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Ana Rita Machado dos Santos

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UMinho|2013

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Dissertação de Mestrado
Mestrado em Ciências da Saúde

Trabalho realizado sob a orientação da
Professora Doutora Luísa Pinto
e co-orientação do
Professor Doutor João Filipe Oliveira

Maio de 2013

Patience, persistence and perspiration make an unbeatable combination for success.

Napoleon Hill

Acknowledgements/Agradecimentos

À Professora Doutora Luísa Pinto, agradeço do fundo do coração pela sábia orientação, pela constante presença durante toda esta etapa, pela amizade, entusiasmo e pela leitura sempre crítica do trabalho, que tornou a execução desta tese um verdadeiro prazer. Ambiciono, um dia, chegar ao nível de excelência com que nos presenteias diariamente.

Ao mestre Dinis Alves, agradeço todo o empenho prestado ao trabalho, todos os serões passados no biotério a planear as tarefas seguintes, toda a organização que tanto me chocava mas que foi tão necessária, a amizade e o bom-humor que foi tão bem-vindo durante este ano.

À mestre Patrícia Patrício, agradeço o apoio prestado, a valiosa e constante ajuda no laboratório, a partilha de conhecimentos, a amizade e a boa-disposição sempre presente no dia-a-dia.

Ao mestre António Pinheiro, agradeço o apoio prestado, a fomentação da discussão dos resultados, a amizade e a partilha de conhecimentos que tanto me ajudaram durante este último ano.

À mestre Mónica Morais, agradeço toda a disponibilidade, ajuda e amizade.

Ao Professor Doutor João Bessa e ao Professor Doutor Nuno Sousa, agradeço a oportunidade de integrar uma equipa fantástica e de desenvolver um trabalho que tanto gozo me deu.

Com uma equipa assim, tudo se torna mais fácil!

Ao Professor Doutor João Filipe Oliveira, agradeço o entusiasmo, disponibilidade, todos os desafios propostos e pela visão crítica sobre o trabalho.

Aos NeRDs, agradeço pelo carinho, pelas sugestões e pela visão crítica sobre o meu trabalho.

Às minhas colegas de laboratório e mestrado Ana Maria Silva, Márcia Santos, Vanessa Sardinha, Magda Reis, Joana Silva e Sofia Lopes, agradeço a boa-disposição, a constante partilha de bons momentos, os sorrisos e as caminhadas. Vocês tornaram-se verdadeiras amigas durante este último ano!

Às minhas colegas de casa Cláudia Miranda, Carla Calçada e Margarida Araújo, agradeço o bom-ambiente que me proporcionaram em Braga, agradeço a ajuda quando tinha menos tempo e, acima de tudo, agradeço a amizade. Braga tornou-se uma cidade mais acolhedora por vossa causa!

Às minhas fiéis amigas Mariana Urbano, Rita Sousa, Filipa Santana e Sofia Brito, agradeço a constante amizade que, apesar de durar tantos anos, continua a alegrar os meus dias e a tornar-me uma pessoa mais feliz e grata por vos ter no meu caminho.

Ao Afonso Costa, tenho a agradecer a amizade, a boa-disposição e a ajuda na formatação desta tese.

À minha família, agradeço a compreensão pelo pouco tempo dispensado e a boa-disposição aos fins-de-semana, que me fizeram sempre querer voltar a casa. À minha mãe agradeço por ser um grande exemplo de força e por nunca me deixar desistir dos meus sonhos.

Ao Amílcar Cordeiro, agradeço com todo o meu coração o apoio constante em todas as etapas da minha vida pessoal e profissional, o amor e carinho dispensados mesmo quando tinha muito trabalho e pouco tempo, a dedicação e a ajuda. Obrigada por me dizeres sempre que sou capaz! Contigo a meu lado, o meu caminho torna-se francamente mais fácil e muito mais feliz!

Abstract

Major depression is a highly prevalent disorder that poses a significant social burden in society nowadays. The pathophysiology of this disease is still poorly understood but growing evidence suggests that impaired neuroplasticity may be a key underlying mechanism for the precipitation of the disorder. This theory is substantiated by the fact that depression leads to a decrease in neurogenesis and several antidepressants (ADs) stimulate hippocampal neurogenesis, but it is still unclear if these pro-neurogenic effects are responsible for their mood-, emotional- and cognitive-improving actions. Recent studies also showed an important role for astrocytes in the pathophysiology of this disorder, which are crucial for neurotransmission and neurovascular coupling, evidenced by astrocytes loss in major depressive disorder (MDD). However, the importance of astrocytes in the precipitation of and recovery from MDD is still largely unknown.

Therefore, we proposed to study the role of astrocytes and adult gliogenesis, more precisely the generation of new astrocytes, in the precipitation of and recovery from depressive-like cognitive behavior in rats both untreated and treated with ADs in a longitudinal manner - at short-term, long-term and recurrence perspective -, using a pre-validated model of depression - the unpredictable chronic mild stress (uCMS).

Regarding the cognitive behavior assessment, although short-term memory seems to be impaired through the course of the disease, at short-term, long-term and recurrence, administration of fluoxetine and imipramine was effective in reverting the cognitive impairments induced by depression. The long-term memory was also highly affected by this disorder in all analyzed time-points, but the treatment with the ADs was not effective in reverting the cognitive impairments observed. Regarding the hippocampal dentate gyrus (DG) astrocytic population, at the long-term perspective of the disease, imipramine, but not fluoxetine, was able to elicit a strong pro-gliogenic effect. The same does not happen at recurrence for both ADs, suggesting that an adaptation to the stressful environment by these specific type of cells might be happening throughout time. Thereby, we provide some consistent evidences for the causative implication of gliogenesis and astrocytes in the pathophysiology of depression, having significant impacts in the long-term development and maintenance of cognitive deficits, as well as in the long-term recovery of those impairments by ADs. Our results endorse the view of the adult hippocampal DG gliogenesis process as a promising therapeutical target in future therapies in the neuropsychiatric field. Moreover, this study shows for the first time that alterations in the hippocampal DG resident astrocytes can be further analyzed as a predictive target for depression.

Resumo

A depressão é uma doença bastante prevalente que, atualmente, representa um peso importante na sociedade. A fisiopatologia desta doença é ainda pouco compreendida, mas evidências crescentes sugerem que o comprometimento da neuroplasticidade pode ser um mecanismo fundamental subjacente à precipitação da doença. Esta teoria é apoiada pelo facto de a depressão induzir uma diminuição da neurogénese e, também, ao facto de vários antidepressivos levarem à estimulação da neurogénese. No entanto, ainda não é claro se esses efeitos pró-neurogénicos são responsáveis pelas melhorias a nível de humor, emocionais e/ou cognitivas, nos pacientes. Estudos recentes demonstraram a importância dos astrócitos na fisiopatologia desta doença, mostrando ser cruciais para a neurotransmissão e acoplamento neurovascular, evidenciada por uma perda de astrócitos em episódios depressivos. Contudo, a importância dos astrócitos na precipitação e recuperação desta doença ainda é, em grande parte, desconhecida.

Por isso, propusemo-nos a estudar o papel dos astrócitos e da gliogénese adulta, mais precisamente da geração de novos astrócitos, na precipitação e recuperação de défices cognitivos associados à depressão. Este estudo foi elaborado com ratos tratados e não tratados com antidepressivos e seguiu uma linha longitudinal no episódio depressivo - a curto prazo, a longo prazo e na recorrência da doença -, usando um modelo pré-validado de depressão – um protocolo de exposição crónica a stress. Quanto à avaliação comportamental a nível cognitivo, embora a memória a curto prazo ser afetada com o decorrer da doença, a administração de fluoxetina e imipramina foi eficaz na reversão dos danos cognitivos induzidos pela depressão. A memória a longo prazo foi igualmente afetada por esta doença em todos os tempos experimentais analisados, mas o tratamento com os antidepressivos não se mostrou eficaz em reverter os défices cognitivos observados. Quanto a alterações da população astrocítica no girus denteado do hipocampo, o tratamento com imipramina, contrariamente ao tratamento com fluoxetina, foi capaz de induzir um efeito pró-gliogénico, a longo termo. O mesmo não acontece na recorrência para ambos os antidepressivos, sugerindo que uma adaptação ao novo ambiente hostil por parte das novas células que estão continuamente a ser geradas, esteja a ocorrer ao longo do tempo. Neste trabalho, fornecemos algumas evidências consistentes do envolvimento ativo da gliogénese e dos astrócitos na fisiopatologia da depressão, tendo impactos significativos no desenvolvimento e manutenção de défices cognitivos a longo termo, bem como na recuperação desses mesmos défices após o tratamento com antidepressivos. Os resultados apresentados apontam para a gliogénese no girus denteado do hipocampo como um alvo terapêutico promissor neste contexto patológico. Mais ainda, este estudo demonstra, pela primeira vez, que alterações a nível da população astrocítica residente no girus denteado do hipocampo poderão ser futuramente analisadas como um alvo preditivo para a depressão.

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Abbreviations

%	Percentage
µm	Micrometre
ADs	Antidepressants
ANPs	Transiently amplifying neural progenitors
BLPB	Brain lipid binding-protein
BPD	Bipolar disease
BrdU	Bromodeoxyuridine
CNS	Central nervous system
CTRL	Control
DAPI	4´-6´- diamidino-2-phenylindole
DG	Dentate gyrus
FLX	Fluoxetine
FST	Forced swimming test
GCL	Granular cell layer
GFAP	Glial fibrillary acidic protein
GLAST	Glutamate aspartate transporter
HPA	Hypothalamic pituitary adrenal
IMIP	Imipramine
LIF	Leukemia inhibitory factor
LT	Long-term
MAM	Methylazoximethanol
MDD	Major depressive disorder
MIN	Minutes
NOR	Novel object recognition
NSC	Neural stem cells
OB	Olfactory bulb
OF	Open field
PDFG	Platelet derived growth factor
PFA	Paraformaldehyde
PFC	Prefrontal córtex
REC	Recurrence
RMS	Rostral migratory stream
RT	Room temperature
SAL	Saline
SEM	Standard error of the mean
SEZ	Subependymal zone

SGZ	Subgranular zone
SPT	Sucrose preference test
SSRI	Selective serotonin reuptake inhibitors
ST	Short-term
TBS	Tris-buffered saline
uCMS	unpredictable chronic mild stress

I. INTRODUCTION

1. Introduction

"Canst thou not minister to a mind diseased, Pluck from the memory a rooted sorrow, Raze out the written troubles of the brain, And with some sweet oblivious antidote Cleanse the stuffed bosom of that perilous stuff Which weighs upon the heart?"

William Shakespeare, ***Macbeth***

1.1. Depression: Current Status, Treatment and Cognitive related aspects

Depression is a complex mood disorder that poses a massive burden in current society and is foreseen as the leading cause for disability during an individual's most productive years. This disorder affects several behavioral domains in patients, such as mood, anxiety and cognition (Bessa, Mesquita, *et al.*, 2009; Clelland *et al.*, 2009) and is characterized by emotion dysregulation – the hallmark of depression - and sustained negative effect. Although impairments in cognitive processes, such as attention and memory, can be correlated with depressive episodes, they can also increase individual´s susceptibility for a first hit and recurrence of this disorder. In fact, more than 75% of the depressed individuals relapse within two years of recovery from the first depressive episode (Gotlib & Joormann, 2010), leading us to believe that there are some specific factors that are empowered to increase patients´ risk for a new depressed episode. Even though there is no knowledge of a real cause for the precipitation of Major depressive disorder (MDD), it is accepted that there is a familial basis for susceptibility to this disorder; however, on the majority of the population only the interplay between a genetic predisposition and some environmental factors (e.g. stress-related factors) are sufficient to cause depression (Nestler *et al.*, 2002). Furthermore, this disorder is characterized by several pathophysiological alterations in the brain such as differences in size of specific brain regions, changes in neuronal morphology, neurochemical and signaling alterations and also changes in genetic and epigenetic regulation. Taking this into account, it is imperative to understand the neural mechanisms behind the onset, maintenance and recurrence of this gradually decadent disorder.

There are currently several leading hypotheses that attempt to elucidate the neural and molecular mechanisms of depression. The monoamine hypothesis of depression has been the most prevalent. In fact, the current treatments in clinics were developed based on the classical monoamine hypothesis of depression (Charney, 1998) as most classic antidepressants (ADs) operate through increasing the levels of serotonin and noradrenaline. It is noticeable that this neurophysiologic theory of depression stands from the drugs that are used to treat it. The most widely ADs used in the clinics are the tricyclic agents (e.g. imipramine), the serotonine-selective reuptake inhibitors (SSRIs; e.g.fluoxetine) and norepinephrine-selective reuptake inhibitors. Despite these drugs have provided the possibility to develop a high range of behavioral tests to allow the validation of phenotypes of depressed-like animals, the number of patients that present a total remission after treatment with these ADs is still far from the desired one – around 50% (Nestler *et al.*, 2002). Furthermore, the monoamine hypothesis theory states that depression is the result of underactivity of monoamines, being almost all ADs monoamine agonists. However, several other hypotheses on the etiology of depression have been put forward - the neurotrophin hypothesis, the cytokine hypothesis, the hypothalamic pituitary adrenal (HPA) axis modulation hypothesis and the neurogenic hypothesis. Importantly, none of these hypotheses are mutually exclusive.

Regarding the several dimensions commonly affected in this neuropsychiatric disease, cognition is, indeed, one of the less marked but still relevant behavioral dimension affected in depressed individuals, being attention and memory the most strongly impaired. In 1976, a cognitive model have emerged and highly contributed to our current knowledge regarding the neuropathophysiology of depression. This model states that early adverse events combined with other intrinsic factors, such as genetic factors, can lead to the self creation of depressive schemas, powered enough to interfere with attention, memory and cognition. By continuously interpreting their experiences in a negative way, these individuals will be susceptible to depression. Actually, the same author claimed that changes in cognition can lead to an amelioration of others symptoms related with this disorder, such as sustained negative affect and anhedonia (Beck, 1976). Anhedonia, commonly defined as the loss of interest or pleasure in all or almost all activities, is an important symptom experienced by depressed patients (Der-Avakian & Markou, 2012). Hereupon, the idea of an interplay and continuity between emotional changes and cognitive impairments has emerged (Swaab *et al.*, 2005; Sotiropoulos *et al.*, 2008). So, tackling this major dimension – cognition – we can possibly revert some of the other symptoms that usually come along with MDD.

It is, though, of major importance to elucidate the cognitive and neurobiological factors that are involved in the onset, resilience and recurrence of this disorder in order to prevent and treat it by developing targeted strategies.

1.2. Neurogenesis in the adult brain: relevance for the pathology of depression

One of the most surprising findings in the context of depression was the involvement of adult neurogenesis imbalances, along with dendritic arborization impairments, in the pathophysiology of MDD, as in the action of ADs, leading to the substantiation of the so called "neurogenic hypothesis of depression" (Warner-Schmidt & Duman, 2006). In a simplified version, this hypothesis states that new neurons in the adult brain are needed for both proper mood control and AD efficacy (Petrik *et al.*, 2012).

Although Cajal´s had claimed that the central nervous system (CNS) was immutable, a great deal of evidences has proved the opposite: CNS features regenerative and plasticity potentials. Moreover, neurogenesis, a process that comprises the generation, differentiation and integration of new neurons in the preexisting brain neuronal networks, occurs in the adult brain and persists throughout life in specific brain locus (Doetsch *et al.*, 1999; Gage, 2002). Nowadays, adult neurogenesis is known to occur mostly in two defined mammalian brain regions: the subependymal zone of the lateral ventricles (SEZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Within these regions, there are located resident progenitor cells, also known as neural stem cells (NSCs). These particular subtype of cells have both morphological and antigenic glial properties, being constantly described as stem cells with glial properties and radial-glia like appearance (Filippov *et al.*, 2003; Rakic, 2003). NSCs can give rise to intermediate progenitor cells, designated as transit amplifying neural progenitors (tANPs, or Type-2 cells) that are mitotically active and divide to give rise to neuroblasts (also known as Type-3 cells). These latter cells will then fully maturate into granule neurons, elongating their axons and making the appropriate axonal connections. The neuroblasts are born in the SEZ and migrate along the rostral migratory stream (RMS) becoming mostly mature GABAergic granule and periglomerular interneurons in the olfactory bulb (OB), whereas those which are born in the adult SGZ migrate into the granular cell layer (GCL) of the DG and differentiate into glutamatergic granule cells. The adult-born neurons become integrated in the pre-existing neuronal network 5 to 7 weeks after their birth (Van Praag *et al.*, 2002; Ambrogini *et al.*, 2004; Espósito *et al.*, 2005; Zhao *et al.*, 2006). Shortly, this complex phenomenon is nothing more than a synchronized orchestra that culminates in the formation of new neurons.

Interestingly, the initial proposal saying that the neurogenic modulatory effects of ADs were responsible for all behavioral improvements observed after chronic treatment with these drugs was an oversimplification, as demonstrated by many studies (Holick *et al.*, 2008; Boldrini *et al.*, 2009; Jayatissa *et al.*, 2009). In fact, it was observed that the short-term mood-improving actions of ADs depend on neuronal remodeling, rather than on neurogenesis (Bessa, Ferreira, *et al.*, 2009). This observation makes all sense since a large number of studies were successful in showing that the time window required by a newborn cell to be fully mature and integrated in the pre-existing neural network is around 5 (Espósito *et al.*, 2005; Zhao *et al.*, 2006) to 7 (Van Praag *et al.*, 2002; Ambrogini *et al.*, 2004) weeks, like mentioned above. Thus, to unravel the importance of neurogenesis in the recovery from depression after treatment with ADs, we need to consider the long-term perspective of this disease, taking in consideration the time that new neurons need to integrate the network and become functional. Indeed, recent data from our group shows, for the first time, that despite triggering an immediate pro-neurogenic response, the neurobiological importance of this ADs effect becomes of particular relevance later on the course of the disease (4 weeks after the treatment with ADs). At this time point, the suppression of cytogenesis (with methylazoximethanol (MAM) administration) significantly compromises behavioral long-term recovery, an effect that is counteracted by ADs treatment (Mateus-Pinheiro *et al.*, 2013). This particular study suggests that slower neuroplastic changes, regarding neurogenesis and remodeling of the neuro-glial networks, are apparently necessary to determine the extent of recovery from depressive symptoms.

1.3. Adult Neurogenesis and Glial Biology: the missing link

Along the entire scientific history, there have always been some secondary actors, forgotten towards the brightest ones. Glial cells are the perfect example of those secondary actors, always obfuscated by neurons. In fact, glia´s central role in cortical and neuronal function was always underestimated and glial cells were frequently seen only as neuron partners, supporting them (Coyle & Schwarz, 2000). However, this particular view has changed dramatically when it was found that NSCs in the developing brain and in the adult neurogenic zones exhibit astroglial properties (Morrens *et al.*, 2012). Hereupon, glial cells immediately jumped to the front line and a vast number of studies were conducted in order to elucidate the role of these cells. Glia was found to interact closely with neuronal cells, participating in brain metabolism, synaptic neurotransmission and in interneuronal communication (Volterra &

Meldolesi, 2005). Moreover, glial cells occupy about half the volume of the brain (Jessen, 2004) and are responsible for actively maintaining the tissue homeostasis (Devinsky *et al.*, 2013). These neuron-partners, the glial cells, are subdivided into distinct classes, possessing different characteristics: Astrocytes, Oligodendrocytes, Microglia and NG2-positive cells.

Starting by describing oligodendrocytes, these mature glial cells are mostly involved in the production of myelin in the brain and spinal cord (Bradl & Lassmann, 2010). Axons are unsheathed with this lipoprotein – Myelin - and, periodically, some gaps are formed, named as Nodes of Ranvier (Morrens *et al.*, 2012), enabling axons to accelerate the conduction of the action potential, by a saltatory mode (Hartline & Colman, 2007). The absence of myelin (demyelination) severely compromises the conduction of the action potential trough the axon, resulting in several neurological deficits (Patel & Balabanov,2012). Due to the absence of myelin, axons can be even more predisposed to severe injury because of the deprivation of the trophic effects of the oligodendrocytes (Trapp *et al.*, 1998; Waxman, 2001).

A great number of oligodendrocyte progenitor cells are generated in the SEZ (Morrens *et al.*, 2012) being, just like adult oligodendrocytes, very vulnerable to certain conditions such as oxidative stress and inflammation. Thus, several pathologies, such as spinal cord injury, Parkinson´s and Alzheimer´s diseases, lead to oligodendrocytes dysfunction or even dead. However, some data reports spontaneous replacement of these cells, under specific conditions (McTigue & Tripathi, 2008).

Regarding Microglia, these parenchymal tissue macrophages constitute about 10% of all cells in the CNS (Aguzzi *et al.*, 2013). Microglia can have a ramified appearance, ordinarily found in the brain parenchyma, or be attached to the vasculature and within the perivascular extracellular matrix (ECM), named as perivascular microglia. Although microglia activation seems not to be pro- or anti-neurogenic *per se*, the molecules secreted by them are the ones responsible for the cellular net outcome (Morrens *et al.*, 2012). These cells can be activated by neurons and are responsible for eliminating and maintaining synapses, thus leading to a normal function of the neural circuit (Aguzzi *et al.*, 2013).

Microglia functions are closely related with phagocytosis, being these cells the ones responsible for eliminating the neural precursor cells and thus regulating adult neurogenesis (Sierra *et al.*, 2010). Several studies regarding the contribution of microglia to disease states have shown beneficial, adverse and dispensable functions of these cells, putting forward an angel/devil perspective of microglia (Morrens *et al.*, 2012). However, more studies are needed to really address the microglial function on both health and disease states.

Mentioning now the NG2 chondroitin Sulfate Proteoglycan expressing cells, the discovery of these cells lead to the existence of a fifth major cell population in the CNS, having as colleagues neurons, astrocytes, mature oligodendrocytes and microglia (Xu *et al.*, 2011). Although NG2 expressing cells are known as oligodendrocyte progenitor cells because of their property to differentiate into oligodendrocytes (Nishiyama *et al.*, 1997; Lu *et al.*, 2002; Zhou & Anderson, 2002; Kitada & Rowitch, 2006; Ligon *et al.*, 2006; Zhu *et al.*, 2008; Komitova *et al.*, 2009), recent data also showed that these specific cells, which are expressed on immature myelinating glia in the CNS, can also give rise to subpopulations of astrocytes during normal development. Besides acting as a plastic progenitor pool for more differentiated cells, this cell population may constitute a unique glial network, constantly interacting with neurons (Jabs *et al.*, 2005; Bergles *et al.*, 2010). NG2 expressing cells represent the most numerous population of proliferating cells in the adult brain (Dawson *et al.*, 2003) and can be found in both neurogenic zones, although in a fewer percentage in the SGZ. Some reports argue about the multipotency of these cells but they seem to be controversial (Nishiyama *et al.*, 2009; Richardson *et al.*, 2011), being the most solid aspect the fact that NG2 positive cells receive synaptic input from neurons and may be involved in glutamate signaling modulation (Bergles *et al.*, 2010; Mangin & Gallo, 2011).

Lastly, regarding astrocytes, the main glial subtype, these cells interact closely with neurons, participating in the regulation of synaptic neurotransmission by releasing chemical transmitters: the so called “tripartide synapse” (Araque *et al.*, 1999) (see Figure 1). These star-shaped cells have functional receptors for neurotransmitters and respond to their stimulation by releasing gliotransmitters, including glutamate. Astrocytes can increase the intracellular calcium ($[Ca2+]_i$) upon an elevation of synaptically released neurotransmitters, resulting in the release of glutamate via regulated exocytosis (Rossi & Volterra, 2009). Data reports that this increase in $[Ca2+]_i$ is extremely important, in a functional view, for astrocyte-astrocyte and also astrocyte-neuron intercellular communication (Sofroniew & Vinters, 2010; Cornell-Bell *et al.*, 1990; Charles *et al.*, 1991).

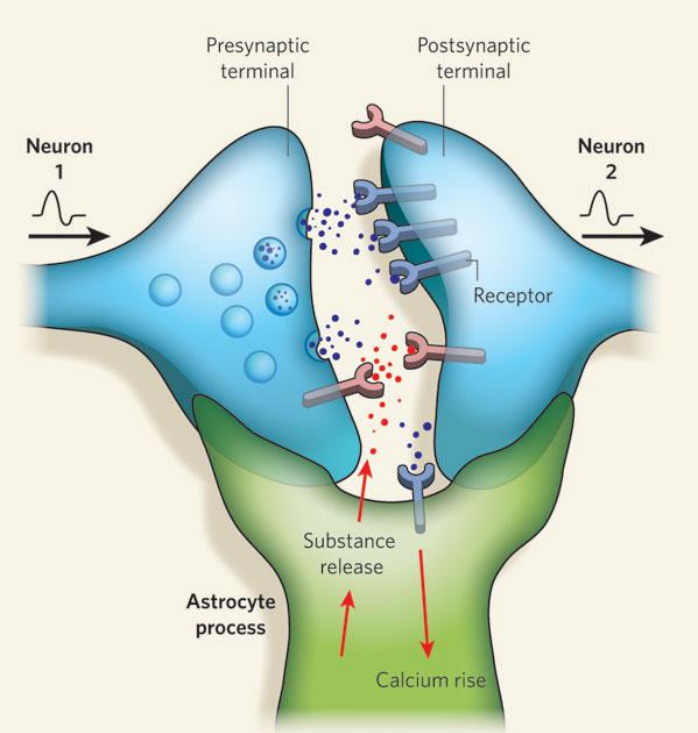


Figure 1. *The tripartide synapse*. Astrocytes express many of the same receptors as neurons. When neurotransmitters are released from the presynaptic terminal of a neuron, astrocytic receptors are thought to be activated, leading to a rise in calcium ions in the astrocyte and the release of various active substances, such as ATP, which act back on neurons to either inhibit or enhance neuronal activity. Astrocytes also release proteins, which control synapse formation, regulate presynaptic function and modulate the response of the postsynaptic neuron to neurotransmitters (Allen & Barres, 2009).

Astrocytes can also couple to neighboring astrocytes through gap junctions and, putting this in a multicellular network perspective, they can play a role in both normal function and CNS disorders (Nedergaard *et al.*, 2003; Seifert *et al.*, 2006). These findings led astrocytes to the spotlight, bringing a new concept of neuron–glia intercommunication where astrocytes play an active role by integrating neuronal inputs and modulating synaptic activity (Rossi & Volterra, 2009). It is also noteworthy that astrocytes are able to synthesize glutamate *de novo* and to store glucose in the form of glycogen, unlike neurons (Hertz & Zielke, 2004), thus contributing to brain metabolism (Hertz *et al.*, 2007). This phenomenon is only possible due to astrocytes´ high oxidative metabolism.

As mentioned above, NSCs express several radial glia and astrocytic markers, including brain lipid binding-protein (BLPB) and the glutamate aspartate transporter - GLAST (Steiner *et al.*, 2006). Although they express the common glial fibrillary acidic protein isoform alpha (GFAP)-a, the progenitor cells also specifically express the GFAP isoform delta and can be isolated based on its specific expression (Van Den Berge *et al.*, 2010). The fact that NSCs in both neurogenic zones of the adult brain (SGZ and SEZ)

have plenty of astroglial properties makes it possible to link adult neurogenesis and glial cells (Morrens *et al.*, 2012). In fact, genetic ablation of GFAP-expressing cells showed to be capable of eliminating adult neurogenesis (A. D. R. Garcia *et al.*, 2004; Imura *et al.*, 2003; Morshead *et al.*, 2003). Moreover, astrocytes from the SEZ and SGZ were shown to promote the proliferation of progenitor cells and their neuronal differentiation *ex vivo* (Lim & Alvarez-Buylla, 1999; Song *et al.*, 2002). Although astroglia seems enough to support synaptic integration and functional maturation of newly born neurons (Hong-jun Song *et al.*, 2002), the same cells derived from a non-neurogenic region fosters glial over neurogenic fate (Lie *et al.*, 2002). *In vivo*, astrocytes seem to provide highly physical support to progenitor cells and newly born neurons (Shapiro *et al.*, 2005; Plümpe *et al.*, 2006), thus also playing a possible role in adult neurogenesis regulation *in vivo* (Morrens *et al.*, 2012).

Citing Ben Barres, an expert in neuron-glial interactions: "Quite possibly saving astrocytes from dying in neurological disease would be a far more effective strategy than trying to save neurons (glia already know how to save neurons, whereas neuroscientists still have no clue)" (Barres, 2008).

1.4. Glial Cell Pathology in the disease context

Astrocytes dysfunction has been related with neural impairments, mostly because of the tripartide synapse disturbance. There is now a growing body of evidences showing that either loss of normal astrocytic functions or gain of abnormal effects can contribute to the progress of several diseases, with these cells playing numerous roles in clinical and pathological mechanisms (Sofroniew, 2005; Seifert *et al.*, 2006; Barres, 2008; De Keyser *et al.*, 2008; Takano *et al.*, 2009).

Focusing in genetic diseases with cognitive delays, astrocytes seem to have a preponderant role, being the alteration of the astrogliogenesis timing more associated with mental impairments, in animal models (Gauthier *et al.*, 2007). Moreover, a recent and specific study focused in Down Syndrome, was successful in showing a gliogenic shift from neural progenitors from Down syndrome patients, with a concomitant decrease in neurogenesis (Lu *et al.*, 2011).

Astrocytes were also found to be related with epilepsy due to their effects both on glutamate transport and release as in buffering potassium and interstitial volume control (Wetherington *et al.*, 2008; De Lanerolle *et al.*, 2010). Astrocytic dysfunction has been shown to be related with abnormal neuronal excitability, regarding adult model systems (Gómez-Gonzalo *et al.*, 2010); additionally, data showed that inducing reactive astrocytosis can lead to the formation of hippocampal epileptic foci

(Ortinski *et al.*, 2010). All these observations have raised several questions, mostly regarding the possibility of increased susceptibility to epileptogenesis resulting from an abnormal astrocyte development (which could result in an alteration of excitatory-inhibitory balance of the developing brain) (Molofsky *et al.*, 2012).

Astrocytes can also see themselves involved in Alzheimer´s disease, being the reactive astrogliosis a well-known feature of this neurodegenerative disease. This specific process seems to be focal in this disease, such that reactive astrocytes are closely associated with amyloid plaques, surrounding them with a high density of processes and acting like neuroprotective barriers (Sofroniew & Vinters, 2010). Some reports state that reactive astrocytes have the capability of taking up and degrade extracellular deposits of a specific form of amyloid beta (Aβ42), leading to the belief on a role for astrocytes in the progression of the disease (Wyss-Coray *et al.*, 2003). Some other studies reported the decrease of astrocyte glutamate transporters in Alzheimer´s disease, suggesting a resulting increased vulnerability of local neurons to excitotoxicity (Simpson *et al.*, 2010).

Giving us a new insight about astrocytes´s role in cognitive functions, an amazing study came out this year, claiming that the engraftment of human glia progenitor cells in mice was enough to differentially enhance both activity-dependent plasticity and learning of the animal (Han *et al.*, 2013). With this study, the authors were able to show that human astrocytes generated within the mouse brain were able to maintain their complex phenotype in a cell-autonomous fashion, suggesting that the specific aspects of human cognition could reflect the course of astrocytic evolution (Oberheim *et al.*, 2006).

To further analyze astrocytes´ specific functions, transgenic mice models have been used along the scientific route, in which some astrocytic functions are blocked or attenuated. An example of this approach is the transgenic mouse model in which the expression of an inositol 1,4,5-trisphosphate absorbent is capable to attenuate astrocytic Ca^{2+} signaling. With this specific model, researchers showed that the attenuated activity of Ca^{2+} was correlated with reduced astrocytic coverage of asymmetric synapses in a specific hippocampal region, resulting in behavioral impairments in reference memory and remote contextual fear memory (Tanaka *et al.*, 2013).

Although controversial, these studies gave as a first clue about the possible role of glial cells and gliogenesis in the disease context, leaving an open window to be further explored.

1.5. Gliogenesis in Depression

Unlike neurons, glial cells retain their ability to proliferate in most brain areas of postnatal and adult subjects (Kraus-Ruppert *et al.*, 1975; Gensert & Goldman, 2001; Kornack & Rakic, 2001). The generation of astrocytes is detectable in the neocortex and the hippocampus of adult human brain and, although the majority of newly generated cells in the adult rat hippocampal DG are neurons (about 75%), there is still around 15% of new cells that are positive for the astrocytic marker GFAP and might be astrocytes. This neuron to glia ratio does not change with AD treatment, indicating that these treatments increase the number of newly generated glial cells in the adult brain (Rajkowska & Miguel-Hidalgo, 2007).

It is well known that there are multiple extrinsic and intrinsic mechanisms acting in concert to repress gliogenesis during the neurogenic period, and further induce gliogenesis when an appropriate number of neurons have been reached (Miller & Gauthier, 2007). However, in contrast to neurogenesis, the function of gliogenesis in the healthy adult brain has so far not been elucidated.

During development, several molecules act together to further determine the fate of multipotent precursor cell, later generating either neurons or glial cells (see Figure 2 for a schematic representation of the gliogenic process).

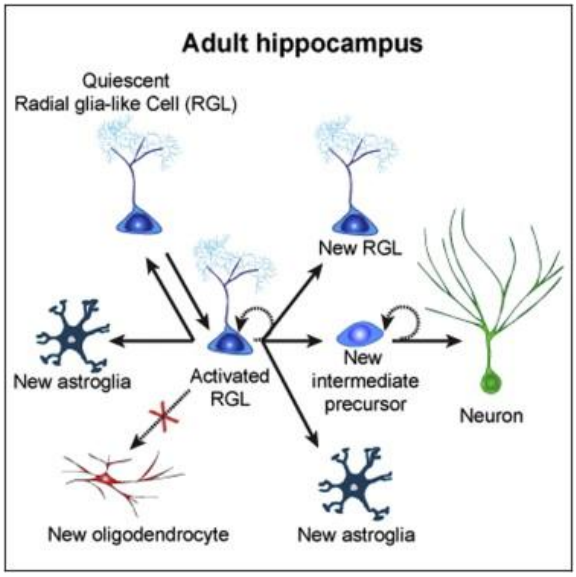


Figure 2. *The Gliogenic Process in the Young Adult Mouse Hippocampus under basal conditions*. There are at least three critical choice points: (1) a radia glia (RGL) cell decides to remain in quiescence or to become activated and enter the cell cycle; (2) an activated RGL can undergo one of three models of self-renewal: (i) symmetric self-renewal to expand the RGL pool, (ii) neurogenic, or (iii) astrogliogenic asymmetric self-renewal to generate a differentiated progeny while maintaining the RGL pool; and (3) The RGL makes a choice between returning to quiescence and maintaining the stemness or differentiating into an astrocyte via transition to astroglia. It is also possible that a quiescent RGL can directly differentiate into an astrocyte without cell division. (Bonaguidi *et al.*, 2011)

After several years of controversy, it is now well accepted that radial glial cells in the developing CNS are multipotent cells that have the capacity to give rise to separate precursors for neurons and mature glial cells (Campbell & Götz, 2002; Malatesta *et al.*, 2003). These glial precursors can differentiate into astrocytes or oligodendrocytes, due to specific factors in the microenvironment of the cell: when exposed to platelet derived growth factor (PDGF) the differentiation culminates in the generation of oligodendrocytes, whereas when exposed to leukemia inhibitory factor (LIF), they produce astrocytes (Bonni *et al.*, 1997; Rajan & McKay, 1998). Moreover, basic FGF (bFGF) has been proved to be relevant in both early neuronal development, maintaining the multipotent precursors, and postnatally, being produced by astrocytes and some neurons and inducing the oligodendrocyte lineage by glial precursors (Rogister *et al.*, 1999; Rajkowska & Miguel-Hidalgo, 2007).

Similarly to the effects on the production of glial cells that occur during development, the factors mentioned above can also act in the proliferative zones of the adult brain and may, according to some reports, participate in the pathophysiology of depression (Horner & Palmer, 2003). Moreover, those factors can be manipulated to further achieve an effective AD action. Regarding this, bFGF, a stimulator of astrocyte and oligodendrocyte proliferation, can be an important factor in the depression field (Skaper & Varon, 1987; Hunter *et al.*, 1993). Depressed patients showed a reduction in the mRNA level of bFGF in the dorsolateral prefrontal cortex, contrarily to ADs treatment that increased bFGF expression in the hippocampus and neocortex in an animal model (Mallei *et al.*, 2002; Evans *et al.*, 2004; Maragnoli *et al.*, 2004).

Besides all these observations, there are still several gliogenic windows that must be further studied and explored in the context of this disease.

Regarding *in vivo* results, data from our lab showed that impairments induced by unpredictable chronic mild stress (uCMS) exposure, a validated animal model of depression (Bessa, Mesquita, *et al.*, 2009), were reversed by both imipramine and fluoxetine ADs. Interestingly, whereas fluoxetine failed to restore working memory when neurogenesis was blocked, the cognitive-improving efficacy of imipramine did not depend on active neurogenesis. Fluoxetine treatment, as previously reported (Boldrini *et al.*, 2009), was more effective at promoting differentiation of newly-born cells into neurons rather than astrocytes, contrarily to imipramine treatment, that showed to elicit a strong pro-gliogenic effect (Mateus-Pinheiro *et al.*, 2013). These striking preliminary results suggest that the efficacy of imipramine in the recovery from depression depends directly on active gliogenesis and not neurogenesis.

However, until now little is known about the molecular changes regulating the decreased adult gliogenesis in depression, as well as about the counteracting mechanisms triggered by ADs. With growing evidence supporting the possible role of glial cells in the ethiopathogenesis of depression and the pro-gliogenic action of ADs, such as imipramine, adult gliogenesis becomes a promising area to study that may help to unravel novel therapeutic options for this pathology.

Since ADs treatment have showed to reverse the reduction in astroglial density in animal models of depression, and being the glial alterations more pronounced than those experienced by neurons, glial cells may represent a good target to give an anticipated and better prognosis of this deleterious disorder.

1.6. Impact of Astrocytes in Depression

During the last years, some studies came up showing the involvement of astrocytes in the pathophysiology of MDD (Hercher *et al.*, 2009). Indeed, as mentioned above, astrocytes have been pointed out as an important player in brain function (Wang & Bordey, 2008; Perea *et al.*, 2009), cross-talking with neurons, complementing and modulating neurotransmission (Araque *et al.*, 1999); and to possess unique phenotypic features that allow them to monitor their neighbourhood, dynamically responding to neurovascular changes (Wang & Bordey, 2008).

In depression, a loss of astrocytes in specific regions of the brain was observed and this phenomenon lead us to an open window that was ill explored (Gosselin *et al.*, 2009). Several studies, conducted in postmortem brain tissue of subjects diagnosed with MDD and/or bipolar disease (BPD), reported prominent decreases in the packing density and number of glial cells in several different frontolimbic areas, including prefrontal and medial prefrontal cortex, the dorsolateral and orbitofrontal cortex, the amygdala and also the hippocampus (Cotter *et al.*, 2001; Harrison, 2002; Rajkowska & Miguel-Hidalgo, 2007; Drevets *et al.*, 2008; Hercher *et al.*, 2009).

However, the opposite pattern - increased glial cell density - was also seen in the GCL of the DG in depressed patients. Actually, this phenomenon could be explained by a reduction on glial processes, rather than a loss of glial cells, which could induce a decrease in hippocampal volume, currently seen in neuroimaging studies in the depression field (Stockmeier *et al.*, 2004). Importantly, the study of glial pathology in mood disorders has not been extensive enough in subcortical structures to draw valid conclusions. It is also crucial to mention that several findings indicate that lower density of astrocytes

and decreased GFAP expression are associated with younger depressed subjects who had early onset of depression. Indeed, some studies indicate that GFAP expression levels are reduced in younger but not older depressed subjects. Thus, an increase in GFAP expression might not simply be related to biological aging, it may also be associated with the progression of cellular changes of depressive illness. This last observation implies that the involvement of GFAP expression is different in early *versus* late life depression. Increasing clinical evidence confirms that late onset depression (first depressive episode when older than 50 years) differs from early-onset depression by its etiology, phenomenology and cerebrovascular pathology (Rajkowska, 2000; Rajkowska *et al.*, 2005).

Besides cell density, it seems that glial cell size and shape also suffer alterations in mood disorders. Some studies reported the increase of the glial cell bodies (Rajkowska *et al.*, 1999, 2001; Chana *et al.*, 2003) in depressed individuals and, regarding this observation, Rajkowska *et al.* proposed the existence of a compensatory mechanism capable of responding to the metabolic needs of the surrounding neurons. Since reduction in glial density was followed by increased glial nuclei, the authors claimed that the functional glial cells (the ones not affected by stress - related mechanisms) would be forced to work, due to the shrinkage of the damaged ones (Rajkowska & Miguel-Hidalgo, 2007). Afterwards, their nuclei would be bigger and with a different conformation. Fascinatingly, this adaptation – more specifically the increased size of glial nuclei - seems to be targeted to depressive disorders, since the glial size was not found to be altered in other disorders, such as schizophrenia (Rajkowska *et al.*, 1998; Selemon *et al.*, 1998).

Although astrocytes represent the most numerous type of glial cell, we cannot forget about the other glial cells, which also suffer alterations in this disease context. Furthermore, specific decreased number of oligodendrocytes was seen in the amygdala in MDD (Hamidi *et al.*, 2004), as additional alterations in the microglial population in BPD (Manji *et al.*, 2000).

With these observations, we can conclude that those alterations currently seen in depression models are not astrocytes – directed, affecting also the other glial populations (despite the astrocytic alteration are the most reported ones).

Lately, an exciting study came out showing that the specific ablation of astroglial cells in the prefrontal cortex (PFC) of adult rats (with L-alpha-aminoadipic acid) was enough to induce a depressive-like behavior on the animals. They presented a phenotype quite similar to those animals that are submitted to uCMS, an animal model of depression (Banasr & Duman, 2008). In order to show that it was specific for astrocytes, the researchers also injected neurotoxic ibotenate but it showed to be harmless to the animals.

Regarding the ADs administration, some studies also suggest that the treatment not only affects neurons, but also activates astrocytes. This activation can lead them to carry out specific functions that result in the reactivation of cortical plasticity and may cause the readjustment of neuronal networks, thus helping depressed individuals to recover (Czéh & Di Benedetto, 2012). Indeed, in a study conducted in *Tupaia belangerie*, a specie phylogenetically close to primates (Martin, 1993), animals were subjected to a chronic stress paradigm (Fuchs & Flügge, 2002) and the reduced number of astroglia found in response to stress was prevented by concomitant fluoxetine treatment (Czéh *et al.*, 2006). Besides treatment with fluoxetine, chronic administration of lithium and antipsychotic medication have led to an increased glial number both in hippocampus and PFC of rats and nonhuman primates (Rocha *et al.*, 1998; Selemon *et al.*, 1999). As seen in other studies, chronic treatment with lithium induced an upregulation of GFAP expression and an alteration of astrocytes original morphology, more specifically in astrocytic orientation.

Taken together, further studies are needed to address the importance of all these glial alterations at the onset, maintenance and recurrence of a depressive episode. It is extremely relevant to determine whether therapies based on gliogenic factors will attenuate all the depressive symptoms. Moreover, it is of major importance to establish a state marker related with glial alterations, during episodes of depression.

1.7. Research Objectives

This project aims to correlate cognitive behavior with alterations in astrocytic cells and gliogenesis in the hippocampal DG of adult rats exposed to uCMS and treated with ADs, in a longitudinal manner: at short-term, long-term and recurrence time-points. Since treatment with the AD imipramine, but not fluoxetine, has been shown to elicit a strong pro-gliogenic effect, we also aim to establish a hippocampal derived-neurospheres culture to further study the differentiation of astrocytes *in vitro,* conditioning the culture with norepinephrine or serotonine (the neurotransmitters that mediates the effect of imipramine and fluoxetine, respectively).

In order to do that, our main objectives are:

- To study the impact of the uCMS model of depression in astrocytic cells;
- To explore how exposition to uCMS and treatment with distinct ADs (fluoxetine and imipramine) modulate hippocampal gliogenesis, more precisely the generation of new astrocytes, at different time-points: immediately after ADs treatment (short-term); 4 weeks after the end of the uCMS protocol and ADs treatment (long-term) and after exposition to a second period of uCMS (recurrence);
- To correlate the cognitive performance of depressive-like rats (rats exposed to uCMS) and AD-treated rats, with astrocytes morphological alterations at short-term, long-term and recurrence;
- To establish a hippocampal-derived neurosphere culture to further assess the proliferation and differentiation of astrocytes *versus* neuronal cells induced by the administration of distinct ADs *in vitro*, conditioning the neurospheres with serotonin or norepinephrine.

II. MATERIALS AND METHODS

2. Materials and Methods

2.1. Animals

Male Wistar rats (Charles-River Laboratories), with 2 months of age and weighing 200-300g 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*.

Forty animals were randomly assigned to five main experimental groups (n=8): one control group (CTRL) not exposed to stress and treated with saline; three groups exposed to uCMS and treated with saline (CMS), fluoxetine (FLX) or imipramine (IMIP); and one group with the same treatment as the ones before but exposed to an extra stress period (DOUBLE).

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

2.2. Chronic mild stress protocol

A protocol of uCMS was applied for 6 weeks as previously validated and described (Willner, 2005; Bessa, Ferreira, *et al.*, 2009; Bessa, Mesquita, *et al.*, 2009). Briefly, the uCMS protocol comprises several 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 4h and noise exposure during 4h) to which animals were random- and uninterruptedly exposed during 6 weeks. In the last 2 weeks of the uCMS protocol, subsets of animals were administered daily with the ADs imipramine or fluoxetine and all other animals were injected daily with saline. On the following 4 weeks, all the animals were allowed to recover due to the stress protocol´s cessation, being kept unexposed to any kind of stressor. After 4 weeks resting, three of the five groups (FLX, IMIP and DOUBLE) were exposed to a second period of uCMS during 6 weeks. The behavioral analysis were performed during the stress protocol (week 4), after the cessation of the stress period (week 6) – short term analysis - ; after the recovery period (week 10) – long term analysis - ; and after the second exposure to the stress protocol (week 16) – recurrence analysis (Figure 3).

For the molecular and cellular analyses, the subsets of rats exposed to only one period of uCMS during 6 weeks and the ones exposed to one period of uCMS that were allowed to recover for additional 4

weeks, were already performed by other researchers and were available for this project for molecular and cellular analyses.

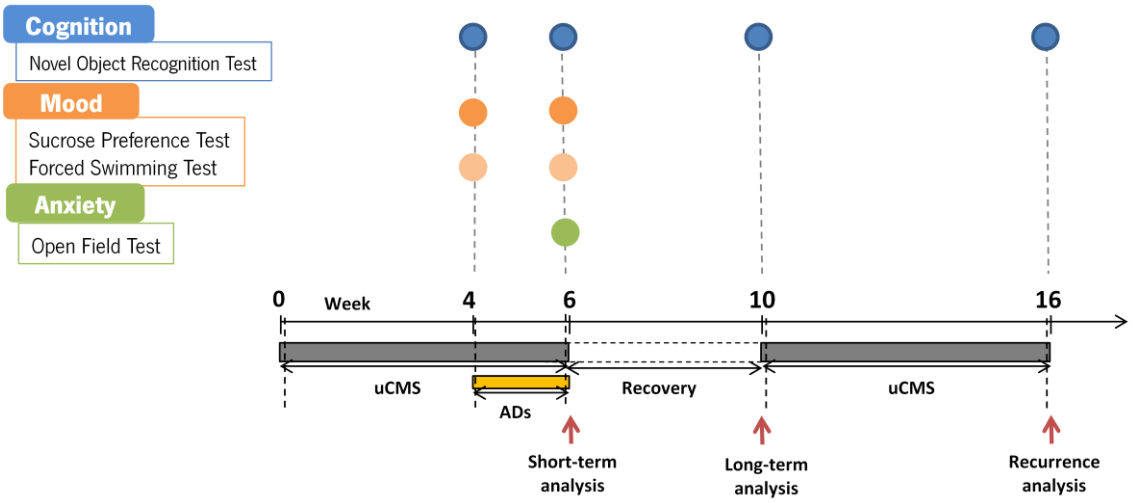


Figure 3. *Schematic representation of the behavior analysis*. The cognitive dimension was evaluated by the novel object recognition test (NOR) in animals exposed to uCMS; the assessment was done at weeks 4, 6, 10 and 16 (after the exposition to the second period of uCMS). The mood dimension was assessed by both Sucrose Preference Test (SPT), giving us an anhedonic information regarding the animals profile, and Forced Swimming Test (FST) at weeks 4 and 6. Anxiety dimension was evaluated trough the Open Field Test (OF) at week 6.

2.3. Drugs

The ADs fluoxetine (10mg.kg^{-1}; Kemprotec, Middlesborough, UK) and imipramine (10mg.kg^{-1}; Sigma-Aldrich, St.Louis, USA) were administered intraperitoneally (i.p.: 1 ml.kg^{-1}). These drugs were dissolved in DMSO (5%; Sigma-Aldrich) and saline (0,9%; B.Braun) and administered daily during the last two weeks of the uCMS protocol (Figure 3).

2.4. BrdU injections

Short-term analysis. BrdU (100mg.Kg^{-1}; i.p.) was administered for one day at the end of the uCMS period.

Long-term analysis. In order to assess cell proliferation, BrdU (50mg.Kg^{-1}; i.p.) was administered daily for five consecutive days at the end of the uCMS period, including the last two days of uCMS protocol and the first three days of the recovery period.

Recurrence analysis. At this time-point, BrdU (50mg.Kg^{-1}; i.p.) was administered daily for seven consecutive days at the end of the first uCMS period, including the last three days of uCMS protocol and the next four days of the recovery period.

2.5. Behavioral Tests

2.5.1. Novel Object Recognition

The cognitive function was assessed longitudinally by the novel object recognition test (NOR) and was performed at weeks 4, 6, 10 and 16 (always with objects different from each other in terms of shape, colour and texture), as schematized on Figure 3 by the blue dots. For this purpose, a black acrylic box (50x50x150cm) with an open field space (51x51x39,5cm) and illuminated with a white lamp (100-140 lux) was used. This test is phased in 4 days and was already tested and described in the literature (Bevins & Besheer, 2006; Dere *et al.*, 2007; Winters *et al.*, 2008; Ennaceur, 2010):

Exploration. On the first day, the animals were allowed to explore the test apparatus without any object during 10 minutes (min).

Sample Phase. On the second day, two identical objects were placed in the back left and right corners of the apparatus and the animal was able to explore them during 10 min. Within an interval of approximately one hour, a second sample object exposure (3 min) was performed. This time, one of the sample objects (left object) was repositioned in a new corner of the apparatus (middle of the left wall). This second trial gives an insight on hippocampus function and it works as a memory "reinforcement" of the sample object.

Choice Phase – Long-term memory. On the third day, the long-term memory was assessed 24 hours after the memory reinforcement (done on the second day of the test) by a 3 min trial with the replacement of one of the sample objects for a novel object.

Choice Phase – Short-term memory. Lastly, on the fourth day, animals were tested for their short-term memory condition. The previous objects were switched for two identical new sample objects and the animal was left for exploration in the apparatus, during 10 min. Within an interval of approximately one hour, a choice phase test was performed. To do this, one of the sample objects was replaced for a

completely new object and, for 3 min, the exploration of the animal was determined in order to test its memory.

Trials were video-recorded and the discrimination index (D) was calculated by the following formula: D = (N-F)/(N+F); being N the time spent exploring the Novel object and F the time spent exploring the Familiar object. For this test, it is crucial to define what we considered as the object exploration by the animal; for that, we assumed exploration of an object as directing the nose to the object at a distance of less than 2 cm or touching it with the nose.

2.5.2. Sucrose Preference Test

To assess anhedonia, the sucrose preference test (SPT) was conducted at week 4 and 6 of the uCMS period (Figure 3). Animals were allowed to habituate to the sucrose solution (2% m/v) 1 week prior to the uCMS protocol, in order to establish the baseline values for sucrose preference. For both assays, animals were food- and water-deprived for 12h during the non-active period. The room was cleaned with ethanol 96% and the test was performed under dimly illumination. Animals were placed in each cage, further covered with both the grid and the lid. Two pre-weighted bottles containing the sucrose solution and tap water were placed simultaneously in the cage and consumption was measured for 1h.
Sucrose preference was calculated by the following formula: sucrose preference=[(sucrose consumption / Total consumption) x 100] like previous described (Bessa, Mesquita, *et al.*, 2009). Anhedonia was defined as a reduction in sucrose preference in relation to the baseline levels.

2.5.3. Forced Swimming Test

Learned-helplessness was assessed through the forced swimming test (FST) and was conducted at week 6 of the uCMS protocol (Figure 3). The test was performed 24h after the 5 min pre-test session, consisting in placing the animals in cylinders filled with water (25°C; 50cm of depth) during 10 min. Trials were video-recorded and both the immobility time and the latency to immobility were measured through the Etholog (vs.2.2) software. Learned-helplessness was defined as an increase in the immobility time and a decrease in the latency to immobility.

2.5.4. Open Field Test

Anxiety-like signs were assessed through the open field (OF) test like some authors have reported (Prut & Belzung, 2003), in the week 6 of the uCMS protocol (Figure 3). The test apparatus consisted of a brightly illuminated square arena of 43.2 x 43.2 cm closed by a wall of 30.5 cm high. Animals were placed individually in the center of the arena and their movement was traced during 5 min, using a two 16-beam infrared system. The resulting data was analysed using the Activity Monitor software (Med Associates,Inc.), considering two previously defined areas: a central and an outer area. Time spent in each of the zones was recorded and analysed, further.

2.6. Measurement of Plasma Corticosterone Levels

Blood samples were collected from the rat's tails in different time-points: 8.00 am and 8.00 pm, at weeks 4 and 6 of the uCMS protocol.

Blood plasma was separated by centrifugation (2500 rpm, 30 min) and corticosterone levels were determined using the Correlate(tm)-EIA ELISA Kits (Assay Design Inc., Ann Arbor, MI, USA).

2.7. Tissue Preparation and Sectioning

Animals were deeply anaesthetized with sodium pentobarbital (20%; Eutasil, Sanofi) and were transcardially perfused through the ascending aorta with saline (0.9% NaCl; B Braun). Brains were dissected from the skull, embedded in Neg-50 Frozen Section Medium (Thermo Scientific) and further frozen in liquid nitrogen. The tissue was processed in series of 20µm cryosections, extending over the entire length of the hippocampus formation. The slides where then maintained at -20°C for immunostaining procedures.

2.8. Immunostainings

2.8.1. Immunofluorescence

Cryosections were immersed in 4% paraformaldeyde (PFA; Sigma-Aldrich) for 30 min at room temperature (RT) and then rinsed in Tris-Buffered Saline (TBS). Eventual pretreatment of the sections is mentioned in the list of used antibodies in Table 1. After 10 min of permeabilization in TBS-Triton (T; Thermo Scientific) 0,2%, sections were incubated with the primary antibody in solution (TBS/10% Fetal Bovine Serum (FBS)) overnight, at 4°C. On the second day, sections were washed 3 times in TBS and incubated with secondary antibody for 2h, at RT. The used secondary antibodies are listed in the Table

2. Sections were rinsed with TBS and further stained with DAPI (1:1000; Invitrogen) and mounted with immune-mounting medium (Immumount, Thermo Scientific) for following analysis by confocal microscopy. Solutions composition is described in Table 3.

For each animal, GFAP-positive cells and GFAP co-localized with BrdU-positive cells within the DG were analyzed in the confocal microscope Olympus FluoViewTM FV1000 (Hamburg, Germany).

Estimation of cell density in the DG was obtained by crossing the $GFAP^+$ cell number values within the corresponding DG areas, determined using the same confocal microscope. Moreover, in order to analyze the density of newborn astrocytes within the area of the DG (from the short-term, long-term and recurrence), cells that co-localized $GFAP^+$ and $BrdU^+$ were selected and analyzed.

For morphologic analysis of the new astrocytes ($BrdU^+GFAP^+$), we used the Neurolucida software, with the AutoNeuron extension module (MBF Bioscience).

2.8.2. DAB (3,3`diaminobenzidine tetrahydrochoride) immunohistochemistry

For the purpose of analyzing the astrocytic morphology, this specific technique was used. Basically, the first day of the protocol is similar to the one explained on the section before, except the blockage of endogenous peroxidases, performed immediately before the primary antibody incubation. This blocking solution consists of TBS with 10% H_2O_2 and it was added for 10 min, shaking. On the second day, the slides were washed with TBS buffer and then incubated with biotinylated secondary antibody (ThermoScientific) for 30 min. After that, streptavidin peroxidase (ThermoScientific) was added for 30 min. A washing step took place with TBS buffer and, before the develop step, the tissue was washed with a Tris-HCl solution for 5 min. The develop solution (0,025 g DAB on 100 ml Tris-HCl solution and 500 µl H_2O_2) was added to the tissue and the reaction was stopped around 5 min after with TBS. The sections were then dehydrated manually, staying 2 minutes in alcohol 96° and 2 min more in alcohol 96° for total tissue dehydration, 2 min in alcohol 100° and 2 final min in xylol.

For each selected astrocyte (localized on the DG of the hippocampus), all processes were reconstructed at 100x (oil) magnification using a motorized microscope (Axioplan2; Carl Zeiss) and Neurolucida software. A three-dimensional analysis of the reconstructed astrocytes was performed using NeuroExplorer software (Microbrightfield).

Table 1. Primary Antibodies.

Prim. Antibody / Specie		Working Dilution	Pretreatment	Company
BrdU	Mouse	1:50	30 min. in HCL (2 M) + 20 min. in pre-heated Cytrate buff. (80°C)	Dako
GFAP	Rabbit	1:200	*None*	Milipore

Table 2. Secondary Antibodies.

Sec. Antibody / Antigenicity		Working Dilution	Pretreatment	Company
Alexa Fluor 488	anti-mouse	1:1000	*None*	Invitrogen
Alexa Fluor 568	anti-rabbit	1:1000	*None*	Invitrogen

Table 3. Solutions Composition.

Solution	Composition
TBS	50 mM Tris base, 150 mM NaCl, pH 7.6
TBS-T	TBS, 0.2% TritonX – 100
PFA 4%	TBS, 4% PFA, pH 7.4
Cytrate Buffer	0.1 M Sodium citrate, pH 6.0

2.9. Neurospheres Culture Set Up

For establishing the neurospheres culture, 10 rats, 6 days old, were sacrificed and their brains removed. The hippocampal DGs were isolated and minced using a bisturi and the dissociated tissue was digested in 0.1% trypsin (Sigma-Aldrich) for 15 min at 37°C. After that, the tissue was triturated with a 5 mL pipette (10 times up and down), and the resulting solution was filtered through a 70 μm strainer (BD Biosciences), followed by a centrifugation at 1300 rpm for 5 min. The resulting pellet was resuspended in 1 ml of neurosphere medium (supplemented with growth factors), and a single cell suspension was achieved by gentle trituration. The growth factors added were 10 ng/ml epidermal growth factor (EGF; Invitrogen) and 10 ng/ml basic fibroblast growth factor (bFGF; Invitrogen). The cells were then plated in a T25 flask at a clonal density of 8-10 cells per μL and the growth factors were added every 2 days.

2.10. Data Analysis

All the statistical analysis was performed with GraphPad Prism 5.01. The unpaired Student´s t-test was used to examine whether data sets differed significantly, when the experimental setup was composed by only two experimental groups, and also to compare the control group with the uCMS group. One-way ANOVA was used in multiple statistical comparisons between groups and with only one level of analysis, with Tukey's multiple comparison test post hoc analysis. Statistical significance was accepted for $p<0.05$.

III. RESULTS

3. Results

3.1. Study of the cognitive behavioral dimension of depressive-like rats treated with the ADs fluoxetine and imipramine at short-term, long-term and recurrence

3.1.1. Establishment and validation of the animal model of depression

As it was reported by our group, the appliance of the uCMS paradigm, the same used in this study, induces typical depressive-like signs in all three behavioral dimensions that are commonly affected by depression – anxiety, mood and cognition (Bessa, Mesquita, *et al.*, 2009). In order to proceed to further tasks, it was imperative to first assess and characterize both the behavioral profile of the animals and the levels of corticosterone as a powerful tool to measure stress induction, since the increased levels of plasma corticosterone can be related with a stressful episode (Kant *et al.*, 1987).

Four weeks after starting the uCMS protocol (described in the 2.2 section of materials and methods), animals were tested in order to evaluate the extension of the psychological damages, which can lead to several affected behavioral dimensions. Therefore, learned helplessness was assessed through the FST, anhedonia was assessed by the SPT and the cognitive domain was evaluated through the NOR test (Figure 4).

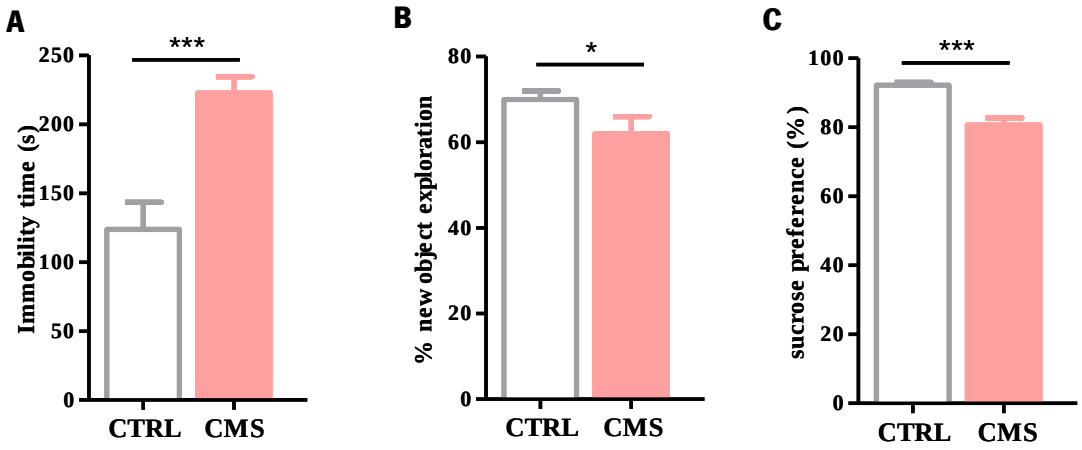


Figure 4. *Behavioral characterization of the animals on the fourth week of the uCMS protocol.* **(A)** Learned-helplessness was assessed through the FST; **(B)** Cognition integrity was assessed through the NOR test; and **(C)** Anhedonia was evaluated with the SPT. Data is represented as mean ± sem. *p<0.05, **<0.01, ***<0.001. Abbreviations used: CTRL – Control Group; CMS – uCMS group.

Consistent with previous results, four weeks after uCMS exposure, the stressed rats presented impairments in the three behavioral dimensions commonly affected in a depressive state. Regarding the FST, this test showed a significant increase in the immobility time of the subgroup of uCMS rats (t_{15}=3.834, p=0.0008) in comparison to the control group, evidencing a depressive-like phenotype of these rats (Figure 4A). Although cognition is one of the dimensions less marked at the first instance, comparing to anxiety and anhedonia, it is already affected after 4 weeks of stress exposure. This was confirmed by a significant decrease in the percentage of time that the uCMS animals spent exploring the novel object (t_{11}=1.873, p=0.0439), when comparing with the control group (Figure 4B). Lastly, stressed animals revealed clear signs of anhedonic behavior corroborated by a significant decrease in the sucrose consumption by the uCMS group (t_{15}=5.978, p<0.0001, Figure 4C).

After administration of the ADs fluoxetine and imipramine daily during the last 2 weeks of the uCMS protocol, we decided to re-validate the model at the behavioral level in order to proceed to further studies. For that, at the sixth week of the uCMS protocol (short-term analysis), animals were tested for the same parameters than in week 4 and also for anxiety, namely for learned helplessness assessed through the FST, anhedonia by the SPT, anxiety by the Open Field Test and the cognitive domain, evaluated through the NOR test (Figure 5).

Once more, results were consistent with the ones obtained at week 4 of the uCMS protocol, suggesting a depressed phenotype of the stressed group. Most of the tested dimensions presented impairments after 6 weeks of uCMS exposure, which is in agreement with previous results regarding a short-term assessment (Bessa, Mesquita, *et al.*, 2009). Animals exposed to uCMS showed increased immobility time (t_{18}=2.564, p=0.0118) in the FST and ADs treatment led to a recovery from learned helplessness ($F_{2,13}$=5.803, p=0.0158). Comparing with stressed rats, that did not receive drug treatment, immobility time was significantly reduced by both fluoxetine (p<0.05) and imipramine (p<0.05, Figure 5A) to levels comparable to control rats. Cognitive evaluation at short-term assessed by the NOR test was successful in revealing significant differences between control and stressed animals in short-term memory (t_{7}=3.396, p=0.058, Figure 5B). This impairment was reversed by AD treatment ($F_{2,9}$=8.731, p=0.0078, Figure 5B) and it was represented by a higher exploration of the new object, similarly to the control rats. It is important to mention that the objects used in the NOR test were all different in terms of color, shape and texture; therefore, we could make this test highly discriminative, making all the results more consistent.

Regarding the SPT, stressed animals revealed clear anhedonic signs, comparing with the control group (t_{14}=4.406, p=0.0003, Figure 5C). Once more, both imipramine and fluoxetine treatments were

able to rescue the depressive phenotype of the uCMS animals, with animals presenting sucrose preference values similar to the ones exhibited by the control group ($F_{2,14}$=6.924, p=0.0081, Figure 5C).

In term of anxiety, animals did not exhibit major differences although there was a tendency for a higher anxiogenic phenotype in uCMS animals, when comparing with control animals. Both ADs showed a tendency to revert the anxiogenic effect and, moreover, the imipramine treatment showed a tendency to be more effective than fluoxetine (Figure 5D).

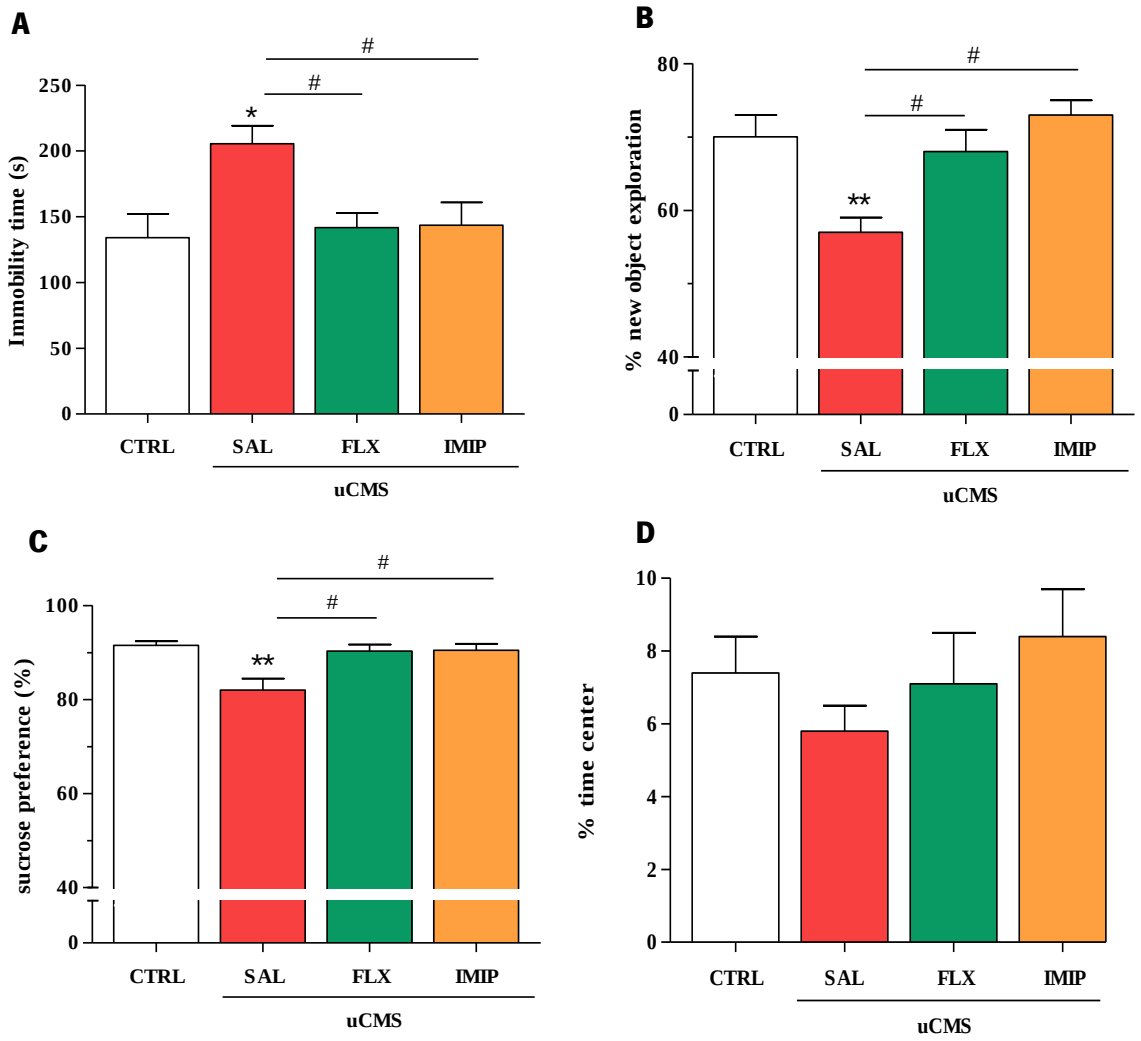


Figure 5. *Behavioral characterization of the animals on the sixth week of the uCMS protocol.* **(A)** Learned-helplessness was assessed through the FST; **(B)** Cognition integrity was assessed through the NOR test; **(C)** Anhedonia was evaluated with the SPT and **(D)** anxiety was assessed by the OF test. Data is represented as mean ± sem. * represents the effect of uCMS; # represents the effect of ADs treatment; *p<0.05, **<0.01, **<0.001. Abbreviations used: CTRL – Control Group, SAL – uCMS Group injected with saline, FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group.

As we also wanted to have a molecular proof of the consistency of our animal model of depression, we have decided to test the plasma corticosterone levels, since it is a powerful tool to correlate with stress episodes. Moreover, it is known that stress is accompanied by hypothalamic-pituitary-adrenal axis

(HPA) hyperactivity, resulting in the release of glucocorticoids into the blood (Schoenfeld & Gould, 2011). For that, animals were tested for corticosterone levels at week 4 (Figure 6A) and week 6 (Figure 6B) of the uCMS protocol.

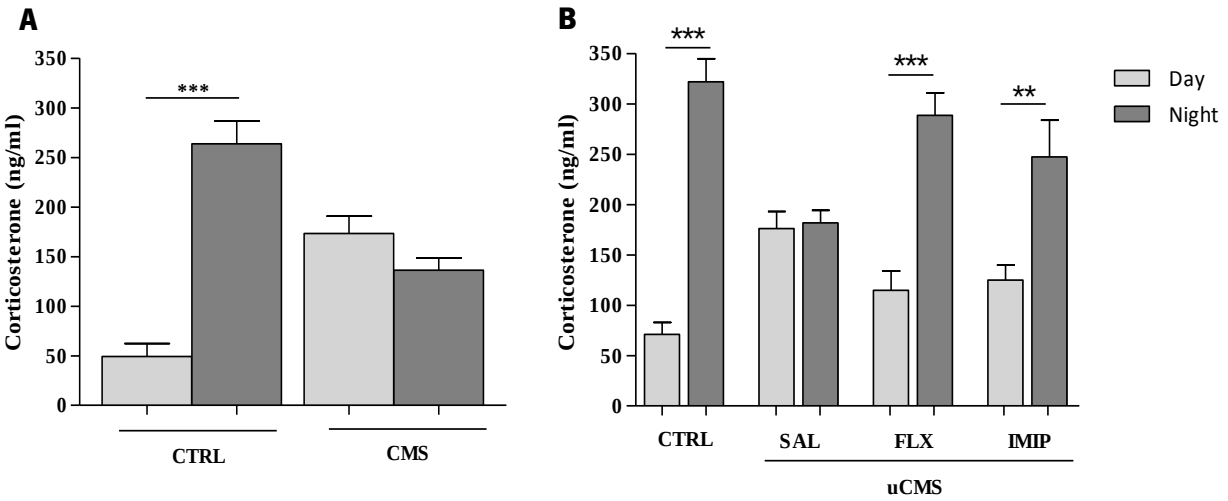


Figure 6. *Effect of stress on corticosterone plasma levels*, **(A)** at 4 weeks after the beginning of uCMS protocol ; **(B)** and at week 6 of uCMS, after the ADs administration. Data is represented as mean ± sem. *p<0.05, **<0.01, **<0.001. Abbreviations used: on Figure A, CTRL – Control Group, CMS – uCMS Group; on Figure B, CTRL – Control Group, SAL – uCMS Group injected with saline, FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group.

Besides all the behavioral information, corticosterone levels showed to be altered already at 4 weeks after the beginning of uCMS. In fact, the day and night corticosterone values of the control group showed to be significantly different, being the night peak the increased one (t_{10}=8.165, p<0.0001, Figure 6A), as previously described (D'Agostino *et al.*, 1982). Moreover, this difference was not found in the uCMS group, suggesting a disruption of the HPA axis (Figure 6A), which has also been described (Ottenweller *et al.*, 1994). After the ADs administration, animals were also tested for corticosterone levels (at 6 weeks of the uCMS protocol) and the same disruption was observed in the uCMS group, comparing with the control animals, that presented again a significantly difference between the night and day peaks (t_{21}=8.300, p<0.0001). The same did not happen in the stressed animals (presenting similar values between night and day, Figure 6B). However, both imipramine (t_{12}=3.445, p=0.0024) and fluoxetine (t_{7}=5.978, p=0.003) were successful in re-establishing the differences between the day and night levels of corticosterone, as observed in the control animals (Figure 6B).

3.1.2. Cognitive evaluation of depressive-like behavior in animals treated with ADs in a longitudinal manner – at short-term, long-term and recurrence

Since cognition is one of the affected dimensions in depressed patients, and being astrocytes lately related with different states of memory impairments, we wanted to understand better the cognitive impairments resulted from the uCMS protocol in a longitudinal manner, at short-term (6 weeks of uCMS), long-term (after 4 weeks of recovery from the 6 weeks of uCMS - week 10 of the protocol) and recurrence (after a second uCMS period of 6 weeks – week 16 of the protocol: see Figure 3). For cognitive evaluation, we used the NOR test with high discriminative objects (see Materials and Methods on section 2.5.1. and Figure 7). In this test, two different measures regarding the memory function arose: the short-term memory (measured in the same day than the objects' image acquisition) and the long-term memory (measures 24h after the objects' image acquisition). Although the short-term memory has been highly affected with stress exposition at 6 weeks, comparing with control animals (t_{7}=3.396, p=0.0058), ADs treatment was able to counteract this effect (Figure 7A). In fact, both fluoxetine and imipramine showed to be effective in reverting the cognitive impairments, at a short-term memory level, experienced by the uCMS group ($F_{2,9}$=8.731, p=0.0078). Regarding the long-term memory (Figure 7B), this dimension was also affected by the stress exposure, showing a significant decrease in comparison to control animals (t_{21}=3.637, p=0.0008). However, fluoxetine and imipramine treatments were not powerful enough to revert the long-term memory impairments caused by the stress exposure, at the short-term time-point (Figure 7B).

In the long-term perspective of the disease (at week 10), the short-term memory was drastically affected on the uCMS group (Figure 7C), whereas animals showed less than half the value of the new object exploration time of the control group (t_{14}=4.030, p=0.0006). Once more, both imipramine and fluoxetine treatments were capable to restore the same new object exploration values of the control animals ($F_{2,14}$=12.97, p=0.0006). Regarding the long-term memory assessment, the stress exposure seemed to not be so drastically affected as observed at short-term (6 weeks), although it was statistically different to the control group (t_{13}=2.356, p=0.0174). In accordance with the results observed at 6 weeks regarding the long-term memory, the ADs were not able to significantly rescue the impairments experienced by the uCMS animals (Figure 7D).

We next analyzed the impact of a recurrence episode of depression (at week 16 of the protocol, see Figure 3 and 7) on rats´ cognitive function.

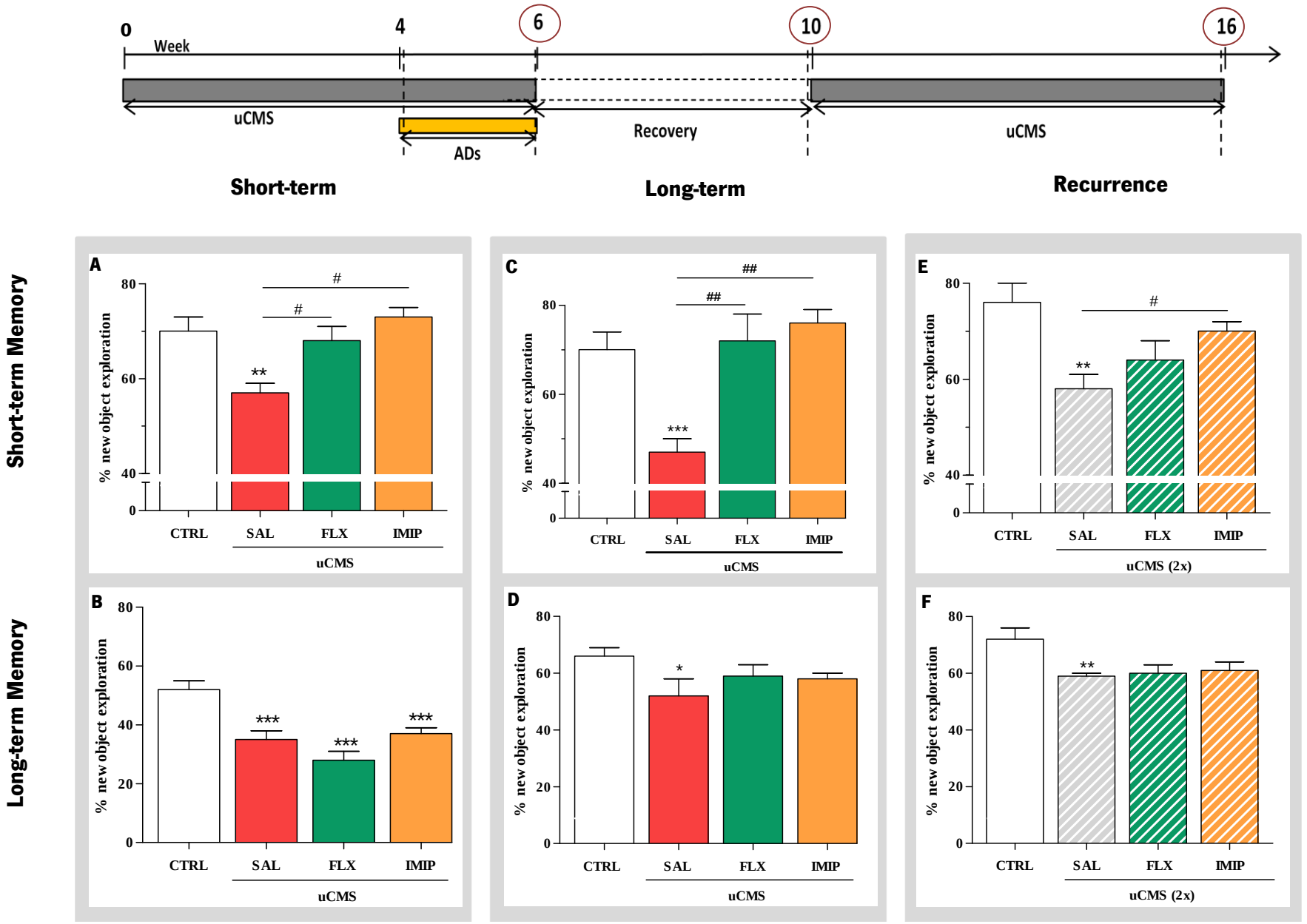


Figure 7. *Longitudinal Cognitive Assessment through the NOR test.* The test was performed at week 6 (Short-term), 10 (Long-term) and 16 (Recurrence) of the uCMS and both short-term and long-term memories were assessed. Data is represented as mean ± sem. * represents the effect of uCMS; # represents the effect of ADs treatment; *p<0.05, **<0.01, **<0.001. Abbreviations used: CTRL – Control Group, SAL – uCMS Group injected with saline, uCMS (2x) – Group submitted to two uCMS protocol; FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group. The striped bars represent the groups that were submitted to a second period of stress.

The second exposure to stress (hereinafter designated as DOUBLE stressed animals) induced long-lasting deficits in short-term memory of uCMS animals (t_{10}=3.600, p=0.0024; Figure 7E). Imipramine administration, but not fluoxetine, counteracted these deficits, increasing the exploration of the new object ($F_{2,14}$=4.259, p=0.0359; Figure 7E). Regarding the long-term memory analysis, the double stressed rats showed a significant decrease in the new object exploration, compared with the control rats (t11=3.392, p=0.030; Figure 7F). However, neither the SSRI drug fluoxetine, nor the trycyclic drug imipramine revealed cognitive improving effects in the recurrence perspective of the disease. Moreover, the ADs treated animals (FLX and IMIP group) showed a similar performance in the NOR test to the stressed rats, regarding the long-term memory assessment (Figure 7F).

3.2. Morphological alterations and total number of astrocytes analyses in the hippocampal DG in depression and after ADs treatment

3.2.1. Total number of astrocytes analyses in depression and after ADs treatment

Several studies reported prominent decreases in the number of glial cells in several different frontolimbic areas, including prefrontal and medial prefrontal cortex, the dorsolateral and orbitofrontal cortex, the amygdala and also the hippocampus of depressed patients (Cotter *et al.*, 2001; Harrison, 2002; Rajkowska & Miguel-Hidalgo, 2007; Drevets *et al.*, 2008; Hercher *et al.*, 2009). Having this fact into account, we have first decided to look at the total number of astrocytes in the hippocampal DG of all the experimental groups at the different time-points on the course of the disease: in the short-term, long-term and recurrence perspective of depression (see Figure 3).

All the cell counts were performed on stainings from hippocampal DG and analyses were performed in the confocal microscope (4DGs per animal, n=3 for each group).

The Figure 8 represents one of the images that were analyzed.

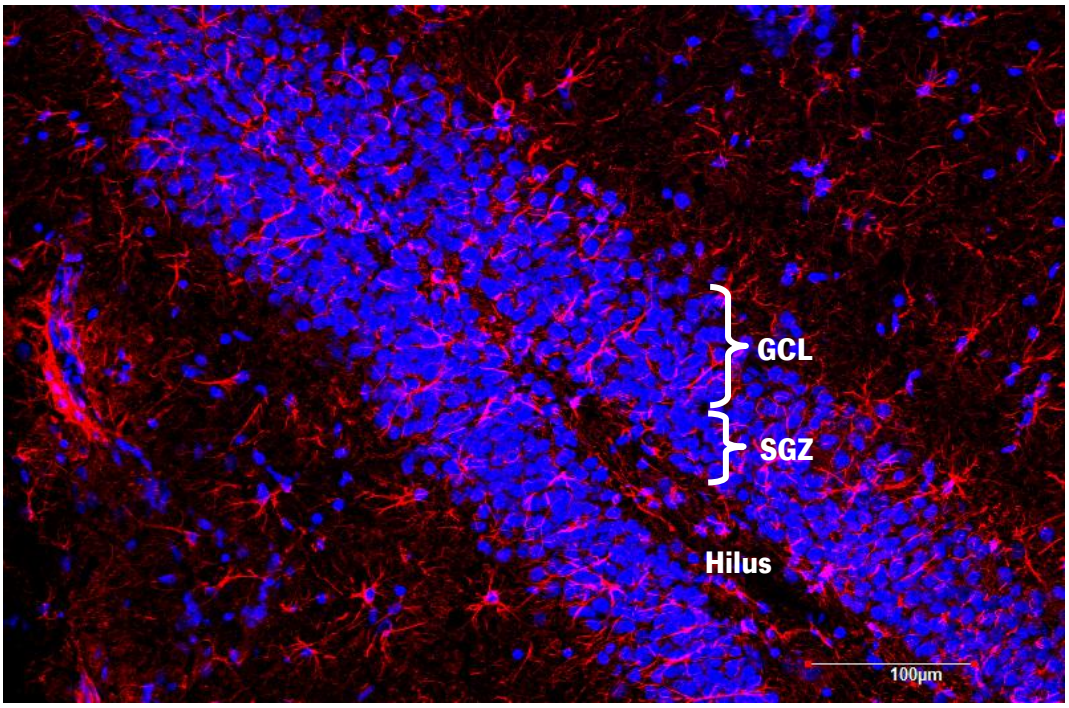


Figure 8. *Total number of astrocytes analysis on the hippocampal DG.* This image represents an immunostaining for GFAP (red), showing several astrocytes present in the hippocampal DG. Dapi (blue) was used to label the nucleus. Abbreviations used: GCL - Granule Cell Layer, SGZ – Subgranular Zone.

On the short-term analysis (week 6), no major differences regarding the total number of GFAP – positive cells per area (100 µm^2) in the hippocampal DG were found, suggesting that the uCMS exposition did not induce alterations on glial number (Figure 9A).

However, on the long-term analysis (week 10), there was a significant increase in the total number of GFAP-positive cells per 100 µm^2 of DG in the uCMS group, comparing with the control animals (t_{22}=3.728, p=0.0006, Figure 9B). Although this result is not in accordance with the studies mentioned above (in the Introduction section), the hippocampal DG is a completely new area that was not contemplated in these studies, so far. Moreover, the imipramine treatment showed to be capable of significantly reduce the number of GFAP – positive cells in the DG ($F_{2,33}$=4.676, p=0.0163, Figure 9B) to levels similar to the control group.

On the recurrence perspective of the disease (week 16), no major differences were found, although there was a high tendency for an increased number of GFAP-positive cells in the DG in the DOUBLE stressed animals, comparing with all other experimental groups (Figure 9C).

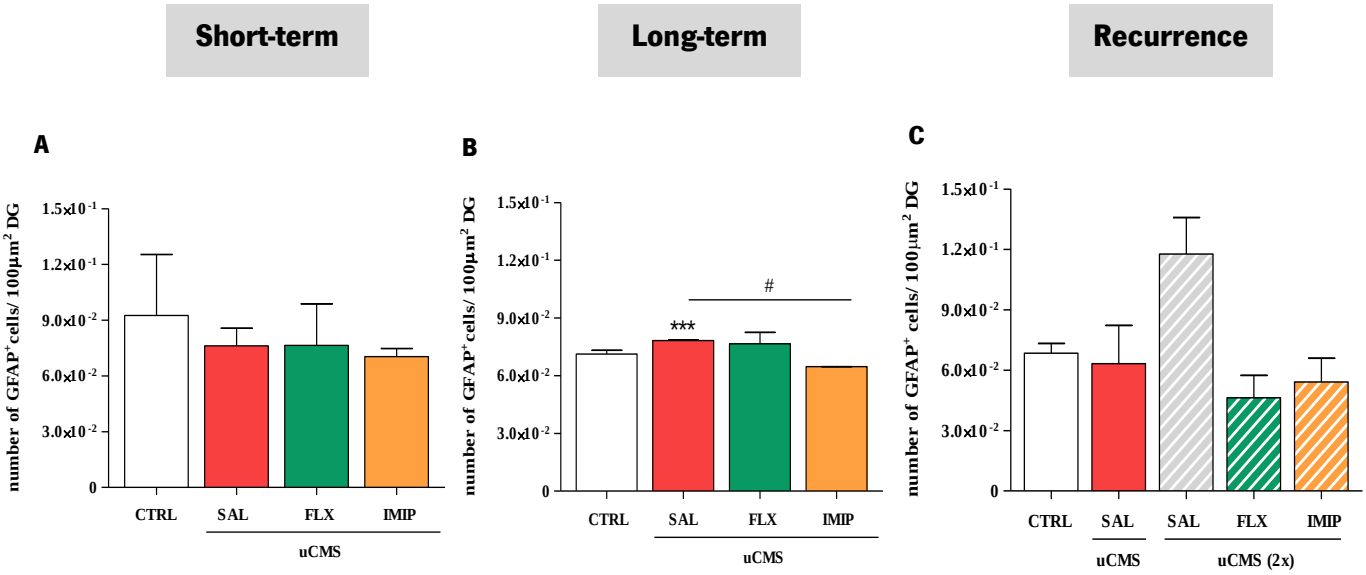


Figure 9. *Total number of astrocytes in the hippocampal DG.* Data is presented in terms of total number of GFAP$^+$ cells per 100 um^2 of DG. * represents the effect of uCMS; # represents the effect of ADs treatment. Data is represented as mean ± sem. *p<0.05, **<0.01, **<0.001. Abbreviations used: CTRL – Control Group, SAL – uCMS Group injected with saline, uCMS (2x) – Group submitted to two uCMS protocol, FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group. The striped bars represent the groups that were submitted to a second period of stress.

3.2.2. Morphological alterations of astrocytes by depression and after ADs treatment

Several studies have reported that astrocytes have the capacity to change their morphology in stressful situations, adding several functions to them (Parpura *et al.*, 2012). Furthermore, recent reports suggest that chronic stress can induce profound atrophy of astrocyte process length and volume (Tynan *et al.*, 2013). Considering those reports, we have decided to study the astrocytic morphology, specifically of the astrocytes present in the hippocampal DG, in all time-points of this study: at short-term, long-term and also recurrence. For that, we used the Neurolucida software and examined astrocytes (8 astrocytes per animal, n=3 for each group) in terms of volume and total processes length.

At short-term, no major differences were found in the total processes length in astrocytes from the hippocampal DG (Figure 10A). However, in terms of volume, astrocytes from the uCMS animals showed a significantly higher volume comparing with control animals (t_{29}=1.988, p=0.0282, Figure 10B). Moreover, both treatments with fluoxetine and imipramine significantly restored the volume value to the control animals levels, being significantly lower than the uCMS values ($F_{2,58}$=5.424, p=0.0069, Figure 10B).

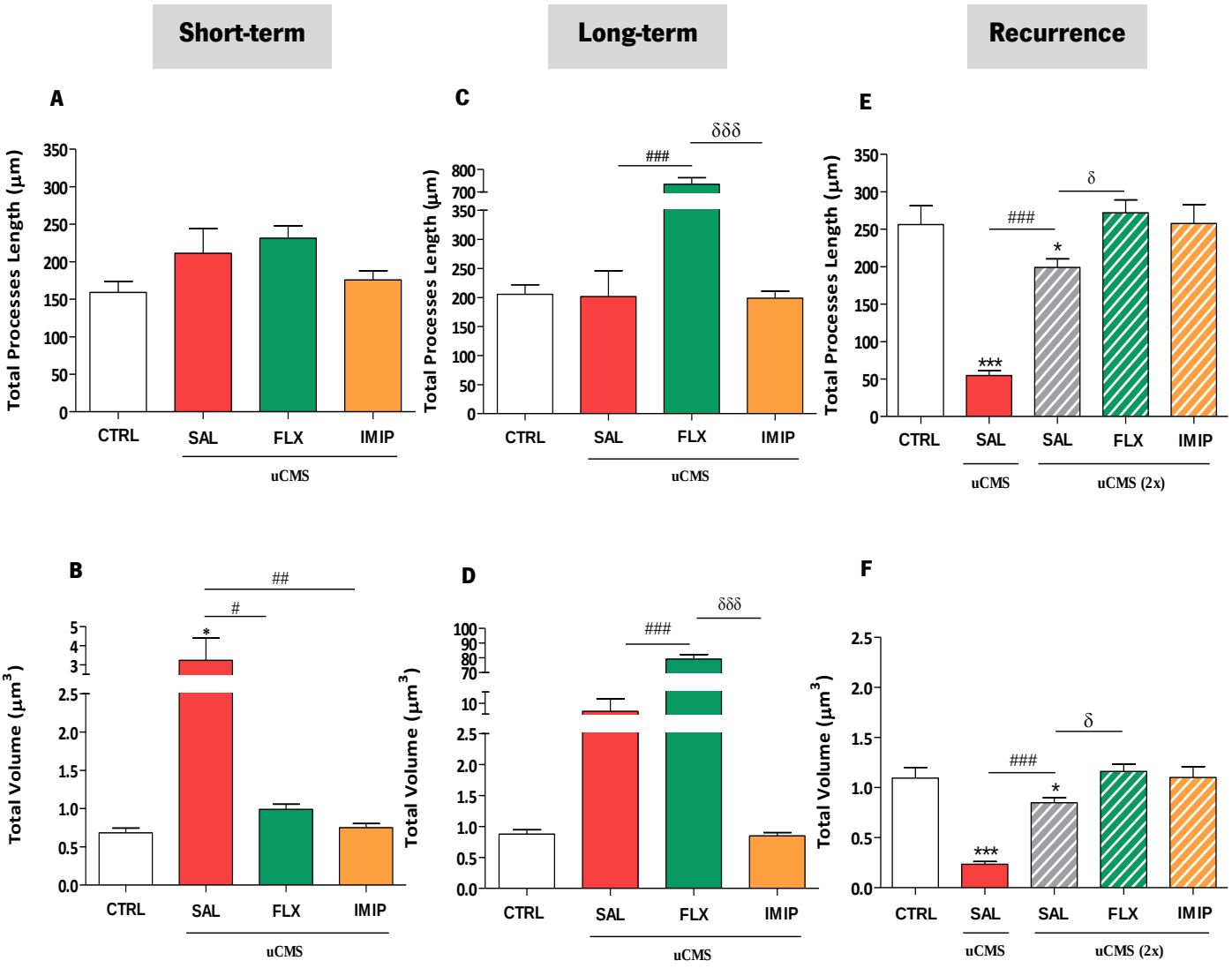
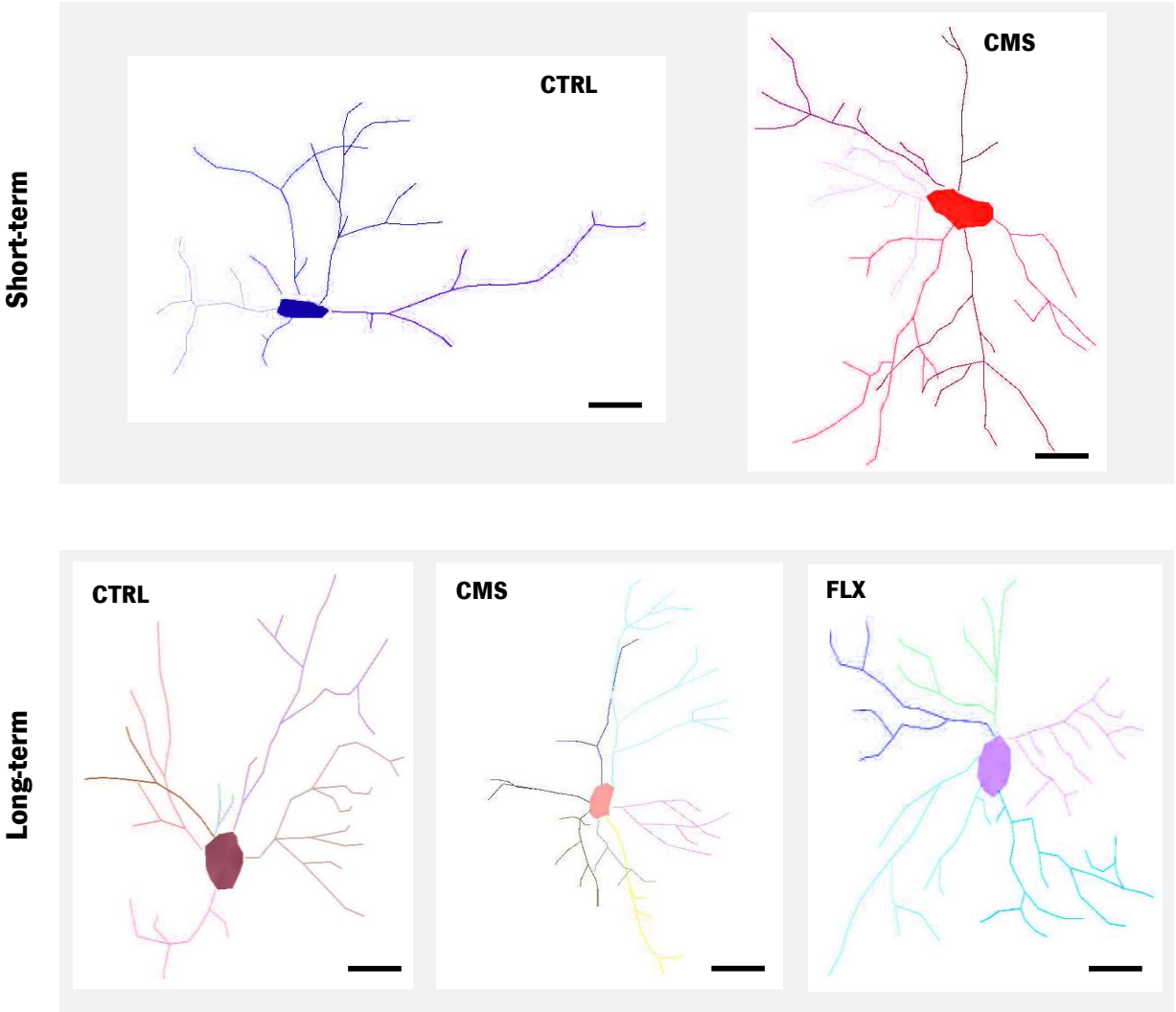


Figure 10. *Morphological assessment of astrocytic population in hippocampal DG.* Data is presented in terms of total processes length (µm²) and total volume (µm³). * represents the effect of uCMS; # represents the effect of ADs treatment and δ represents the effect between treatments. Data is represented as mean ± sem. *p<0.05, **<0.01, **<0.001. Abbreviations used: CTRL – Control Group, SAL – uCMS Group injected with saline, uCMS (2x) – Group submitted to two uCMS protocol, FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group. The striped bars represent the groups that were submitted to a second period of stress.

Interestingly, at long-term, the fluoxetine treatment promoted a significant increase in the astrocytic process length, in comparison to the stressed animals (t_{22}=0.06273, p=0.4753, Figure 10C). This effect was significantly different from the values reached by the animals treated with imipramine ($F_{2,37}$=60.66, p<0.0001), being these animals´astrocytes restored to values similar to the control group (Figure 10C). In terms of astrocytic volume, fluoxetine treatment showed consistency with the values regarding the process length, being significantly increased in comparison with both the uCMS animals (t_{22}=0.6743, p=0.2536) and imipramine treated animals ($F_{2,37}$=82.27, p<0.0001, Figure 10D).

Regarding the recurrence analysis, both the uCMS animals and DOUBLE stressed animals showed a significant decrease in the total process length, comparing with the control animals. Furthermore, the astrocytic process length of the DOUBLE stressed animals revealed a significant increase in comparison to the uCMS group ($F_{2,41}$=45.89, p<0.0001, Figure 10E). The fluoxetine treatment induced a significantly higher value of the astrocytic processes length, when comparing with the DOUBLE group ($F_{2,39}$=4.466, p=0.0179; Figure 10E). In terms of total astrocytic volume, the same differences were found. The astrocytes from the DOUBLE stressed animals showed a significant higher volume than the ones from the uCMS animals, and both presented a significantly decreased volume, comparing with the control group ($F_{2,41}$=45.89, p<0.0001; Figure 10F). Once more, the fluoxetine treated animals showed a significant higher astrocytic volume in comparison with the DOUBLE animals ($F_{2,39}$=4.467,p=0.0179, Figure 10F).

In Figure 11, some 3D visualization examples of the astrocytes that were manually analyzed with the Neurolucida software are represented.



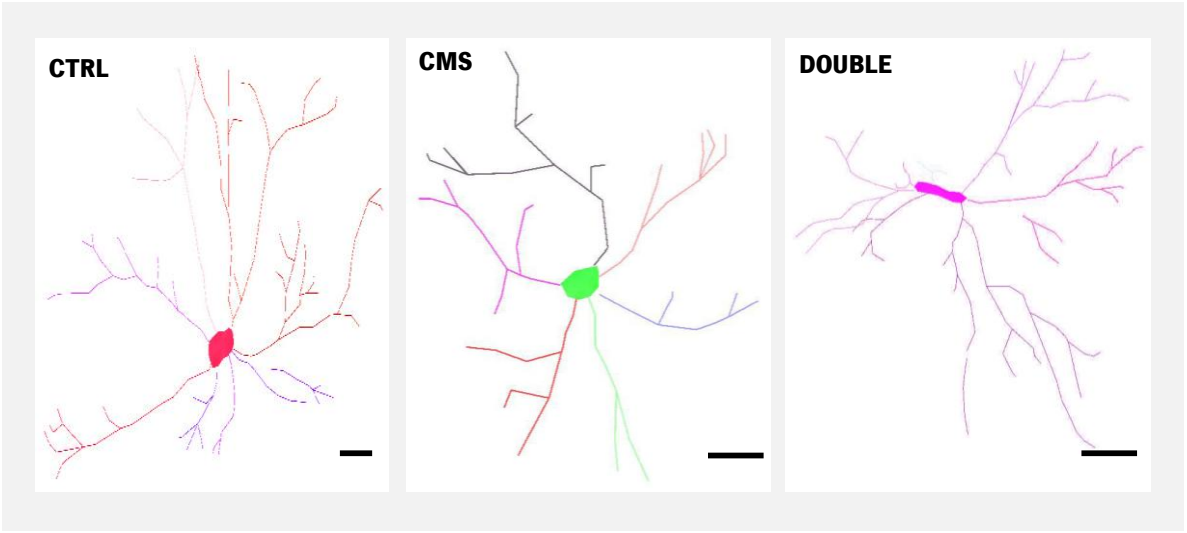


Figure 11. *3D visualization of astrocytes analyzed with the Neurolucida software, at short-term, long-term and recurrence perspective of depression.* Abbreviations used: CTRL – Control Group, CMS – uCMS Group, FLX – Fluoxetine treated Group, DOUBLE – Double stressed Group.

3.3. Impact of adult Gliogenesis in depression and after ADs treatment

3.3.1. Cell-fate and density of newborn glial cells analysis on hippocampal DG

Since one of our main interests was to understand the impact of hippocampal gliogenesis in depressive animals, further treated with ADs, to correlate with cognitive performance, we decided to characterize and quantify at the different time-points (short-term, long-term and recurrence) the newborn astrocytes (double stained with GFAP and BrdU, Figure 12) in all the conditions: control group, stress group and AD treated groups (4 DGs per animal, n=3 per group). With that information we can better understand what is the contribution of newborn astrocytes to the neurodegenerative processes and cognitive behavioral impairments, observed in depression (Figure 13).

All the cell counts were performed on stainings from hippocampal DG.

The Figure 12 represents one of the images that were analyzed.

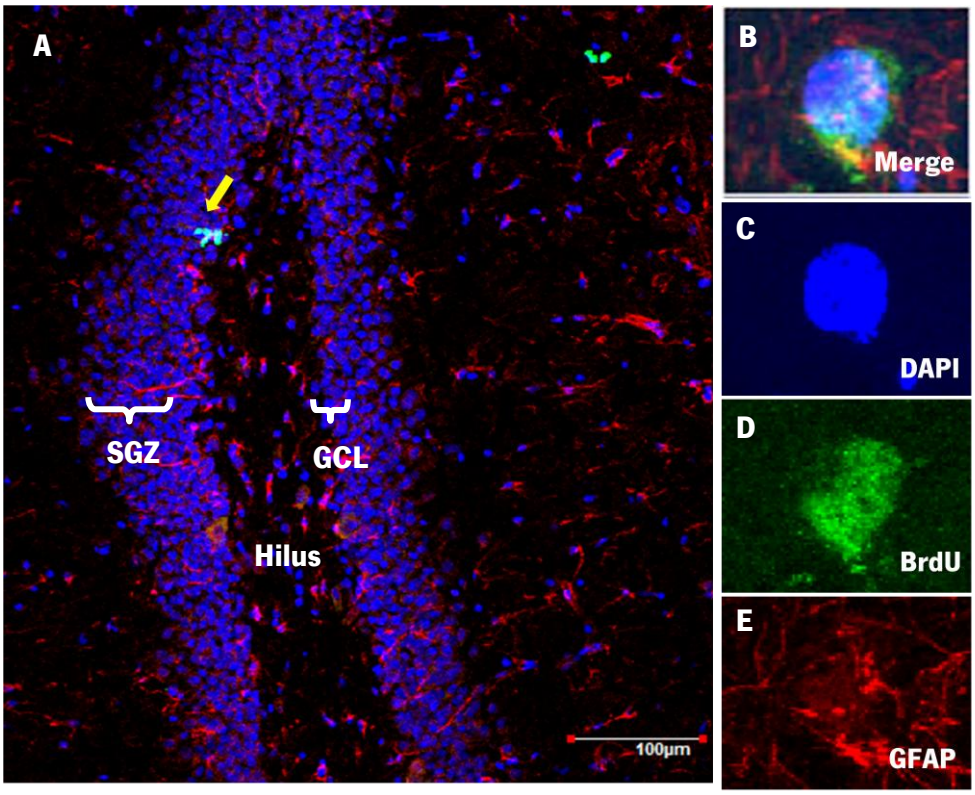


Figure 12. *Glial differentiation in the hippocampal DG, 4 weeks after the recovery (week 10 of the protocol, see Figure 1).* Rats were injected with BrdU for a period encompassing the last 2 days of uCMS and the first 3 days of recovery. Micrographs from **A** to **E** describe immunostaining for Dapi (blue, to label all nuclei), BrdU (green) and GFAP (red), showing that proliferative cells have differentiated into astrocytes that successfully survived and incorporated in the hippocampal DG. Micrographs from **B** to **E** represent one cell co-localizing BrdU (green) and GFAP (red). Dapi (blue) was used to label the nucleus. Abbreviations used: GCL - Granule Cell Layer, SGZ – Subgranular Zone.

After analyzing the different time-points of the disease, several differences were observed. Regarding the short-term analysis (week 6), no major differences were presented neither relative to the percentage of GFAP and BrdU – double positive cells per total BrdU positive-cells (named as glial fate from now on; Figure 13A), nor to the number of GFAP and BrdU – double positive cells per area (100 μm^2) of the DG (named as density of newborn glial cells hereinafter) analyzed (Figure 13B). Though, already a tendency for both an increased glial fate and newborn glial density was observed in animals treated with the trycyclic drug imipramine (Figure 13A and B).

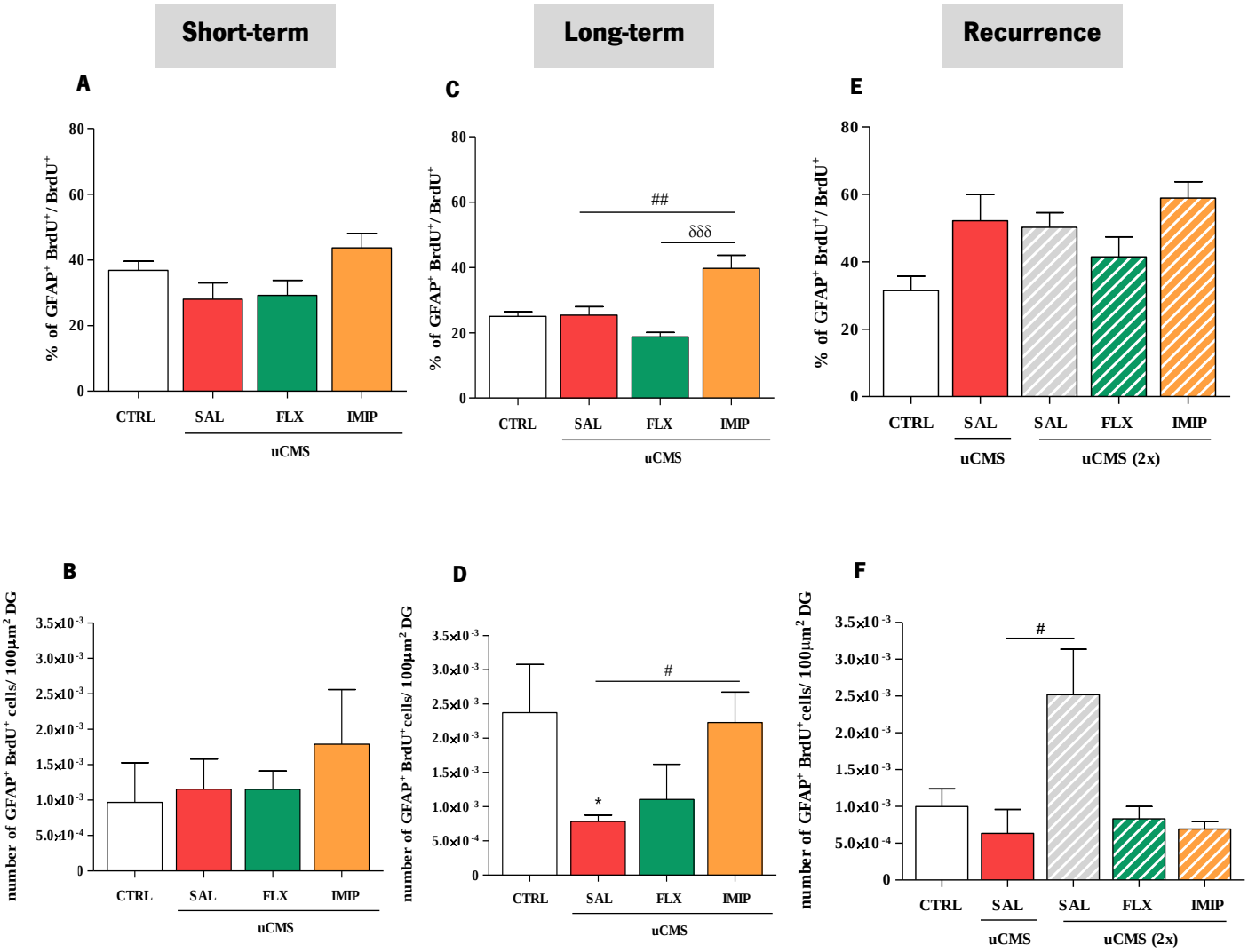


Figure 13. *Glial Cell-fate and density of newborn glial cells analysis on hippocampal DG.* Data is presented in terms of GFAP+BrdU+ double positive cells per total number of BrdU+ cells and number of GFAP+BrdU+ double positive cells per 100 um² of DG. * represents the effect of uCMS; # represents the effect of ADs treatment and δ represents the effect between treatments. Data is represented as mean ± sem. *p<0.05, **<0.01, **<0.001. Abbreviations used: CTRL – Control Group, SAL – uCMS Group, uCMS (2x) – Group submitted to two uCMS protocol, FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group. The striped bars represent the groups that were submitted to a second period of stress.

Focusing now on the long-term analysis (week 10), a strong and significant pro-gliogenic effect was observed with the imipramine treatment in the glial fate analysis, traduced in an increased percentage of GFAP and BrdU – double positive cells per total BrdU – positive cells, comparing with the uCMS group (Figure 13C). This result is in accordance with recent studies performed by our group (Mateus-Pinheiro *et al.*, 2013). Moreover, that increase was significantly different from the animals that received the fluoxetine treatment ($F_{2,33}$=13.99, p<0.0001, Figure 13C). This difference was traduced in a concomitant significant increase in the density of newborn glial cells in the imipramine treated animals ($F_{2,33}$=3.662, p=0.0366, Figure 13D), comparing with the uCMS group. Moreover, the density of

newborn glial cells of the stressed animals was significantly decreased in comparison to the control animals (t_{22}=2.241, p=0.0177), resulting in a reduced number of GFAP and BrdU- double positive cells per 100 μm² of DG (Figure 13D).

Regarding the recurrence analysis, no major differences were observed in terms of glial fate, although a tendency for a decreased percentage of GFAP and BrdU – double positive cells per total BrdU – positive cells was observed in the stressed groups (Figure 13E). Moreover, a significant increase in density of newborn glial cells was observed in the DOUBLE stressed animals ($F_{2,6}$=0.2186, p=0.8097, Figure 13F), traduced in approximately twice the value presented by the control group.

3.3.2. Morphological alterations of newly born astrocytes by depression and after ADs treatment, at long-term

Furthermore, we decided to look to the morphology of the newly born astrocytes that would integrate the hippocampal DG, at long-term, which means after 4 weeks of recover from the uCMS protocol. This parameter could give as a clue about the impact of stress and ADs treatment in the morphology of the new astrocytes. For that purpose, we selected astrocytes with double staining for BrdU and GFAP and analyzed them with the Neurolucida software, using the AutoNeuron extension module. This analysis gave us the information regarding both the processes length and total volume of the astrocytes from the hippocampal DG (Figure 14, 15A and 15B). The next figure (Figure 14) shows one of the astrocytes that were submitted to the AutoNeuron analysis.

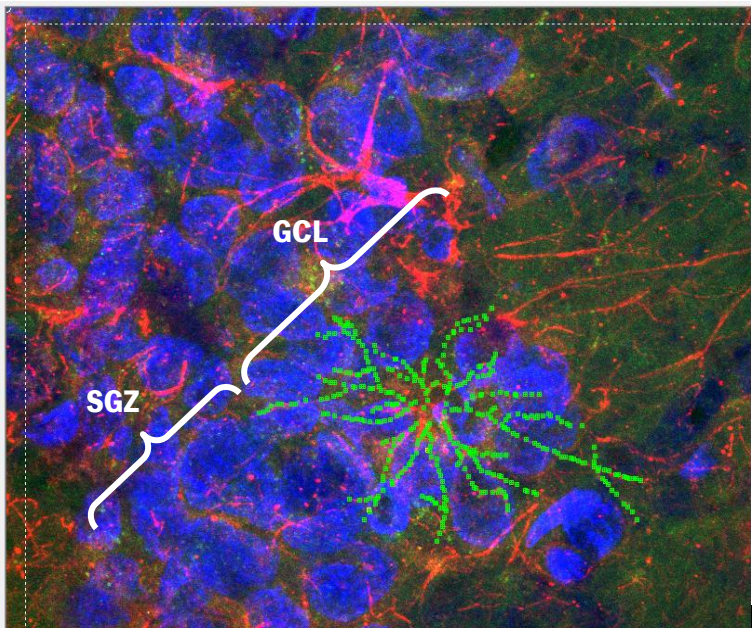


Figure 14. *Morphologic characterization of the BrdU·GFAP· cells present in the hippocampal DG, at long-term.* Selected astrocytes that co-localized the BrdU (green) and GFAP(red) markers were subjected to AutoNeuron extension module analysis, which resulted in automatic total process length and total volume analysis. The label in green represents the seeds applied by the Autoneuron module into the astrocyte processes to analyze the processes length and volume of that precise astrocyte. Abbreviations used: GCL - Granule Cell Layer, SGZ – Subgranular Zone.

In terms of total processes length, the newly born astrocytes from the uCMS group showed significantly higher processes length in comparison to the control animals (t_{17}=3.634, p=0.0010, Figure 15A). The imipramine treatment triggered a significant decrease of the astrocytic processes length, when compared with the stressed animals, to the levels of the control group. Moreover, this reduction was not seen on animals that were treated with the SSRI fluoxetine, showing a significant increase of the processes length of the astrocytes, contrasting with the effects of imipramine on the newborn astrocytes ($F_{2,20}$=8.477,p=0.0022, Figure 15A).

Regarding the total volume analysis, fluoxetine treated animals exhibited an increased astrocytic volume, comparing with the uCMS group. Moreover, this increased volume effect was also significantly higher than the one belonging to astrocytes from animals treated with imipramine ($F_{2,20}$=5.004,p=0.0173, Figure 15B), evidencing once again the contrasting effect of both ADs on astrocytes and gliogenesis.

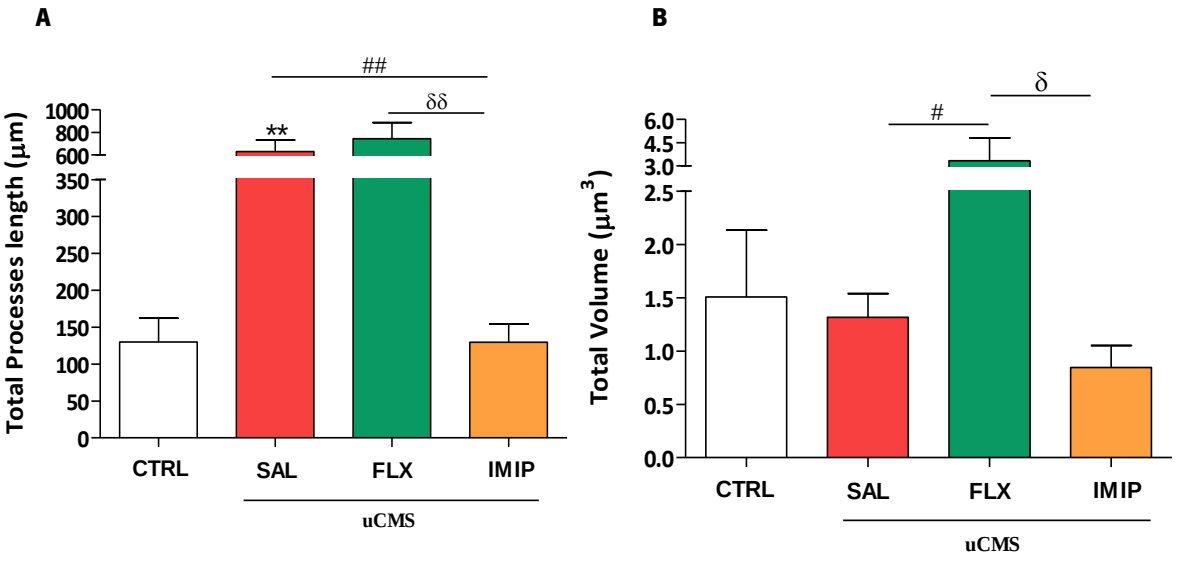


Figure 15. *Morphological assessment of newly-born astrocytes in the hippocampal DG, at long-term.* Positive cells for both BrdU and GFAP markers were selected from the DG and further analyzed in the Neurolucida with the Autoneuron extension module. Data is presented in terms of total processes length (µm²) and total volume (µm³). * represents the effect of uCMS; # represents the effect of ADs treatment and δ represents the effect between treatments. Data is represented as mean ± sem. *p<0.05, **<0.01, **<0.001. Abreviations used: CTRL – Control Group, SAL – uCMS Group, FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group.

Taken together, in order to correlate all alterations in pre-existing and newborn astrocytes (adult gliogenesis process) with the behavioral cognitive changes induced by stress and by ADs treatment at

short-term, long-term and recurrence, we included the Table 4 to summarize all results described and obtained with this work.

		Short -Term			Long – Term			Recurrence			
		uCMS vs CTRL	FLX vs uCMS	IMIP vs uCMS	uCMS vs CTRL	FLX vs uCMS	IMIP vs uCMS	uCMS vs CTRL	2xuCMS vs CTRL	FLX vs uCMS	IMIP vs uCMS
Cognition	ST Memory	↓	Rescue	Rescue	↓	Rescue	Rescue	↓	↓	=	Rescue
	LT Memory	↓	=	=	↓	=	=	↓	↓	=	=
Astrocytes	Total density	=	=	=	↑	=	Rescue	=	=	=	=
	Processes length	=	=	=	=	↑	=	↓	↓	Rescue	=
	Volume	↑	Rescue	Rescue	=	↑	=	↓	↓	Rescue	=
Gliogenesis	Glial fate	=	=	=	=	=	↑	=	=	=	=
	Density of new glia	=	=	=	↓	=	↑	=	↑	=	=
	Processes length				↑	=	Rescue				
	Volume				=	↑	=				

Table 4. Schematic representation of all the results regarding the cognition assessment, the astrocytic alterations and adult gliogenesis processes. ↑ represents an increase in each evaluated parameter, comparing between the groups in each column; ↓ represents a decrease in each evaluated parameter, comparing between the groups in each column; = represents no change seen in each evaluated parameter, comparing between the groups in each column; "Rescue" represents a recovery, after ADs administration, re-establishing the values obtained by the control group. All the comparisons are made between AD treated animals and stressed animals, except the 1st column of each time-point, which is composed by the comparison between control animals and stressed animals. Abreviations used: CTRL – Control Group, uCMS – uCMS Group, FLX – Fluoxetine treated Group, IMIP – Imipramine treated Group; vs – versus; ST Memory– short-term memory; LT Memory- long-term memory.

3.3.3. Study of glial proliferation and differentiation *in vitro*, using neurosphere cultures

Evaluation of ADs treatment effects have been widely performed *in vitro* by using hippocampal-derived neurospheres cultures. Since treatment with the AD imipramine, but not fluoxetine, has been shown to elicit a strong pro-gliogenic effect, we also wanted to study the differentiation of astrocytes *in vitro* using hippocampal derived-neurospheres cultures conditioned with norepinephrine and compare

with serotonine (the neurotransmitters that mediates the effect of imipramine and fluoxetine, respectively). For that purpose, we established a neurospheres culture in 2 weeks (Figure 17), being able to observe the progression of rat hippocampal DG neural stem cells from a single cell to a neurosphere (Figure 17A and B, respectively) during a period of 10 days. We also differentiated the neurospheres and although we did not perform a specific staining for neurons, astrocytes and oligodendrocytes, to prove that neurons and glial cells were present, we could see by their morphology that neurospheres were multipotent and successfully differentiated into different cell types (Figure 18A and 18B). This observation supports the conclusion that the neurosphere cultures were well established.

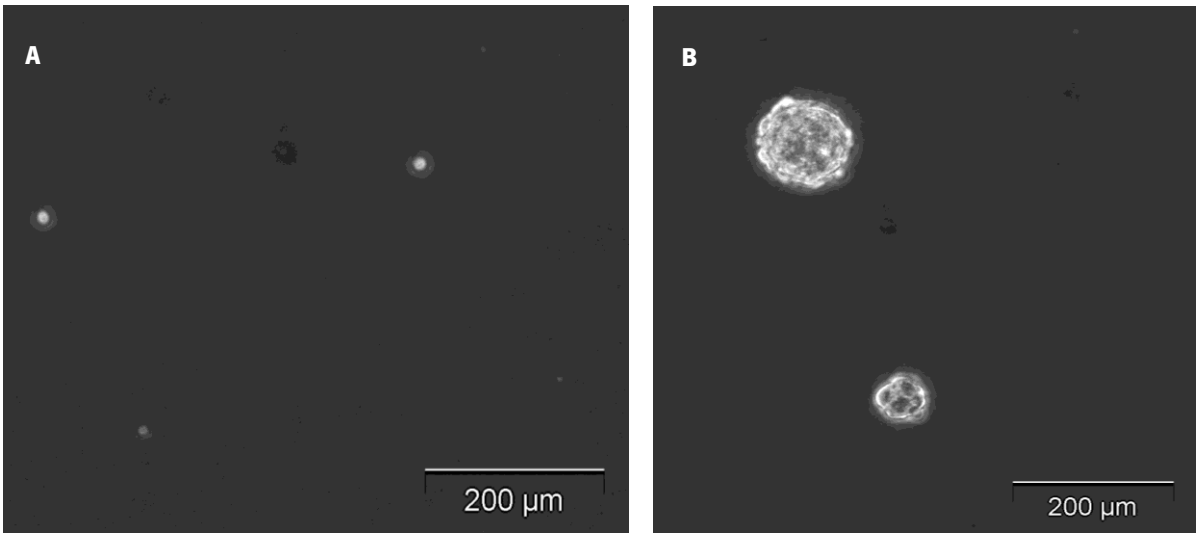


Figure 17. *Neurosphere culture was established after 10 days,* making possible the observation of rat hippocampal DG neural stem cells progression from (A) a single cell stage to (B) a neurosphere. Scale bar: 200 µm.

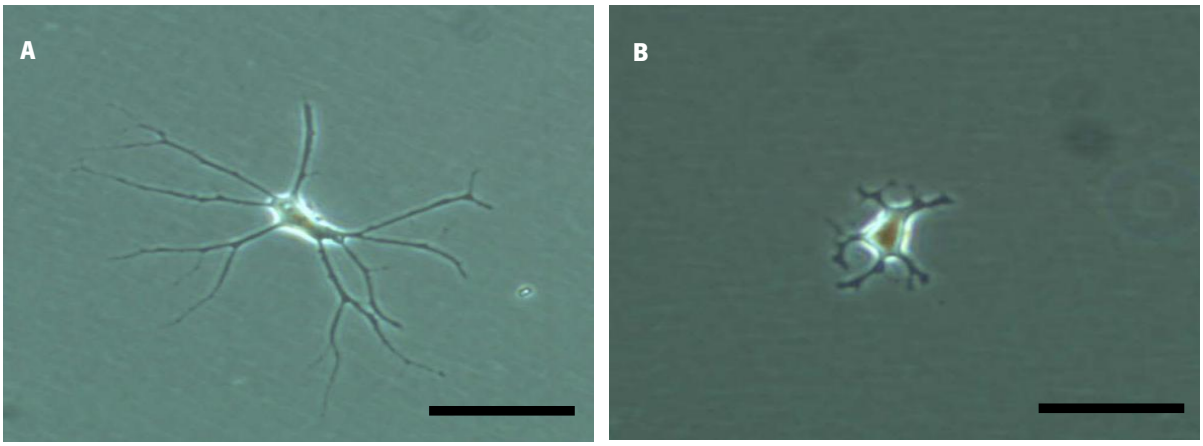


Figure 18. *Neurospheres were successfully differentiated into different cell types,* represented on Figures (A) and (B). Scale bar: 200 µm.

Further studies will include the administration of dexamethazone to mimic stress exposure and serotonin and norepinephrine to the cultures in order to evaluate the effects of these neurotransmitters, which are known to be implicated in the ADs actions, on cellular differentiation. Serotonin and norepinephrine will be used to study *in vitro* the effect of fluoxetine and imipramine, respectively, on glial differentiation and thus correlate with our analyses *in vivo*. Proliferation will be assessed by immunocytochemistry using markers for ki67. Cellular differentiation will be assessed by immunocytochemistry, using markers for both neurons (MAP2, NeuN) and astrocytes (GFAP, GS, S100β). Cell counts will be performed in a fluorescence microscope.

IV. DISCUSSION

4. Discussion

It is well known and proved that there are alterations of the brain network function on depressed individuals, on the onset and maintenance of the disease. Although the main focus around the mechanisms underlying this disorder has been put into neurogenesis, due to the reports showing that deficits in adult neuroplasticity are present in depressed individuals (Sahay & Hen, 2007; Bessa, Ferreira, *et al.*, 2009; Mateus-Pinheiro *et al.*, 2013), the process of gliogenesis seems now to be a new mechanism influencing certain cellular processes in the brain of these individuals (Rajkowska & Miguel-Hidalgo, 2007).

In fact, glial cells have been forgotten towards neurons along the neuroscience history. However, these cells have been related with several preponderant functions in the brain, interacting with neuronal cells and supporting them. Moreover, glial cells are known to actively participate in brain metabolism, synaptic transmission, interneuronal communication and are also responsible for maintaining the tissue homeostasis (Volterra & Meldolesi, 2005; Wang & Bordey, 2008; Devinsky *et al.*, 2013). Specifically in the case of astrocytes, the main glial subtype, the interaction with neurons was shown to be really close. In fact, these cells were found to participate in the regulation of synaptic neurotransmission by sensing the activity of vicinal neurons, propagating calcium signals and releasing chemical transmitters: the gliotransmitters (Araque *et al.*, 1999; Perea *et al.*, 2009). Furthermore, studies *in vivo* suggested a possible role for astrocytes in adult neurogenesis, where they reported that astrocytes seem to provide highly physical support to progenitor cells and newly born neurons (Shapiro *et al.*, 2005; Plümpe *et al.*, 2006; Morrens *et al.*, 2012).

Gliogenesis, the process that comprehends the generation of non-neuronal glia populations derived from neural stem cells (NSCs), has been recently involved in the pathophysiology of depression. In fact, several studies reported alterations in the astrocytes number after stress exposure (Rajkowska & Miguel-Hidalgo, 2007) and, moreover, glial loss in the prefrontal cortex has been proved to induce depressive-like behavior (Banasr & Duman, 2008). Furthermore, astrocytic dysfunction has been implied in cognitive impairments, when an attenuated astrocytic Ca^{2+} signaling led to behavioral impairments in reference memory and remote contextual fear memory (Tanaka *et al.*, 2013). Altogether, these studies might support an underlying mechanism to the precipitation of depression, related with astrocytic dysfunction that apparently leads to cognitive deficits, commonly observed in depressed subjects.

Taking these results into account, the main objective of this work was to study the function of gliogenesis and astrocytes in the pathophysiology of depression, using a pre- validated animal model of depression (Bessa, Mesquita, *et al.*, 2009). Moreover, we wanted to correlate the cognitive performance of the depressed animals and animals treated with antidepressants (ADs) with their possible dysregulation in astrocytic morphology and density, as well as in gliogenesis/glial cell fate at the hippocampal dentate gyrus (DG). All these parameters were assessed longitudinally in several time-points on the course of the disease, more precisely at short-term (immediately after the uCMS protocol and AD treatment), long-term (after 4 weeks of the uCMS protocol) and recurrence (after exposition to a second uCMS protocol).

To evaluate the function of gliogenesis and pre-existing astrocytes in the precipitation of- and recover from depression, we had first to validate the uCMS protocol applied to our animals. The obtained data confirmed the validity of the employed uCMS paradigm in both analyzed time-points: 4 and 6 weeks after the beginning of the uCMS exposure. In fact, already at 4 weeks after the uCMS protocol exposure, the stressed animals revealed clear signs of depressive-like mood (assessed trough the Forced Swimmimg Test (FST)), anhedonic behavior (assessed through the Sucrose Preference Test (SPT)) and also impairments in the cognitive domain (assessed by the Novel Object Recognition test (NOR)). These alterations on the cognitive performance go along with our expectations based on previous studies (Beck, 1976), since the mechanisms related with cognitive impairments are much more complex than those related with anxiety alterations, for instance. Additionally, these behavior alterations were correlated with the disruption of the corticosterone levels during day and night in the uCMS animals, a measure for the hyperactivity of the Hypothalamic-Pituitary-Adrenal (HPA) axis by the stress exposition, as previous described (Kant *et al.*, 1987).

After 6 weeks of uCMS exposition, our results were also successful in confirming, once more, the depressive phenotype, traduced in general impairments in all the domains commonly affected in depression – mood, anxiety and cognition. In terms of the cognitive evaluation, stressed animals showed marked deficits, comparing with the results after 4 weeks of stress exposure. Moreover, stressed rats were able to completely recover from a depressive-state with the administration of some of the most commonly used ADs in the clinics: the tricyclic agent imipramine and the serotonine-selective reuptake inhibitor (SSRI) fluoxetine, restoring all the experienced deficits to normal values (observed for control animals).

Once again, corticosterone levels of the uCMS animals showed the same disruption between the levels during day and night as previously observed, at week 4, which were re-established by imipramine and fluoxetine administration.

In terms of cognitive assessment, stress seems to affect the animals throughout the time, at short-term, long-term and also recurrence perspective of this disorder. The same statement can be applied to the different types of memory that were assessed – short-term memory and long-term memory.

Regarding the disease perspective, at short-term, several cognitive deficits were experienced by the stressed animals, reflected in both short- and long-term memory impairments. It is though important to mention that the hippocampus is responsible for long-term object recognition, whereas the short-term memory is a cognitive function that involves prefrontal cortex (PFC) activity (Reger *et al.*, 2009). These observations might suggest that not only the hippocampal cell populations are affected by the uCMS exposition, but also a decreased population of newborn cells that integrates the hippocampal formation is not able to establish projections with the PFC, under stress conditions. An additional hypothesis is that both the hippocampus and PFC cells are affected by the uCMS, thus also leading to disruption of the normal connectivity between both regions and impairments in short- and long-term memories. In fact, several studies already reported altered cell numbers and morphological changes in the hippocampus and PFC of stressed animals (Cerqueira, Taipa, *et al.*, 2007; Bessa, Ferreira, *et al.*, 2009), which may lead to an increased difficulty in the communication between these two regions (Cerqueira, Mailliet, *et al.*, 2007; Oliveira *et al.*, 2013) and can explain our results. However, it is still important to clarify which is the cell type (or cell types) essential for the regulation of these connectivity impairments between the hippocampus and PFC under depressive-like conditions that lead to such marked cognitive dysregulations. In an astrocytic perspective, we can think of a deficient support provided by astrocytes to the pre-existing and newborn neurons that can be causing all these connectivity alterations and short-term memory impairments, seen not only at short-term but also throughout the disease periods taken in consideration in this study. It is noticeable that the short-term memory, but not the long-term memory, deficits were markedly rescued by the administration of either fluoxetine or imipramine. This interesting result suggests that both ADs might be acting on the reestablishment of the neural circuits connecting the hippocampus and the PFC, but locally at the hippocampus they are not enough to rescue all cellular and molecular changes induced by depression.

From our results, these stress-induced impairments in short- and long-term memory seem to be related with alterations in the volume of resident astrocytes at the hippocampal DG, which was drastically increased with the stress exposure. Moreover, with the ADs administration, there was both a

rescue in short-term memory deficits and in astrocytic volume, whose values were restored to values similar to the ones presented by the control animals. Furthermore, the long-term memory does not seem to be related with volumetric alterations of the pre-existing astrocytes of the DG, since the rescue of the astrocytic volume was not accompanied by a rescue of the long-term memory deficits (caused by the stress exposition). These volume alterations can be explained by an astrocytic effort/increased activity to re-establish the system or compensate lost functions after the stress assault. Actually, it was already reported that, among several factors, stress exposure and stress hormones were the first regulatory factors acknowledged to modulate DG neurogenesis (Cameron & Gould, 1994; McEwen, 1997; Sapolsky, 2000). Moreover, since stress exposure induces decreases in hippocampal neurogenesis (Duman *et al.*, 2001), the neuronal atrophy can be counteracted by an increased astrocytic action reflected in terms of volume, perhaps to support the debilitated neuronal network and improve functioning. Probably astrocytes can become activated when their partners need an extra help, like in major depressive disorder (MDD).

In contrast with the volume alterations registered in the pre-existing astrocytes, gliogenesis do not seem to be related with any alterations seen immediately after the stress exposure - at short-term. Indeed, neither the density of astrocytes nor the glial fate was altered when animals were exposed to uCMS and further treated with ADs. Nonetheless, it is important to notice that, at short-term, animals were injected with BrdU 24 hours before the sacrifice. Therefore, at this time-point we are still evaluating the progenitor cells (which are described to be GFAP-positive (Casper & McCarthy, 2006), not allowing us to detect the effect of stress in glial or neuronal fate. Interestingly, this result also shows us that the population of GFAP expressing progenitors is not affected by stress, neither by the ADs treatment.

At the long-term perspective of the disease, after allowing the animals to recover from the stress exposition during four weeks, cognition seems to be affected in the same extension, as it is at short-term. Actually, the stress exposure affected both the short- and long-term memories, as previously observed at short-term. Likewise, the impairments in the short-term memory experienced by the stressed animals were reversed by both fluoxetine and imipramine administration. Once more, the long-term memory deficits were not counteracted by the ADs administration. These cognitive deficits were accompanied by several alterations related with both astrocytic alterations and gliogenesis *per se*. Regarding the astrocytic alterations, an increase in the total density of astrocytes seemed to be directly correlated with short-term memory deficits, in the stressed animals. Moreover, the fluoxetine treatment induced profound alterations in the pre-existing astrocytes, reflected in a large increase of the astrocytic

volume and processes length of these cells. These alterations induced by the treatment with fluoxetine can be related with astrocytes´activation by the stress disturbance of the system, leading to a readjustment of the neuronal network. Indeed, several studies suggest that ADs treatment can activate astrocytes, leading them to a state of over-working, in an attempt to re-establish the neuronal network, thus helping depressed individuals to recover (Czéh & Di Benedetto, 2012). This state of over-working can cause profound alterations in astrocytic morphology, resulting in an increased volume and processes length. Interestingly, in contrast to the fluoxetine treated animals, imipramine treatment did not induce alterations in pre-existent astrocytes neither in terms of volume, nor in processes length. However, one of the mechanisms that according to our results seems to be helping the hippocampal DG system to recover after treatment with imipramine is through a rescue in the density of astrocytes in this brain region (Mateus-Pinheiro *et al.*, 2013).

When we proceeded to the gliogenesis analysis at the long-term perspective, a brand new story arose. Indeed, the new cells that are born in the adult neurogenic regions take between 5 to 7 weeks to fully differentiate and establish new contacts with the neighbor cells. Thus, at this time-point of our analyses, it makes sense that newborn astrocytes are already differentiated and attempting to establish new contacts with nearby cells, becoming more important in the contribution to the normal functioning of the system. Therefore, the gliogenic process becomes an important factor in this long-term perspective of depression, as previously pointed out by our group (Mateus-Pinheiro *et al.*, 2013). Indeed, stress induces a decrease in the density of newborn astrocytes, traduced in less GFAP and BrdU – double positive cells in the hippocampal DG. Although less glial cells seem to born in the DG after the stress exposure, it was also reported recently that the total number of $BrdU^+$ cells is decreased, at long-term, in the uCMS animals (Mateus-Pinheiro *et al.*, 2013). These results are then traduced in an unaltered glial-fate in stressed animals when compared with the control group. Moreover, those newborn glial cells that are analyzed at long-term show an increased processes length, which can also be related with an attempt to compensate the decreased density of newly generated glia, by establishing more contacts with the neuronal network. It is also important to mention that for these gliogenesis analyses at long-term, BrdU administration was performed during 5 days, and the proliferation/differentiation results reflect the proliferative status of the cell population immediately after the end of uCMS protocol as well as after the conclusion of the AD treatment, encompassing the 3 initial days of the recovery period.

Although fluoxetine treatment had induced several morphological alterations in the pre-existent astrocytes (like mentioned above), the same does not happen with the gliogenic process. Indeed, this

AD did not rescue the alterations in the density of newborn astrocytes and total process length of the same cells and thus the recover of the cognitive performance (short-term memory) induced by fluoxetine might not be related with gliogenesis. In fact, several studies reported that fluoxetine treatment seems to be more related with neurogenesis rather than with gliogenesis (Wang *et al.*, 2008; Xi *et al.*, 2011; Mateus-Pinheiro *et al.*, 2013). However, it is also important to notice here, that the newborn glial cells in the DG after treatment with fluoxetine show an increased volume that can also be a mechanism through which these glial cells try to compensate the decreased density of newly generated glia and be able to rescue short-term memory.

Contrarily to fluoxetine, imipramine treatment showed to elicit a strong pro-gliogenic effect, reflected in a positive staining for GFAP in around 40% of the newborn cells (BrdU-positive). Moreover, this pro-gliogenic effect caused by the imipramine administration can be associated with an increase in the density of new glial cells to compensate the decreased number of these newly generated cells reached after the stress exposure, compensating in this way the lost function. Furthermore, the increase in the astrocytic processes length seen in the uCMS animals was re-established to values similar to control animals, after imipramine treatment, suggesting that the neuronal network could be already restored and astrocytes were no longer needed to establish more contacts with the neighbor cells. Again, contrarily to fluoxetine treatment, no astrocytic alterations were seen regarding the total volume analysis of the newborn cells.

Altogether these evidences suggest that, at the long-term perspective of depression, the cognitive rescue promoted by imipramine administration is related with underlying astrocytic density and gliogenic mechanisms. On the contrary, the mechanisms that might mediate the cognitive improvements induced by fluoxetine treatment are more neurogenesis-dependent, as previously suggested (Mateus-Pinheiro *et al.*, 2013), and related with morphological alterations of astrocytes.

These evidences also indicate that stress exposure leaves persistent morphological and behavioral scars on AD-treated animals. In this context, it is conceivable that the severity of the effects of a first depressive episode on cytogenic and remodeling processes may determine the rate and extent of relapse and recurrence of a subsequent depressive episode. Moreover, and taking into account the previous statement, in the first 5 years after recovery, around 50-70% of depressed patients suffer recurrent episodes (Burcusa & Iacono, 2007), so it becomes crucial to understand the neurobiological mechanisms behind this phenomenon of recurrence. It is also of the upmost importance to study the impact of treatment with typical ADs and uncover strong indicators of susceptibility to experiment recurrent depression.

Therefore, we included in our study the recurrence perspective of this disorder, by subjecting our animals to a second exposition to uCMS after the 4 weeks of recovery. Regarding the cognitive performance of our animals, both the short- and long- term memories were strongly affected with the second exposure to uCMS. Interestingly, imipramine treatment showed to be effective in promoting the recovery of the short-term, but not long-term, memory deficits caused by the stress exposition, and therefore seems to be resilient for a second hit of stress. However, fluoxetine treated animals were not able to rescue the cognitive impairments induced by stress exposure, in both analyzed dimensions, thus not being capable of preventing a relapse episode at the cognitive level.

Taking in consideration the impact on the astrocytic population residing in the hippocampal DG, after the second exposition to uCMS, we observed a decrease in both processes length and total volume of the resident astrocytes. These results can be explained by a higher susceptibility of astrocytes for a second hit of stress, being supported by the fact that, at the short-term perspective of depression, only an increase in terms of volume was observed in the astrocytes (when astrocytes were exposed to the first hit of stress). Thus, the DG resident astrocytes when exposed to a first episode of stress are resistant and get activated to help in the recover of the affected neural circuits. However, if astrocytes encounter a new stressful event, they become more susceptible and probably less active in the recover of the hippocampal DG system, thus contributing to the persisting cognitive impairments observed in recurrence. On the other hand, an increase in the density of newly generated astrocytic cells apparently took place in the DG at recurrence. As we do not observe an increased number of total astrocytic cells, this result might rather be explained by an increased expression of GFAP in the newborn cells, like sustained by several studies (Rajkowska, 2000; Rajkowska *et al.*, 2005). Indeed, the GFAP expression has been related with the progression of cellular changes by depressive illness, which might be happening with the double stressed animals resulting in an increased number of GFAP and BrdU – double positive cells in the hippocampal DG.

In the case of ADs treatment, after a second exposition to uCMS, fluoxetine seems to lose efficacy in recurrence, like previously mentioned. The explanation for that fact does not rely neither in astrocytic nor gliogenic factors, but might rely on the adult neurogenic process, as observed for the long-term perspective of the disease. The same conclusion can be applied to the imipramine treatment in recurrence, as this AD induced the recovery of the cognitive deficits, in short-term memory, not by means of astrocytic changes or gliogenesis - related processes, once both processes were impaired similarly to the double stressed group. However, the imipramine was inefficient in the recovery from the long-term memory deficits observed by the double stressed animals. This loss of efficacy in this specific

cognitive dimension could be explained by the impossibility of imipramine to rescue the morphological alterations in the resident and newly generated astrocytes induced by the second exposition to stress. These morphological alterations might be interfering with the local hippocampal neural circuits, impeding their normal activity and thus interfering with the long-term object recognition.

Altogether, having all these results into account, it seems that both astrocytic changes and gliogenesis alterations in the hippocampal DG are more relevant for the cognitive rescue at long-term. Moreover, it seems that the different ADs treatment (with fluoxetine or imipramine) induce differential effects in the glial populations, which might be reflected in the final outcome. Taking in consideration the cognitive dimension of depression, fluoxetine seems to be efficient in the recovery of the impairments observed in stressed animals at long-term, through neurogenic-dependent mechanisms. However, this AD seems to be more susceptible to a second stressful episode, loosing its cognitive efficacy, again via neurogenic-dependent processes (Figure 19). On the other hand, imipramine seems to be a more complex AD in terms of mechanisms of action, since at long-term the cognitive improvements observed are gliogenic-dependent, but in recurrence, its action seems to be more neurogenic-dependent (Figure 19). Importantly, imipramine treatment is more resilient to a second hit of stress at some cognitive levels, thus probably indicating that this AD might be more efficient to treat the cognitive dimension of depression in the clinics.

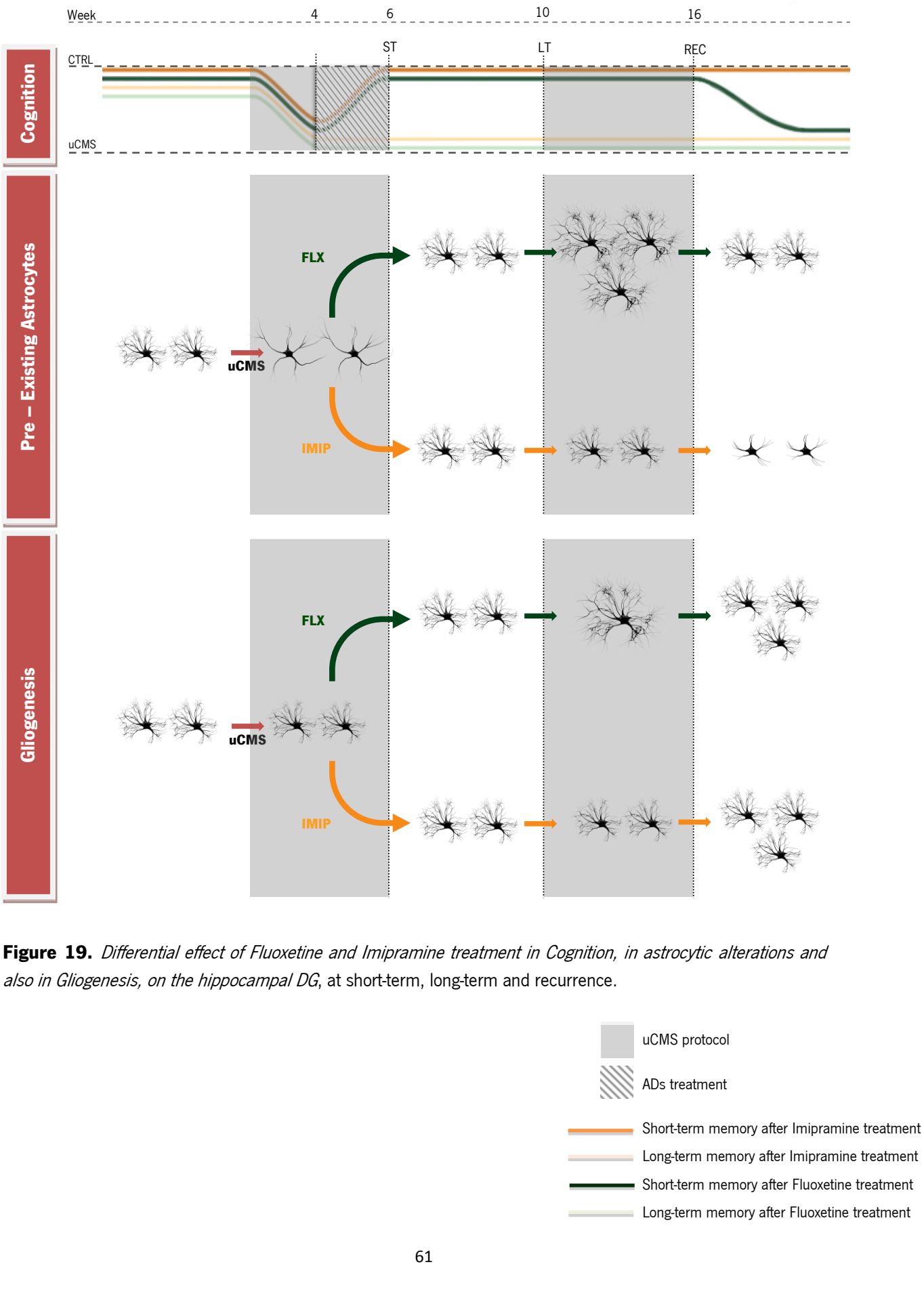


Figure 19. *Differential effect of Fluoxetine and Imipramine treatment in Cognition, in astrocytic alterations and also in Gliogenesis, on the hippocampal DG*, at short-term, long-term and recurrence.

V. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

5. Concluding Remarks and Future Perspectives

In the last decades, significant efforts have been done in the attempt to understand the complex pathological basis that underlie several neuropsychiatric disorders, such as MDD. Since neurogenesis has not been able to unravel all the causative neurobiological mechanisms of depression, we have put gliogenesis forward as a process underlying this disorder. In fact, some reports suggested that astrocytes might be responsible for the precipitation of the disease, presenting several impairments both in terms of basic functions as also in alterations of their morphology.

In this work, we have been able to show that cognition seems to be affected in both components examined (short – and long- term memories) throughout the course of the disease. Short-term memory seems to be the only one that can be rescued by the ADs treatment, contrarily to the long-term memory, whose deficits seem to persist even after ADs administration. Moreover, these cognitive deficits seem to be correlated with alterations in the morphology and density of the hippocampal DG resident astrocytes as with the gliogenesis process. On the recurrence perspective of the disease, ADs treatment seems not to be related with gliogenesis *per se*, but rather with alterations of the pre-existing astrocytes, which might also be reflecting the neurons state. However, at long-term, the ADs treatment show differential actions, being imipramine the only one capable of reverting the cognitive and gliogenic deficits that arose after the stress exposure. Indeed, imipramine was demonstrated to elicit a strong pro-gliogenic effect in the newborn cells of the hippocampal DG, at long-term. This effect was accompanied by a rescue in the number of the resident astrocytes (Figure 19). It is tough possible that a deficient function of the neuronal network can lead to a readjustment of astrocytic number and functions, in order to compensate the system failure.

Regarding the fluoxetine treatment, this AD induced a state of astrocytic activation, in which the gliogenesis process was not involved. Rather, a morphologic alteration in terms of volume and processes length of the pre-existing astrocytes was the strategy of fluoxetine action (Figure 19). This activation can be related with an attempt to readjust the neuronal network by inducing a state of over-working in the pre-existing astrocytes.

Thereby, we provide consistent evidence for the causative implication of gliogenesis and astrocytes in the pathophysiology of depression, having significant impacts in the long-term development and maintenance of cognitive deficits, as well as in the long-term recovery of those impairments promoted

by ADs treatment. Thus, our results strongly endorse the view of the hippocampal gliogenesis process as a promising therapeutic target in future therapies in the neuropsychiatric field. Moreover, this study shows for the first time that alterations in the hippocampal DG resident astrocytes can be further analyzed as a predictive target for depression.

Further studies will include the evaluation of ADs treatment effects *in vitro*, using hippocampal-derived neurospheres culture conditioned with norepinephrine or serotonine to prove all our *in vivo* results.

It will also include the addition of S100 β and other markers for mature astrocytes in glial morphology and density analysis, which will complement the results presented here by adding this additional characterization of the astrocytic population. Furthermore, it will be important to study the differential effects of imipramine and fluoxetine, looking at the neurotransmitter level, and further correlate with the cognitive performance.

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6. REFERENCES

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