dimensional morphometric analysis of Golgi-impregnated neurons using computer-assisted reconstructions of dentate gyrus and prefrontal cortex (PFC). (a) Total dendritic length of neurons in the dentate gyrus of the hippocampus. (b) Representative neurons of different experimental groups. (c) Representative sholl dendritic analysis of a dentate gyrus neuron, dendritic density was measured by placing a series of concentric circles, spaced at 20µm intervals centered on the soma. (d) Sholl analysis of dendritic distribution of neurons in the dentate gyrus. (e) Total dendritic length of neurons in the PFC. (f) Representative neurons of different experimental groups. (g) Representative sholl dendritic analysis of a PFC neuron, dendritic density was measured by placing a series of concentric circles, spaced at 20µm intervals centered on the soma. (h) Sholl analysis of dendritic distribution of neurons in the PFC. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals. #,*p<0,05; ##,**p<0,01;***p<0,001. n=6 animals per group.

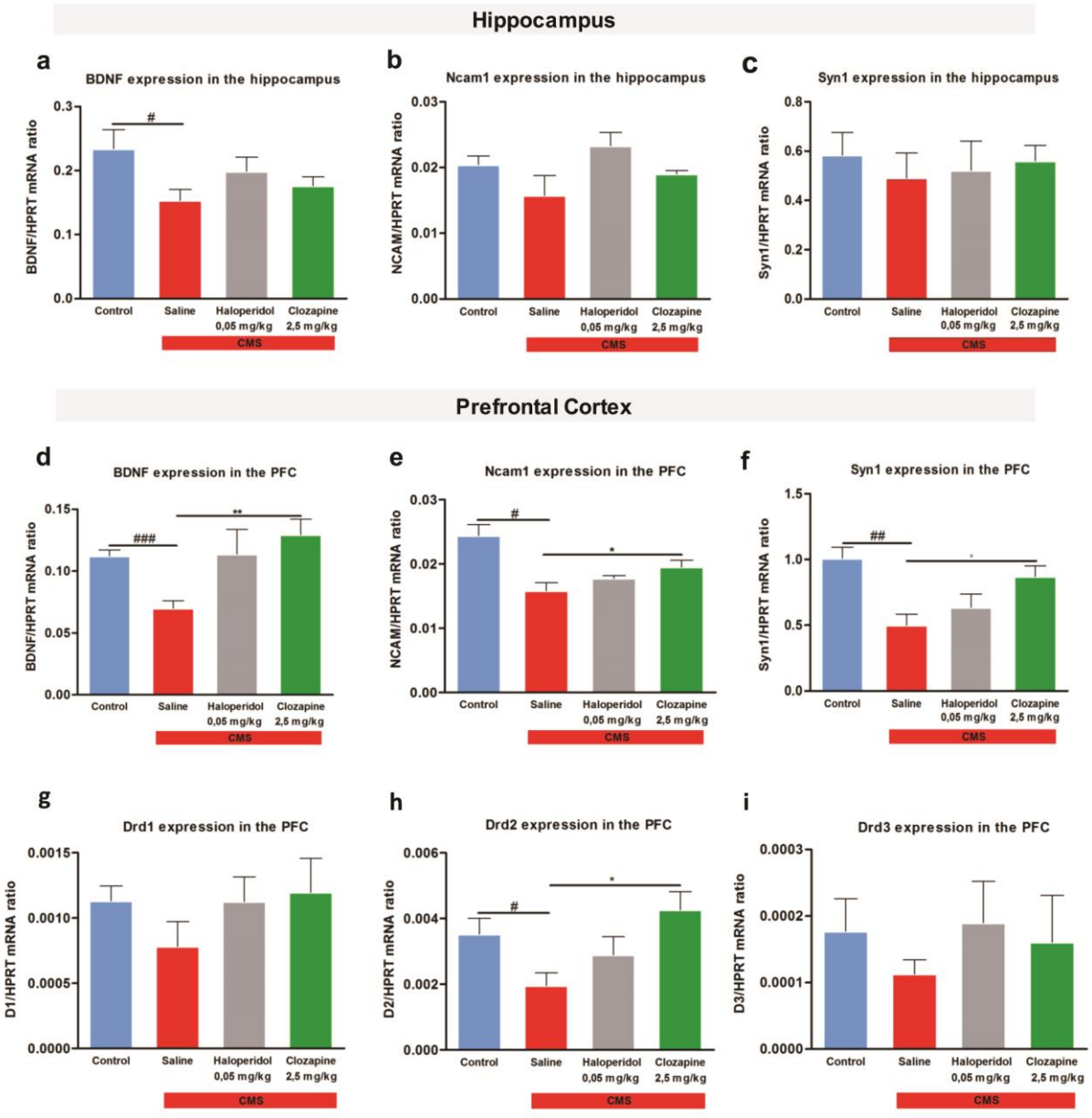


Figure 4- Gene expression analysis of the hippocampus and prefrontal cortex (PFC) by RT-PCR. In the hippocampus we measure the gene expression levels of different neuroplastic markers such as (a) BDNF (b) Ncam1 and (c) Syn1. The same markers of neuroplasticity were measure in the PFC (d) BDNF (e) Ncam1 and (f) Syn1. In the PFC the levels of expression of different dopamine receptors (Dr) D1, D2, and D3 (g) Drd1, (h) Drd2 and (i) Drd3. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals. $^{\#,*}p<0,05$; $^{\#\#,**}p<0,01$;$^{\#\#\#}p<0,001$. n=5 animals per group.

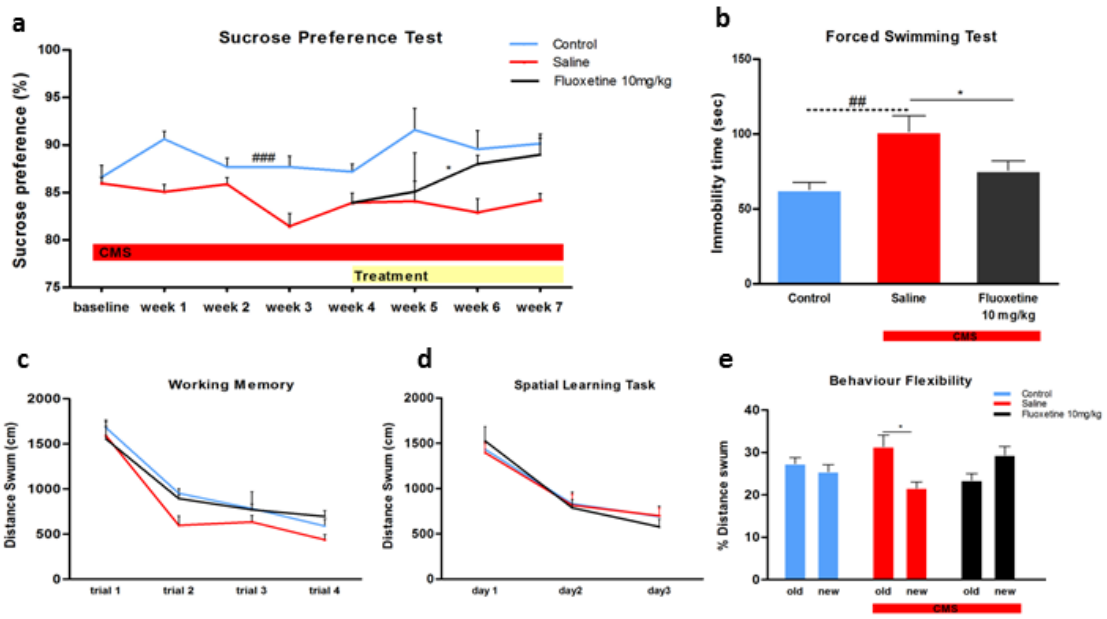
Supplementary figures and table

S1

Gene	Sense	Antisense	Produc size
Drd1	TCCTTCAAGAGGGAGACGAA	CCACAAACACATCGAAGG	168bp
Drd2	ATGTGCTGGTGTGCATGGCT	CACCCACCACCTCCAGGTAGAC	142bp
Drd3	GGGGTGACTGTCCTGGTCTA	TGGCCCTTATTGAAAACTGC	169bp
Ncam1	AAAGGATGGGGAACCCATAG	TAGGTGATTTTGGGCTTTGC	195bp
Syn1	CACCGACTGGGCAAAATACT	TCCGAACTTCCATGTCC	140bp
BDNF	CCTGGATGCCGCAAACATGTCTAT	CGCTGTGACCCACTCGCTAAT	103bp
B2M	GCTTGCCATTCAGAAAACTCC	AGGTGGGTGGAACTGAGACA	136bp

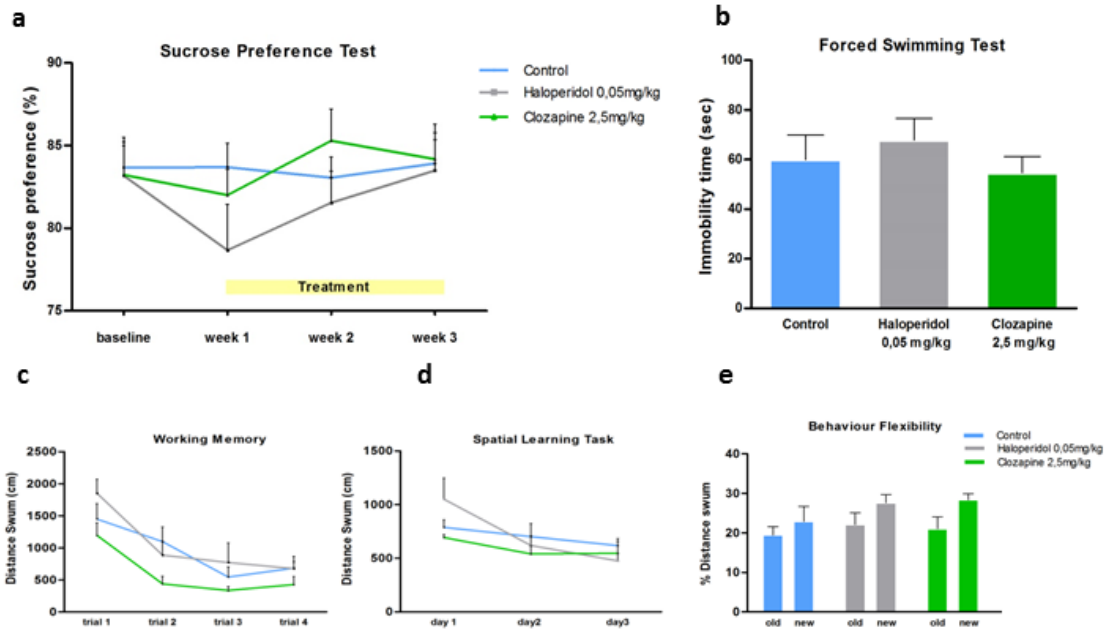
Supplemental table S1- Sense and antisense sequences of oligonucleotide primers used in the RT-PCR.

S2



Supplemental Figure S$_2$- Behavioral effects of the chronic mild stress (CMS) and fluoxetine treatment on mood and cognition. CMS protocol was applied to the rats for 7 weeks; fluoxetine treatment was administrated in the last three weeks of the CMS protocol. (a) Sucrose Preference Test was performed during all experimental protocol to evaluate anhedonia. (b) Learned helplessness was evaluated in the Forced Swim Test. Cognition was analysed in the different tasks of the Morris Water Maze (c) Working Memory (d) Spatial Learning Task and (e) Behaviour Flexibility. Data represented as mean + sem. #denotes the effect of CMS-exposure; *denotes the effect of antipsychotic compared with CMS non treated animals.*p<0,05; ##p<0,01. n=15-17 animals per group.

S3



Supplemental Figure S_3- Behavioral effects of the chronic treatment with antipsychotics in control animals on mood and cognition. Control animals were treated during three weeks (a) Sucrose Preference Test was performed once a week during all treatment period to evaluate anhedonia. (b) Learned helplessness was evaluated in the Forced Swim Test. Cognition was analysed in the different tasks of the Morris Water Maze (c) Working Memory (d) Spatial Learning Task and (e) Behaviour Flexibility. Data represented as mean + sem. n= 6 animals per group.

5th CHAPTER

The impact of antipsychotic drugs in behavior and cell genesis:

New perspectives from the MAM rodent model of Schizophrenia

Mónica Morais, Joana Pereira, Shilan Aslani, Patrícia Patrício, Dinis Alves, Joana Correia, Ana Rita Santos, António Pinheiro, Carlos Portugal, Nadine Santos, Luísa Pinto, Joana Palha, Nuno Sousa, João M. Bessa

The impact of antipsychotic drugs in behavior and cell genesis:
New perspectives from the MAM rodent model of Schizophrenia

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Abstract

Schizophrenia is a complex psychiatric disorder of unknown etiology involving gene-environment interactions that affects about 1% of the population worldwide. In the 1950´s, the serendipitous discovery of drugs with antipsychotic effects revolutionized the treatment and outcome of schizophrenia. However, the pharmacological treatment of schizophrenia remains a challenge and the mechanisms involved in the therapeutic effects of antipsychotic drugs in the different behavioral dimensions of schizophrenia are still largely unknown. To address this question, an animal model of schizophrenia was used, induced by the administration of methylazoxymethanol acetate (MAM) in pregnant dams. Male offspring (3 months old) were subsequently treated with the classic antipsychotic drug haloperidol (0,05mg/kg/day), and three different atypical antipsychotics clozapine (2,5mg/kg/day), risperidone (0,25mg/kg/day) and aripiprazole (1mg/kg/day) for a period of five weeks. During the last two weeks of treatment a battery of behavior tests were used to assess mood, anxiety, cognition and social. Furthermore, adult hippocampal neurogenesis and gliogenesis were analysed in the brain of the animals after sacrifice. Our data indicates that animals exposed to MAM *in utero* present significant impairments in social interaction. The classical antipsychotic drug haloperidol had no significant effects on sociability but the atypical antipsychotic drugs clozapine, risperidone and aripiprazole were able to revert the impairment on sociability induced by MAM exposure. Furthermore, MAM animals exposed to MAM revealed significant impairments in recognition memory. The classical antipsychotic drug haloperidol had no significant cognitive effects in these animals but the atypical antipsychotic drugs clozapine and risperidone were able to revert the cognitive impairment induced by MAM exposure. Interestingly, while no significant effects were observed in adult neurogenesis, a striking downregulation of adult gliogenesis was observed in MAM animals. Moreover, all the atypical antipsychotic drugs used were able to promote an increase in gliogenesis while haloperidol revealed an opposite effect. In summary, using a neurodevelopmental animal model of schizophrenia we identify specific cognitive deficits and social impairments that are differentially modulated by antipsychotic drugs. The classical haloperidol presented no beneficial effects in these behavioral dimensions. On the other hand, the atypical clozapine and risperidone showed a positive effect on both dimensions with aripiprazole presenting a significant effect exclusively in social behavior. Finally, the analysis of adult neurogenesis and gliogenesis suggests that the formation of new GFAP cells could be implicated in these observations.

Introduction

Schizophrenia is a complex neurodevelopmental psychiatric disorder of unknown etiology involving gene-environment interactions affecting about 1% of the population worldwide (Owen *et al*, 2016a). Patients with schizophrenia show abnormal mental functions that have been categorized into: positive, negative and cognitive symptoms. Positive symptoms reflect an excess or distortion of thoughts and perceptions, typically characterized by the development of delusions and hallucinations. Negative symptoms include social withdrawal, loss of motivation, affective blunting and anhedonia, which also characterize other mood disorders such as major depression and account for the significant suicide rates in schizophrenic patients (Freedman, 2003; Owen *et al*, 2016b). Cognitive symptoms involve multiple deficits in cognitive and executive processes.

In the 1950´s, the serendipitous discovery of drugs with antipsychotic effects revolutionized the treatment and outcome of schizophrenia (Miyamoto *et al*, 2012). Antipsychotic drugs are generally classified into classical (or first generation of antipsychotics) or atypical (or second generation of antipsychotics). The classical antipsychotic drugs (e.g. Haloperidol) with a mechanism of action based in dopamine receptor D2 antagonism, proved to be effective in positive symptoms. However, besides the side effects of eliciting extrapyramidal symptoms (EPS), hyperprolactinemia and metabolic changes, these drugs exacerbate the negative and cognitive symptoms that often lead to treatment discontinuation and relapse of symptoms. In contrast, atypical antipsychotics, with less potent D2 antagonism and with modulation of serotonin and noradrenaline receptors, maintain their effectiveness against positive symptoms, with fewer EPS and with beneficial effects on cognitive functions and negative symptoms (Gallhofer *et al*, 1996). Clozapine is usually considered to be the most effective atypical antipsychotic drug (Essock *et al*, 1996; McEvoy *et al*, 2006). However, considering the serious side effects associated with clozapine treatment (such as agranulocytosis), its prescription was restricted to refractory schizophrenia (Kilian *et al*, 1999; Rajagopal, 2005; Wong and Delva, 2007). Risperidone (Leysen *et al*, 1988), like clozapine, presents higher affinity for serotonin 5-HT_2 receptors than dopamine D2 receptors, but they differ in other pharmacologic properties and side effects. Aripiprazole is also an atypical agent with a different mechanism of action, reducing dopaminergic neurotransmission through D2 partial agonism, being considered a third-generation antipsychotic. As an atypical drug it also modulates receptors of the serotonin system: 5-HT_{1A} partial agonist and serotonin 5-HT_{2A} antagonist. Despite all the therapeutic benefits provided by the atypical antipsychotics, attention has been drawn considering the risk of developing metabolic complications and body weight gain.

The phenomenon of adult cell genesis has grown progressive interest based on studies that implicate adult neurogenesis in the etiopathology of different psychiatric disorders, including schizophrenia and depression (Mateus-Pinheiro *et al*, 2013; Morais *et al*, 2014). In 2006, Reif and colleges published a study in brain tissue of schizophrenic patients, revealing a significant reduction in hippocampal cell proliferation (Reif *et al*, 2006). Further post-mortem studies demonstrated neuronal abnormalities in the olfactory bulb, indicating a possible disturbance of cell proliferation in the subependymal zone (SEZ) (Arnold *et al*, 2001). In fact, the majority of the patients with schizophrenia show defects in olfaction, and first-degree relatives of schizophrenia patients also show such olfactory defects (Moberg *et al*, 1999). Regarding the effects of antipsychotic drugs on adult cell genesis the literature is not consensual. Previous studies have shown that haloperidol has no effect on hippocampal neurogenesis (Halim *et al*, 2004; Malberg *et al*, 2000). In the case of atypical antipsychotics, it was reported that risperidone and olanzapine increase neurogenesis in the SEZ, but not in the hippocampus (Wakade *et al*, 2002). However, another study has shown that olanzapine increases hippocampal neurogenesis (Kodama *et al*, 2004). The hippocampus is a brain region with a critical role in cognitive function (learning and memory) and emotional processing (Fanselow and Dong, 2010; Kheirbek and Hen, 2010); and several studies have described a positive role of hippocampal cell genesis in emotional and cognitive domains (9) (corrigir REF). Thus, the idea that atypical antipsychotics in contrast with classical antipsychotics potentiate adult hippocampal cell genesis is particularly attractive and may possibly be one of the mechanisms contributing to the action of atypical antipsychotics in the negative and cognitive symptoms.

In the present study, we evaluated the behavioral effects of different classes of antipsychotics in an animal model of schizophrenia: methylazoxymethanol acetate (MAM) model E17. To induce this model, MAM was administrated to pregnant dams (Wistar rats prenatally treated at gestational day 17, 20mg/kg). The offspring of these animals were subsequently treated in early adulthood with the classic antipsychotic drug haloperidol (0,05mg/kg/day), and three different atypical antipsychotics clozapine (2,5mg/kg/day), risperidone (0,25mg/kg/day) and aripiprazole (1mg/kg/day) for a period of five weeks (daily administered). During the last two weeks of treatment a battery of behavior tests to assess mood (forced swimming test (FST)), anxiety (elevated-plus maze test (EPM)), cognition (Morris water maze (MWM) and novel object recognition (NOR) test) and social interaction (three-chamber sociability test) were performed. To explore the role of cell genesis in this context we examined the impact of MAM and antipsychotics in this process. Using this integrative approach, we hope to understand the role of

newly born cells in the behavioral actions of antipsychotic drugs.

Materials and Methods

Animals

Prenatal exposure to MAM

Pregnant females (Wistar rats) were injected with methylazoxymethanol acetate (MAM 20,0 mg/kg; National Cancer Institute, Midwest Research Institute, Kansas City, MO, USA) or saline at gestational day 17 (GD17). Only the male pups were included in this study. Subsequently, the MAM group was subdivided into five experimental groups treated at the age of three months for five weeks with vehicle, haloperidol, clozapine, risperidone or aripiprazole.

Drugs

The antipsychotics used in this study were haloperidol (0,05 mg/kg; Sigma-Aldrich, St Louis, MO, USA), clozapine (2,5 mg/kg; Kemprotec, Middlesborough, UK), risperidone (0,25 mg/kg; Kemprotec, Middlesborough, UK) and aripiprazole (1 mg/kg; Kemprotec, Middlesborough, UK). Compounds were dissolved in distilled water and administered intraperitoneally (i.p.) (1 ml/kg) during 5 weeks.

Behavioral Tests

Prepulse inhibition test (PPI)

To perform this test, the animals were placed in Plexiglas cylinders with 16 cm length and a diameter of 9 cm The cylinders were set onto a horizontal plate equipped with a transducer that allows the detection of startle response in a sound attenuated chamber. This test measures the acoustic startle reflex, reflecting the sensorimotor gating, as the exposure to the pre-stimulus inhibits the startle response to a strong auditory stimulus. After an acclimatization period of five minutes with white noise [70dB(A)], five startle trials of 120 dB bursts of white noise were delivered, during 40 ms. The session consisted in the presentation of ten startle trials of 120 dB, followed by prepulse intensities of 2, 4, 8 and 16 dB(A) above background level, respectively PP72, PP74, PP78 and PP86 with a duration of 20 ms. The startle amplitude was measured as the mean of ten startle trials applied. PPI (in percentage) was calculated as follows: [100 – (Mean of all startle amplitudes on prepulse trials/Basal startle amplitude) x 100].

Mood:

Sucrose preference test (SPT)

To assess anhedonia, the SPT was conducted weekly during all the experimental procedure. Briefly, animals were allowed to habituate to the sucrose solution for 1 week before the CMS protocol to establish baseline values for sucrose preference. To test sucrose preference, animals that were subjected to food and water deprivation for 24h and then presented with two pre-weighed bottles containing 2% of sucrose solution or tap water for a period of 1h. Sucrose preference was calculated according to the formula: sucrose preference = [sucrose intake/(sucrose intake + water intake)] × 100, as previously described (Bessa *et al*, 2009). Anhedonia was defined as a reduction in sucrose preference relative to baseline levels.

Forced swimming test (FST)

Behavior despair was assessed through the FST. Twenty-four hours after a pre-test session (10 min), the FST was conducted by placing rats in cylinders filled with water (25 °C; depth 30 cm) for a period of 5min. Test sessions were assessed using a camera connected to a video tracking system (Viewpoint); the system automatically calculated immobility time and latency to immobility. Behavior despair was defined as an increase in time of immobility and a decrease in latency to immobility.

Anxiety:

Novelty suppressed feeding (NSF)

To characterize anxiety-like behavior, NSF was assessed. Based on previous studies, the animals were deprived of food for 23 h before being placed in a novel environment for 10 min (an open-field arena; MedAssociates Inc.); a single food pellet was placed in the center of the arena. Upon reaching the pellet, animals were returned to their home cages and presented with preweighed food over a period of 5 min. Latency to feeding in the open field was used as an index of anxiety-like behavior; the amount of food consumed in the home cage provided a measure of appetitive drive.

Cognition:

Morris Water Maze (MWM)

Cognitive function was evaluated in the MWM. The MWM was conducted in a circular black tank (diameter: 170 cm; depth: 50 cm), divided in quadrants by imaginary lines, and filled with water (22°C)

to a depth of 31 cm. During testing, a black platform (12 × 12 cm; invisible to the rats) was placed at a height of 30 cm. The room was dimly lit and extrinsic visual clues were glued to the walls. Data were collected using a video tracking system (Viewpoint) (Cerqueira *et al*, 2007).

The working memory task was used to evaluate the cognitive domain that relies on the interplay between the hippocampal and PFC function (Cerqueira *et al*, 2007). In this task the position of the platform is kept constant during the four trials of each day, but varies on each successive day such that all four quadrants are used. Rats are placed, facing the wall of the maze, at a different starting point (north, east, south, or west) at the beginning of each of the four daily trials. A trial is considered complete when the rat escapes onto the platform; when this escape fails to occur within 120 s, the animal is gently guided to the platform and an escape latency of 120 s is recorded for that trial. Rats are allowed to spend 30s on the escape platform before being positioned at a new starting point. Length of the path described (distance swam) and time spent to reach the platform (escape latency) are recorded in the consecutive trials. The total distance swum was evaluated as a measure of locomotor activity. All behaviour data analysis was performed with the experimenter blinded to the group under analysis.

Novel object recognition (NOR)

Cognitive function was assessed using the NOR test. Rats were first familiarized to the testing arena consisting of a black acrylic box (50 x 50 x 150 cm) with an open field space, for 8 minutes and with no objects presentation. On the following day, animals were allowed to explore two identical objects for 10 minutes. Twenty-four hours later, animals returned to the arena for 3 minutes, in which one of the objects was replaced by a novel one. The familiar and novel objects differed on size, shape, texture and color. The NOR arena was cleaned with ethanol (10%) between trials to avoid odour cues. All sessions were videotaped and the time spent exploring both objects was determined manually and blindly. The percentage of time spent exploring the novel object was used as a measure of long-term memory performance.

Social:

Three chamber test

Social behaviour was assessed using the three chamber test. This test is performed in three sessions within a three-chambered box. The test starts with the habituation to the empty box (5min.). Then, rats

are given a choice between spending time with another rat (never-before-met) under one container and an empty container (10 min.; first part of the test). After this, the tested rat encounters the first intruder ("familiar") as well as a new unfamiliar rat under another container (10 min.; second part of the test). Social interaction was determined by measuring the amount of time spends with the rat (first part of the test); and the time spends with the first intruder ("now-familiar") and with the unfamiliar rat (stranger).

Tissue processing and immunohistochemical analysis

Animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Safoni) and perfused with saline and rapidly decapitated. Serial coronal sections (20 μm) were cut on a cryostat and stored at -20°C. We evaluate the impact of antipsychotics on neurogenesis by counting the number of Ki-67^{+} cells (#AB9260; rabbit, 1:300; Millipore) and *doublecortin* (DCX, goat, 1:300, Abcam) in the hippocampus using a confocal microscope (Olympus FV1000).

Statistical analysis

After confirming the homogeneity of the data distribution, the appropriate statistical tests were performed using SPSS software. Equality of variances was tested with an F test. A repeated measure ANOVA was used to analyse working memory. One-way ANOVA was used to evaluate the impact of CMS and antipsychotic treatment in the SPT, FST, NSF and immunostaining results. Differences between groups were then determined by Tukey's honestly significant difference test (Tukey HSD) post hoc analysis. All values were calculated as means + standard error of the mean (SEM). Statistical significance was accepted for $P < 0.05$.

Results

Behavioral results

Our data indicates that animals exposed to MAM *in utero* present no alterations in the PPI (Figure 1b), SPT (Figure 1c), FST (Figure 1d) and EPM (Figure 1e).

Cognitive function was evaluated using different tests: the MWM to assess spatial working memory and the NOR to assess recognition memory. In the working memory task, we observed no differences between all the groups analysed. In the NOR test, animals exposed to MAM in the prenatal period spend less time exploring a novel object revealing impairments in cognition ($F_{1,14}$=10,804; p=0,006, Figure 2b, 2c) revealing an impairment in the ability to discriminate between an old and a novel object. The chronic treatment with antipsychotics induces an overall effect ($F_{4,40}$=5,417; P=0,001, Figure 2b, 2c). The classical antipsychotic drug haloperidol had no significant cognitive effects in these animals (p=0,823). The atypical antipsychotic drugs clozapine (p=0,017) and risperidone (p=0,003) were able to revert the cognitive impairments induced by MAM while the effects of aripiprazole on cognition were not significant (p=0,517).

To assess social interaction we used the three chamber test. In the first part of the test we analyzed the time that the tested animal spends sniffing (direct interaction) a stranger rat trapped in a cage. MAM animals presented a decrease in the time spent with another rat ($F_{1,27}$=7,168; p=0,013, Figure 3b) showing a deficit in social interaction. Regarding the impact of antipsychotic drugs in this behavioural dimension, an overall effect was observed ($F_{4,68}$=9,017,p<0,001, Figure 3b). The classical antipsychotic drug haloperidol had no significant effects on sociability (p=0,246). In contrast, all the atypical antipsychotic drugs clozapine (p=0,010), risperidone (p<0,001) and aripiprazole (p=0,002) were able to revert the impairment on sociability induced by MAM exposure. In the second part of the test, we assessed the time spent with a “new” and with a “familiar” animal. Regarding the time spendt with the “new” animal, no differences were observed between all the groups analysed (Figure 3c). In terms of time spent with the “familiar” animal, MAM animals presented a decrease in the time spent interacting ($F_{1,27}$=7,592;p=0,011; figure 3c). The classical antipsychotic drug haloperidol had no significant effects on this dimension (p=0,317) while the atypical antipsychotic drugs clozapine (p=0,038) and aripiprazole (p=0,041) were able to increase the time spent with a familiar animal. The atypical risperidone (p=0,482) was not able to reverse the impact on time spends with familiar animal.

Cell genesis

The possible modulation of adult hippocampal cell genesis (including neurogenesis and gliogenesis) by MAM and antipsychotic treatment was analyzed after 5 weeks of chronic treatment. To determine the cell fate of the ki-67^{+} cells, these cells were co-labelled with cell-specific markers, including DCX and GFAP to assess neurogenesis and gliogenesis, respectively. In the case of neurogenesis, the percentage of ki-67^{+} cells that co-labelled with DCX was not reduced ($F_{1,9}$=0,421; p=0,535, Figure 4a, b) in rats exposed to MAM during gestation. Regarding the effect of chronic treatment with antipsychotics, only haloperidol induced a significant reduction on neurogenesis ($F_{4,29}$=3,101; p=0,033, Figure 4a, b). Gliogenesis was measured by the percentage of ki-67^{+} cells that co-labelled with GFAP, a method that may include a small percentage of neural progenitor cells. A significant reduction of gliogenesis was observed in MAM animals ($F_{1,11}$=34.454, p<0.00,1, Figure 4c, d). Regarding the effect of chronic treatment with antipsychotics an overall effect was observed ($F_{4,29}$=7,482, p<0.001, Figure 4c, d), with clozapine (p=0,009), risperidone (p=0,02) and aripiprazole (p=0,001) presenting an increase, and no effect induced by chronic treatment with haloperidol (p=0,930).

Discussion

In the present study we use an animal model of schizophrenia, induced by the prenatal administration of the cytostatic agent MAM agent in day 17, to investigate the effects of different classes of antipsychotics in the negative and cognitive symptoms of this neuropsychiatric disorder in which hippocampal cell genesis has been implicated.

Social deficits are present in some psychiatric disorders with particular relevance in schizophrenia. To determine whether MAM rats display social deficits, we tested our animal in the three-chambered apparatus, where the social approach of a rat toward a stranger rat (trapped in a wire cage) can be measured. Animals exposed to MAM presented an impairment in social interaction (measured by time spent sniffing) as observed by the reduced preference for exploring a stranger rat compared with the control group. In the second part of the test we measured the preference for social novelty and no differences were observed between MAM and control animals. These results indicate that MAM animals, as controls, display normal social novelty recognition or social anxiety. In accordance, other studies have already reported deficits in (IRSp53$^{-/-}$) mice in the first part of the three chamber test, with no deficits in the second part (time spent with a "novel" animal) (Chung *et al*, 2015). Regarding the time interacting with the "familiar" animal (in the second part of the test), MAM animals revealed a significant impairment. We hypothesize that this behavioural phenotype may represent a correlate of reduced affect or emotional blunting, a common symptom in schizophrenic patients (de Leon *et al*, 1993). Furthermore, chronic treatment with clozapine, risperidone and aripiprazole was able to reverse the impairment on sociability induced by MAM exposure.

The NOR test is widely used to evaluate recognition memory in rodents and is based on the natural preference of rodents for exploring novel objects (Antunes and Biala, 2012). This cognitive test (in contrast with MWM) is particularly attractive because it requires no external motivation and reward (Silvers *et al*, 2007). As expected, control animals spent significantly more time exploring the novel objected. In contrast, MAM animals explored during the same time the old and new object with no ability to discriminate between them. Haloperidol treatment was not able to produce a beneficial effect on cognition. The atypical antipsychotic drugs clozapine and risperidone were able to reverse the cognitive impairments induced by MAM exposure while the effects of aripiprazole on cognition were not significant. Again, these data highlight the beneficial effect of some atypical in comparison with classical antipsychotics.

Considering the neuroplastic phenomena analysed, MAM animals presented a decrease in hippocampal gliogenesis (new GFAP cells) with no significant effects in hippocampal neurogenesis. This reduction could be involved in the cognitive and emotional deficits observed in these animals. In fact, normal recognition performance depends on the integrity of the hippocampus; and animals with reduced levels of cell genesis are impaired in some cognitive tasks (Jessberger *et al*, 2009; Mateus-Pinheiro *et al*, 2013). The chronic treatment with haloperidol induced no effects on gliogenesis and a significant reduction in neurogenesis. In terms of behaviour, haloperidol treated animals were not able to recover from the cognitive and emotional deficits induced by MAM exposure. This goes in line with previous reports describing that haloperidol treatment acts mainly against the positive symptoms of schizophrenia, exacerbating in some cases the negative and cognitive symptoms.

All the antipsychotics used in this study were not able to promote alterations on adult hippocampal neurogenesis; this could be attributed to the fact that MAM animals presented no deficits in this neuroplastic phenomenon. On the other hand, the formation of new GFAP cells was promoted by clozapine, risperidone and aripiprazole treatment. These results suggest a possible role of the serotonergic system (modulated by the atypical drugs) in the positive effects on the formation of newly GFAP cells.

An important dimension in social interaction is social communication, and rodents emit ultrasonic vocalizations under social contexts. In the future, we are planning to analyse the ultrasonic vocalizations emitted during the three chamber test to appreciate the emotional status of the animals during the test. Additionally, we are planning to study other neuroplastic phenomena beyond cell genesis namely the structural analysis of the DG neurons to understand if the exposition to MAM during gestation and the chronic treatment with antipsychotics are able to induce alterations in the dendritic length and neuronal complexity.

In conclusion, in the present study we have demonstrated that exposition to MAM during gestation is a good model to study the negative and cognitive symptoms of schizophrenia. Adult neurogenesis is not affected in MAM animals. In contrast, the formation of new GFAP cells is decreased in these animals. Regarding antipsychotic action on neuro/gliogenesis, we observed a positive effect on gliogenesis promoted by the atypical agents. By contrast, haloperidol induces a negative effect on neurogenesis.

The present observations should be further explored to understand if these neuroplastic alterations are implicated in the behavior actions of antipsychotics. Altogether, the present data suggest adult cell genesis as a possible target to be addressed for further therapeutic interventions in schizophrenia.

References

Antunes M, Biala G (2012). The novel object recognition memory: neurobiology, test procedure, and its modifications. *Cognitive processing* **13**(2): 93-110.

Arnold SE, Han LY, Moberg PJ, Turetsky BI, Gur RE, Trojanowski JQ*, et al* (2001). Dysregulation of olfactory receptor neuron lineage in schizophrenia. *Archives of general psychiatry* **58**(9): 829-835.

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA*, et al* (2009). The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Molecular psychiatry* **14**(8): 764-773, 739.

Cerqueira JJ, Mailliet F, Almeida OF, Jay TM, Sousa N (2007). The prefrontal cortex as a key target of the maladaptive response to stress. *J Neurosci* **27**(11): 2781-2787.

Chung W, Choi SY, Lee E, Park H, Kang J, Park H*, et al* (2015). Social deficits in IRSp53 mutant mice improved by NMDAR and mGluR5 suppression. *Nat Neurosci* **18**(3): 435-443.

de Leon J, Peralta V, Cuesta MJ (1993). Negative symptoms and emotional blunting in schizophrenic patients. *The Journal of clinical psychiatry* **54**(3): 103-108.

Essock SM, Hargreaves WA, Covell NH, Goethe J (1996). Clozapine's effectiveness for patients in state hospitals: results from a randomized trial. *Psychopharmacology bulletin* **32**(4): 683-697.

Fanselow MS, Dong H-W (2010). Are the Dorsal and Ventral Hippocampus Functionally Distinct Structures? *Neuron* **65**(1): 7-19.

Freedman R (2003). Schizophrenia. *The New England journal of medicine* **349**(18): 1738-1749.

Gallhofer B, Bauer U, Lis S, Krieger S, Gruppe H (1996). Cognitive dysfunction in schizophrenia: comparison of treatment with atypical antipsychotic agents and conventional neuroleptic drugs. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* **6 Suppl 2**: S13-20.

Halim ND, Weickert CS, McClintock BW, Weinberger DR, Lipska BK (2004). Effects of chronic haloperidol and clozapine treatment on neurogenesis in the adult rat hippocampus. *Neuropsychopharmacology* **29**(6): 1063-1069.

Jessberger S, Clark RE, Broadbent NJ, Clemenson GD, Jr., Consiglio A, Lie DC*, et al* (2009). Dentate gyrus-specific knockdown of adult neurogenesis impairs spatial and object recognition memory in adult rats. *Learning & memory (Cold Spring Harbor, NY)* **16**(2): 147-154.

Kheirbek MA, Hen R (2010). Dorsal vs Ventral Hippocampal Neurogenesis: Implications for Cognition and Mood. *Neuropsychopharmacology* **36**(1): 373-374.

Kilian JG, Kerr K, Lawrence C, Celermajer DS (1999). Myocarditis and cardiomyopathy associated with clozapine. *Lancet (London, England)* **354**(9193): 1841-1845.

Kodama M, Fujioka T, Duman RS (2004). Chronic olanzapine or fluoxetine administration increases cell proliferation in hippocampus and prefrontal cortex of adult rat. *Biological psychiatry* **56**(8): 570-580.

Leysen JE, Gommeren W, Eens A, de Chaffoy de Courcelles D, Stoof JC, Janssen PA (1988). Biochemical profile of risperidone, a new antipsychotic. *The Journal of pharmacology and experimental therapeutics* **247**(2): 661-670.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* **20**(24): 9104-9110.

Mateus-Pinheiro A, Pinto L, Bessa JM, Morais M, Alves ND, Monteiro S*, et al* (2013). Sustained remission from depressive-like behavior depends on hippocampal neurogenesis. *Translational psychiatry* **3**: e210.

McEvoy JP, Lieberman JA, Stroup TS, Davis SM, Meltzer HY, Rosenheck RA*, et al* (2006). Effectiveness of clozapine versus olanzapine, quetiapine, and risperidone in patients with chronic schizophrenia who did not respond to prior atypical antipsychotic treatment. *The American journal of psychiatry* **163**(4): 600-610.

Miyamoto S, Miyake N, Jarskog LF, Fleischhacker WW, Lieberman JA (2012). Pharmacological treatment of schizophrenia: a critical review of the pharmacology and clinical effects of current and future therapeutic agents. *Molecular psychiatry* **17**(12): 1206-1227.

Moberg PJ, Agrin R, Gur RE, Gur RC, Turetsky BI, Doty RL (1999). Olfactory dysfunction in schizophrenia: a qualitative and quantitative review. *Neuropsychopharmacology* **21**(3): 325-340.

Morais M, Santos PA, Mateus-Pinheiro A, Patricio P, Pinto L, Sousa N*, et al* (2014). The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition. *Journal of psychopharmacology (Oxford, England)* **28**(12): 1178-1183.

Owen MJ, Sawa A, Mortensen PB (2016a). Schizophrenia. *Lancet (London, England)* **388**(10039): 86-97.

Owen MJ, Sawa A, Mortensen PB (2016b). Schizophrenia. *The Lancet* **388**(10039): 86-97.

Rajagopal S (2005). Clozapine, agranulocytosis, and benign ethnic neutropenia. *Postgraduate medical journal* **81**(959): 545-546.

Reif A, Fritzen S, Finger M, Strobel A, Lauer M, Schmitt A*, et al* (2006). Neural stem cell proliferation is decreased in schizophrenia, but not in depression. *Molecular psychiatry* **11**(5): 514-522.

Silvers JM, Harrod SB, Mactutus CF, Booze RM (2007). Automation of the novel object recognition task for use in adolescent rats. *Journal of neuroscience methods* **166**(1): 99-103.

Wakade CG, Mahadik SP, Waller JL, Chiu FC (2002). Atypical neuroleptics stimulate neurogenesis in adult rat brain. *Journal of neuroscience research* **69**(1): 72-79.

Wong J, Delva N (2007). Clozapine-induced seizures: recognition and treatment. *Canadian journal of psychiatry Revue canadienne de psychiatrie* **52**(7): 457-463.

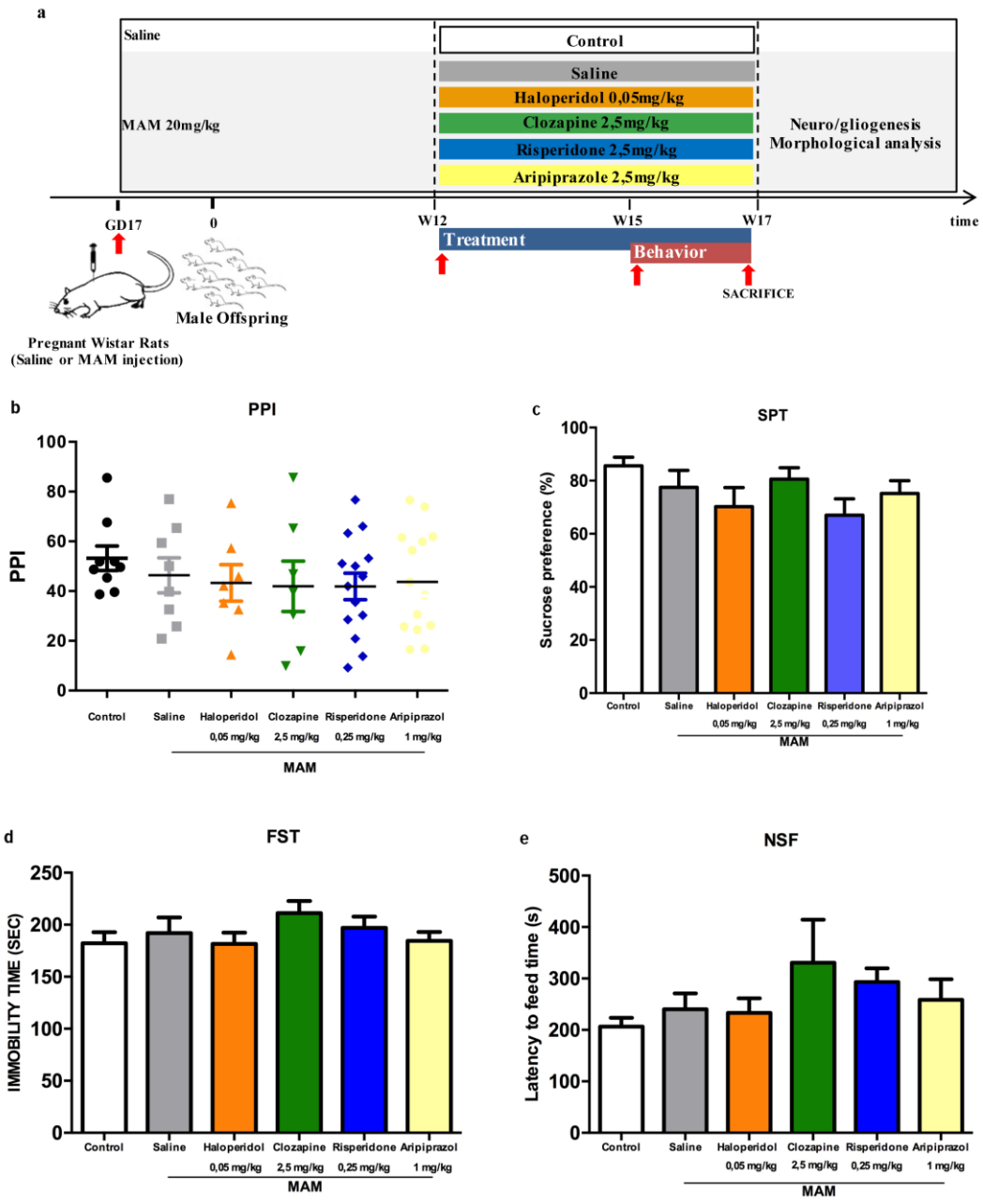

Figure 1- Behavioural effects of the MAM model and antipsychotic treatment on PPI, mood (SPT, FST) and anxiety (NSF). (a) Schizophrenia animal model was induced by the administration of MAM in pregnant females at day 17 (MAM E17); four different antipsychotics (haloperidol, clozapine, risperidone and aripiprazole) were administrated during five weeks. (b) PPI. (c) Anhedonia was evaluated on Sucrose Preference Test (SPT). (d) Learned helplessness was evaluated in the Forced Swim Test (FST). (e) Anxiety was evaluated in the novelty supressed feeding (NSF). Data represented as mean + sem.

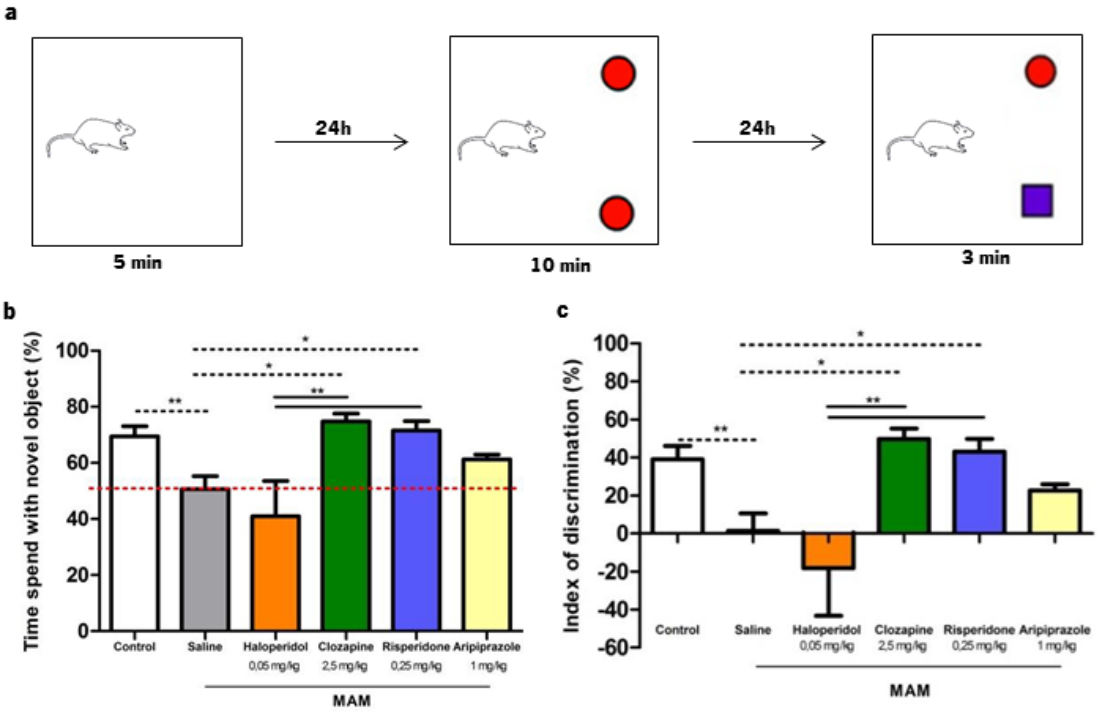

Figure 2- Behavioral effects of the MAM model and antipsychotic treatment on cognition (NOR). (a) Cognitive function was assessed using the NOR test. Rats were first familiarized to the testing arena for 8 minutes and with no objects presentation. On the following day, animals were allowed to explore two identical objects for 10 minutes. Twenty-four hours later, animals returned to the arena for 3 minutes, in which one of the objects was replaced by a novel one. (b) Time spend exploring the novel object. (c) Index of discriminatio. Data represented as mean + sem. *p<0,05; **p<0,01.

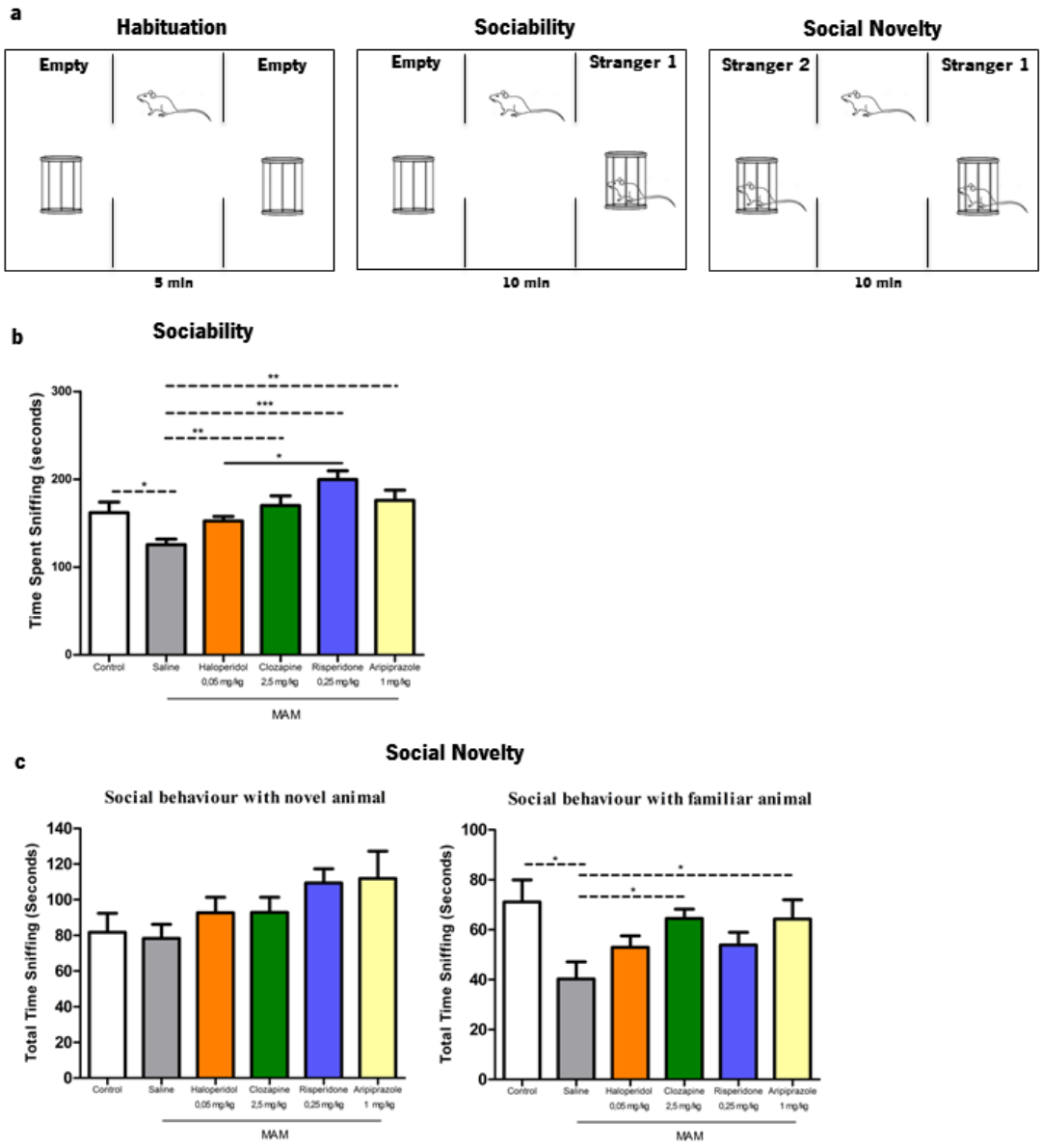

Figure 3- Behavioral effects of the MAM model and antipsychotic treatment on social behaviour. (a) Social behaviour was assessed using the three chamber test. This test is performed in three sessions within a three-chambered box. The test starts with the habituation to the empty box (5min.). Then, rats are given a choice between spending time with another rat (never-before-met) under one container and an empty container (10 min.). After this, the tested rat encounters the first intruder ("familiar") as well as a "new" unfamiliar rat under another container (10 min.). (b) Social interaction was determined by measuring the amount of time spends with the rat; (c) and the time spends with the "new" animal and and with the "now-familiar" animal. Data represented as mean + sem. *p<0,05; **p<0,01;***p<0,001.

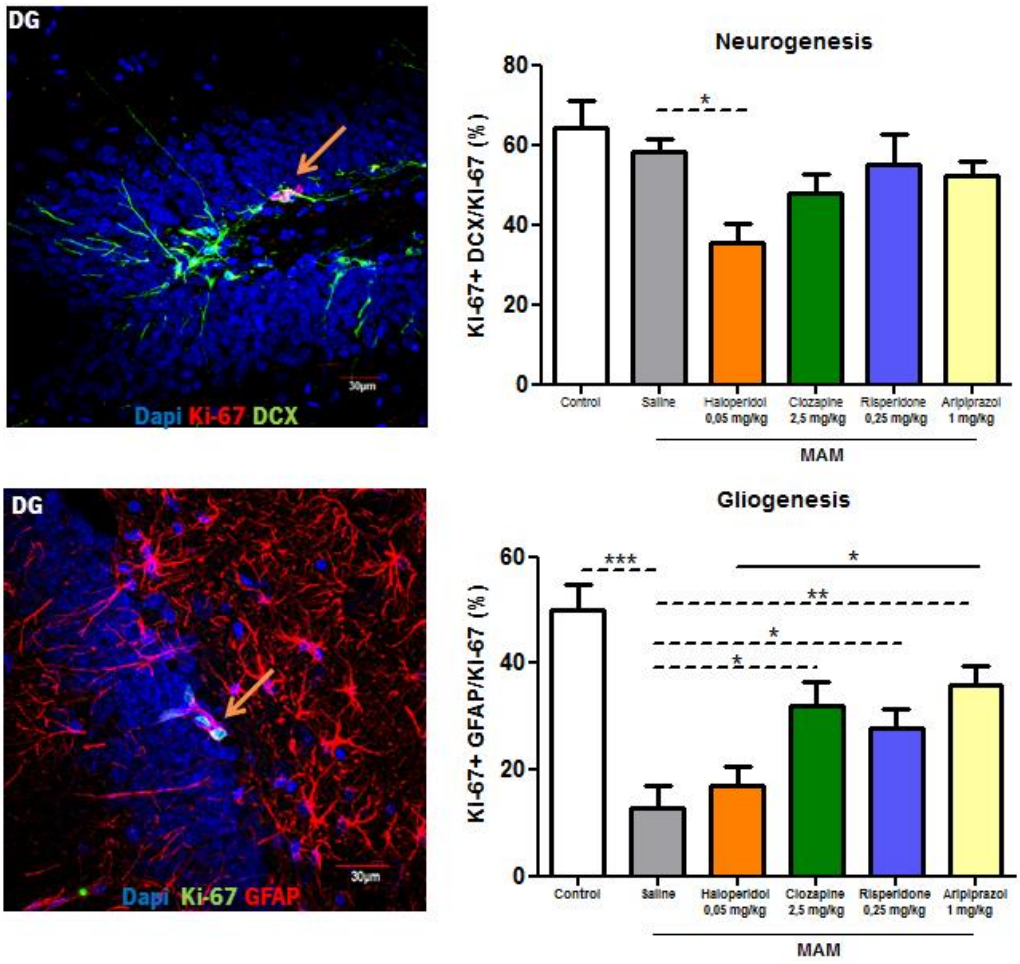

Figure 4- Effects of MAM and antipsychotic treatment on the newly born cells. (a) The percentage of ki-67^{+} cells that was co-labelled with the antibody against DCX. (b) Percentage of ki-67^{+} cells that was co-labelled with glial marker GFAP in the SGZ. Data represented as mean + sem. *p<0,05; **p<0,01;***p<0,001.

SECTION III – DISCUSSION AND CONCLUDING REMARKS

6th CHAPTER

Overall discussion and conclusions

General discussion

Over the last 50 years, different categories of antidepressant and antipsychotic drugs have been fortuitously discovered (Pittenger *et al*, 2008) leading to a prevailing neuro-chemical hypothesis of depression and schizophrenia in which these disorders became considered "states" of chemical imbalance in the brain that were corrected by the pharmacological actions of these drugs (Lopez-Munoz and Alamo, 2009). Therefore, all currently available drugs to treat these disorders are "monoamine-based therapies". However, these drugs are far from ideal regarding their efficacy and tolerability and much remains to be understood regarding the pathophysiology of depression and schizophrenia. Increasing evidence demonstrates that more complex neuroplastic events are associated to the onset of depression and antidepressant treatment (Bessa *et al*, 2009a; Mateus-Pinheiro *et al*, 2013; Morais *et al*, 2014; Patricio *et al*, 2015). In fact, structural neuroplasticity, including dendritic remodelling and cell genesis is disrupted not only in depression but also in schizophrenia. In this thesis we have focused on structural plasticity in these two psychiatric disorders.

Despite advances in our understanding of the neurobiology of major depressive disorder, no established mechanism can explain all the aspects of the disease. Using an animal model of depression - the unpredictable chronic mild stress (uCMS) model - with a clear impact on structural neuroplasticity (neurons with reduced dendritic complexity and length and decreased number of new cells in the hippocampus) we proved that monoamine oxidase subtype A (MAO-A) selective inhibition (like other classes of antidepressant drugs) reverses the deleterious neuroplastic effects of chronic stress in the hippocampus by restoring adult neurogenesis and by rescuing dendritic atrophy of granule neurons (Morais *et al*, 2014). We extended our analysis to the more recently described neurogenic region - the hypothalamus - showing for the first time that chronic stress and antidepressant treatment modulate this phenomenon. Even with major achievements on the comprehension of depression pathology, a great proportion of patients are still resistant to currently available antidepressant. New strategies have been adopted in these patients, such as the use of antipsychotics (Papakostas *et al*, 2007; Shelton *et al*, 2008). Exploring neuroplasticity as a possible mechanism involved in their action we found that atypical antipsychotics (in contrast with classical) are able to reverse depressive-like behaviour induced by the uCMS protocol and promote an increase in structural plasticity.

Antipsychotics are first line to treat schizophrenia and crescent evidence indicates disrupted neuroplasticity in this pathology. Based on these evidences we explored the neuroplastic role of

antipsychotics in the negative and cognitive symptoms in an animal model of schizophrenia. Animals prenatally exposed to MAM presented deficits in recognition memory and social interaction. These behavioural changes were accompanied by significant decreases in the levels of hippocampal gliogenesis. Strikingly, the atypical antipsychotics were able to promote an increase on gliogenesis but not on neurogenesis. In this work, we demonstrate that classical and atypical antipsychotics differentially modulate hippocampal cell genesis possibly contributing to different behavioural actions in hippocampal dependent functions (chapter 4 and 5).

In the first study of this thesis (chapter 2) we investigated the effects of an antidepressant (pirlindole) that acts through the selective inhibition of MAO-A on neuroplasticity. The potentiation of adult cell genesis in the dentate gyrus of the hippocampus has been extensively described as a common action observed in all the different classes of antidepressant drugs (Bessa *et al*, 2009a; Malberg *et al*, 2000; Morais *et al*, 2014; Sairanen *et al*, 2005; Santarelli *et al*, 2003). However, the effects of antidepressants in the reversal of stress-induced morphological changes of granule neurons in the hippocampus have only been described with tricyclics and SSRIs (Bessa *et al*, 2009a; Jayatissa *et al*, 2006; Surget *et al*, 2011). Considering the importance of neuronal remodelling in the mood improving actions of antidepressants we explored the actions of a MAO-A inhibitor in the modulation of this phenomenon. In fact, several studies in patients with depression revealed significant volumetric reductions in specific brain regions: hippocampus and prefrontal cortex (Botteron *et al*, 2002; Coryell *et al*, 2005; Frodl *et al*, 2002; Sheline *et al*, 2003) while treatment with antidepressants is able to reverse this atrophy (Frodl *et al*, 2008; Sheline *et al*, 2003). Our data demonstrates that pirlindole (a MAO-A inhibitor) is able to reverse the behavioural effects of stress exposure potentiating hippocampal adult neurogenesis and rescuing the stress-induced dendritic atrophy of granule neurons in the dentate gyrus of the hippocampus (Morais *et al*, 2014). These results further reinforce the notion that modulation of monoaminergic neurotransmission is involved in the neuroplastic effects of currently available antidepressant drugs. However, it also highlights the fact that antidepressant treatment is not exclusively dependent on the ability to modulate monoamines and neuroplasticity as observed by high proportion of non-remitted patients. In fact, despite the significant advance in understanding the neurobiology of depression no widely accepted biomarkers are available to assist diagnostics or treatment choice for individual patients (Labermaier *et al*, 2013). This could be attributed to the complexity of this pathology. Thus, multiple biological measures may be needed to refine our

understanding and ultimately extract useful information to personalize the treatment with antidepressants. In fact, translating these research questions from the clinical setting to preclinical models*, from bed to bench and back*, still remains a challenge.

The discovery of neural stem cells in the mature adult brain opened a new chapter in the neuroscience field being considered an important player in neural plasticity. Adult neurogenesis is particularly well defined and characterized in two specific brain regions: the hippocampus and the SEZ (Ernst and Frisen, 2015; Ming and Song, 2011). In the second study of this thesis (chapter 3), we focused our attention on neuroplasticity in a distinct brain region: the hypothalamus. This region is involved in a variety of functions including energy balance regulation and has been recently described as a novel neurogenic brain region (Kokoeva *et al*, 2005; Lee *et al*, 2012; Lee *et al*, 2014). Since altered feeding behaviour is a common symptom of depression, we explored the possibility that hypothalamic neurogenesis may play a role in the alterations of energy-balance and appetite observed in the onset of, and the recovery from depression. These analyses were performed in the arcuate and median eminence nuclei of the hypothalamus (hypothalamic regions implicated in energy balance regulation). In fact, ME nuclei was only recently discovered to be implicated in body weight regulation (Lee *et al*, 2012). Our results demonstrated that uCMS and antidepressant treatment can modulate hypothalamic neurogenesis in these two hypothalamic nuclei involved in energy homeostasis. Furthermore, different classes of antidepressants, with an opposite action on body weight gain, differentially modify hypothalamic neurogenesis. Our present data has opened new questions, regarding the function of these new-born cells. In the future, we are also planning to analyse the survival of these cells.

Modulation of neuroplasticity is a critical factor involved in depression pathology and antidepressant treatment (Pittenger *et al*, 2008). At short-term (immediately after chronic treatment) neuronal remodelling seems to be critical to the mood improving actions of antidepressants (Bessa *et al*, 2009a). However, for the sustained remission (long-term antidepressant effects) hippocampal neurogenesis is a critical factor (promoted at short-term) beyond neuronal remodelling (Mateus-Pinheiro *et al*, 2013). Our present data is in agreement with this hypothesis, with efficient antidepressants (reverting *depressive* phenotype) promoting neuronal remodelling and adult neurogenesis. Additionally, we have demonstrated that stress and antidepressants modulates adult neurogenesis not only in the hippocampus (a critical region involved in the mood and cognitive dimension) but also in the

hypothalamus, a brain region involved in the regulation of vegetative symptoms, such as appetite. Currently, the research on adult mammalian neurogenesis has been almost exclusively focused on the dentate gyrus of the hippocampus and in the SEZ of the lateral ventricles. Hypothalamic neurogenesis has recently emerged as a third glio/neurogenic niche in the central nervous system. This neurogenic niche has been regarded with some scepticism, in part due to the remaining open questions: 1) what are the potential sources of hypothalamic neurogenesis? Very little information is available about the hypothalamic neural stem cell (NSC) niche. Some experiments have demonstrated that a population of specialized radial glial cells called tanycytes are characterized by the expression of some neural progenitors and stem cell markers. Additionally, they have gliogenic and neurogenic properties (Rizzoti and Lovell-Badge, 2016; Rodriguez *et al*, 2005). However, more studies are still required regarding the identity and location of adult hypothalamic stem and/or progenitor cells; 2) what is the functional significance of the postnatal hypothalamic neurogenesis? Hypothalamic neurogenesis has been associated with energy balance regulation (namely regulation of appetite), in part grounded by the expression of different neuropeptides (implicated in appetite) by these newly born neurons. In fact, the expression of neuropeptide Y (NPY), proopiomelanocortin (POMC) by these newly born cells has been previously described (Kokoeva *et al*, 2005; Lee *et al*, 2012). However, it remains unclear if other functional roles are associated with these recently formed cells. The nature of this new site of adult cell genesis is still poorly studied and requires further investigation. Ablating adult born hypothalamic neurons will probably give more insight into their function. Additionally, the study of drugs that modulate the appetite could give us important clues to understand the mechanism implicated in the regulation of hypothalamic neurogenesis. Considering the hypothalamus as a complex brain structure composed by multiple nuclei involved in different physiological functions, more studies should also be performed to understand if neurogenesis can take place in other hypothalamic nuclei with consequences in other brain functions (Rizzoti *et al*, 2016).

In the third study of this thesis (chapter 4), we studied the neuroplastic impact of a different class of drugs, the antipsychotics. Clinical studies have previously highlighted the potential beneficial effects of atypical antipsychotics in treatment-resistant depression (Papakostas *et al*, 2007; Sagud *et al*, 2006; Shelton *et al*, 2008). In this chapter we explored for the first time the role of antipsychotic drugs in the uCMS model on behaviour and neuroplasticity. Based on evidences describing the key role of neuroplasticity in the therapeutic actions of antidepressants, we hypothesized that atypical

antipsychotics may exerted their action through the modulation of these same phenomena. Our data demonstrates that the atypical antipsychotic clozapine improved measures of depressive-like behavior while haloperidol had no beneficial effect, aggravating learned helplessness in the forced swimming test and behavior flexibility in a cognitive task. Importantly, an upregulation of adult neurogenesis and neuronal survival was observed in animals treated with clozapine while haloperidol promoted a downregulation of these processes. Furthermore, clozapine was able to re-establish the stress-induced impairments in neuronal structure in the hippocampus and prefrontal cortex. These results demonstrate that the atypical antipsychotic clozapine is able to reverse the behavioral effects of chronic stress by improving adult neurogenesis, cell survival and neuronal reorganization.

Antipsychotics are primarily used in the treatment of schizophrenia. These drugs are highly effective in reducing the positive symptoms of schizophrenia, with negative and cognitive symptoms being more problematic to treat (King, 1998; Singh *et al*, 2010). These non-treated impairments lead to profound consequences in terms of ability to function in areas such as work and social relationships. In the last study of this thesis (chapter 5), we explored the neuroplastic effects of antipsychotic drugs in a well-established animal model of schizophrenia. To achieve this we explored possible deficits in negative (mood and social behaviour) and cognitive domains (spatial memory and recognition memory) in animals prenatally exposed to MAM, followed by analysis of hippocampal cell genesis. Hippocampus is a key brain region involved in the emotional and cognitive processes (normal recognition performance depends on the integrity of the hippocampus) (Rubin *et al*, 2014). In fact, MAM animals presented specific deficits in cognition (recognition memory) and social behaviour. Interestingly, these animals presented decreased levels of gliogenesis in the hippocampus, being one of the possible factors contributing to the negative impact on cognitive behaviour. Regarding the action of antipsychotics, four different agents were used in this study: the classical haloperidol and the atypical clozapine, risperidone and aripiprazole. All these agents share the modulation of the levels of dopamine through distinct mechanisms of action: haloperidol acting as a pure antagonist of the D2 receptors, clozapine and risperidone presenting this same D2 antagonism combined with the modulation of the serotonergic system and aripiprazole acting as a D2 partial agonist with modulating effects on the serotonergic system. While the classical antipsychotic drug haloperidol revealed no significant cognitive effects in these animals, the atypical antipsychotic drugs clozapine and risperidone were able to reverse the cognitive impairment induced by prenatal MAM exposure while the effects of aripiprazole on cognition

were not significant. Our present results suggest the potential importance of serotonergic and noradrenergic system modulation in the beneficial effects of atypical antipsychotics in cognitive behaviour. We also assessed cognition using the Morris water maze (MWM). Our MAM animals present no deficits in spatial memory. In fact, there is no consensual in the literature if these animals presented deficits in this dimension (Flagstad *et al*, 2005; Gastambide *et al*, 2015; Hazane *et al*, 2009). One possible explanation is the use of different strains of rats between studies. Regarding social interaction, two different analyses were performed: (a) time spent interacting with a new animal (b) time spent with a "familiar" vs "new" animal. In the first part (a) the classical antipsychotic drug haloperidol had no significant effects on sociability. All the atypical antipsychotic drugs were able to revert the impairment on sociability induced by MAM exposure. Once again, the present data indicates the critical impact of serotonergic system (modulated by the atypical drugs) to the positive effect on sociability. In the second part of the test (b) we found no differences between MAM and control animals regarding the time spent with the "new" stranger, suggesting normal social novelty recognition or social anxiety in MAM animals. In terms of time spent with the "familiar" animal, control animals spend significantly more time interacting than MAM. We hypothesized that MAM animals display reduced affect or emotional blunting (a common symptom of schizophrenia) that possibly is represented in the second part of the three chamber test. Only clozapine and aripiprazole presented a beneficial effect in this dimension, increasing the time spend with the "familiar" animal. Regarding the chronic treatment with haloperidol no effects were observed on cognition and social behaviour. This goes in line with previous reports describing that haloperidol treatment acts mainly against the positive symptoms of schizophrenia, exacerbating in some cases the negative and cognitive symptoms (Miyamoto *et al*, 2005). Additionally, neurogenesis and gliogenesis were not modulated by haloperidol treatment.

The formations of new neurons has been critically appreciated in the context of psychiatric disorders, with the majority of studies discarding the possible involvement of new astrocytes. However, astrocytic dysfunction and glial pathology have been also associated to the regulation of emotional and cognitive behaviour (Banasr *et al*, 2011). In fact, we have recently observed that imipramine treatment promotes the generation and differentiation of new hippocampal cells into astrocytes (Mateus-Pinheiro *et al*, 2013). Additionally, our present data shows that clozapine, risperidone and aripiprazole promotes the formation of new GFAP cells in an animal model of schizophrenia. However, the same was not observed

after clozapine treatment in the uCMS model. In fact, we only observed a reduction in the formation of new GFAP cells in our animal model of schizophrenia, with no effects induced by the uCMS model.
In 2006, a remarkable post-mortem study by Reif and colleagues reported that proliferating cells are significantly reduced in the dentate gyrus of the hippocampus in brains of schizophrenic patients (Reif *et al*, 2006), suggesting a possible involvement of adult cell genesis in the aetiology of schizophrenia. Interestingly, animals exposed to MAM presented alterations on gliogenesis. All the antipsychotics are able to promote an increase in the formation of new GFAP cells. The present results further reinforce the notion that astrocytes could be critical players in the aetiology and treatment of psychiatric disorders. However, new questions arise from these observations: "Is gliogenesis more important than neurogenesis in schizophrenia field?" "Is the modulation of gliogenesis implicated in the therapeutic effects of antipsychotics?". The use of different animal models, with clear effects on neuroplasticity, could give us important clues regarding the ability to promote recovery, with or without neurogenic promotion. Interestingly, DISC1 knockdown mice (genetic animal model of schizophrenia) presented deficits in gliogenesis that are reversed by increase expression levels of DISC1 (Wang *et al*, 2016), confirming glial cell genesis as a potential target for schizophrenia.

Despite the diverse pharmacological profiles (monoamine oxidase inhibitors, tricyclic antidepressants, serotonin-selective reuptake inhibitors and serotonin–norepinephrine reuptake inhibitors), all antidepressant drugs result in similar behavioral and neuroplastic outcomes, suggesting similar mechanisms of action. In contrast, classical and atypical antipsychotics are strikingly different as evidenced by their actions, mechanisms, effects and side effects. For instance, the atypical antipsychotic clozapine has a more complex pharmacological action than the classical haloperidol, presenting binding affinities for various neurotransmitter receptors, including several serotonin and noradrenaline receptors (Meltzer *et al*, 1989; Miyamoto *et al*, 2005). Our present results suggest the potential importance of serotonergic and noradrenergic system modulation in the beneficial effects of atypical antipsychotics on neuroplasticity that should be addressed in future studies.

The oversimplification of the pathophysiology of depression as a neurochemical imbalance disorder has biased the research of new antidepressants for decades and currently available treatment for depression is often inadequate for many patients. In fact, promising hypothesis for depression and antidepressant treatment has failed to be applied in a number of patients, indicating that much remains

to be understood regarding the pathophysiology of depression. Probably, the complexity of this pathology needs a more careful attention in order to understand which hypothesis is more correctly associated to which individual patient or if different hypothesis are implicated in a specific patient. In the present thesis we have been particularly interested in the "neurogenic hypothesis of depression", claiming that stress (as an etiological factor) decreases the production and survival of newly born cells and treatments with antidepressants increase them. In fact, blocking the formation of these newly born cells prevents the long-term beneficial effects of antidepressants in several behavioural paradigms. However, the precise mechanism by which newly born neurons influence the antidepressant response remains unclear and more studies are still required. Regarding schizophrenia, more studies should be performed addressing neuroplasticity as a target for antipsychotic treatment.

Together, these findings contribute to expand our knowledge on the role of psychopharmacological agents (including antidepressants and antipsychotics) on the modulation of different neuroplastic events, including cell genesis and neuronal remodelling. In future, this knowledge may contribute to new therapeutic interventions both in depression and schizophrenia.

Conclusions

In the present thesis we showed that:

1- Pirlindole (a MAO-A drug) is able to reverse the behavioural effects of stress exposure potentiating at the same time hippocampal adult neurogenesis and rescuing the stress-induced dendritic atrophy of granule neurons in the dentate gyrus of the hippocampus (Chapter 2);

2- uCMS and antidepressant treatment can modulate hypothalamic neurogenesis. Different classes of antidepressants, with an opposite action on body weight gain, modulate differentially hypothalamic neurogenesis (Chapter 3).

3- The modulation of adult neuroplasticity (adult neurogenesis, cell survival and neuronal reorganization) is involved in the mood improving actions of atypical antipsychotics in an animal model of depression (Chapter 4)

4- Prenatal MAM exposure induces specific cognitive deficits and social impairments. The classical haloperidol presents no beneficial effects in these behavior dimensions. The atypical clozapine and risperidone have a positive effect on both dimensions with aripiprazole presenting only a statistic effect in the social measure. Adult gliogenesis is affected in MAM animals and is modulated by some atypical antipsychotics (Chapter 5).

References

Banasr M, Dwyer JM, Duman RS (2011). Cell atrophy and loss in depression: reversal by antidepressant treatment. *Current opinion in cell biology* **23**(6): 730-737.

Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA*, et al* (2009). The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. *Molecular psychiatry* **14**(8): 764-773, 739.

Botteron KN, Raichle ME, Drevets WC, Heath AC, Todd RD (2002). Volumetric reduction in left subgenual prefrontal cortex in early onset depression. *Biological psychiatry* **51**(4): 342-344.

Coryell W, Nopoulos P, Drevets W, Wilson T, Andreasen NC (2005). Subgenual prefrontal cortex volumes in major depressive disorder and schizophrenia: diagnostic specificity and prognostic implications. *The American journal of psychiatry* **162**(9): 1706-1712.

Ernst A, Frisen J (2015). Adult neurogenesis in humans- common and unique traits in mammals. *PLoS Biol* **13**(1): e1002045.

Flagstad P, Glenthoj BY, Didriksen M (2005). Cognitive deficits caused by late gestational disruption of neurogenesis in rats: a preclinical model of schizophrenia. *Neuropsychopharmacology* **30**(2): 250-260.

Frodl T, Jager M, Smajstrlova I, Born C, Bottlender R, Palladino T*, et al* (2008). Effect of hippocampal and amygdala volumes on clinical outcomes in major depression: a 3-year prospective magnetic resonance imaging study. *Journal of psychiatry & neuroscience : JPN* **33**(5): 423-430.

Frodl T, Meisenzahl EM, Zetzsche T, Born C, Groll C, Jager M*, et al* (2002). Hippocampal changes in patients with a first episode of major depression. *The American journal of psychiatry* **159**(7): 1112-1118.

Gastambide F, Taylor AM, Palmer C, Svard H, Karjalainen M, Janhunen SK*, et al* (2015). Alterations in spatial memory and anxiety in the MAM E17 rat model of hippocampal pathology in schizophrenia. *Psychopharmacology* **232**(21-22): 4099-4112.

Hazane F, Krebs MO, Jay TM, Le Pen G (2009). Behavioral perturbations after prenatal neurogenesis disturbance in female rat. *Neurotoxicity research* **15**(4): 311-320.

Jayatissa MN, Bisgaard C, Tingstrom A, Papp M, Wiborg O (2006). Hippocampal cytogenesis correlates to escitalopram-mediated recovery in a chronic mild stress rat model of depression. *Neuropsychopharmacology* **31**(11): 2395-2404.

King DJ (1998). Drug treatment of the negative symptoms of schizophrenia. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology* **8**(1): 33-42.

Kokoeva MV, Yin H, Flier JS (2005). Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science* **310**(5748): 679-683.

Labermaier C, Masana M, Muller MB (2013). Biomarkers predicting antidepressant treatment response: how can we advance the field? *Disease markers* **35**(1): 23-31.

Lee DA, Bedont JL, Pak T, Wang H, Song J, Miranda-Angulo A*, et al* (2012). Tanycytes of the hypothalamic median eminence form a diet-responsive neurogenic niche. *Nat Neurosci* **15**(5): 700-702.

Lee DA, Yoo S, Pak T, Salvatierra J, Velarde E, Aja S*, et al* (2014). Dietary and sex-specific factors regulate hypothalamic neurogenesis in young adult mice. *Frontiers in neuroscience* **8**: 157.

Lopez-Munoz F, Alamo C (2009). Monoaminergic neurotransmission: the history of the discovery of antidepressants from 1950s until today. *Current pharmaceutical design* **15**(14): 1563-1586.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* **20**(24): 9104-9110.

Mateus-Pinheiro A, Pinto L, Bessa JM, Morais M, Alves ND, Monteiro S*, et al* (2013). Sustained remission from depressive-like behavior depends on hippocampal neurogenesis. *Translational psychiatry* **3**: e210.

Meltzer HY, Bastani B, Ramirez L, Matsubara S (1989). Clozapine: New research on efficacy and mechanism of action. *European archives of psychiatry and neurological sciences* **238**(5): 332-339.

Ming GL, Song H (2011). Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron* **70**(4): 687-702.

Miyamoto S, Duncan GE, Marx CE, Lieberman JA (2005). Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. *Molecular psychiatry* **10**(1): 79-104.

Morais M, Santos PA, Mateus-Pinheiro A, Patricio P, Pinto L, Sousa N*, et al* (2014). The effects of chronic stress on hippocampal adult neurogenesis and dendritic plasticity are reversed by selective MAO-A inhibition. *Journal of psychopharmacology (Oxford, England)* **28**(12): 1178-1183.

Papakostas GI, Shelton RC, Smith J, Fava M (2007). Augmentation of antidepressants with atypical antipsychotic medications for treatment-resistant major depressive disorder: a meta-analysis. *The Journal of clinical psychiatry* **68**(6): 826-831.

Patricio P, Mateus-Pinheiro A, Irmler M, Alves ND, Machado-Santos AR, Morais M*, et al* (2015). Differential and converging molecular mechanisms of antidepressants' action in the hippocampal dentate gyrus. *Neuropsychopharmacology* **40**(2): 338-349.

Pittenger C, Duman RS (2008). Stress, depression, and neuroplasticity: a convergence of mechanisms. *Neuropsychopharmacology* **33**(1): 88-109.

Reif A, Fritzen S, Finger M, Strobel A, Lauer M, Schmitt A*, et al* (2006). Neural stem cell proliferation is decreased in schizophrenia, but not in depression. *Molecular psychiatry* **11**(5): 514-522.

Rizzoti K, Lovell-Badge R (2016). Pivotal role of median eminence tanycytes for hypothalamic function and neurogenesis. *Molecular and cellular endocrinology*.

Rodriguez EM, Blazquez JL, Pastor FE, Pelaez B, Pena P, Peruzzo B*, et al* (2005). Hypothalamic tanycytes: a key component of brain-endocrine interaction. *International review of cytology* **247**: 89-164.

Rubin RD, Watson PD, Duff MC, Cohen NJ (2014). The role of the hippocampus in flexible cognition and social behavior. *Frontiers in human neuroscience* **8**: 742.

Sagud M, Mihaljevic-Peles A, Muck-Seler D, Jakovljevic M, Pivac N (2006). Quetiapine augmentation in treatment-resistant depression: a naturalistic study. *Psychopharmacology* **187**(4): 511-514.

Sairanen M, Lucas G, Ernfors P, Castren M, Castren E (2005). Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. *J Neurosci* **25**(5): 1089-1094.

Santarelli L, Saxe M, Gross C, Surget A, Battaglia F, Dulawa S*, et al* (2003). Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants. *Science* **301**(5634): 805-809.

Sheline YI, Gado MH, Kraemer HC (2003). Untreated depression and hippocampal volume loss. *The American journal of psychiatry* **160**(8): 1516-1518.

Shelton RC, Papakostas GI (2008). Augmentation of antidepressants with atypical antipsychotics for treatment-resistant major depressive disorder. *Acta Psychiatr Scand* **117**(4): 253-259.

Singh SP, Singh V, Kar N, Chan K (2010). Efficacy of antidepressants in treating the negative symptoms of chronic schizophrenia: meta-analysis. *The British journal of psychiatry : the journal of mental science* **197**(3): 174-179.

Surget A, Tanti A, Leonardo ED, Laugeray A, Rainer Q, Touma C*, et al* (2011). Antidepressants recruit new neurons to improve stress response regulation. *Molecular psychiatry* **16**(12): 1177-1188.

Wang S, Liang Q, Qiao H, Li H, Shen T, Ji F*, et al* (2016). DISC1 regulates astrogenesis in the embryonic brain via modulation of RAS/MEK/ERK signaling through RASSF7. *Development (Cambridge, England)* **143**(15): 2732-2740.